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Cellular and Molecular Approaches to Regeneration and Repair

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Springer Series in Translational Stroke Research

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ISSN 2363-958X ISSN 2363-9598 (electronic)
Springer Series in Translational Stroke Research
ISBN 978-3-319-66678-5 ISBN 978-3-319-66679-2 (eBook)
https://doi.org/10.1007/978-3-319-66679-2

Library of Congress Control Number: 2017957214

Printed on acid-free paper

This Springer imprint is published by Springer Nature
The registered company is Springer International Publishing AG
The registered company address is: Gewerbestrasse 11, 6330 Cham, Switzerland

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Part I
Stem Cells

Chapter 1
Stroke: Cytoprotection, Repair and Regeneration—The Continuum of Patient Care

Paul A. Lapchak

Abstract The ischemic penumbra is now defined as tissue at risk of becoming fully involved in the evolving neurodegenerative process following an embolic stroke. After an ischemic core is quickly developed following vascular occlusion, there is slow spreading of the ischemic injury from the core to areas immediately surrounding the core until full recruitment is achieved. Forty years ago, seminal electrophysiological studies forming the basis of the penumbral hypothesis were conducted in a large animal model, baboons, an animal that is now rarely used in translational stroke research because it can no longer be justified! Thereafter, the rabbit embolic stroke model led the way for approval of Alteplase® (tissue plasminogen activator, tPA, rt-PA) to treat acute ischemic stroke.

Stroke research continues to evolve with the use of rodents primarily mice, rats, Oryctolagus cuniculus (rabbits), and occasionally non-human primates, but recent scientific expert statements have now suggested that non-human primates are not essential for stroke therapy development. One commonality amongst all species used historically is the documented presence of a core and penumbra following vascular occlusion, whether it be an artificial suture or clip occlusion or a blood clot.

This article reviews the historical basis for a few select mechanisms that are currently being targeted for cytoprotection, the rationale for target engagement to arrest penumbral growth and reduce clinical deficits, and it also sets a basis for the future of regeneration strategies to treat stroke patients.

Keywords Translational • Neuroprotection • Neuroprotective • Cytoprotection • Brain • Stroke • Embolic • Hemorrhage • Clinical trial • NIHSS • Stem cell • regeneration

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P.A. Lapchak, J.H. Zhang (eds.), *Cellular and Molecular Approaches to Regeneration and Repair*, Springer Series in Translational Stroke Research,
https://doi.org/10.1007/978-3-319-66679-2_1

Abbreviations

ACTION	Effect of Natalizumab on Infarct Volume in Acute Ischemic Stroke
ALS	Amyotrophic lateral sclerosis
ATP	Adenosine triphosphate
AU	Arbitrary units
ESCAPE	Endovascular Treatment for Small Core and Proximal Occlusion Ischemic Stroke
EXTEND-IA	Trial and Extending the Time for Thrombolysis in Emergency Neurological Deficits-Intra-Arterial
FDA	Food and Drug Administration
FRONTIER	Field Randomization of NA-1 Therapy in Early Responders
H_2O_2	Hydrogen peroxide
HO•	Hydroxyl radical
ICB	Intracerebral
IV	Intravenous
M1 or M2	Macrophage
MASTERS	Multipotent adult progenitor cells in acute ischemic stroke
MR CLEAN	Multicenter Randomized Clinical Trial of Endovascular Treatment for Acute Ischemic Stroke in the Netherlands
mRS	Modified Rankin scale
Nd:YaG	Neodymium-doped yttrium aluminum garnet
NADPH	Nicotinamide adenine dinucleotide phosphate
NADH	Nicotinamide adenosine dinucleotide
NEST	NeuroThera® Efficacy and Safety Trial
NIH	National Institutes of Health
NIHSS	National Institutes of Health Stroke Scale
NINDS	National Institute of Neurological Disorders and Stroke
NMDA	N-methyl-D-aspartate
nNOS	Neuronal Nitric oxide synthase
OPB	2-Oxo-3-(phenylhydrazone)-butanoic acid
O_2•−	Superoxide anion radical
PISCES	Human neural stem cells in patients with chronic ischaemic stroke
PSD-95	PSD-95 (postsynaptic density protein 95
PDZ1-2	Postsynaptic density-95, discs large 1, zonula occludens-1
REVASCAT	Endovascular Revascularization With Solitaire Device Versus Best Medical Therapy in Anterior Circulation Stroke Within 8 Hours
SWIFT PRIME	Solitaire With the Intention For Thrombectomy as PRIMary Endovascular Treatment
TCA cycle	Tricarboxylic acid cycle
TIGAR	TP53-inducible regulator of glycolysis and apoptosis

TLT	Transcranial laser therapy
tPA	Tissue plasminogen activator
THRACE	THRombectomie des Arteres CErebrales
TTC	Triphenyl tetrazolium chloride
UV	Ultraviolet
VLA-4	Very late antigen-4

1 Introduction

The existence of an ischemic stroke "penumbra" was first hypothesized by Astrup et al. in 1977 by demonstrating that there was a continuum of "threshold of ischemia" measured by electrical failure (potassium gradient) and reduced cerebral blood flow in the baboon cortical grey matter [1]. The gradient indicated an ischemic core, which is now known to be a mass of dead, unrecoverable tissue at the center of the ischemic infarct. The core is surrounded by **oligemic tissue**, defined as "Oligemia" tissue with reduced blood flow, but function is unaltered, and **ischemic tissue** with reduced blood flow. The **penumbral tissue**, is "at risk" of death tissue with altered potassium release, altered electrical failure and dysfunctional. In 1983, Olsen and colleagues demonstrated that an ischemic penumbra also existed in stroke patients; there was differential distribution of blood flow in non-ischemic, ischemic and hyperemic tissues [2]. The Ischemic Penumbra and Time is Penumbra have been reviewed in some detail by Heiss [3–14] and Donnan [15–26], and two main publications in association with receiving the Johann Jacob Wepfer Award [4, 16].

On this occasion, the 40th anniversary of describing the penumbra, we will take a brief look back at the origin of the stroke penumbra, and forward to review current clinically relevant targets that may arrest penumbral recruitment, and the consequences of such detrimental recruitment. We will also briefly discuss the potential need for multiple forms of therapeutic interventions to maximally promote both short and long term recovery in patients.

2 Cellular Measures of Metabolism and Penumbra

First, we will describe a novel technique to study the penumbral threshold in the rabbit embolic stroke model before addressing drug targets to attempt to arrest penumbra. In Fig. 1.1 we provide a topographical map of the rabbit cerebral cortex following an embolic stroke, comparing unstained fresh brain tissue (Fig. 1.1a) to a Nicotinamide Adenosine dinucleotide (NADH) map (Fig. 1.1c) of the brain followed by standard Triphenyl tetrazolium chloride (TTC) staining (Fig. 1.1b) to demarcate core (umbra) vs. viable tissue [27, 28].

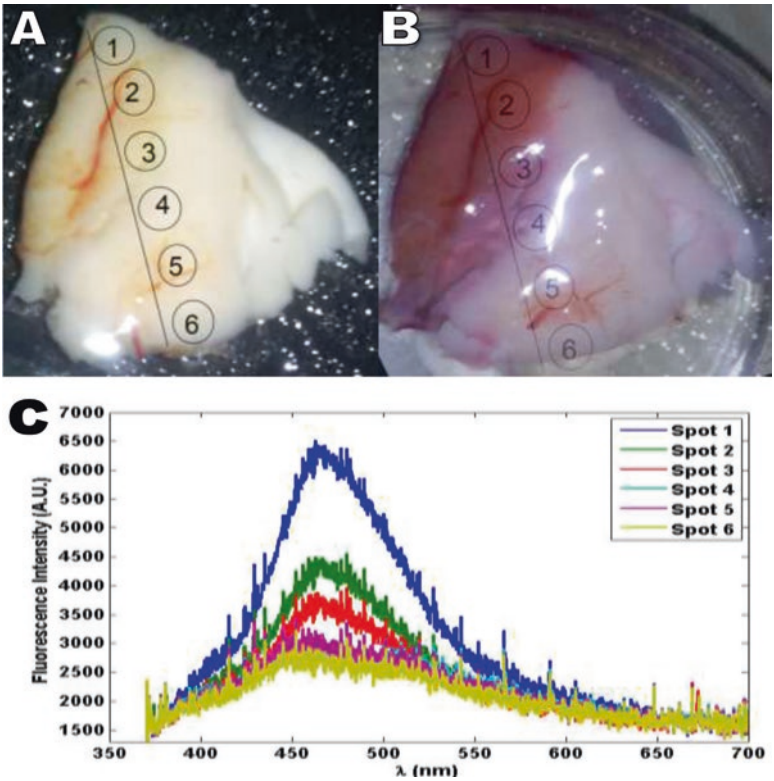

Fig. 1.1 (Credit: USPTO Patent 9404870 B2) Time-resolved laser-induced fluorescence spectroscopy systems and uses thereof [113]. Rabbits were prepared and embolized as described previously [60, 82, 114–118]. Fresh brain tissue was removed 3 h post-embolization for core (umbral) and penumbral NADH measurement. (**a**) Fresh rabbit cortex in oxygenated Krebs medium; (**b**) TTC-stained rabbit cortex; (**c**) Fluorescence measurement of NADH following an embolic stroke

2.1 Nicotinamide Adenosine Dinucleotide (NADH) Penumbra Mapping: A New Technique

NADH is produced in mitochondria from glucose during glycolysis [29]; it is one of the main coenzymes involved in the redox reaction for adenosine triphosphate (ATP) production in aerobic respiration. NADH is oxidized to NAD+ at the mitochondrial membrane by combining the hydrogen to oxygen thus forming H_2O, and in the process, produces ATP. In hypoxia, NADH accumulates inside the cell until there is shut down of tricarboxylic acid cycle (TCA). Alterations in the proton gradient result in further ATP depletion by the catabolic effects of ATP synthase [30]. If the oxygen depletion persists for a long duration, there may be cell death. These variations in NADH level provide us a brief window into the viability and vulnerability of cells under ischemic condition at a fixed time point. One way to evaluate

these fluctuations in NADH level is to measure the fluorescence emission from NADH. NAD+ and NADH both have a strong absorption in ultraviolet (UV) spectrum [31–36], but they differ in their fluorescence characteristics NADH [31–36]. Importantly, NADH demonstrates a strong fluorescence in the violet/blue band around 440/460 nm, and this is dependent upon its bound versus free-state. By measuring NADH fluorescence in real time it is possible to monitor the changes in the NADH level and assess the metabolic status of NADH [31–36], as well as the status of the cell.

We have used a Q-switched neodymium-doped yttrium aluminum garnet (Nd:YaG) laser emitting at a wavelength of 350 nm, running at 1 kHz with a pulse width (Full-Width Half-Maximum, FWHM) of 400 ps (Teem Photonics PNVM02510). The total energy per pulse did not exceed 5 μJ to prevent photobleaching of NADH. The excitation light is delivered to the tissue using a custom made trifurcated optical probe, which consists of a central 600μ fiber for delivering the excitation light surrounded by 12 x 200μ fibers to collect the fluorescence. Every other fiber from the twelve collection fibers are bundled together forming two channels. One collection channel/bundle goes to a spectrometer (Ocean Optics, Maya), which measure the fluorescence spectrum every 100 ms.

As shown in Fig. 1.1c, there was a continuum of Fluorescence Intensity in arbitrary units (AU) as the probe was moved from position 1–6 along the surface of the cortex (see Fig. 1.1a, b), and NADH levels were recorded across all wavelengths between 350 and 700 nm. Notably, the peak is at approximately 465 nm, and Position 1, which had the highest levels of NADH was “normal” tissue compared to position 6, which had the lowest NADH levels “infarct core”. Throughout probe placement positions 2–5, various states of the penumbra, there was a gradual decrease in NADH levels measured. This map is reminiscent of the original Astrup et al. [37] electrical failure map with one difference: the current NADH measurement correlates with the ability of cells to synthesize ATP, whereas the Astrup and colleagues measured tissue potassium (K^+) levels. Nevertheless, separated in time by 40 years, we still have a great interest in the stroke penumbra, and both how to measure it and save it!

3 Cytoprotection: What a Time for Intervention?

We have recently reviewed cell death in brain following an ischemic event such as an embolic stroke or ischemic insult [38] (see also [39, 40]) leading us to hypothesize that there are multiple opportunities to provide pharmacological interventions to reduce the primary and secondary consequences of stroke; targets to “attack” in order to reduce widespread cellular death. But when there is widespread cellular death due to inadequate recovery following thrombolysis, embolectomy or new interventions, the stroke patient will move from the neurologist or interventionalist to the transplant neurosurgeon, if intracranial injections of stem cells prove to be superior to other methods of administration.

To recapitulate on recent and current knowledge, basic research demonstrates that neurons are first and foremost affected by ischemia, and die rapidly followed ischemia. Thereafter, brain endothelial cells (i.e.: forming the basis of the blood brain barrier), pericytes (i.e.: contractile cells that wrap around the endothelial cells of capillaries and venules), microglia (i.e.: brain resident macrophage; type M1 or M2), and then astrocytes (microglia) [41–43]. However, all cell types are intricately co-mingled and can directly or indirectly influence each other. This is an important observation and it should form the basis for meaningful and clinically relevant cyto-protection research.

4 The Stroke Cascade and Current Intervention Attempts Toward Efficacy

Recently, stroke interventionalists have advanced endovascular procedures, embolectomy with stentrievers or thromboaspiration catheters to a technical level where statistically significant efficacy was demonstrated in a sextet of positive clinical trials enrolling large vessel-occluded patients with substantial penumbral substrate (See Table 1.1): (1) Multicenter Randomized Clinical Trial of Endovascular Treatment for Acute Ischemic Stroke in the Netherlands (**MR. CLEAN**) [44]; (2) Endovascular Treatment for Small Core and Proximal Occlusion Ischemic Stroke (**ESCAPE**) [45]; (3) Endovascular Revascularization With Solitaire Device Versus Best Medical Therapy in Anterior Circulation Stroke Within 8 Hours (**REVASCAT**) [46]; (4) Solitaire With the Intention For Thrombectomy as PRIMary Endovascular Treatment (**SWIFT PRIME**) [47]; (5) Trial and Extending the Time for Thrombolysis in Emergency Neurological Deficits-Intra-Arterial (**EXTEND-IA**)

Table 1.1 Population efficacy of reperfusion therapies compared to NINDS rt-PA

		No Symptoms----------►Death						
Study	**Treatment**	**0**	**1**	**2**	**3**	**4**	**5**	**6**
MR. CLEAN	**Control (267)**	**0**	**6**	**13**	16	30	12	22
	Intervention (233)	**3**	**9**	**21**	18	22	6	21
ESCAPE	**Control (150)**	**7**	**10**	**12**	15	24	12	19
	Intervention (165)	**15**	**21**	**18**	16	13	7	10
REVASCAT	**Control (103)**	**5.8**	**6.8**	**15.5**	19.4	16.5	20.4	15.5
	Intervention (103)	**6.8**	**17.5**	**19.4**	18.4	7.8	11.7	18.4
SWIFT PRIME	**Control (98)**	**9**	**11**	**16**	17	22	26	
	Intervention (98)	**17**	**26**	**17**	12	15	12	
EXTEND-IA	**Control (35)**	**17**	**11**	**11**	11	17	11	20
	Intervention (35)	**26**	**26**	**20**	17	3	0	9
THRACE	**Control (202)**	**11.9**	**16.3**	**13.9**	12.4	27.7	4.5	13.4
	Intervention (200)	**15.5**	**19.5**	**18.0**	12.5	17	5.5	12
NINDS rt-PA	**Control (312)**	**26**		**25**		27		21
	Intervention (312)	**39**		**21**		23		17

Efficacy Analysis: mRS outcome (90 Day shown as (%) per tier; Highlighted Boxes indicat mRS 0-2 functional independence.

[48]; (6) THRombectomie des Arteres CErebrales (**THRACE**) [49], which were completed and published almost 22 years after the "positive" National Institute of Neurological Disorders and Stroke (NINDS) tissue plasminogen activator (tPA) Stroke Study Group trial and publication [50], and subsequent approval of tPA by the Food and Drug Administration (FDA).

Table 1.1 presents the 90-day outcome data for modified Rankin scale (mRS) for 6 endovascular trials compared to the original rt-PA NINDS trial. Every trial indicates that "intervention", thrombolysis plus an endovascular procedure is better than the endovascular procedure alone, when the procedures are done within approximately 8 h of the stroke. Now both reperfusion procedures used independently or in combination are commonplace in US medical centers and hospitals, but recent treatment estimates suggest that only 5–10% of stroke patients are eligible for the procedures, and less than half have full recovery [38, 46, 47, 51–53] (see Fig. 1.2 for study outcome results).

While rational neuroprotection and cytoprotection trials are still evolving after many failed attempts, no specific strategy has realized efficacy in any form of ischemic stroke [54, 55]. Thus, unfortunately, there remains a need for efficacious cytoprotection and regeneration/repair procedures.

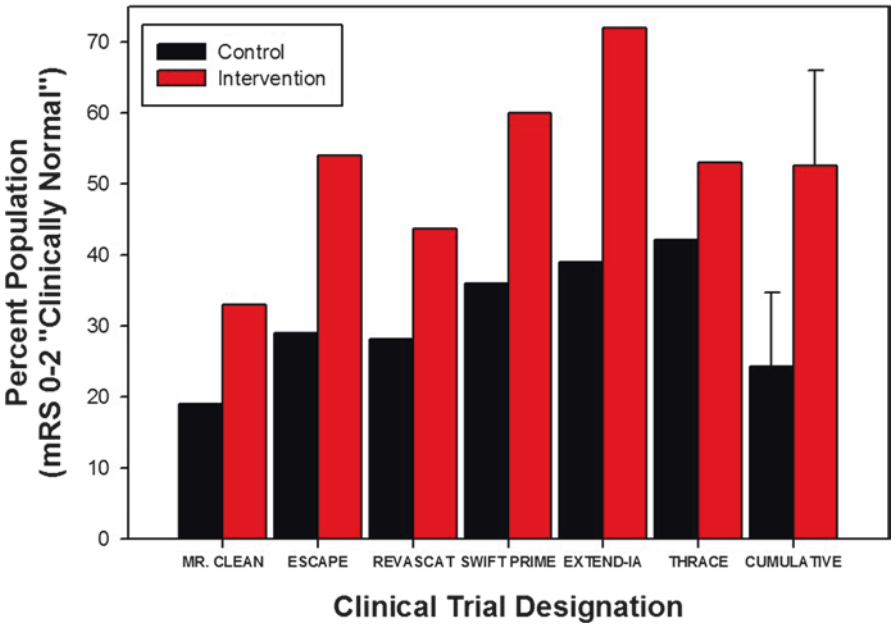

Fig. 1.2 Direct comparison of the patient population that is mRS 0-2 or normal, with functional independence in six recent embolectomy trials [(**MR. CLEAN**) [44]; (**ESCAPE**) [45]; (**REVASCAT**) [46]; (**SWIFT PRIME**) [47]; (**EXTEND-IA**) [48]; and (**THRACE**) [49]. In the trials, there was an a significant improvement in combined thrombolytic/endovascular procedure therapy (intervention) compared to control (endovascular procedure alone). All bars are Mean values, and the CUMULATIVE bars are the Mean ± SD for all patients mRS 0-2 showing that intervention more than doubles the population mRS 0-2. Note: Early enrollment is required for tPA and embolectomy within 8 h

5 Critical Targets for Rapid Intervention

The stroke ischemic cascade has many point of intervention, but there are few targets as important as cellular metabolism and excitotoxicity. As shown in Fig. 1.3, these mechanisms set the stage for cell death because they are the first in the sequence of events.

5.1 Energy Depletion and Cell Death

Early in the 1970s, Siesjo et al. recognized many important features of ischemia [56–59] related to rapid energy depletion by cells and metabolic changes that are either irreversible or reversible. In fact, in Fig. 1.1, we have taken advantage of some of the metabolic changes so that we can map the ubmra and penumbra. Two interesting and potentially important therapeutic approaches at this level have been documented over the last 10–15 years. First, transcranial laser therapy (TLT) was

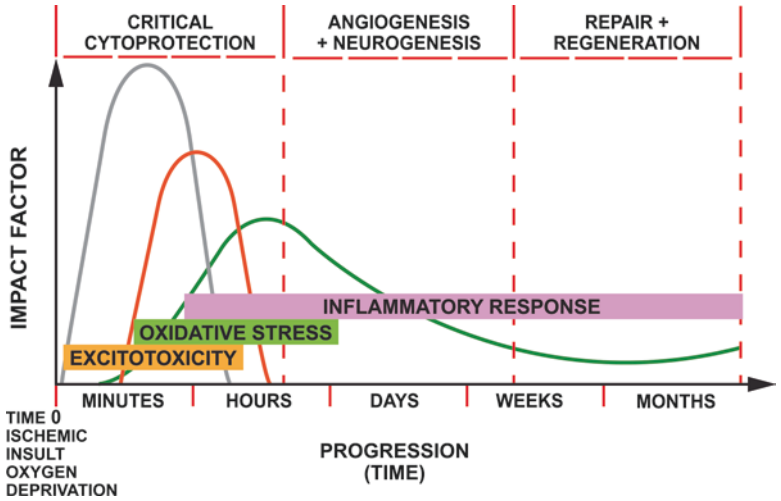

Fig. 1.3 The stroke cascade. Following an ischemic stroke, reduced blood blow and depleted ATP levels cause rapid death of neurons followed by initiation of a sequence of event in cells directly surrounding the core. There are numeours potential intervention points for therapy in the cascade, including reperfusion therapy conducted early after the ischemic event. The temporal profile for therapeutic intervention in stroke patients. The *graph* indicates therapeutic windows for mechanism-based acute pharmacological therapies, potentiating neurogenesis and angiogenesis, and then providing cell restoration or replenishment therapies. None of the interventions are mutually exclusive and optimal cytoprotection may require the use of combined therapies

attempted as an approach to enhance metabolism and increase cerebral blood flow; this worked well in multiple species [60–68], and this was linked to enhanced mitochondrial function and ATP levels [67, 68]. However, in stroke patients, reproducible clinical efficacy could not be achieved [69–71]. Because it has become clear that TLT was not sufficiently optimized before proceeding to the (NeuroThera® Efficacy and Safety Trial) NEST trials, TLT should be revived as a potentially useful approach [65]. New concepts are under development to deliver photons locally to promote cytoprotection.

More recently, Li et al. [72] used nicotinamide adenine dinucleotide phosphate (NADPH) as a therapy based upon initial observations that the TP53-inducible regulator of glycolysis and apoptosis (TIGAR) or fructose-2,6-bisphosphatase TIGAR plays a role in cell survival by increasing the flux of pentose phosphate pathway. The strategy of administering NADPH was successful in animals, where ATP levels were increased, stroke volume decreased and behavior improved statistically.

5.2 Excitotoxicity

Excitatory amino acid release (EAA), primarily glutamate is considered the first acute insult as reported by Jorgensen and Diemer in 1982 [73], and reviewed by Schwarz and Meldrum [74]. While many classic attempts at antagonism at EAA receptors with high affinity ligands have failed [54, 55], Tymianski and Cook [75, 76] have made a significant advance in the field by custom designing a therapeutic molecule targeting PSD-95 (postsynaptic density protein 95); the PSD-95 inhibitor Tat-NR2B9c which has shown potential using a non-human primate ischemia model and has advanced to the Field Randomization of NA-1 Therapy in Early Responders (FRONTIER) clinical trial [77]. The article by Bratane et al. referred to this form of neuroprotection as “freezing ischemic penumbra evolution” [78], which if this can be achieved, should significantly attenuate some of the deleterious steps within the temporally-dependent ischemic cascade. Clearly, the problem with the approach is that intervention is required as soon as possible after a stroke to attenuate cascade activation.

Moreover, Avilex Inc. has continued with this line of research and have developed a series of small molecules and peptidomimetics targeting the PSD-95 complex. AVLX-144 is the companies lead compound for the treatment of stroke. PSD-95 inhibitors targeting the postsynaptic density-95, discs large 1, zonula occludens-1 (PDZ1-2) domains of PSD-95 effectively block the formation of the ternary nNOS/PSD-95/NMDA receptor complex and uncouple the NMDA receptor pathway [79–81]. This is a fertile and competitive area of research that may result in identification of a therapeutic to use as a first line of defense in patients, and in patients undergoing thrombectomy.

5.3 Oxidative Stress

Free radicals are a valid target for therapeutic intervention for the treatment of AIS [82–85], because oxidative stress is a major early component of the ischemic stroke cascade [84, 86, 87]. Thus, free radical scavengers have been repeatedly tested in stroke and ischemia models, and have been reviewed by leading stroke experts as part of the ischemic cascade [38, 54, 83, 88, 89].

Oxidative stress following an ischemic stroke results in the production of various reactive oxygen species (ROS): hydrogen peroxide (H_2O_2), hydroxyl radical (HO•) and superoxide anion radical (O_2•−) that can induce membrane lipid peroxidation and vascular endothelial cell injury [90–94].

Recently the FDA formally approved edaravone (3-methyl-1-phenyl-2-pyrazolin-5-one; Radicava™) to treat patients with amyotrophic lateral sclerosis (ALS), Lou Gehrig's disease [95]. Edaravone (Radicut™) is a free radical scavenger marketed in Japan by Mitsubishi Tanabe Pharma Corporation to treat acute ischemic stroke patients presenting within 24 h of the attack. For stroke, there are two forms of edaravone currently available. Injectable edaravone ampoules (30 mg b.i.d, i.v. for 14 days) were first approved on May 23, 2001, and then on January 19th 2010, the Radicut BAG was approved by the Japanese Ministry of Health and Welfare and is used as an i.v. infusion. The demonstrated efficacy of Radicut has been limited to the Asian population (China, India and Japan) and there has still not been a randomized clinical trial conducted in North America. The clinical efficacy of Radicut in stroke patients was reviewed in detail by Lapchak [96].

The primary focus of edaravone research has revolved around its potential to scavenge hyrdoxyl, peroxyl and superoxide radicals that mediate neuronal and vascular damage [97–100]. At physiological pH, edaravone is present in an anionic form, and due to electron transfer, electrons released from the edaravone anion can effectively bind radical species containing a free electron. For example, the lipid peroxyl radical (OOL•) that can be formed after free radical extraction of a proton from an unsaturated fatty acid. The transfer of electrons from edaravone to a reactive oxygen species produces an edaravone radical, which then forms a peroxyl radical of edaravone. The result is the formation of a, a 4,5-dione (i.e. 3-methyl-1-phenyl-2-pyrazolin-4,5-dione) and eventually 2-oxo-3-(phenylhydrazone)-butanoic acid (OPB).

With Radicut now given orphan drug status in the United States for ALS, perhaps the near future will see the use of the antioxidant in stroke victims in North America.

5.4 Immunotherapy

The immune response following a stroke may present the longest therapeutic window for intervention, but may also be the most problematic pharmacological target because of the diversity of mediators and mechanisms, and the dual role of many

mediators. Moreover, the possibility exists that both central and peripheral immune systems can be mediated to modulate brain activity following a stroke [101, 102]. The immune or inflammatory response is normally characterized by the production of a vast array of inflammatory mediators including lymphokines, cytokines, and necrosis factors (i.e.: tumor necrosis factor). There is also recruitment and accumulation of "inflammatory cells", leukocytes, activation of microglia, and distribution of macrophage to the lesion area [103–105]. Natalizumab, a humanized monoclonal antibody against alpha$_4$-integrin (CD49d), specifically targeting α4 integrin within the adhesion molecule very late antigen-4 (VLA-4), is being tested in a clinical trial based upon limited efficacy in animal models [106]. A review of the preclinical literature shows that there is not unanimous neuroprotection or improvement in documented preclinical studies [106–108]. However, since the antibody attenuates the transmigration of leukocytes across the endothelium, it is hypothesized to block lymphocyte and adhesion and subsequent transmigration into the CNS, this attenuating the immune reaction. Preliminary results from the ACTION trial [106] indicate that "Natalizumab administered up to 9 h after stroke onset did not reduce infarct growth. Treatment-associated benefits on functional outcomes might warrant further investigation".

6 Regeneration, Repair and Repopulation

Novel methods of efficacious cytoprotection in stroke patients continues to be a challenge to both the translational and clinical stroke researcher. Based upon the cumulative data from the endovascular trials that were discussed earlier in this chapter, we can estimate that 5–10% of stroke patients will benefit from reperfusion procedures [109], and that only a small fraction of all stroke patients will be eligible for the procedures. Notably, patients with high National Institutes of Health Stroke Scale (NIHSS) scores have reduced probability of returning to normal (mRS 0-2) at 90 days [109], or thereafter. It is further estimated that 16–19% of patients will be dependent for the remainder of their lives [110]. With these assumptions, even if a cytoprotective agent was developed and FDA-approved, the overall shift in the "normal" patient population would be unacceptably small, but it is critical to attempt to intervene at all stages following a stroke.

6.1 What Happens to the Stroke Victim When Penumbra No Longer Exists as a Target for Intervention?

There would also be a need for strategies to regenerate and repair neuronal pathways in some stroke patients, especially those with long-term disability. This section will focus on three first-in-man stem cell clinical trials (Table 1.2).

Table 1.2 Stem-cell based therapy: results of three trials

Study	Cell type	Route	Trial design and result
MASTERS [109]	Multipotent adult progenitor cells	IV	**Design**: Randomized, double-blind, placebo-controlled **Result**: Safe. No difference in global stroke recovery scores at 90 days
PISCES [110]	Immortalized human neural stem-cell line CTX0E03	Intracerebral Stereotaxic	**Design**: Open-label, single center, dose-escalation. **Result**: Safe, no adverse events. ➢ Highly variable changes in clinical function measured over 24 months
SB623 [111]	Bone-marrow-derived mesenchymal cells - SB623 cells transfected with human Notch-1 intracellular domain	Intracerebral Stereotaxic	**Design**: Unblinded, non-randomized **Result**: Safe, but patients did experience treatment emergent adverse events. ➢ Improvement on European Stroke Scale; NIHSS; Fugl-Meter total score; Fugl-Meyer motor function score, but no changes in mRS measured over 12 months

The results of preliminary stem-cell based therapy trials suggest that certain cells can be administered safely, and that a potentially useful intravenous (IV) or intracerebral (ICB) route may be used in patients. Clearly, all published trials were preliminary safety trials, the focus was to be "first", and they were not designed to adequately or comprehensively determine efficacy on standardized stroke scales. More importantly, they were not rigorous [112]. Nevertheless, the three first-in-man clinical trials for stroke will eventually lead to meaningful rigorous blinded, controlled, randomized patient population investigation in this area.

7 Conclusion

In conclusion, translational stroke research continues to evolve from neuroprotection to cytoprotection, and now delves into regeneration and repair. Thrombolysis and embolectomy are now proven efficacious therapeutic interventions, but the procedures can be improved with adjunct therapy. The future of translational stroke research will continue to progress, and patients will be offered multiple forms of therapy depending on the clinical response to initial therapeutic intervention, their initial and follow-up NIHSS and mRS scores, and overall level of functional disability.

Acknowledgments and Funding This article was written without direct financial support from government sources (PAL). PAL is supported by pharmaceutical industry grants and philanthropic donations. P. Butte is thanked for assistance with NADH measurements.

Conflict of Interest Statements: PAL is Editor-in-Chief, Journal of Neurology & Neurophysiology and Associate Editor, Translational Stroke Research.

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Chapter 2
Interdisciplinary Advances Towards Understanding and Enhancing the Therapeutic Potential of Stem Cell-Based Therapies for Ischaemic Stroke

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Abstract Worldwide, stroke is the second single most common cause of death and is a major cause of permanent disability. Moreover, the highest incidence of these pathologies is observed in the elderly, increasing the socioeconomic burden in an aging population. Current available therapies lead to insufficient functional improvement or are not applicable to all patients. This stresses the urgent need for alternative strategies in treating stroke patients, for example cell-based therapies. These cells showed great preclinical potential although the underlying therapeutic mechanisms, preferential route of administration and most suitable stem cell-subtype are unknown. Mechanisms of action include neuroprotection, cell replacement, neurogenesis, immunomodulation and the promotion of both neuroplasticity and angiogenesis in damaged central nervous system regions. Moreover, stem cells have been genetically engineered to enhance their beneficial effects after transplantation. Additionally, noninvasive imaging can be used to provide detailed spatial and functional information on the donor cell fate and the response of the host microenvironment. This chapter provides an overview of recent advances in (bio-)medical research using or manipulating stem cell-based therapies for ischaemic stroke with a focus on their neuroprotective, neuroregenerative and immunomodulatory properties. Additionally, the use of noninvasive imaging to allow temporospatial evaluation of stem cell fate following transplantation in animal stroke models will be discussed.

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P.A. Lapchak, J.H. Zhang (eds.), *Cellular and Molecular Approaches to Regeneration and Repair*, Springer Series in Translational Stroke Research,
https://doi.org/10.1007/978-3-319-66679-2_2

Keywords Stem cells • Ischaemic stroke • Noninvasive imaging • Gene therapy • Regenerative medicine • Immunomodulation

Abbreviation

AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid
Ang-1	Angiopoietin-1
ASC	Adipose-derived stem cell
ATP	Adenosine triphosphate
BBB	Blood-brain barrier
BDNF	Brain-derived neurotrophic factor
bFGF	Basic fibroblast growth factor
BLI	Bioluminescence imaging
BM-MNC	Bone marrow-derived mononuclear cells
BMMSC	Bone marrow-derived MSC
CCR2	C-C chemokine receptor type 2
CT	Computed tomography
CXCR4	C-X-C chemokine receptor type 4
DAMPs	Danger associated molecular pattern molecules
DPSC	Dental pulp stem cell
EC	Endothelial cells
ECM	Extracellular matrix
EGF	Epidermal growth factor
ESC	Embryonic stem cell
EVs	Extracellular vesicles
FGF	Fibroblast growth factor
FLI	Fluorescence imaging
GDNF	Glial-derived neurotrophic factor
GFAP	Glial fibrillary acid protein
hESC	Human embryonic stem cell
HGF	Hepatocyte growth factor
ICAM-1	Intercellular Adhesion Molecule 1
IDO	Indoleamine 2,3-dioxygenase
IFN-γ	Interferon-gamma
IGF-1	Insulin-like growth factor 1
IL	Interleukin
iPSC	Induced pluripotent stem cell
MCAO	Middle cerebral artery occlusion
MCP-1	Monocyte chemotactic protein 1
MHC	Major histocompatibility complex
MLR	Mixed lymphocyte reaction
MMP	Matrix metalloproteinase

MRI	Magnetic resonance imaging
MSC	Mesenchymal stem cell
NF-κB	Nuclear factor kappa B
NGF	Nerve growth factor
NK cells	Natural killer cells
NMDA	N-methyl-D-aspartic acid
NO	Nitric Oxide
NSC	Neural stem cell
OGD	Oxygen-glucose deprivation
PDGF-BB	Platelet-derived growth factor BB
PET	Positron emission tomography
PGE2	Prostaglandin E2
ROS	Reactive oxygen species
SDF-1α	stromal cell-derived factor 1 α
SGZ	Subgranular zone
SPECT	Single-photon emission computed tomography
SPIO	Superparamagnetic iron oxide
STAT3	Signal transducer and activator of transcription 3
SVZ	Subventricular zone
TGF-β	Transforming growth factor beta
TIMP	Tissue inhibitor of metalloproteinase
TNF-α	Tumour necrosis factor alfa
Treg	Regulatory T cell
VEGF	Vascular endothelial growth factor

1 Introduction

The pathophysiology of stroke is defined as a neurologic dysfunction of vascular origin with the rapid occurrence of symptoms and signs corresponding to the involvement of focal areas in the brain [1]. Two different types of stroke can occur: ischaemic stroke (80–85%) and haemorrhagic stroke (15–20%). Ischaemic stroke is most frequently caused by thromboembolisms while haemorrhagic stroke most often results from vessel wall pathology associated with hypertension and microaneurysms [2]. This chapter will only focus on ischaemic stroke as the main pathology.

Worldwide, stroke is the second most common cause of death and is a major cause of permanent disability [3, 4]. Moreover, the highest incidence of these pathologies is observed in the elderly, increasing the socioeconomic burden in an ageing population [4]. In ischaemic stroke, the blood supply to certain areas of the brain is compromised which triggers a cascade of deleterious events ultimately leading to neuronal cell death [5]. This in turn triggers the acute immune response which can have a persistent and detrimental effect on stroke outcome [6]. The resulting severe neurological

dysfunction is clinically translated into symptoms such as paralysis, sensory disturbances, aphasia, urinary incontinence and cognitive impairment. Limited stroke-induced endogenous neurogenesis can be observed in patients but adequate functional recovery is not achieved [7]. Recombinant tissue plasminogen activator is the only FDA-approved pharmacological treatment for stroke but comes with many restrictions. Administration should be started within a time window of 4.5 h post-ischaemia, limiting its use to merely 2–4% of the patients and leading to an insufficient functional improvement [8]. These indications highlight the urgent need for alternative strategies in treating stroke patients.

Stem cell therapy is a promising approach to minimize neurological damage and enhance functional recovery after stroke. Preclinical studies in animal stroke models using for instance neural stem cells (NSC) [9], mesenchymal stem cells (MSC), induced pluripotent stem cell (iPSC)-derived cells delivered encouraging results (See Table I and Table II in [10]). However, the optimal stem cell-source, mechanisms of action, cell fate and optimal treatment protocol remain to be elucidated. Mechanisms of action include neuroprotection, cell replacement, immunomodulation and the promotion of both neuroplasticity and angiogenesis in damaged central nervous system regions [10]. Moreover, stem cells that were genetically engineered to overproduce growth factors such as brain-derived neurotrophic factor (BDNF), nerve growth factor (NGF), Noggin and angiopoietin-1 (Ang-1) have been previously shown to enhance post-stroke recovery [11–13]. Additionally, noninvasive imaging can be used to provide detailed spatial and functional information on the donor cell fate and the response of the host microenvironment following cell-transplantation into animal stroke models [10].

This chapter aims to provide an up to date overview of current interdisciplinary advances in preclinical stroke research, focussing on neuroregeneration, neuroprotection and immunomodulation supported by noninvasive imaging opportunities.

2 Therapeutic Approaches and Evaluation of the Post-stroke Microenvironment

The multiple mechanisms that have been proposed for stem cell-mediated therapies include brain protection, cell replacement, immunomodulation and promoting both brain plasticity and angiogenesis in damaged brain regions (Fig. 2.1) [10]. Interestingly, these mechanisms are mainly thought to be mediated by the paracrine effect of the transplanted cells on endogenous stem cells and on the host microenvironment instead of directly replacing the lost cells, although encouraging results have also been achieved with cell-replacement studies. Therefore, the engrafted cells can be seen as a vehicle for persistent growth factor delivery at the stroke lesion which can also respond dynamically to changes in the local microenvironment. In addition, the transplanted cells directly or indirectly influence extracellular matrix (ECM) remodelling and glial scar formation.

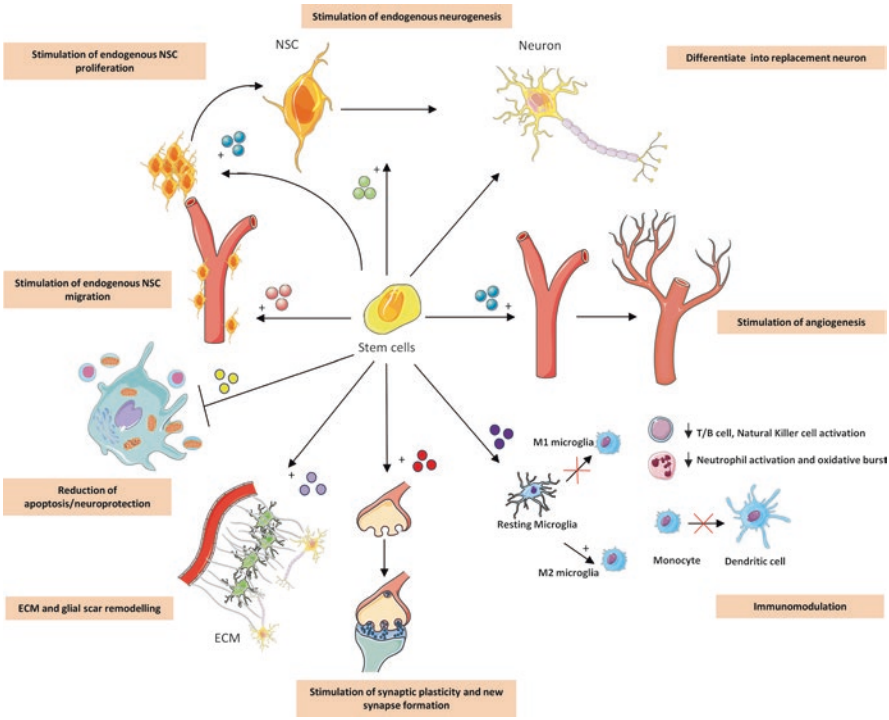

Fig. 2.1 Mechanisms of action of cell-based therapies in ischaemic stroke. The poststroke microenvironment can be influenced by exogenously delivered stem cells by multiple mechanisms to trigger tissue repair. Stem cells contribute to poststroke recovery by stimulating endogenous NSC migration toward the stroke lesion, where proliferation and differentiation toward replacement neurons can be triggered. Additionally, transplanted stem cells are thought to be able to replace the lost neurons themselves. Moreover, the formation and attraction of novel blood vessels toward the ischaemic lesion and the stimulation of synaptogenesis and synaptoplasticity contribute to brain repair. In addition to directly stimulating the formation of new brain tissue, the degradation of resident cells is inhibited by neuroprotective mechanisms and transplanted cells can influence the extent of glial scar formation. Immunomodulatory effects are also observed and include the inhibition of neutrophil activation and migration, effector T-cell and B-cell inhibition, reducing the activation and attraction of peripheral dendritic cells, and stimulating the M2 microglial phenotype. These effects are predominantly caused by the soluble factors released by the stem cells, but also cell–cell interactions appear to play a role. Image was adapted from [10] with permission

2.1 The Neuroprotective Effect of Stem Cell-Based Therapies

The ideal therapeutic approach for stroke would be to prevent neuronal cell death induced by the ischaemic insult, thereby minimizing neurological damage and stroke severity. Any strategy that aims to inhibit or antagonize the pathophysiological cascade of biochemical events resulting in irreversible cell damage and neuronal cell death is considered a neuroprotective approach [14].

2.1.1 The Complexity of the Ischaemic Cascade

Neuronal cells located in the ischaemic core die within minutes after stroke onset, whereas peripheral cells residing in the penumbra provided with limited collateral blood flow become dysfunctional but do not undergo acute cell death. However, if left untreated, the neuronal cells in the penumbra are likely to progress into delayed neuronal cell death hours to days after the ischaemic insult [15]. Therefore, a restricted time window exists wherein reversibly damaged neuronal cells can be salvaged from cell death in order to limit infarct size and improve functional outcome after stroke.

In the ischaemic core and penumbra, a series of neurochemical events occur, described as the ischaemic cascade [16]. The brain depends on oxygen and glucose to assure normal neuronal cell function and maintain ionic homeostasis. Impairment of cerebral blood flow causes disturbances in these vital energetic processes [5]. Ischaemia leads to dysfunction of ATP-dependent ion pumps, including the Na^+/K^+ pump, which results in alterations in the membrane potential and depolarisation of neurons and glial cells [17]. Subsequently, voltage-dependent Ca^{2+} channels are activated and excitatory neurotransmitters, including glutamate, are released into the extracellular space. This accumulation of glutamate in the extracellular space leads to the stimulation of α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA) and N-methyl-D-aspartic acid (NMDA)-type glutamate receptors on adjacent neurons. Consequently, these neurons become depolarized, which results in additional Ca^{2+} influx and glutamate release, causing an exacerbation of the initial ischaemic insult. Due to the ionic imbalance, intracellular water accumulation occurs, which is responsible for the formation of cytotoxic oedema [5, 16]. Furthermore, the increase in intracellular Ca^{2+} leads to the activation of Ca^{2+}-dependent enzymes, including proteases, endonucleases, phospholipases and cyclooxygenases. These enzymes cause extensive cell damage and are partially responsible for the generation of reactive oxygen species (ROS). ROS are important mediators of cellular damage by inducing DNA damage, lipid peroxidation and protein denaturation, which ultimately results in mitochondrial failure and membrane disruption [5, 16, 17]. The outcome of these detrimental molecular events is cell death via necrosis or apoptosis, depending on the degree of ischaemic injury [17, 18].

2.1.2 Stem Cell-Mediated Neuroprotection

Numerous neuroprotective agents which target different components of the pathophysiological cascade have been investigated in the context of stroke (reviewed by Moretti et al. [19]). These molecular targets comprise various mechanisms of calcium influx, glutamate excitotoxicity, ROS scavenging, NO metabolism and apoptosis. Despite promising preclinical results, no neuroprotective agent has passed clinical trials in stroke patients. Alternatively, approaches like therapeutic hypothermia and decompressive craniectomy have been explored to minimize neurological damage. Both these approaches seem to result in mild improvements in

neurological scores in stroke patients. However, these patients still have a poor functional outcome after 6 months [20, 21].

Stem cell-based therapy is considered a promising treatment strategy to minimize neurological damage and enhance functional recovery following stroke. By secreting neurotrophic and anti-apoptotic factors, stem cells can provide support to reversibly damaged neurons present in the penumbra. In this way, the stem cells can exert neuroprotective effects and rescue neurons which otherwise would progress into delayed cell death, thereby preventing additional neuronal damage [22, 23]. It has been demonstrated that MSC, including bone-marrow-derived MSC (BMMSC) and dental pulp stem cells (DPSC), secrete a plethora of paracrine factors comprising BDNF, NGF, neurotrophin-3 (NT-3) and glial cell-derived neurotrophic factor (GDNF), which are considered hallmark neurotrophic and neuroregulatory factors [24–27].

Neuroprotective effects mediated by stem cells have been observed in numerous *in vitro* studies performed by independent research groups. It has been shown that MSC protect neuroblastoma cells against hypoxia and glutamate excitotoxicity [28–30]. Furthermore, MSC are able to rescue primary cortical neurons which are exposed to oxygen-glucose deprivation and trophic factor withdrawal [25, 31]. Additionally, MSC can also prevent cell death in ischaemic human astrocytes [32]. Extensive evidence of neuroprotective effects mediated by MSC has also been provided in *in vivo* stroke models. Injection of the conditioned medium of stem cells as well as the stem cells as such have proven to exert neuroprotective effects in experimental stroke models [28, 33–37]. The paracrine factors secreted by the stem cells are believed to be responsible for this neuroprotective effect, since no or very limited integration of the stem cells in the lesion site is observed. These studies comprised the use of MSC derived from different sources, including adipose-derived stem cells, umbilical tissue-derived stem cells, BMMSC and DPSC. Furthermore, different routes of administration, cell dosages and time points of transplantation have been used in these studies.

2.2 Regeneration of Endogenous Tissue by Stem Cells After Ischaemic Stroke

In the 1990–2000s, MSC therapy gained a lot of attention to be used as therapy after stroke because of their assumed ability to transdifferentiate into neuronal cells, endothelial cells and glial cells. Numerous *in vitro* experiments demonstrating this so-called transdifferentiation potential sparked hope to use these stem cells for neuronal tissue replacement. However, these properties are merely induced under certain artificial cell culture conditions, not representing their endogenous properties. Another disadvantage is that this processes turned out to be time-consuming and very inefficient. Despite of this, transplantation of undifferentiated MSC has been explored with the rationale that they would locally replace the neural tissue. Intracranial transplantation of MSC showed that the cells migrated towards the

infarct region, survived in the host brain and stimulated functional recovery [38]. However as already mentioned above, numerous studies indicated that only a small percentage of the transplanted MSC survived and locally differentiated preferentially towards astroglial cells instead of the desired neurons [10, 34, 39]. It is generally accepted that functional replacement of the lost neurons is not the main mode of action of MSC.

Embryonic stem cells (ESC) and iPSC have the tremendous potential to differentiate in all possible cells from the nervous system including glial cells and neurons. However, achieving mature neurons from these pluripotent stem cells is even *in vitro* very time-consuming, costly and inefficient. Also their possible tumorigenicity when used in undifferentiated state is a major disadvantage [40, 41]. Therefore, iPSC are irreversibly pre-differentiated *in vitro* in order to minimize tumour formation and improve the functional outcome as only the undifferentiated iPSC form teratomas. The true potential of iPSC and ESC in stroke research is to apply them *in vitro* as a patient-in-a-dish model, trying to understand disease mechanisms and for drug discovery [42]. Especially the novel method culturing cerebral organoids resembling the 3D brain structure is a big step forward closing the gap between 2D cell cultures and animal models [43, 44].

When it comes to the use of stem cells for neuronal tissue regeneration as well as for neuroprotection, the current focus lays on the angiogenic and neurotrophic factors, cytokines and chemokines that are able to enhance the endogenous repair mechanisms after cerebral ischaemia, i.e. vascular remodelling, activation of endogenous neuroregeneration and remodelling of the extracellular matrix.

2.2.1 Vascular Remodelling After Stroke

Cerebral ischaemia leads to increased vascular remodelling in both the acute and chronic phase [45]. During acute blood flow obstruction, arteriogenesis also referred to as collateralisation can occur. This development of a functional blood flow from pre-existing arterial anastomoses is induced by mechanical forces such as shear stress and is independent of a hypoxia state [46]. By contrast, angiogenesis, the development of new capillaries sprouting from existing small blood vessels, is a key endogenous process induced by chronic hypoxia. The angiogenic process is a complex cascade of events, involving breakdown of the extracellular matrix, activation of endothelial cells (EC), followed by the proliferation, migration of EC. In a final step, pericytes are recruited towards the formed tubular network of EC resulting in mature blood vessels. A multitude of angiogenic factors and signalling molecules such as vascular endothelial cell growth factor (VEGF), fibroblast growth factor (FGF), angiopoietin-1 (Ang-1), Platelet-derived growth factor BB (PDGF-BB), nitric oxide (NO) have to co-operate in concert with spatiotemporal precision [47, 48]. Although distinct triggering mechanisms induce either collateralisation or angiogenesis, similar growth factors, cytokines and signalling molecules are shared by both modes of vascular remodelling [45]. A lot of angiogenic therapies applying recombinant proteins gained disappointing results in clinical trials [49, 50]. This can

be partly explained by the fact that the most studied angiogenic factor VEGF, is linked to the generation of immature and unstable vessels leading to oedema and vessel regression over time, aggravating stroke progression. In addition, the majority of angiogenic therapy regimens only involved administration of a single angiogenic protein. In that respect, MSC which have been showed to secrete numerous cytokines and angiogenic factors and as a surplus can act as a pericyte, can create the right angiogenic microenvironment [51]. Furthermore, numerous animal stroke studies support the pro-angiogenic properties of MSC obtained from different tissues and that the reported improved recovery is associated with increased blood vessel density. For example, increased VEGF and VEGFR2 expression was observed after intravenous injection of human BMMSC in a rat model of ischaemic stroke [52]. Another research group reported elevation of Ang-1 and Ang-2 mRNA levels in BMMSC treated rats [53]. Wakabayashi et al. showed that intravenous injection human MSC in a rat middle cerebral artery occlusion (MCAO) model induced functional improvement and reduced infarct volume by producing angiogenic factors. Moreover, MSC locally secreted IGF-1 in the ischaemic core and interestingly, this IGF-1 production was only detected *in vivo*, suggesting its specific induction by the ischaemic environment. In addition, the transplanted MSC affected the host cells, as endogenous VEGF, EGF, and bFGF levels were significantly elevated in stem cell-treated rats 7 days after injury [54]. Not only the stem cells themselves have a significant therapeutic potential in ischaemic stroke but also their extracts were shown to have a therapeutic effect. Intraperitoneal injection of a cell-free extract derived from MSC was shown to dramatically decrease the ischaemic volume and improve motor function after stroke [55].

2.2.2 Endogenous Regeneration After Stroke

For a long time, cells of the adult central nervous system were considered to be incapable of regeneration. However, it was demonstrated that human adult NSC reside in the dentate gyrus of the hippocampus and in the subventricular zone (SVZ) [56, 57]. Under normal physiologic conditions, adult NSC predominantly produce neurons, interneurons of the olfactory bulb for SVZ-derived cells, and dentate granule cell neurons for SGZ-derived cells. Ischaemic stroke enhances proliferation of the SVZ cell population and these cells migrate towards the lesion and differentiate into mature striatal neurons and replace damaged neurons. SDF-1α/CXCR4 and MCP-1/CCR2 receptor signalling has been shown to regulate the directed migration of these NSC to the injured area [58, 59]. NSC themselves have been shown to produce matrix metalloproteinase (MMP)-3 and MMP-9 in response to these extrinsic signals. Blocking the expression of MMP-3 or MMP-9 in NSC interferes with both their differentiation and migration [60], suggesting a prominent role of these MMPs in the endogenous NSC response. The leading fraction of the migrating NSC is closely associated with blood vessels, suggesting that this interaction provides a scaffold to direct the NSC towards the damaged brain region [61]. However, the amount of endogenous cells generated is considered to be too low to have a significant impact

on functional recovery after stroke. Nevertheless, various preclinical trials have been performed to enhance neurogenesis after stroke using EGF, VEGF, erythropoietin and statins [62, 63]. These investigations have also formed the basis to investigate the ability of cell transplantation to activate NSC and to mediate their differentiation towards neurons.

Neurotrophins/growth factors found in the MSC secretome include GDNF, NT-3, NGF and BDNF [10, 24, 30, 64]. To our knowledge, the effect of MSC on NSC migration and/or differentiation has not yet been tested *in vitro*. CM of DPSC, BMMSC and Wharton Jelly MSC have shown to enhance neuronal maturation of a pre-differentiated neuroblastoma cell line SH-SY5Y cells [65, 66]. In addition, the MSC secretome has been demonstrated to enhance neurite outgrowth in various types of primary neurons including, dopaminergic, primary cortical neurons and neurons derived from dorsal root and the retinal ganglia [10, 24, 30, 64]. In contrast to the overwhelming preclinical evidence on the induction of angiogenesis by MSC transplantation, only few reports on activation of endogenous NSC proliferation, migration and maturation are available. Munoz et al. showed that hMSC injected stereotactically induced migration of BrdUrd-labeled endogenous cells throughout the dorsal hippocampus, which were doublecortin-positive, and expressed markers for astrocytes as well as for neural or oligodendrocyte progenitors 7 days after treatment. In addition, 30 days after implantation, the newly generated NSC expressed markers for more mature neurons and astrocytes [67]. Another study in a rat stroke model, demonstrated that human MSC transplanted intracranially induce proliferation of endogenous NSC and subsequent migration as shown by double staining of BrdU and doublecortin at 1 and 2 weeks after MCAO induction [63]. Recent work in the setting of traumatic brain injury (TBI) showed that transplanted exogenous MSC are able to guide the migration of endogenous cells from the neurogenic site to the area of injury in the cortex via the formation of a 'biobridge' between the neurogenic and ischaemic site. This biobridge, visualized immunohistochemically and laser captured, corresponded to an area between the neurogenic SVZ and the injured cortex and consists of an altered endogenous expression of MMPs and ECM [68]. Despite the fact that MSC transplantations have been shown to induce both proliferation and differentiation of SVZ-derived NSC, neuronal differentiation rates were very low. As a consequence, there is a controversy on the fact whether or not MSC-induced enhancement of endogenous neurogenesis significantly contributes to an enhanced post stroke recovery [69, 70].

2.2.3 ECM and Scar Tissue Remodelling

After ischaemia, gliosis also referred to as scar formation is strongly induced at the infarct boundary. Damaged neurons initially interact with the adjacent astrocytes, which become activated and show increased expression GFAP, musashi-1 and secrete inflammatory cytokines [71, 72]. These triggered astrocytes in turn rapidly surround the infarct with fibrils [73]. The possible role of this demarcation consisting of activated astrocytes and ECM, but also microglia and oligodendrocytes is to separate the necrotic tissue

from viable brain and avoid further spreading of damage. Furthermore, this seal has also shown to play a role in maintenance of ion and fluid balance, preventing further inflammation, free radical scavenging and increasing tropic and metabolic support of the nerve tissue and for blood vessel ingrowth. On the other hand, it has a devastating effect on functional recovery as it impedes axonal regeneration [71, 72]. The ECM compound represents a physical barrier for new regenerating axons to cross. In addition, the reactive astrocytes secrete growth-inhibitory molecules such as Nogo [74]. According to this rationale, therapies that are able to reduce gliosis would thus be beneficial and enhance stroke recovery [72]. Several studies indicate that MSC secrete MMPs that cleave the ECM and would play a role in scar tissue destruction. BMMSC were able to produce active MMP-2, MMP-3 and also membrane-bound MT1-MMP [75, 76]. MSC are also able to activate exogenous proMMP-2 and proMMP-13. Interestingly, a recent study showed that the majority of the MMP activity is associated with the MSC cell surface while they secrete high levels of TIMPs, which strongly inhibits soluble MMPs. Since they bind and activate MMPs at their surfaces, the net result is a very controlled pericellular localization of MMP activities by MSC [77]. However, in the context of stroke, the contribution of this MMP production and the beneficial effects of MSC treatment remains to be elucidated. Only a few reports are available that studied the effect of stem cells on scar tissue formation. MSC treatment reduced the thickness of the scar wall and reduced the number of microglia/macrophages within the scar wall 4 months after surgery in a rat MCAO stroke model [71]. The same research group reported that long-term follow up (more than 1 year) of BMMSC injected in the carotid artery 1 day after MCAO significantly reduced thickness of the lesion scar wall and the number of Nogo-positive cells [78]. The exact molecular mechanisms behind this reduction of the scar wall thickness remain to be elucidated.

Although several studies emphasize that reactive astrocytes after CNS injury induce glial scar formation, which inhibits axon regeneration and impedes functional recovery, others indicated a neuroprotective role of astrocytes in CNS injury [72]. In that respect, it is worth to study the impact of stem cells on astrocyte survival. Indeed, MSCs suppress astrocyte apoptosis induced by OGD *in vitro*, an effect that has been attributed through the MSC-induced activation of IL-6 signalling in injured astrocytes [32, 79]. In addition, Song et al. showed that both BMMSCs as wells as DPSCs attenuated OGD-induced GFAP, nestin, and musashi-1 expression and inhibited OGD-induced ROS and interleukin-1β production in activated astrocytes *in vitro* [32].

2.3 Immunomodulatory Properties of Candidate Stem Cell-Based Therapies for Ischaemic Stroke

Whereas the neuroregenerative and neuroprotective effect of transplanted stem cells on the stroke-affected microenvironment has been studied thoroughly as described in the previous sections, the effect of the transplanted cells on the immune system and the infiltrating immune cells remains to be fully characterized.

2.3.1 Introduction to Stroke Immunology

The immune system and inflammation play a key role in the pathophysiology of stroke and can greatly influence stroke outcome [80]. Moreover, as a response to the ischaemic insult, the brain exerts a suppressive effect on the systemic immune system which leads to systemic lymphocytopenia [81]. This makes patients more susceptible to infections and is a major cause of stroke-associated morbidity and mortality [82, 83].

The various elements of the immune system are involved in all stages of ischaemia-induced brain loss. Early vascular events after arterial occlusion initiate inflammation where hypoxia, the production of ROS and changes in blood flow trigger the coagulation cascade, blood platelets and complement [84–86]. These events are followed by the upregulation of adhesion molecules on the platelet—and EC surface such as P-selectin, E-selectin and intercellular adhesion molecule 1 (ICAM-1) [87]. Moreover, the production of pro-inflammatory signals/cytokines is increased as well as the production of the vasodilator nitric oxide (NO) [86, 87]. Ultimately, EC junctions are weakened which allows protein and cellular extravasation into the perivascular space where mast cells and macrophages are activated and secrete proteases and pro-inflammatory mediators leading to blood-brain barrier (BBB) damage and leukocyte infiltration [86, 88].

In the subsequent phase of ischaemic cell death, the dying neuronal cells send out danger signals that activate the immune system [89]. These so-called danger associated molecular pattern molecules (DAMPs) include extracellular ATP or other nucleotides [90], heat-shock proteins, ECM breakdown proteins [91] and the high mobility group box 1 protein (HMGB1) [92] which are released from dying brain tissue following stroke [89, 93]. These DAMPs activate ionotropic purine receptors and scavenger—or pattern recognition receptors on inflammatory cells, leading to the production of pro-inflammatory mediators by resident brain cells and infiltrating leukocytes (for in-depth DAMP signalling, see review Gelderblom et al. [93]).

Stroke-induced inflammation eventually diminishes and triggers several pathways needed for the repair and reorganisation of the injured brain. This switch from a tissue-damaging pro-inflammatory stroke microenvironment to an anti-inflammatory, repair-stimulating environment remains poorly understood, but is coordinated by an intertwined cascade of inter- and intracellular signalling [86]. In this transition, macrophages and microglia switch from a pro-inflammatory M1 phenotype to an M2 phenotype that stimulates repair processes and attenuates the inflammatory response [94, 95]. Dead cells and debris attract and activate infiltrating macrophages and microglia which subsequently phagocytize the lost tissue. Phagocytosis induces the production of cytokines such as transforming growth factor beta (TGF-β), IGF-1 and IL-10 which were shown to have a neuroprotective and/or an anti-inflammatory effect [96–98].

In addition to the innate immune system, the adaptive immune system was also shown to contribute to inflammation-induced neuronal damage. DAMPs from damaged cells can also function as antigens that are presented to cells of the adaptive immune system, leading to immunity against these antigens [99]. Although the

damage to the post-ischaemic brain does not appear to be caused by an autoimmune response, the observed injury also does not fit the profile of classical adaptive immunity due to the temporal profile of the cellular infiltrate [86]. Blocking postischaemic trafficking of T cells 24–48 h after ischaemia provides a neuroprotective effect [100], whereas the classical adaptive immune response takes up to 1 week to develop and damage the ischaemic tissue. Interestingly, B cells do not significantly contribute to brain injury [101] and T-cell mediated damage is associated with γδ T cells which release the pro-inflammatory cytokine interleukin-17 (IL-17) [102, 103] and blocking the IL-17 signalling axis was shown to decrease neutrophil infiltration and ameliorate stroke outcome [103]. The contribution of natural killer (NK) cells and NK T cells to stroke injury remains to be elucidated [100].

This brief introduction in post-ischaemic inflammation provides several targets for stem cell-directed therapies for immunomodulation. Starting at the early onset, for example ROS scavenging can reduce the initial ischaemia-reperfusion injury, whereas stem cell-mediated therapies can also influence stroke outcome by modulating other aspects of the inflammatory cascade, as will be discussed next.

2.3.2 Mechanisms of Stem Cell-Mediated Immunomodulation

Although stem cell survival can be influenced by the host immune system, the transplanted cells themselves are believed to possess immunomodulatory properties [104, 105]. When considering immunomodulation as a stem cell-based therapy for ischaemic stroke, the post-stroke systemic immunosuppression needs to be taken into account. Additional systemic immunosuppression by cell-based therapies could worsen stroke outcome. Fortunately, no adverse effects on systemic cytokine levels were observed following syngeneic BMMSC transplantation in a mouse model of ischaemic stroke [105]. Moreover, the majority of completed clinical pilot studies with autologous MSC showed no adverse effects and improved clinical outcome, although post-stroke immunosuppression was not investigated [106, 107].

When considering mechanisms of stem cell-mediated immunomodulation, several *in vitro* reports are available. BMMSC and their extracellular vesicles (EVs) were shown to suppress T-cell proliferation [108, 109], BMMSC and adipose-derived stem cells (ASC) suppressed lymphocyte proliferation and the mixed lymphocyte reaction (MLR) [110]. Similarly, DPSC possess immunomodulatory properties [111]. Demircan et al. showed that the suppressive actions on T cells were mediated via paracrine effects by means of a transwell and MLR assay [112]. Increased levels of hepatocyte growth factor (HGF), TGF-β, ICAM-1, IL-6, IL-10, VEGF and human leukocyte antigen-G were found in DPSC/T cell co-cultures [112], the latter factor was additionally shown to suppress T cell and NK cell function and induce regulatory T cell function when secreted by BMMSC [113]. Moreover, the expression of pro-inflammatory cytokines by T-cells such as interferon-gamma (IFN-γ), IL-2, IL-12, IL-17A and tumour necrosis factor alpha (TNF-α) were decreased in the transwell system whereas the expression of the anti-inflammatory cytokine inducible protein-10 was upregulated [112]. Interestingly,

the expression of the regulatory T cell (Treg) markers CD4, CD25 and Foxp3 was increased. T cell apoptosis was increased after 24 h incubation with DPSC [112]. A similar paracrine mediated immunosuppression was exerted by the secretome of porcine and human DPSC [114–116]. In addition to T cells, BMMSC were shown to inhibit NK cell activation by producing prostaglandin E2 (PGE2) and indoleamine 2,3-dioxygenase (IDO) [117]. The proliferation, activation, maturation and antigen presentation of dendritic cells was also inhibited by MSC subtypes [118–122] and macrophage/microglia polarization was shifted towards an M2 phenotype after exposure to MSC, their secretome or EVs [120–125]. This effect was presumably mediated by PGE2 [124] and by inhibiting the nuclear factor kappa B (NF-κB) pathway and stimulating the signal transducer and activator of transcription 3 (STAT3) pathway [126]. These reports include both paracrine-mediated immunomodulation [124–126] as direct co-cultures [120, 121, 123, 124, 126]. M2 polarized macrophages increased the production of the anti-inflammatory cytokines IL-10 and IL-6 and of arginase-1 while production of TNF-α was decreased [120, 123, 126]. Interestingly, pro-inflammatory cytokines were shown to stimulate MSC to secrete PGE2 and IDO and upregulate the expression of cell adhesion molecules, thereby stimulating the immunomodulatory capacity of MSC [127–129].

In addition to MSC, iPSC were also shown to modulate the immune system. Naïve major histocompatibility complex (MHC)-matched and/or -mismatched iPSC were reported to have superior immunomodulatory properties compared to MHC-matched and/or -mismatched MSC [130]. iPSC- or ESC-derived MSC acquired similar immunomodulatory properties as adult MSC and were able to inhibit lymphocyte proliferation and function [131–133] and NK cell function [132]. Soluble factors secreted by bone marrow-derived mononuclear cells (BM-MNC) were able to prevent macrophage-microglia induced neuronal cell death and ROS induced neurotoxicity [134]. Conversely, NSC were shown to upregulate arginase-1 production when exposed to inflammatory cytokines [135]. Whereas most *in vitro* proof for the immunomodulatory properties of stem cell-based therapies comes from MSC-centred research, proof for NSC-mediated immunomodulation mostly comes from *in vivo* data [136] and is mainly thought to be an effect of the transplanted cells on peripheral immunosuppression after intravenous administration [137, 138].

Studies that specifically focused on stem cell-based immunomodulation in stroke after transplantation *in vivo* are scarce. Similar to NSC, systemically injected BMMSC home towards the spleen, where TNF-α production is diminished. Moreover, the percentage of MHC-II-activated immune cells in the brain is reduced [139]. Intracranial ASC administration decreased the number of Iba-1$^+$ cells in the brain [140] and IL-10 production was increased after stem cell transplantation in a monkey stroke model [141]. Interestingly, intravenous delivered BMMSC-derived EVs had a similar functional outcome than BMMSC transplantation and attenuated postischaemic immunosuppression in the peripheral blood without altering the number of brain-infiltrating immune cells [142].

Although several encouraging results have been achieved and underlying mechanisms of stem cell-mediated immunomodulation were elucidated *in vitro*, the systemic and local effect of stem cells or stem cell-derived therapies such as EVs on

immunomodulation *in vivo* remains elusive. In accordance with previous sections, noninvasive imaging modalities can be applied to acquire insight in underlying immune mechanisms in ischaemic stroke.

3 Noninvasive Monitoring of Stem Cells in the Stroke Microenvironment

For many years, medical imaging focused on the anatomical changes taking place following ischaemic stroke. During the last decade, however, there has been a major shift in medical imaging towards the molecular processes underlying these anatomical changes. A large number of noninvasive imaging methods have now been developed to study molecular processes such as metabolic changes, gene expression and cell migration. Visualizing these processes can be of great benefit in the diagnosis and treatment follow-up in ischaemic stroke.

These noninvasive imaging methods comprise magnetic resonance imaging (MRI), computed tomography (CT), positron emission tomography (PET), single-photon emission computed tomography (SPECT) and optical methods such as bioluminescence imaging (BLI) and fluorescence imaging (FLI).

In order to noninvasively track stem cell therapy *in vivo*, cells need to be labelled with a specific imaging probe. This can be done by means of incubation of the cells *in vitro* with contrast agents or radioactive tracer molecules prior to injection, which is referred to as direct cell labelling. Contrast dilution and leakage of these agents from the cells hampers long-term imaging, which has led to the development of indirect cell labelling methods. Hence, so-called "imaging reporter genes" are introduced into the cells and their expression enables the accumulation of imaging probes on a cellular level. This enables repeated stem cell visualization *in vivo* over time within the same subject [143].

Imaging stem cells in the field of ischaemic stroke research is mainly focused on determining the optimal injection route, cell dosing, engraftment, survival and effect on the lesion volume. ESC-derived NSC were imaged with BLI and MRI after labelling with superparamagnetic iron oxide (SPIO) particles, and migration as well as differentiation of the NSC towards neural lineages was confirmed [144]. MRI has also been used in a clinical setting of stem cell transplantation. For example, Zhu et al. have tracked autologous SPIO-labelled NSC transplanted in a patient with brain trauma. Cells migrated towards the lesion site but the signal disappeared 7 weeks after the transplantation [145].

ESC-derived NSC were genetically engineered to express the herpes simplex virus type 1 thymidine kinase (HSV1-tk) reporter gene for PET and labelled with SPIO for MRI. 3 months after stroke, PET and MRI showed a decrease infarct size and functional engraftment of the transplanted cells [146].

Intra-arterial injection of stem cells for the treatment of ischaemic stroke seems to be a favourable injection route. MRI tracking of ASC in a rat MCAO model has

shown that the neuroprotective effect might be due to the secretion of trophic factors. Intra-arterially transplanted cells actively migrated towards the lesion sites, but only a low number of cells survived 8 weeks post transplantation [147]. Human umbilical cord blood-derived stem cells were labelled for MRI and injected intra-arterially 60 min after stroke. Researchers found an improved cerebrovascular function, a reduced infarct size and improvement in behavioural deficits [148]. Furthermore, Grudzenski et al. have shown using MRI that not only the injection route, but also the number of cells injected is important in the treatment of ischaemic stroke [149].

As all imaging modalities have their specific strengths and weaknesses, modern molecular imaging often combines several modalities. Multimodal imaging of MSC transplanted in rats with ischaemic stroke has combined MRI together with SPECT and FLI, using one single tri-modal probe (^{125}I-fSiO4@SPIOs). MSC transplanted intracerebrally or intravenously both improved neurobehavioral outcomes of these stroke animals [150].

4 Conclusion

Multiple advances have been made to understand stem cell-mediated mechanisms of brain regeneration following ischaemic stroke. Stem cell-based therapies applied via various administration routes have shown great promise in *in vitro* and *in vivo* models of ischaemic stroke focussing on a plethora of regenerative mechanisms including neuroregeneration, ECM and vascular remodelling and/or angiogenesis, stimulating endogenous repair and immunomodulation. However, these studies were not able to pierce the veil and pinpoint precise mechanisms of action of the transplanted cells. Nonetheless, harmonized stroke- and stem cell research will continue to contribute to the discovery of new targets and modulable pathways potential therapeutic approaches could be directed at. Moreover, noninvasive imaging methods allow changes in host microenvironment caused by the transplanted cells or cell-derived therapies to be connected with functional improvement.

Acknowledgements Pascal Gervois, Esther Wolfs, Jessica Ratajczak, Tim Vangansewinkel, Petra Hilkens, Yörg Dillen and Annelies Bronckaers are funded by Fonds Wetenschappelijk Onderzoek Vlaanderen by grants 12U7718N, G0A7514N, G089213N, G029112N, 12D8516N, 1134717N and 1508015N respectively. Greet Merckx and Melissa Lo Monaco are funded by Bijzonder Onderzoeksfonds grants BOF16DOC06 and BOF16DOCNA02 respectively.

Conflict of Interest: The authors declare that they have no conflict of interest.

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Chapter 3
Stem Cell Transplants in the Aged Stroke Brain: Microenvironment Factors

Aurel Popa-Wagner and Mario Di Napoli

Abstract In aged humans, stroke is a major cause of disability for which no neuroprotective measures are available. The incidence of stroke increases significantly with age both in men and women with incidence rates accelerating above 70 years. Since stroke afflicts mostly the elderly comorbid patients it is highly desirable to test the efficacy of cell therapies in an appropriate animal stroke model. It has been noted that the potential for neurogenesis is also preserved in aged, stroke-injured brains and the environment of the aged brain is not hostile to cell therapies. However, there remain significant developmental and translational issues that remain to be resolved in future studies such as (1) Understanding the differentiation into specific phenotypes. Upon transplantation, the differentiated cells often de-differentiate; (2) Tumorigenesis remains a significant concern; (3) Anti-neuroinflammatory therapies is a potential target to promote regeneration and repair after brain injury and neurodegenerative conditions by stem cell therapy; (4) Efficacy of cell therapy can be enhanced by physical rehabilitation; (5) One potential weakness of the preclinical dataset is, however, the lack of proof in aged subjects. It is in fact a general drawback of preclinical evaluations of candidate stroke drugs that due to cost effectiveness and practicability most studies were done in young animals. A lack of data from aged subjects in preclinical studies may at least in part explain the failure of candidate neuroprotective drugs in clinical trials. The aged brain has compared to the young brain, an enhanced susceptibility to stroke and displays a limited recovery from an ischemic injury. Finally, a better understanding of potential risks of stem

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P.A. Lapchak, J.H. Zhang (eds.), *Cellular and Molecular Approaches to Regeneration and Repair*, Springer Series in Translational Stroke Research,
https://doi.org/10.1007/978-3-319-66679-2_3

cell therapies in stroke shall make the translation of cell therapies safer. Likewise, awareness of may help improve their efficacy to achieve therapeutic success.

Keywords Aging • Stroke • Therapies • Stem cells • G-CSF • BM-MSC • BM-MNC

Abbreviations

BBB	Blood–brain barrier
BMECs	Brain microvascular endothelial cells
BM-MNC	Bone marrow-derived mononuclear cells
BM-MSC	Bone marrow mesenchymal cells
ECA	External carotid artery
EPC	Endothelial progenitor cells
ESCs	Embryonic stem cells
G-CSF	(Granulocyte-Colony Stimulating Factor) Hematopoietic factor
hBMMSCs	Mesenchymal cells of human origin
HSPC	Hematopoietic stem/progenitor cells
iPSC	Human-derived inducible pluripotent cells
MCA	Middle cerebral artery
MCAO	Middle cerebral artery occlusion
MRI	Magnetic Resonance Imaging
MSCs	Mesenchymal stem cells
NSCs	Neural stem cells
SVZ	Subventricular zone
UCB	Umbilical-cord blood
VEGF	Vascular endothelial growth factor
SHR	Spontaneously hypertensive rat model

1 Introduction

Stroke is globally the second cause of death by 2015, just 1.5% less prevalent than ischemic heart disease (13.2%). From 1990 to 2010, the age-standardised incidence of stroke significantly decreased by 12% in high-income countries, while in low- and middle-income countries, it increased, although non-significantly, by about 12%, while mortality decreased significantly in both (mean 37% vs. 20%, respectively), of which 31% (with about 80% of it in low- and middle-income countries) were in children and young adults (below 65 years) [1].

Stroke patients are at the highest risk of death in the first week after the event, and between 20% and 50% die within the first month depending on type, severity, age, co-morbidity and effectiveness of treatment of complications. Stroke burden as

measured by the disability-adjusted life-years has risen from the fifth place in 1990 to the third in [2]. Considerable spontaneous recovery occurs up to 6 months. However, patients who survive may be left with mild, moderate or severe disability [1]. Moreover, patients with a history of stroke are at risk of a subsequent event of around 10% in the first year and 5% per year thereafter [2].

The negative consequences of stroke extend well beyond the victims themselves, ultimately including families, caregivers, social networks and employers. The consequences of stroke are often devastating and a significant proportion of survivors may suffer from disabilities requiring a temporary or lifelong assistance. The proportion of patients achieving independence in self-care by 1 year after a stroke ranges from around 60% to 83%. This wide variation relates to whether the studies are community based or hospital based, which activities are considered in estimating independence, and the methods used to rate ability. In established marked economies, depending on the organization of hospital services, between 10% and 15% of survivors are resident in an institution at 1 year [3].

Primary prevention does not diminish the risk of cerebrovascular events, but only postpones the onset of the first stroke in later ages [4]. In demographically developed countries, the average age at which stroke occurs is around 73 years reflecting the older age structure of these countries. This could explain why after a first decrease of stroke incidence in developed countries, there is now a stabilization and a new increase due to ageing of the population (for an increase of at risk people). The increase in life expectancy after stroke mainly due to stroke medicine to reduce fatal outcomes and its progression through increasingly disabled states will produce an expansion of stroke morbidity worldwide. Stroke survivors will survive for longer, and therefore, the period of time that they spend in a state of chronic ill-health and disability at the end of life will increase. In less developed regions, the lower average age of stroke onset will increase further this period of ill-health and disability in the population if age-specific prevalence rates remain constant as the population ages.

2 Age Is the Principal Risk Factor for Stroke

The incidence of stroke increases significantly with age both in men and women with incidence rates accelerating exponentially above 70 years [5]. However, there are gender differences in the incidence by age subgroups. Men aged up to 75 years old are more likely to be hit by stroke than women. The risk to have a stroke then becomes higher in women than men aged 85 years or older [5]. This may be attributed to sex-related differences in life expectancy of women and the development of age-related atherosclerosis. It should be noted that the age-associated decline in functional reserve is most pronounced after the age of 85, and implies an impaired response to stressors and illnesses. Importantly, age-associated changes show great variability among individuals, which may be modulated by genetic and long-term lifestyle factors [5–7].

3 Stroke Commorbidities

Since stroke afflicts mostly the elderly comorbid patients, it is highly desirable to test the efficacy of stroke therapies in an appropriate animal stroke model. Animal models of stroke often ignore age and comorbidities frequently associated with senescence, and this could be one of the explanations for unsuccessful bench-to-bedside translation of neuroprotective strategies.

Worldwide stroke is increasing in parallel with modernization, changes in lifestyle, and the growing elderly population. In particular, rates in Eastern Europe have been increasing, such that currently the highest rates are found in countries such as Bulgaria, Romania, and Hungary. Women and men individuals with a low-risk lifestyle (smoking, exercising daily, consuming a prudent diet including moderate alcohol) and having a healthy weight during mid-life had a significantly lower risk of stroke than individuals without a low-risk lifestyle. Therefore, the relatively high incidence of stroke may be due, in part, to the impact of numerous known risk factors: arterial hypertension, diabetes, high cholesterol, smoking, alcoholism, obesity, stress, and a sedentary lifestyle [8].

Comorbidities such as diabetes, arterial hypertension or hypercholesterolemia, are common in elderly persons and are associated with a higher risk of stroke, increased mortality and disability [9]. Moreover, simultaneous presence of vascular diabetic complications-associated comorbidities like hypertension and chronic diabetes, significantly increase the level of ischemic damage in humans and animal models [10, 11].

High blood pressure is a major risk factor for stroke. Large clinical trials have shown that ACE inhibitors reduce the incidence of stroke by up to 43% [12]. However, because normotensive patients also benefit from ACE inhibition it has been suggested that these effects may also be independent of the blood pressure-lowering effects of ACE inhibition [13]. Indeed, neither short (7 days) nor long-term (42 days) administration of ACE inhibitors to SHR prior to stroke, reduced the infarct size despite lowering the blood pressure while WKY normotensive rats showed, paradoxically, marked reductions in infarct volume [14].

By Magnetic Resonance Imaging MRI, hyperglycemia was also shown to accelerate infarct progression in cortical areas [15]. However, the mechanisms of hyperglycemia-associated infarct progression remain unclear. It could be that hyperglycemia aggravates brain infarction by hemorrhagic transformation that leads to blood–brain barrier (BBB) disruption and neuronal cell death [16, 17].

Our knowledge about the molecular and cellular mechanisms underlying accelerated infarct progression in subjects with metabolic syndrome is still poor. Some studies report a strong connection between nutrition and body weight, on one hand, and increased oxidative stress or pro-inflammatory changes in the brain, which promote neural imbalance and glucose level elevation, on the other hand [18]. Zhang and colleagues suggested that metabolic inflammatory changes in the brain are linked to the inflammatory IKK/NF-kB signaling pathway [19]. Moreover, the patient's weight may show a complex relation with stroke outcomes, leading to

more deaths among individuals with dramatically lower BMIs (obesity paradox) [22]. However, this may reflect undernourishment by consciously avoiding essential nutrients in daily meals [23].

Observational studies have shown a strong correlation between increased blood lipid levels and stroke [20]. In animal models, it could be shown that vascular endothelial growth factor (VEGF)-induced angiogenesis is compromised by hyperlipidemia and provided an explanation of the poor efficacy of pro-angiogenic therapies is animal models of hyperlipidemia [21]. Over-nutrition and hypercholesterolemia may not only be responsible for metabolic inflammation of the brain but can also induce mitochondrial dysfunction and increased oxidative stress [18, 22]. In this light, a better understanding of molecular factors and signaling pathways underlying the metabolic syndrome as well as the contribution of comorbidities to stroke-induced sequelae, may be translated into more successful treatments or prevention therapies against age-associated diseases which, in turn, would extend lifespan and improve lifespan quality.

The effect of age and gender on stroke incidence, functional recovery and mortality has not only been shown in humans but also in animal models [23, 24]. Indeed, the age-dependent increase in the infarct volume strongly suggests that age accounts for the variability in tissue outcome in acute human stroke [25].

4 Stroke Models Using Aged Animals Are Clinically More Relevant

Over the past 10 years, a variety of models of middle cerebral artery occlusion (MCAO) have been established in rodents [26]. MCAO in aged rodents has been produced with permanent or transient occlusion for 30–120 min using (1) MCA ligation after cranioectomy [27]; intraluminal thread occlusion [28]; using a hook attached to a micromanipulator [29]; cauterization [30, 31]; photothrombosis [32]; endothelin injection [33, 34], injection of a thrombus via external carotid artery (ECA) [35], or intraluminal thrombus formation by thrombin injection using occlusion of distal branches of the middle cerebral artery (MCA).

Since focal cerebral ischemia is technically difficult to perform in very old rats and since based on epidemiological studies human stroke occurs more often in late middle aged (60–70 years old) subjects [36], it is advisable to use middle aged instead of very old animals for stroke research [37].

4.1 Spontaneous Stroke Recovery in Aged Patients and Animals

Stroke patients regain some of their lost neurological functions during the first weeks or months after the stroke. In clinical practice recovery is thought to occur via recruitment of neighbouring neuronal circuitries and physical therapy is widely

Table 3.1 Recent therapies for acute ischemic stroke in humans

Therapy	Effects on recovery	Effect on survival	Reference
Endovascular thrombectomy	1. Lower degrees of disability at 3 months	1. Increases survival rate	[44]
Recombinant tissue plasminogen activator	1. Improves learning and memory 2. Promotes neurite outgrowth, synaptic plasticity	1. Increases survival rate	[45]
CTX0E03 (human neural stem-cells)	1. Improved neurological function	1. Improves quality of life 2. Prevents reoccurrence of stroke	[46]
Cortexin	1. Normalization of the quality of life in the early rehabilitation petriod		[47]

used to exploit this phenomenon for stimulating post stroke brain recovery [38–41].

In animal models of stroke, complete spontaneous recovery may occur in young rats, depending on the size and location of the ischemic lesion. Under normal conditions, young rats begin to show improvements of neurological function starting by day 2 post stroke, whereas in aged rats, neurological recovery is hardly detectable before days 4–5, with about 75% of the functional improvement observed in young rats. However, stroke recovery is delayed and often incomplete in aged rats [42]. Therefore, unlike stroke therapies in humans (Table 3.1) most of recent therapies involving young animals are reportedly successful (Table 3.2).

Housing experimental animals in an enriched environment enhances the recovery from brain damage both in young and aged animals [42]. When aged rats were allowed to recover in an enriched environment, the delay period was shortened and behavioural performance was significantly improved. The improvement in task performance positively correlated with slower infarct development, fewer proliferating astrocytes and smaller size of the glial scar [42]. It has been hypothesized that older brains may be more vulnerable to stroke because of decreased rates of compensatory oligodendrogenesis due to an age-related decline in cyclic AMP response element-binding protein (CREB)-mediated oligodendrogenesis after brain injury [43]. Even more effective rehabilitation of the contralateral forelimb could be achieved by Corbett and colleagues by combining enriched environment with training [44].

Spontaneous recovery is common if the infarct is located in striatum, a subcortical structure that exhibits activity-dependent plasticity and is important for controlling movement and motor learning. The enhanced recovery was associated with structural and synaptic plasticity in the contralesional striatum [45]. This may explain why patients with subcortical lacunar stroke are more likely to have early functional recovery after stroke [46, 47]. Other studies suggest that the beneficial

Table 3.2 Recent therapies for ischemic stroke in animal models

Therapy	Molecular targets	Effects on recovery	Effect on stroke area	Reference
Thiamet G	1. Inhibits β-N-acetylglucosaminidase (OGA) 2. Modulates the expression of pro-inflammatory and anti-inflammatory cytokines 3. Decreases the expression of iNOS and COX2 mainly by suppressing NF-κB p65 signaling	Improved outcome in neurobehavioral tests	Reduces the infarct volume	[48]
Ruscogenin	1. Down-regulation of intercellular adhesion molecule-1 (ICAM-1) 2. Involved in nuclear factor-κB (NF-κB) activation in anti-inflammatory pathways 3. Suppresses inducible nitric oxide synthase (iNOS), cyclooxygenase (COX-2), tumor necrosis factor-α (TNF-α) and interleukin-1β (IL-1β)	Improved neurological deficits	Decreased the infarct volume Reduced brainwater content	[49]
Curcumin	1. Inhibits the activation of TLR2/4-NF-κB signaling pathway 2. Attenuates the release of TNF-α and IL-1β in blood	Reduced neurological deficit scores	Decreased the infarct volume Reduced brainwater content	[50]
Xanthotoxol	1. Inhibits the neutrophil infiltration, 2. Decreases the expression of ICAM-1 and E-selectin 3. Attenuates Brain Blood Barrier disruption 4. Reduces the IL-1β, TNF-α, IL-8 and NO level, and attenuates the iNOS activity	Reduced neurological deficit scores	Reduced brain edema	[51]
Leonurine	1. Attenuates mitochondrial membrane swelling, 2. Restores the mitochondrial membrane potential and content of cytochrome c (Cyt-C) in mitochondria from ischemic cortex 3. Decreases the expression of Bax and increases the expression of Bcl-2 4. Decreases reactive oxygen species (ROS) level	Improved neurological outcome	Decreased the infarct volume	[52]

(continued)

Table 3.2 (continued)

Therapy	Molecular targets	Effects on recovery	Effect on stroke area	Reference
Liraglutide	1. Prevents apoptosis by increasing the anti-apoptotic protein Bcl-2 expression and decreases pro-apoptotic protein Bax expression 2. Decreases oxidative stress.	Attenuated neurological deficit	Decreased the infarct volume	[53]
Scutellarin	1. Has an inhibitory effect on the ACE/Ang II/AT1 axis 2. Proinflammation inhibition	Attenuated neurological deficit	Decreased the infarct volume	[54]
Endothelial cells-derived microvesicles	1. Improves local cerebral blood flow 2. Modulates astrocyte functions, Brain Blood Barier integrity	Attenuated neurological deficit	Reduced infarct volume	[55]

effect could be due to in situ secretion of neuroprotective factors by the transplanted cells. For example, human-derived inducible pluripotent cells (iPSC) implanted into striatum of young animals at 1 week after MCAO protected substantia nigra from atrophy, probably through a trophic effect [48].

5 Stroke Therapy in Aged Subjects Using G-CSF

The hematopoietic factor G-CSF (Granulocyte-Colony Stimulating Factor) effectively reduces infarct size and improves functional outcome after various types of experimental stroke [49–52]. G-CSF exerts a wide range of potential effects and can reduce the number of fatal hemorrhages after experimental thrombolysis in young animals models of stroke [55]. Under ischemic conditions, G-CSF inhibits programmed neuronal cell death [53] and stimulates neural progenitor cell differentiation. These mechanisms and others, including immunomodulation, anti-apoptotic properties, blood vessel plasticity, and by reducing excitotoxicity-driven penumbral apoptosis are currently thought to be responsible for infarct size reduction and improved functional outcome in young-adult rodent stroke models treated with G-CSF [54, 56–58]. Indeed, G-CSF was once viewed as one of the best preclinically studied candidate stroke drugs of the recent years that was translated into clinical development [59].

One potential weakness of the preclinical dataset is, however, the lack of proof in aged subjects. A lack of data from aged subjects in preclinical studies may at least in part explain the failure of candidate neuroprotective drugs in clinical trials. The aged brain has compared to the young brain an enhanced susceptibility to stroke and displays a limited recovery from an ischemic injury [29, 31, 42, 60].

G-CSF treatment was recently shown to increase substantially the number of neural progenitor cells and immature neurons in subcortical regions adjacent to the infarcted area. G-CSF also increased neurogenesis in the dentate gyrus of the hippocampus. This cell-regenerative effect in young adult animals could be reconfirmed to some extent in the aged rats that have been treated with G-CSF for 14 days after stroke [61]. Although G-CSF treatment in aged rats increased the number of proliferating cells in the dentate gyrus and in the subventricular zone (SVZ), in aged rats there were more newborn neurons only in the SVZ of the damaged hemisphere. Likewise, G-CSF treatment in aged rats after stroke enhances survival, functional neurological recovery [61, 72].

6 Cell Therapy of Stroke Using a Combination Therapy

Cellular therapy can enhance the endogenous restorative mechanisms of the injured brain by supporting processes of neovascularization, neurogenesis, neural reorganization and functional recovery (Fig. 3.1).

Despite some positive impact of G-CSF on the post-stroke aged brain, a monotherapy may have, nevertheless, limited effects on tissue and functional recovery after ischemic stroke. Therefore, treating post-stroke aged rats with a combination of bone marrow-derived mononuclear cells (BM-MNC) and G-CSF might improve the long term (56 days) functional outcome by compensating the delay before G-CSF comes to full effect. To this end, 1×10^6 syngeneic BM-MNC per kg bodyweight in combination with G-CSF (50 µg/kg, intraperitoneal application, continued for 28 days) were administered via the jugular vein to aged Sprague-Dawley rats at 6 h post-stroke. Infarct volume was measured by magnetic resonance imaging at 3 and 48 days post-stroke and additionally by immunohistochemistry at day 56 (Fig. 3.2). Functional recovery was tested during the entire post-stroke survival period. Daily G-CSF treatment led to robust and consistent improvement of neurological function, but did not alter final infarct volumes. This result was unexpected since benefits of G-CSF and BM-MNC treatment paradigms in stroke, independently

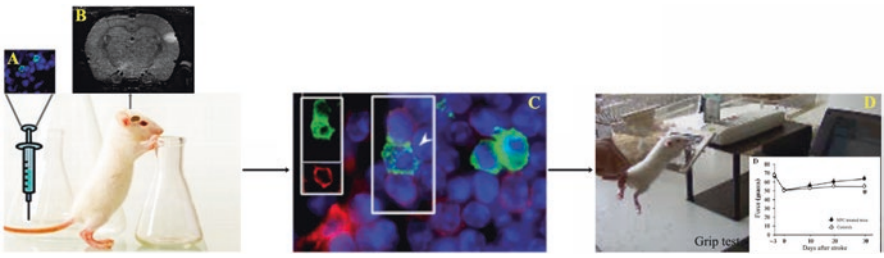

Fig. 3.1 Cellular therapy can enhance the endogenous restorative mechanisms of the injured brain by supporting processes of neovascularization, neurogenesis, neural reorganization and functional recovery

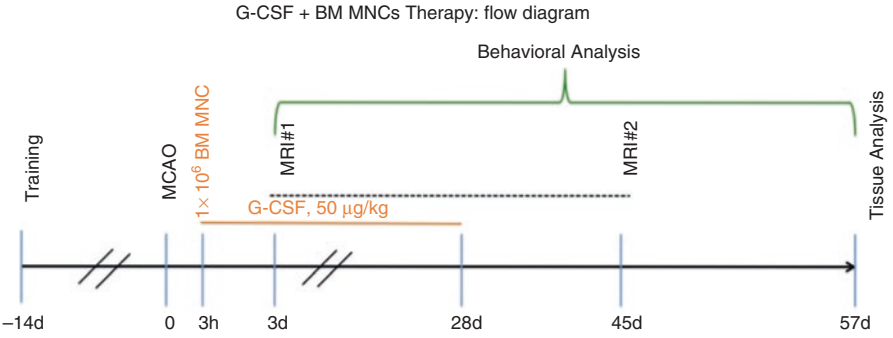

Fig. 3.2 Bone marrow derived mononuclear cells (BM-MNC) and G-CSF might improve the long term (56 days) functional outcome by compensating the delay before G-CSF comes to full effect. To this end, 1×10^6 syngeneic BM-MNC per kg bodyweight in combination with G-CSF (50 µg/kg, intraperitoneal application, continued for 28 days) were administered via the jugular vein to aged Sprague-Dawley rats at 6 h post-stroke. Infarct volume was measured by magnetic resonance imaging at 3 and 48 days post-stroke and additionally by immunohistochemistry at day 56

from each other, have been repeatedly reported by independent experiments and groups were hypothesized to work synergistically especially in the aged, stroke-lesioned brain. The lack of an additional benefit may be due to an hitherto not well investigated interaction between both approaches and, to a minor extent, to the insensitivity of the aged brains to regenerative mechanisms. Also considering recent findings on other tandem approaches involving G-CSF in animal models featuring relevant co-morbidities, we conclude that such combination therapies are not the optimal approach to treat the acutely injured aged brain.

Current knowledge suggests that administered BM-MNC provide indirect neuroprotection leading to infarct size reduction after ischemic damage in a time window of up to 1 month [62]. G-CSF in turn, induces BM MNC mobilization while the SDF-1/CXCR4 mechanisms, causing BM-MNC to invade the ischemic brain [51, 63], where they are believed to exert therapeutic effects. However, the initiation of this potentially beneficial action may take simply too much time: although a granulocyte boost is seen after about 48 h, peaking G-CSF-based mobilization can take up to 9 days [64], which is beyond the therapeutic time window for BM-MNC. Since endogenous G-CSF is not available in sufficient concentrations directly at the lesion site after the ischemic event [57], a combination therapy providing (1) G-CSF in sufficient amounts to act neuroprotectively and (2) exogenous BM-MNC early enough to bridge the time gap until G-CSF-based endogenous BM-MNC mobilization comes to full effect, seemed promising—but failed to fulfill the expectations. One may assume that either the lesioned and aged rat brain environment was insensitive to regenerative mechanisms by BM-MNC or cell treatment has been mainly ineffective. Indeed, the aggravated impact of ischemic damage on the aged brain is well known while potential detrimental effects of ageing on BM-MNC have been anticipated [37, 65, 66]. Moreover, technical complications may come into play as well: a limited influence of long-term cryopreservation on the therapeutic efficacy

of umbilical cord blood MNC, a population being very similar to BM-MNC, has been discussed recently [67]. However, deriving syngeneic cells from young animals and limiting cryopreservation to no more than 4 weeks might have limited effects on the donor side due ageing and cryopreservation process itself. An alternative explanation for the reduced efficacy of the combination treatment could be interference between both treatment regimes.

A recent study in hypertensive animals demonstrated that intravenously administered BM-MNC challenges the splenic granulocyte clearance capacities for apoptotic cells [81]. This clearance system usually removes apoptotic granulocytes from the circulation, which represents an important anti-inflammatory mechanism [82]. Being already compromised by externally administered BM-MNC, the newly generated granulocyte boost from the BM by G-CSF may have completely exhausted the clearance system after treatment. This detrimental interaction may have caused a sustained systemic and central pro-inflammatory bias, leading to subtle additional damage, not enhancing, but partly reducing the neuroprotective G-CSF effect.

7 Co-administration of G-CSF and BM-MSC in the Microenvironment of the Post-stroke Aged Rats

Due to the ethical concerns and limited availability of using pluripotent embryonic stem cells (ESCs) and iPS in the clinic, the emphasis was placed on mesenchymal stem cells (MSCs), which are free of both ethical concerns and teratoma formation.

Recent studies suggest that modified (MSCs) are able to form a "biobridge" between neurogenic subventricular zone (SVZ) and the ischemic cortex area (penumbra). Using this road, endogenous stem cells can migrate from the neurogenic area to the site of lesion and may ameliorate outcome in experimental models of cerebral ischemia [39, 68–70]. Several studies showed that grafting bone marrow derived stem cells in the peripheral circulation improved functional neurological outcome and reduced the infarct volume. Most of these studies used bone marrow mesenchymal cells (BM-MSC) [38, 71].

Mesenchymal stem cells and hematopoietic stem/progenitor cells (HSPC) that are most frequently used in preclinical and clinical neurorestorative studies after stroke, augments this endogenous response. MSC can also be obtained from adipose tissue [72]. HSPC can be isolated from bone marrow or from umbilical-cord blood (UCB), or can be mobilized into the blood by the administration of G-CSF.

Amniotic fluid has been investigated as a new cell source for mesenchymal stem cells in the development of cell-based transplantation. Earlier studies have demonstrated the ability of amniotic fluid-derived stem cells to differentiate along a neurogenic pathway [73].

The combination of mesenchymal stem cells and neural stem cells (NSCs) could improve also functional recovery after stroke if given prior stroke. To this end, a mix of MSCs isolated from the femurs and tibias of rats and NSCs isolated from rat

embryo ganglion eminence were labeled with PKH26-GL and administered 1 day before stroke into the lateral ventricle and neurological recovery evaluated for 28 days after stroke. The results suggest that this combination cell therapy is more efficient in promoting brain recovery after stroke than each stem cell alone [74].

More recently, triple cells co-transplantation with a mixture of rat NSCs, astrocytes and brain microvascular endothelial cells (BMECs) have been attempted. After grafting these cells into the ischemic brain it was found that the learning and memory ability of these rat improved to some extend. Moreover, rats with triple cells transplantation did perform better than those who grafted with two cells only. Rats grafted only one cell showed least improvement. From this experiment, it was concluded that co-transplantation of NSCs with astrocyte and BMECs can improve learning and memory in the water maze test, probably due to the microenvironment improvement by the transplanted astrocytes and BMECs [75].

In another approach, the combination therapy was given in sequence. First, mesenchymal stem cells were transplanted during the acute phase after stroke (1 day) in an attempt to diminish the inflammation and provide an appropriate microenvironment for regeneration after ischemia. Then, the neural stem cells were transplanted at 7 days after stroke to help regeneration by differentiation into neurons, oligodendrocytes or astrocytes [74].

Finally, experiments aimed at improving long term functional outcome in aged rodents by grafting pre-differentiated BM-MSC in G-CSF-treated animals, have been performed. To this end, rat BM-MSC isolated from young Sprague-Dawley rats were administered a single dose of BM-MSCs (10^6/kg) given in combination with G-CSF (50 μg/kg) via the jugular vein or intrathecally at 6 h post-stroke. The phenotypes of BMSCs used in this study were positive for CD105, CD166, CD29, and CD44 [92]. Cells tested negative for CD14, CD34 and CD45. Prior to transplantation the cells were tested for purity by flow cytometry and for their ability to differentiate into osteogenic, chondrogenic and adipogenic lineages. The control groups received daily injections of either G-CSF 50 μg/kg or vehicle (5% glucose) for 28 days. To investigate the localization of injected cells, a separate group of aged animals were injected with mesenchymal cells of human origin (hBMMSCs). Although hBMMSCs are poorly immunogenic [76] the animals were given cyclosporine A (s.c., Sandimmun, Novartis, 10 mg/kg) diluted in Chremophor EL, Sigma, to prevent graft rejection.

Infarct volume was measured by MRI at 3- and 48 days post-stroke and additionally by immunhistochemistry at day 56. Functional recovery was tested during the entire post-stroke survival period of 56 days. Daily treatment of post-stroke aged rats with G-CSF led to a robust and consistent improvement of neurological function. The combination therapy, GG-CSF + BM MSC in aged rats showed, surprinsingly, no additional improvement in recuperation of the sensory function (adhesive tape), although recuperation of more complex motor (rotating pole) and spatial reference-memory tasks was improved both by G-CSF and the combination. Paradoxically, MCAO rats swam slightly faster than unoperated animals, probably due to a post-stroke excitatory state, an observation confirmed in previous studies [77].

Of the treated groups, the best recovery rate was seen for the G-CSF group which showed significant improvement in the watermaze spatial reference-memory task between days 21 and 42, suggesting that the beneficial effect of the G-CSF treatment is restricted to the G-CSF treatment period [61]. Probably, the improved functional recuperation of the G-CSF group may have been helped by the stimulation of endogenous neurogenesis by G-CSF as previously reported [61]. In the combined treatment study it was found an increased cellularity in the formerly infarct core of the G-CSF + BM MSC group at day 56 post-stroke and intact neurogenesis in the lateral ventricle region. However, there was a clear regional separation of the DCX^+ cells which emanated from the ventricular wall, and BrdU-labeled nuclei which were localized mainly in the vascular network of the lateral ventricle. Since BrdU was administered for the first 14 days after stroke, it seems likely that at 2 months post-stroke, DCX^+ cells with BrdU nuclei did not survive. Instead, $BrdU^+$ nuclei survived most likely in endothelial cells of the vascular wall [78]. Further, the combination therapy significantly improved recuperation and microvessel density in the formerly infarct core and beyond. Finally, it was found that the aged brain environment is permissive for the migration of human BMMSCs toward the lesion site. Finally, it was suggested that, in a real clinical situation involving older post-stroke patients, successful regenerative therapies may have to be delivered throughout a prolonged period, perhaps for 6–12 months.

8 Functional Neurological Recovery and Tissue Repair After Neural Tissue Transplantation

Although rehabilitation is important for improving functional recovery in the early stages after stroke, it does not provide a replacement of lost tissue. Moreover, if and how the aged brain responds to grafted neural tissue is largely unknown. For example mouse fetal hippocampal NSCs implanted into the injured hippocampus of 24-months-old rats, exhibited limited neuronal plasticity, robust astrocytic differentiation, and impaired migration [96].

Most clinical studies conducted so far used neural cells derived from human fetal donors. The techniques to achieve effective survival and growth of neuronal tissues transplanted into the CNS are meanwhile will established [79]. Even though effective, neural grafting has, however, not become a standard treatment for several reasons including the limited supply of fetal tissue of human origin and the beneficial effects have also been controversial [80]. Of the various options, stem cell therapy presents us with a viable alternative [81]. In order to enable the replacement of lost tissue, cell replacement strategies were used in human stroke patients. However, these early clinical studies lacked appropriate control groups [82, 83].

9 Stem Cell Therapy in Subcortical Stroke. Role of Endogenous Neurogenesis and Aging

The ultimate goal of stroke treatment is restoration of neurological function. Stroke is a heavily undertreated disease demanding a vigorous search for new therapies. Despite of improving knowledge about stroke pathology, therapeutical benefits for stroke patients are limited. Distinguished by a necrotic core surrounded by the ischemic area (penumbra), stroke is still the largest cause of disability in stroke survivors. Crucial for recovery phase are the first days and weeks after stroke. Studies before showed that even years after stroke is possible "remodeling" of the brain by neuroplasticity. However, despite the recent progression in stroke research, the major problems to be solved for stroke survivors remains the restorative process.

Spontaneous recovery is common, whenever the infarct is located in the striatum, a subcortical structure that exhibits activity-dependent plasticity and is important for controlling movement and motor learning. Neurological recovery is associated with structural dendritic and synaptic plasticity in the contralesional striatum [45] and axonal plasticity in contralesional motor cortex [84], which may explain why patients with subcortical stroke are likely to exhibit functional neurological recovery [46, 47].

Cell-based therapy augments this endogenous response. Thus, human iPSCs implanted into striatum of young-adult animals at 1 week after MCAO protected substantia nigra from atrophy, probably through a trophic effect via release of survival-promoting growth factors [48]. However, how cells are transplanted and where they are placed after stroke are important issues in graft survival and efficacy in promoting behavioural recovery. Data from many groups have shown that stroke increases proliferation of neuronal progenitors in the ipsilateral subventricular region of young-adult rodents with a maximum at 1–2 weeks, and the newly generated neuroblasts migrate to the damaged area in the peri-infarcted striatum over a period of several months. Eventually the neuroblasts differentiate into medium size spiny neurons and may become part of the neuronal network [85–89]. It seems that the injected cells itself can also stimulate neurogenesis in the SVZ [32, 88].

It has been shown that intracerebral transplantation of NSI-566RSC, a spinal cord-derived NSC line, at two sites within the striatum reduced behavioral deficits associated with ischemic stroke. Significant improvements in both motor and neurological tests were detected in the NSI-566RSC-treated stroke animals. In addition, the results revealed significant dose-dependent differences in the behavioral improvement across treatment groups at post-transplantation periods with the highest NSI-566RSC dose showing the most significant improvement in both motor and neurological tests. These results have demonstrated the safety and efficacy of NSI-566RSC in a subacute model of ischemic stroke in rats [70].

However, the proportion of surviving neurons is discouragingly low [85, 90, 91]. In animal models, the number of new striatal neurons in aged rodents after stroke was similar to that in young animals [92, 93] despite 50% decline in neurogenesis in the subventricular zone of elderly rodents compared to young adult animals [94, 95]. Similar findings have been reported in humans [87, 96–98]. Earlier studies on postmortem human brains provided evidence that there might be SVZ cell proliferation and

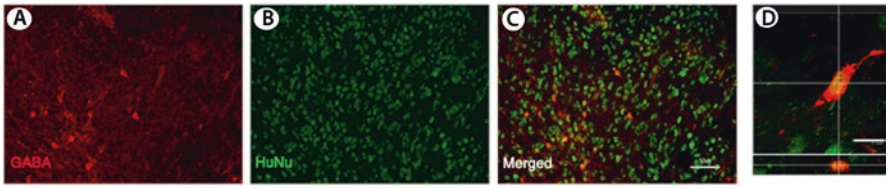

Fig. 3.3 Human iPSC survived and differentiated into neurons after intracortical transplantation in aged rats with cortical stroke and also improved functional recovery in cylinder test at 4 and 7 weeks

neuroblast formation after stroke even in aged patients [87, 97, 99]. The finding that new neurons are continuously added in the adult human striatum along with the presence of an increased number of putative neuroblasts in the human striatum after stroke lends support to this hypothesis [97]. However, whether endogenous neurogenesis contributes to spontaneous recovery after stroke has not yet been established. In addition, age, co-morbidities, physical condition of the patient and severity of disease could substantially influence these steps and, therefore, the outcome of the healing process.

The establishment of iPSCs offers new prospects for stroke treatment. iPSCs can be generated from a patient, avoiding both ethical problems and immune rejection and a limited differentiation potential of adult stem cells [100]. However, if and how the aged brain responds to grafted cells is largely unknown. Experiments done so far yielded conflicting results. For example, mouse fetal hippocampal NSCs implanted into the injured hippocampus of 24-months-old rats, exhibited limited neuronal plasticity, robust astrocytic differentiation, and impaired migration. Still another study using NSCs transplanted into young-adult (3-month-old) and aged (24-month-old) rat brains at 1 day after stroke reportedly reduced ischemic brain injury in aged rats [101]. In stroke models, hiPSC-lt-NES cells derived from a young adult male have the potential to survive, differentiate into immature and mature neurons, and migrate to the peri-infarct area of aged rats. The treated aged rats showed improved behavioral recovery after implantation into the stroke-injured striatum and cortex of adult rats [102, 103]. In a recent study, it could be shown that human iPSC survived and differentiated into neurons after intracortical transplantation in aged rats with cortical stroke and also improved functional recovery in cylinder test at 4 and 7 weeks (Fig. 3.3) [104].

Recent studies indicate that inducible pluripotent cells (iPSCs) can also be generated from aged humans and differentiate into specific cell types [104–106]. Moreover it seems that the re-differentiation efficiency of human fibroblasts via iPSCs into functional motor neurons is the same as in 29–82 year old individuals [107].

10 Mesenchymal Cells Can Be Used as Drug Carriers for Stroke Therapy

Cyclin-dependent kinase-5 (Cdk5) is over-expressed in both neurons and microvessels in hypoxic regions of stroke tissue and has a significant pathological role following hyper-phosphorylation leading to calpain-induced cell death. Recently, the

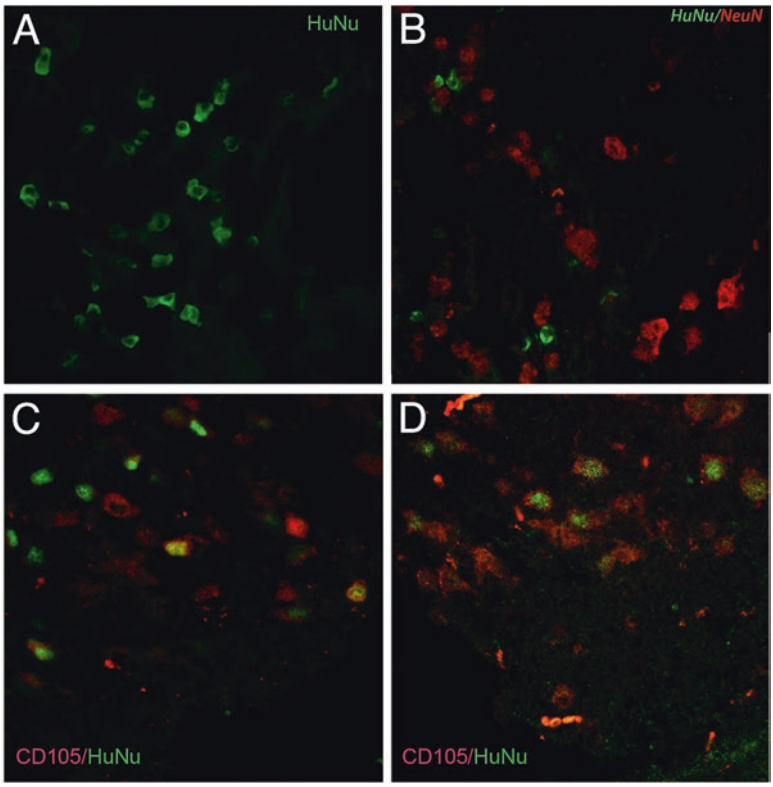

Fig. 3.4 Intracortical administration of mesenchymal harboring the CIP-peptide to middle aged rats with stroke increased survival of transplanted cells in the perinfarct area as shown by double immunofluorescence

neuronal cyto-protective potential of a natural small peptide (CIP-peptide) was demonstrated after neurotoxic stress. CIP is a derived-p35 cleavage peptide, which selectively targets Cdk5/p25 activity without affecting Cdk5/p35 signalling. In hypoxia, insertion of Cdk5/p25-inhibitory peptide (CIP) vector preserved and enhanced in vitro angiogenesis [108]. Indeed, intracortical administration of mesenchymal harboring the CIP-peptide to middle aged rats with stroke increased survival of transplanted cells in the perinfarct area as shown by double immunofluorescence (Fig. 3.4).

11 Angiogenesis After Stroke in the Aged Brains

Recuperative therapeutic strategies for stroke are focused on revascularization, neuroprotection and neuroregeneration, but most of the strategies that have been clinically tested failed to show benefit in humans. Post-stroke vascular remodeling is an essential event with crucial importance for neuroregeneration, but unfortunately this

process is still incompletely understood and therefore not exploited for therapeutic purposes [109–112].

Impaired neovascularization was described in elderly, but the effect of aging on angiogenesis and vascular remodeling after stroke has not been studied in detail. Previous studies from our group showed that, following insult to the brain, old rats are still capable of upregulating genes that are active during development, but the response is often blunted and temporarily uncoordinated [113].

Understanding mechanisms underlying angiogenesis and vascular remodeling after stroke in the elderly is crucial for developing new treatment strategies to improve the functional outcome after stroke in aged patients. Unfortunately, the molecular mechanisms regulating angiogenesis and vascular remodeling in aging brains are still poorly understood. Recently the regenerative potential of endothelial progenitor cells (EPC) has been under intense investigation. Endothelial progenitor cells are likely to promote vasculogenesis after cerebral ischemia. Therefore the regenerative potential of endothelial progenitor cells (EPCs) has been under intense investigation [114]. Many angiogenic factors, such as VEGF, IGFs or FGFs, are involved in the mobilization of EPCs and the differentiation and increased levels of EPCs were correlated with increased plasma VEGF levels in stroke patients [108]. However, currently there is no effective and safe stem-cell based therapy for stroke [115].

Other studies have established that bone marrow-derived (EPCs) are present in the systemic circulation, and they are able to differentiate into mature endothelial cells in the ischemic area, but the number of these cells is reduced by aging [116, 117]. The cytokine-induced generation of bone marrow-derived EPCs can be enhanced by the administration of G-CSF, and leads to improved functional outcome after stroke in aged rats [61].

Few studies have investigated human post-stroke angiogenesis at the molecular level. Thus, Krupinski and colleagues [118] noted active angiogenesis in the penumbral areas of patients who survived from several days to weeks after cerebral stroke, as well as a positive correlation between microvessel density and patient survival. In subsequent studies, the authors demonstrated an increased synthesis of angiogenic growth factors such as FGF-2, PDGF, VEGF and their receptors within hours of stroke that correlated with blood vessel growth in the penumbra [119, 120].

The literature on gene expression profiles after stroke in humans also is limited. In this regard, Vikman and Edvinsson [121] have shown similarities in gene expression profiles between human strokes and those in animal models, and reported new genes that support the dynamic changes that occur in the middle cerebral artery branches supplying the ischemic region. Also, promising results of blood genomic profiling in human stroke have been obtained in pilot studies [122–124].

These results argue for the utility of proangiogenic therapies in stroke, given the potential effects consisting of increasing blood flow, decreasing infarct size and supporting the restoration and recovery of neurovascular networks after ischemia [61].

Despite the obvious clinical significance of post-stroke angiogenesis in aged subjects, a detailed transcriptomic analysis of post-stroke angiogenesis has not yet been undertaken in an aged experimental model. By combining stroke transcrip-

tomics with immunohistochemistry in aged rats and post-stroke patients, an attempt was made to identify an age-specific gene expression pattern that may characterize the angiogenetic process after stroke. It was found that both young and old infarcted rats initiated vigorous angiogenesis. However, the young rats had a higher vascular density by day 14 post-stroke. "New-for-stroke" genes that were linked to the increased vasculature density in young animals included *Angpt2*, *Angptl2, Angptl4, Cib1, Ccr2, Col4a2, Cxcl1, Lef1, Hhex, Lamc1, Nid2, Pcam1, Plod2, Runx3, Scpep1, S100a4, Tgfbi* and *Wnt4*, which are required for sprouting angiogenesis, reconstruction of the basal lamina and the resolution phase. The vast majority of genes involved in sprouting angiogenesis (*Angpt2*, *Angptl4, Cib1, Col8a1, Nrp1, Pcam1, Pttg1ip, Rac2, Runx1, Tnp4, Wnt4*)*;* reconstruction of a new basal lamina *(Col4a2, Lamc1, Plod2) or* tube formation and maturation *(Angpt1, Gpc3, Igfbp7, SPARC, Tie2, Tnfsf10)*, had however, a delayed upregulation in the aged rats. The angiogenetic response in aged rats was further diminished by the persistent upregulation of "inflammatory" genes (*Cxcl12, Mmp8, Mmp12, Mmp14, Mpeg1, Tnfrsf1a, Tnfrsf1b)* and vigorous expression of genes required for the build-up of the fibrotic scar (*Cthrc1, Il6ra, Il13ar1, Il18, Mmp2, Rassf4, Tgfb1, Tgfbr2, Timp1*). Beyond this barrier angiogenesis in the aged brains was similar to that in young brains. It was also reported that the aged human brain is capable of mounting a vigorous angiogenic response after stroke, which most likely reflects the remaining brain plasticity of the aged brain [125].

12 Conclusions

Recent results using a variety of drug, cell therapy or combination thereof suggest that, (1) G-CSF in aged rats has primarily a beneficial effect on functional outcome most likely via supportive cellular processes such as neurogenesis; (2) the combination therapy, G-CSF with mesenchymal cells (G-CSF + BM-MSC or G-CSF + BM-MNC) did not further improve behavioral indices, neurogenesis or infarct volume as compared to G-CSF alone in aged animals; (3) better results with regard to integration of transplanted cells in the aged rat environment have been obtained using iPS of human origin; (4) mesenchymal cells may be used as drug carriers for the aged post-stroke brains. Finally, while the middle aged brain does not seem to impair drug and cell therapies, in a real clinical practice involving older post-stroke patients, successful regenerative therapies would have to be carried out for a much longer time.

Acknowledgments This study has been supported by a UEFISCDI grant PN-II-RU-TE-2014-4-0705, "The impact of microglia phagocytosis of live neurons on the efficacy of stem cell therapy of stroke "No 165/2015.

Conflict of Interest: The author confirms that he has no conflict of interest to declare for this publication.

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Chapter 4
Modulating Endogenous Adult Neural Stem Cells to Improve Regeneration in Stroke Brain

Fucheng Luo and Yu Luo

Abstract Stroke is a major cause of death and disability globally. Experimental and clinical stroke studies have demonstrated that endogenous brain repair processes could be activated in the brain following stroke. However, the spontaneous brain repair process is constrained with limited improvement of neurological outcome. Neurogenesis, oligodendrogenesis, angiogenesis, axonal outgrowth, and synaptogenesis are major brain repair processes during stroke recovery. In adult rodents and human, there are endogenous neural stem cells that generate new neurons, astrocyte, oligodendrocyte, and NG2-glia under physiological or pathological conditions. Much progress has been made in preclinical studies on the roles of endogenous neural stem cells in brain repair processes in response to stroke. In this review, we will summarize recent progress on the cellular and molecular mechanisms underlying how endogenous adult neural stem cells contribute to neurogenesis and oligodendrogenesis, and their modulatory effects on angiogenesis and inflammation, which may play critical roles in brain repair and leads to improvement of neurological function after stroke.

Keywords Stroke • Neural stem cells • Neurogenesis • Oligodendrogenesis • Brain repair

Abbreviations

3V	Third ventricle
ANg	Angiopoietin
BDNF	Brain-derived neurotrophic factor
BMP	Bone morphogenetic protein

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P.A. Lapchak, J.H. Zhang (eds.), *Cellular and Molecular Approaches to Regeneration and Repair*, Springer Series in Translational Stroke Research,
https://doi.org/10.1007/978-3-319-66679-2_4

CB2R	Cannabinoid type-2 receptor
CCR2	C-C chemokine receptor type 2
ChAT	Choline acetyl-transferase
CNS	Central nervous system
CNTF	Ciliary neurotrophic factor
CSPGs	Chondroitin sulfate proteoglycans
CX3CR1	CX3C chemokine receptor 1
CXCL12	C-X-C motif chemokine 12
CXCR4	C-X-C chemokine receptor type 4
DARPP-32	cAMP-regulated neuronal phosphoprotein
DCX	Doublecortin
DG	Dentate gyrus
ECM	Extracellular matrix
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
FGF10	Fibroblast growth factor 10
FGF2	Fibroblast growth factor 2
GABA	Gamma aminobutyric acid
GAD67	Glutamic acid decarboxylase
GAP43	Growth Associated Protein 43
GSK-3β	Glycogen synthase kinase-3β
HDACs	Histone deacetylases
IGF-1	Insulin-like growth factor 1
MCAO	Middle cerebral artery occlusion
MCP-1	Monocyte chemoattractant protein 1
MMPs	Matrix metalloproteases
mTORC1	Mechanistic target of rapamycin complex 1
Nf1	Neurofibromatosis type 1
NPCs	Neural progenitor cells
NSCs	Neural stem cells
OB	Olfactory bulb
P57kip2	Cyclin-dependent kinase inhibitor 1C
PDGF	Platelet-derived growth factor
PDGFR	Platelet-derived growth factor receptor α
Ptc-1	Patched 1
PV	Parvalbumin
RMS	Rostral migratory stream
Robo	Roundabout
ROCK	Rho-associated kinase
SDF-1	Stromal cell-derived factor 1
SGZ	Subgranular zone
Shh	Sonic hedgehog
siRNA	Short interfering ribonucleic acid
Smo	Smoothened

SVZ	Subventricular zone
TGF-α	Transforming growth factor-alpha
TIA	Transient ischemic attack
Tregs	Regulatory T cells
Usp9x	Ubiquitin-specific peptidase 9, X-linked
VEGF	Vascular endothelial growth factor

1 Introduction

Globally, stroke is the second leading cause of death and the third most common cause of disability [1]. There are three types of stroke: ischemic stroke, hemorrhagic stroke, and transient ischemic attack (TIA, also called a "mini-stroke"). Ischemic stroke is caused by obstruction within a blood vessel and accounts for 87% of all stroke cases, while hemorrhagic stroke occurs when blood vessel rupture. TIAs are caused by a transient clot or blockage in the brain. Although TIAs last only a few minutes and causes no permanent damage to the brain, they are indicative of the likelihood of a coming stroke and should be taken seriously. Only a small percentage of stroke patients benefit from thrombolysis and endovascular thrombectomy treatments due to the short window (4.5–6 h) of these treatments. As a result, a large population of stroke patients still suffer from permanent severe neurological deficits in stroke survivors. Thus, there is an urgent need to develop new therapies for stroke to enhance functional recovery.

Studies from experimental stroke and patients with stroke show that some degree of spontaneous neurological recovery occurs after stroke. However, this endogenous brain self-repair is not sufficient to restore neurological function after stroke [2, 3]. Endogenous brain repair involves a set of highly interactive processes during stroke recovery, such as neurogenesis and oligodendrogenesis, which is induced mostly by endogenous neural stem cells (NSCs). Coupling of neurogenesis and angiogenesis has been implicated in some recent stroke studies [2]. In addition, stroke-induced inflammation, which is characterized by the activation of resident microglia and infiltration of monocytes and lymphocytes, is a major causative factor for neurological deficits [4]. Recent studies also suggest that there is cross-talk between neural stem cells and immune cells in response to brain injury [5]. Therefore, a promising field of investigation is to focus on modulating endogenous adult neural stem cells and their interactions with other cellular processes such as angiogenesis and neuroinflammation to improve functional recovery following stroke. Understanding how endogenous stem cells are activated, differentiate, migrate, integrate, and restore neuronal circuitry will help us develop less invasive therapeutic interventions. Elucidation of the interactions of neurogenesis with other cellular processes such as angiogenesis and inflammation after stroke will provide additional information needed to modulate this process to improve brain recovery after stroke. In this review, we will provide an update on the recent

findings on the mechanisms underlying endogenous NSC-mediated neurogenesis and oligodendrogenesis and their modulatory effects on angiogenesis and inflammation after stroke.

2 NSCs Responses in Adult Brain Following Stroke

NSCs are multipotent stem cells that can self-renew, divide, and differentiate into new mature neurons, astrocytes, and oligodendrocytes. In the adult brain, there are three main neurogenic niches containing NSCs: the subventricular zone (SVZ) of the lateral ventricle, the subgranular zone (SGZ) in the dentate gyrus of the hippocampus, and the recently discovered hypothalamic stem cell niche [6] (Fig. 4.1). In these regions, there is a basal rate of neurogenesis in normal conditions. In response to stroke, endogenous NSCs are activated and participate in brain repair processes.

2.1 Radial Glial Cells (Type B Cells) in SVZ

The NSCs in the SVZ are termed as Type B cells. They divide slowly to generate transit-amplifying type C cells, which proliferate actively and further differentiate into neuroblasts (also named type A cells). Finally, these neuroblasts form chains and migrate via the rostral migratory stream into the olfactory bulb (OB), where they differentiate into granule cells or periglomerular interneurons. Adult NSCs in the SVZ also generate NG2-glia that migrates toward the gray and white matter. Focal cerebral ischemia stimulates SVZ NSC proliferation and neurogenesis in adult rodent, monkeys, and even human brains [7–10]. Augmented neuroblasts could migrate from the SVZ to ischemic sites and differentiate into neurons in

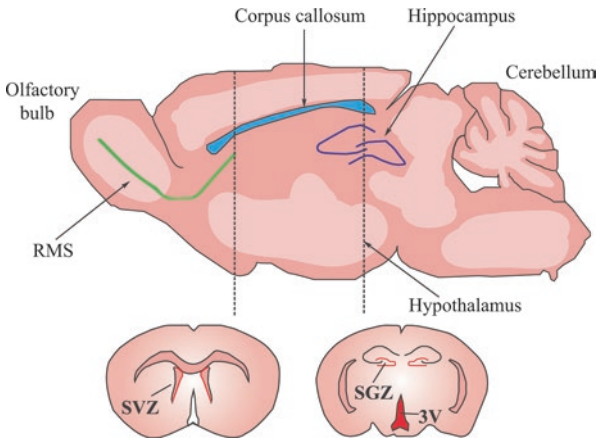

Fig. 4.1 Sagittal and coronal view of the adult rodent brain, illustrating the three niches where adult NSCs reside: the subventricular zone (SVZ), the subgranular zone (SGZ), and the recently described hypothalamic NSCs niche around the third ventricle

rodent middle cerebral artery occlusion (MCAO) models [11, 12]. In addition, stroke also induces oligodendrogenesis in the SVZ and the generated NG2-glia can migrate to the lesion site and differentiate into myelinating oligodendrocytes [13, 14]. Furthermore, activated SVZ NSCs give rise to a subpopulation of reactive astrocytes in the cortex that contribute to astrogliosis and scar formation [15]. Altogether, these data indicate that SVZ NSCs are a major therapeutic target for improving functional recovery after stroke.

2.2 Radial Glia-Like Cells (Type-1 Cells) in SGZ

SGZ NSCs are also known as type-1 cells or radial glia-like cells. These cells divide slowly and give rise to type-2 cells or transit-amplifying progenitors that could differentiate into neurons and astrocytes [16]. However, it is still a matter of debate whether these cells can spontaneously, that is without any exogenous manipulation, give rise to oligodendroglial cells. Indeed, either ectopical and elevated Ascl1 expression or inactivation of p57kip2, Nf1, Drosha, or Usp9x induce oligodendrogenesis in SGZ NSCs [17–21]. The function of neurogenesis derived from SGZ NSCs is associated with learning, memory, and cognition. Following a stroke, there is significantly enhanced proliferation of NSCs and neurogenesis in the SGZ of many species, such as rats, mice, monkeys, and humans [22]. Generally, the increased proliferation starts bilaterally at 3–4 days post-ischemia, peaks at 7–10 days, and returns to control levels by 3–5 weeks after the ischemia [22]. Recent studies show that hippocampal neurogenesis is responsible for some aspects of recovery following brain ischemia, such as learning and memory [23]. These data suggest that target SGZ NSCs might help to improve functional recovery after stroke.

2.3 Tanycytes

It has been recently demonstrated that NSCs also reside in the adult hypothalamus. The NSCs/NPCs of this region are termed as tanycytes, which express classical markers of neural precursor cells and multipotent cell markers, such as nestin, Sox2, UGS148, and FGF10 [6]. These tanycytes belong to ependymal glial cells and surround the lateral walls of the infundibular recess of the third ventricle. In response to peripheral signaling (i.e. CNTF, Leptin and high-fat diet), tanycytes are able to proliferate, migrate, and differentiate into neurons, such as arcuate pro-opiomelanocortin neurons and orexigenic and anorexigenic neurons [6, 24–27]. Importantly, tanycytes exhibited increased proliferation on the infarcted side on day 4 after ischemic stroke injury (MCAO model in rats) [28]. However, the functional role of tanycyte proliferation after stroke is still largely unknown.

3 Promoting Neurogenesis of Endogenous NSCs

Neurogenesis is a multistep process that includes proliferation, fate determination, migration, maturation, and survival of NSCs. Understanding the molecular mechanisms regulating these processes is essential for developing therapies to improve neurological recovery (Fig. 4.2). Many factors are involved in the regulation of adult NSCs, including growth factors, neurotransmitters, and chemokines. We will briefly summarize them in this review.

3.1 Proliferation

The initial response of NSCs following stroke is to increase proliferation, a process that is regulated by various intrinsic and extrinsic factors. The mechanism underlying stroke-induced proliferation of NSCs is unclear. Several hypotheses have been suggested as potential mechanisms to regulate proliferation of NSCs. Adult rodent stroke studies have shown that quiescent adult neural stem cells can be activated and recruited to an active pool to increase neurogenesis. As a response to stroke, an increased neurogenesis might result from transiently switching neural progenitors division from asymmetric to symmetric and from a reduction of the length of the cell cycle [29, 30]. Stroke can trigger the early expansion of the progenitor cell pool by shortening the cell-cycle length and retaining daughter cells within the cell cycle at an early stage after stroke. At a later stage, lengthening the cell cycle and the G1 phase leads to the daughter cells exiting the cell cycle and differentiating into neurons [31]. Several important pathways that may regulate the proliferation of NSCs and their early progeny have been identified.

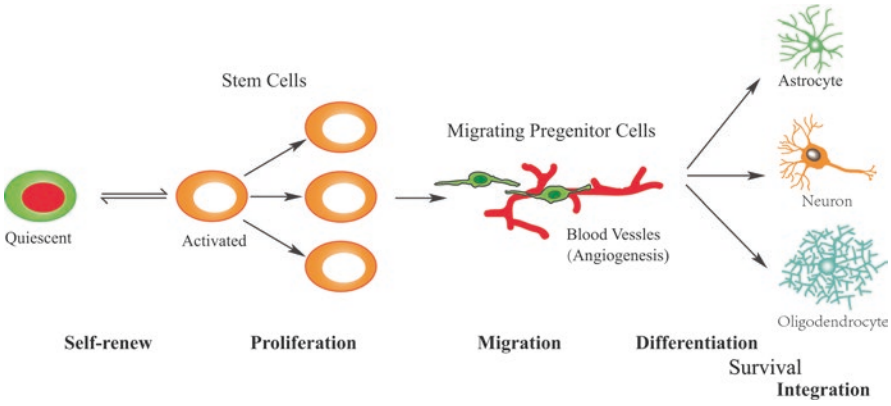

Fig. 4.2 Cellular and molecular processes that are involved in the maintenance of adult NSCs, generation of different lineages of cells and their integration in the brain after stroke

3.1.1 Sonic Hedgehog (Shh)

Shh is a secreted glycoprotein. It binds to its receptor Patched (Ptc-1) to de-repress Smoothened (Smo) and activate transcription factors of the Gli family. Shh signaling is required for SVZ NSC maintenance as conditional deletion of smoothened gene in adult SVZ NSC leads to decreased BrdU-positive cells and DCX+ neuroblasts in the SVZ [32]. Studies have found that stroke upregulates Shh signal in multiple cell types, such as neurons, reactive astrocyte, and SVZ neural progenitor cells [33, 34]. In vivo, blockage of the Shh signaling pathway with cyclopamine, a specific inhibitor of Smo, suppressed ischemia-induced proliferation of subgranular NPCs in the hippocampus [34]. Conditional deletion of shh genes in nestin-expressing cells leads to significantly more severe behavioral deficits in a cortical ischemic model [33]. Administration of cyclopamine also abolished carbamylated erythropoietin-induced neurogenesis [35]. These data suggests that Shh signaling is a key factor for NSC self-renewal or proliferation. Interestingly, at a lower dosage, delayed post-stroke treatment of Shh agonist improves functional recovery by enhancing survival of newly born neurons and angiogenesis [36] but not by increasing BrdU-positive cells at the NSC niche, suggesting that Shh signaling might play multiple roles in ischemia-induced neurogenesis and whether it enhances the proliferation or survival of the newly generated NSC progeny is dose-dependent.

3.1.2 Epidermal Growth Factor (EGF)/Fibroblast Growth Factors 2 (FGF2)

Studies have reported that FGF-2 and EGF expression in the brain increased significantly after ischemic stroke [37, 38]. Importantly, cerebral ischemia resulted in an increase in the number of EGF receptor (EGFR)-positive transit-amplifying cells (type C cells) in the SVZ [39]. Overexpression of FGF-2 significantly increased the proliferation of progenitor cells after ischemic stroke in both FGF-2-deficient mice and wild-type mice [40]. Meanwhile, in vivo infusion of EGF into adult mouse forebrain for 6 consecutive days resulted in a dramatic increase in the proliferation and the total number of subependymal cells and induced their migration away from the lateral ventricle walls into adjacent parenchyma [41]. Furthermore, infusion of EGF together with FGF-2 into the brain of adult rats was found to promote dentate gyrus (DG) and SVZ NPC proliferation after focal ischemic stroke [42, 43].

3.1.3 Insulin-Like Growth Factor 1 (IGF-1)

The progenitors in both the SVZ and DG show IGF-1 receptor expression [44]. In vitro studies demonstrate that IGF-1 stimulated the proliferation of cultured NPCs via activating the PI-3-kinase/Akt signaling pathway [45]. Following ischemic stroke, IGF-1 expression is increased in the activated astrocytes in the ischemic penumbra [44]. Inhibiting IGF-1 activity by intracerebroventricular infusion of

IGF-1 antibody significantly blocked the ischemia-induced neural progenitor proliferation [44]. *Exogenous* IGF-1 injection after ischemic stroke promoted neurogenesis [46]. Meanwhile, post-ischemic IGF-1 gene transfer in the peri-infarct region potently promoted neural and vascular regeneration in the chronic stage of cerebral infarction [47]. These results suggest that IGF-1 formed in the ischemic penumbra might be one of the endogenous diffusible factors that mediate post-ischemic neural progenitor proliferation.

3.1.4 Notch Signaling Pathway

Notch signaling is an evolutionarily conserved pathway that regulates cell-fate determination during development and maintains adult tissue homeostasis. Recent studies have shown that stroke increases the expression of Notch1 and Hes1 in SVZ cells [48]. Transient administration of Notch ligands to the brain of adult rats increases the numbers of newly generated precursor cells and improves motor skills after ischemic injury [49], while the blockage of the Notch pathway by short interfering ribonucleic acid (siRNA) against Notch or a gamma secretase inhibitor significantly blocked ischemia-induced cell proliferation in the SVZ [50]. These data suggest that the Notch signaling pathway mediates adult SVZ neural progenitor cell proliferation after stroke. Interestingly, it has recently been shown that striatal astrocytes can turn on nestin expression and generate neurons in stroke model through downregulation of Notch signaling, suggesting that Notch signaling might also suppress "NSC status" in mature astrocytes [51].

3.1.5 Other Regulators

Finally, other potential mediators of stroke-induced proliferation of NSCs in the neurogenic niches have been described. These include vascular endothelial growth factor (VEGF) [52], glial cell-derived neurotrophic factor (GDNF) [53], brain-derived neurotrophic factor (BDNF) [54], Wnt signaling, retinoic acid [55], bone morphogenetic protein [56], and microRNA [57, 58]. In addition, the communication between NSCs and other cell types also affects NSC proliferation after stroke. It has been reported that M2 phenotype microglia-derived transforming growth factor-alpha (TGF-α) is one of the key factors to enhance proliferation and neural differentiation of NSPCs after ischemic stroke [59]. Activated regulatory T cells (Tregs) enhanced SVZ NSC proliferation in normal and ischemic mice; blockage of IL-10 abolished Tregs-mediated NSC proliferation in vivo and in vitro [60]. Furthermore, astrocytic calcium waves are long-range signals capable of transmitting the occurrence of a brain injury to the SVZ, where they stimulate NSC proliferation and self-renewal and increase the migratory potential of NSPCs. It is shown that the Notch signaling pathway mediates effects of elevated calcium levels on NSPCs [61].

3.2 Migration

After stroke, following NSC proliferation, another critical biological process is the migration of these NSCs from neurogenic niches to the ischemic region. In the normal adult brain, SVZ neuroblasts migrate along the rostral migratory stream to the olfactory bulb. Lateral migration into the striatum and parenchyma is not observed in the rodent brain under normal condition. However, in the ischemic damaged brain, neuroblasts will migrate laterally into the ischemic injury region [11]. Although little is known about the molecular mechanisms underlying stroke-induced redirected migration, several potential mediators have been identified. These include stromal cell-derived factor 1 (SDF-1), monocyte chemoattractant protein 1 (MCP-1/CCL2), matrix metalloproteases (MMPs), cannabinoid type-2 receptor (CB2R), and β1 integrin. Further, the neurovascular niche within the SVZ and SDG is also a key regulator of neuroblast migration following stroke.

3.2.1 Stromal Cell-Derived Factor 1 (SDF-1)

SDF-1, also known as C-X-C motif chemokine 12 (CXCL12), is a chemokine protein that exerts biological functions by binding to its receptors CXCR4 and CXCR7. SDF-1 (CXCL12) is a member of the alpha (CXC) chemokine family which are involved in inflammatory responses [62]. SDF-1 and its receptor CXCR4 have been demonstrated to play an important role in the mobilization and homing of stem cells to bone marrow [63, 64]. Neuroblasts are reported to express CXCR4 [65]. During adult neurogenesis, SDF-1 is secreted by vascular endothelial cells and plays a role in the directional migration of neuroblasts in the CNS [65]. Following stroke, SDF-1 is upregulated by activated astrocyte and endothelial cells, subsequently guiding neuroblast migration toward the injured tissue [66–68]. In contrast, CXCR4 blockade blocks this pathology-directed chain migration [69].

3.2.2 Monocyte Chemoattractant Protein-1 (MCP-1)

MCP-1 is a member of the C-C chemokine family that regulates migration and infiltration of monocytes/macrophages [70]. Following cerebral ischemia, MCP-1 is induced in activated astrocytes and neurons within the injured tissue [71, 72]. The migrating neuroblasts in the ischemic brain express MCP-1 receptor CCR2. Infusion of MCP-1 into the normal striatum induced neuroblast migration to the infusion site [73]. In knockout mice that lacked either MCP-1 or its receptor CCR2, there was a significant decrease in the number of migrating neuroblasts from the SVZ to the ischemic striatum [73].

3.2.3 Matrix Metalloproteinases (MMPs)

Matrix metalloproteinases (MMPs) are members of the metzincin group of proteases that participate in several physiological processes, such as bone remodeling, angiogenesis, immunity, and wound healing [74]. Recent studies suggest that MMPs are involved in guiding neuroblast migration from the neurogenic region to the ischemic boundary [75]. Neuroblasts express MMP-3 and MMP−9. Inhibition of MMPs diminishes neuroblast migration after stroke [76, 77]. Moreover, MMP2 and MMP9 secreted by endothelial cells are also associated with neuroblast migration after stroke [78].

3.2.4 CB2R

The endocannabinoids (eCBs) 2-arachidonoylglycerol and anandamide are lipid signaling messengers involved in the homeostatic control of a large variety of functions of the nervous system through binding cannabinoid type-1 receptor (CB1R) and cannabinoid type-2 receptor (CB2R) [79]. CB2R is expressed in resident microglia, NG2-glia, and NSCs. CB2R is neuroprotective in acute experimental stroke by anti-inflammatory mechanisms [80]. *In vitro* studies show that CB2R promotes NSC proliferation via mTORC1 signaling [81]. Furthermore, in stroke, CB2R is required for neurogenesis by promoting neuroblast migration toward the injured brain tissue [82].

3.2.5 β1 Integrin

β1-class integrins are transmembrane receptors for several extracellular matrix (ECM) proteins such as laminin [83]. Under normal conditions, migrating neuroblasts generated in the adult SVZ express β1 integrin, which is required for their chain formation during RMS migration [84, 85]. Following stroke, laminin-β1 integrin signaling enables neuroblasts to form chains and migrate efficiently along vascular scaffolding in the post-stroke brain [86].

3.2.6 Neurovascular Niche

Stroke-induced directional migration of neuroblasts is closely associated with thin astrocytic processes and blood vessels, suggesting that blood vessels may act as a scaffold for neuroblast migration [87, 88]. Virally labeled SVZ NPCs were observed to migrate along both newly formed and pre-existing blood vessels toward the ischemic injured area. Live imaging showed that migrating SVZ NPCs have their leading process closely associated with blood vessels, suggesting that this interaction provides directional guidance for the NPCs [89]. In addition, vasculature promotes neuroblast migration via secreting various growth and chemotactic factors, including BDNF, MMPs, angiopoietins, and SDF-1 [22].

3.2.7 Other Regulators

Wnt3a, Angiopoitin (ANg)-1 and its receptor Tie 2, and Slit and its receptor (ROBO) also promote post-stroke neuroblast migration and behavioral recovery [66, 90, 91]. It also should be noted that stroke also induces inhibitory molecules to block the migration of neuroblasts. Glycogen synthase kinase-3β (GSK-3β) inhibition promoted proliferation of neural stem cells (NSCs) and migration of nascent doublecortin (DCX+) neuroblasts from the SVZ to the lesioned cortex [92]. Inhibition of Na^+-K^+-Cl^--co-transporter can increase migration of neuroblasts in the SVZ towards the infarct areas and improve sensorimotor recovery [93]. Stroke also induces chondroitin sulfate proteoglycans (CSPGs), which could block neuroblast migration through Rho-associated kinase (ROCK) activation [94, 95].

3.3 Survival, Differentiation, and Integration of Newborn Neurons

The long-term survival and functional maturation of newborn neurons following stroke are also crucial for neurological recovery. However, there are fewer studies that have examined the survival, differentiation, and integration of newborn neurons in the ischemic brain. The migration of SVZ neuroblasts to the lesion sites may persist for up to 1 year after ischemia [96], thus offering a long-term window for therapeutic manipulations. Ischemia-induced newly generated cells in the damaged areas express medium-size spiny neuronal marker dopamine- and cAMP-regulated neuronal phosphoprotein (DARPP-32) or neurotransmitter synthesizing enzymes such as glutamic acid decarboxylase (GAD67) and choline acetyl-transferase (ChAT) [11, 97, 98]. Moreover, ischemia-induced newly formed striatal GABAergic and cholinergic neurons could exhibit electrophysiological activity and functional synapses [97]. These data indicate that proliferating neuroblasts that migrate into the damaged areas following stroke are able to differentiate into a variety of functional neuronal cells. Compared to our knowledge of factors that promote adult neural precursor cell proliferation or migration, there is comparatively little known about factors that promote newborn neuron survival and integration in stroke. Intraventricular administration of EGF and albumin enhance the differentiation of newly born immature neurons into mature PV-expressing neurons, replacing more than 20% of PV+ interneurons lost after cerebral ischemia [99]. Complement-derived peptide C3a regulates neural progenitor cell migration and differentiation in vitro and C3a receptor signaling stimulates neurogenesis in unchallenged adult mice. Daily intranasal treatment of wild-type mice with C3a beginning 7 days after stroke induction robustly increased synaptic density and expression of Growth Associated Protein 43 (GAP43) in the peri-infarct cortex [100]. Post-stroke p53

inhibitor enhances the survival of NSCs and their progeny by inhibition of apoptosis in these cells through PUMA gene regulation [101]. Similarly, Shh agonist, delivered after stroke at a lower dose that did not affect BrdU-positive cells in SVZ and SGZ improved the long-term survival of YFP-labeled NSCs and their progeny in stroke model [36]. Some knowledge has been gained regarding factors that enhance newborn neuron integration and survival under normal physiological conditions, including the RhoA family of small GTPases, suppressor of cytokine signaling-2, neurotrophins, neurotransmitters (GABA and glutamate), and semaphorins [102]. Logically, we might get some implication from those factors and explore their roles after ischemic injury. However, it should be noted that the stroke-affected CNS environment is quite an inhibitory environment for newborn cell survival. Experimental studies have shown that only a small proportion of cells survive long enough to integrate into the damaged parenchyma after stroke [46, 68, 102, 103]. Thus, neuroprotective or anti-inflammatory strategies might need to be included with therapy to improve newborn neuron maturation, integration, or plasticity in stroke treatment.

4 Endogenous NSCs and Oligodendrogenesis

NG2-glia, also called oligodendrocyte precursor/progenitor cells and polydendrocytes, characterized by expression of chondroitin sulfate proteoglycan NG2 and platelet-derived growth factor receptor α (PDGFR α). It is widely distributed in the brain and can continuously produce differentiating and mature oligodendrocytes in the central neural system throughout the lifespan of the animals. Myelination of axons in the adult brain is critical for saltatory conduction, axonal integrity, neural plasticity, and circuitry function, which are important for functional recovery after stroke [104]. Stroke acutely leads to mature oligodendrocyte damage, resulting in myelin loss, which is associated with a loss of axons. Oligodendrogenesis is induced in the regions surrounding the lateral ventricles and peri-infarct areas during stroke recovery [105–107]. Studies demonstrate that stroke not only activates resident NG2-glia in white and gray matter, but also increases NG2-glia generation in the SVZ and attracts them to the ischemic area [108–111]. The newly generated NG2-glia could differentiate into mature myelinating oligodendrocytes in the peri-infarct areas, which is involved in the brain repair process [106]. Therefore, these studies suggest that NG2-glia generated by adult NSCs contribute to oligodendrogenesis after stroke. The process of SVZ NSC-mediated oligodendrogenesis is regulated by many intrinsic and extrinsic factors, therefore offering many pathways for potential therapeutic interventions to promote functional recovery following stroke.

4.1 Extrinsic Factors for Oligodendrogenesis

4.1.1 Shh

In addition to neurogenesis, Shh signaling regulates oligodendrogenesis by inducing transcription factor olig2 expression [112]. In the SVZ, there is a dorsal Shh-dependent domain producing many oligodendroglial lineage cells [113]. The blockage of Shh signal with cyclopamine could abolish cerebrolysin-enhanced oligodendrogenesis in stroke [114]. Bone marrow stromal cell transplantation stimulates oligodendrogenesis by activation of Shh/Gli1 pathway, which mediates subsequent functional recovery after stroke [115]. Thus, these data suggest that Shh signaling in SVZ plays an important role in mediating oligodendrogenesis in the ischemic brain.

4.1.2 Stromal-Derived Factor 1 (SDF-1)

SDF-1, has been shown to be able to promote neurogenesis and angiogenesis, leading to functional recovery in ischemic mice [116, 117]. Through binding with CXCR4 in NG2-glia, SDF-1 activates their proliferation, migration, and differentiation [118–120]. SDF-1 gene therapy at 1 week after ischemia promotes NG2-glia proliferation in the SVZ and further enhances their migration to the ischemic lesion area [121]. These data support that in addition to enhancing neurogenesis, SDF-1 promotes oligodendrogenesis as well after stroke.

4.1.3 Vascular Endothelial Growth Factor (VEGF)

VEGF is a signaling protein that is important for vasculogenesis and angiogenesis. The administration of VEGF improves neurological performance through mediating angiogenesis and survival of newborn neurons in the rat MCAO model [122]. Studies have shown that VEGF-C stimulates NG2-glia proliferation [123] while VEGF-A can induce NG2-glia migration via ROS and FAK-dependent mechanisms, but did not affect their proliferation and differentiation [124]. In the neonatal hypoxia-ischemia rat model, VEGF-A and VEGF-C are induced in the SVZ. Moreover, VEGF-C promotes the proliferation of both early and late oligodendrocyte progenitors through VEGFR-3 receptor [108]. These data suggest that besides promoting angiogenesis and neurogenesis, VEGF signaling is also involved in oligodendrogenesis after stroke.

4.1.4 Brain-Derived Neurotrophic Factor (BDNF)

BDNF is a well-known member of a neurotrophin family that regulates neuronal survival, synaptic plasticity, learning, and memory. Recent studies show that BDNF could promote the proliferation and differentiation of NG2-glia and is required for normal CNS myelination [125–127]. Astrocyte-derived BDNF supports oligodendrogenesis and regeneration after white matter ischemic injury or cuprizone-induced demyelination [128, 129]. BDNF administration improves functional recovery and promoting oligodendrogenesis and remyelination in rats subjected to ischemic stroke [130]. These data suggest that in addition to neuroprotective effects, BDNF plays important roles in white matter protection and remyelination after stroke.

4.1.5 Other Factors

There are many other factors regulating oligodendrogenesis under normal and pathological conditions [131]. For example, neuregulin-1 promotes NG2-glia survival and maintains NG2-glia in an immature state [132]. Platelet-derived growth factor (PDGF) is an important factor for maintaining NG2-glia proliferation and stimulating their differentiation into mature oligodendrocytes [133]. PDGF signaling in the SVZ promotes oligodendrocyte generation [134]. Insulin-like growth factor (IGF)-1 could promote the differentiation of adult NSCs into oligodendrocyte lineage cells through inhibiting BMP signaling [135]. Epidermal growth factor (EGF) induces the progeny of SVZ NSCs to migrate and differentiate into oligodendrocytes [136]. It has been reported that these above growth factors play positive roles in functional recovery after stroke [137]. However, the contribution of ischemia-induced oligodendrogenesis to functional recovery in stroke by these growth factors remains to be established.

4.2 Epigenetic Modulators and Stroke-Induced Oligodendrogenesis

Epigenetics is defined as the heritable changes in gene expression without a change in the DNA sequence [138]. Recent studies have shown that the multiple steps of oligodendrocyte generation (i.e., specific cell fates, proliferation, differentiation, and myelination) can be regulated through epigenetic mechanisms [139–141]. The epigenetic modulators of gene expression include post-translational modulations of nucleosomal histones, histone modification, chromatin remodeling enzymes, DNA methylation, and microRNAs [142]. Among them, we will mainly focus on miRNA and histone deacetylases (HDACs) in this review.

4.2.1 microRNAs

A number of miRNAs have been found to play a critical role in the proliferation or differentiation of OPCs into mature oligodendrocytes as well as myelination [143, 144]. miR-219 and miR-338 could promote NG2-glia differentiation into mature oligodendrocytes through suppressing the expression of PDGFRa, Sox6, Zfp238, FoxJ3, and NeuroD1 [145]. Stroke considerably increased miR-146a density in the corpus callosum and SVZ of the lateral ventricle of the ischemic hemisphere. In vitro, overexpression of miR-146a in neural progenitor cells (NPCs) significantly increased their differentiation into O4+ NG2-glia [146]. During development, miR17-92 cluster can regulate proliferation and survival of NG2-glia in the brain. In stroke, the miR17-92 cluster was significantly up-regulated in SVZ neural progenitor cells [147]. It could mediate the proliferation and survival of SVZ NPCs in the ischemic brain [148]. miR17-92 cluster-enriched exosomes could increase neural plasticity and functional recovery after stroke [149]. In addition, miR-23a, miR-9, and miR-200b are also likely involved in stroke-induced oligodendrogenesis by regulating serum response factor [150, 151]. Collectively, these findings suggest that miRNAs play an important role in stroke-induced oligodendrogenesis.

4.2.2 Histone Deacetylases (HDACs)

The administration of HDACs inhibitor suberoylanilide hydroxamic acid or TSA can confer protection against ischemia-induced brain injury [152, 153]. HDAC1 and HDAC2 are associated with oligodendrocyte differentiation and remyelination during brain development and disease [154–157]. In ischemic brains, there is increased expression of HDAC1 and HDAC2 proteins in NG2-glia [158]. In addition, blockage of HDACs with valproic acid considerably increased OPCs and new oligodendrocytes after stroke [159]. HDACs clearly play important roles in stroke-induced oligodendrogenesis.

5 The Implicating Effects of NSC-Mediated Neurogenesis and Oligodendrogenesis on Angiogenesis and Inflammation

Stroke continuously induces neuroblasts, which migrate to peri-infarct regions for at least 1 year [160]. The ablation of neuroblasts after stroke reduces ischemic brain repair and exacerbates functional recovery [161]. Experimental studies show that only a small fraction of neuroblasts derived from endogenous NSCs in the peri-infarct regions differentiate into mature neurons and survive [162–164]. Meanwhile, there is increased production of NG2-glia and some of them in the peri-infarct regions generate into mature myelinating oligodendrocytes after stroke [165–167].

These data suggest that stroke-induced neurogenesis and oligodendrogenesis might provide additional beneficial effects that are independent of cellular replacement of dead neurons and myelinating oligodendrocyte production to re-wire neuronal circuitry.

5.1 Angiogenesis

Angiogenesis is characterized by the formation of new vessels from existing blood vessels. Coupling and bi-directional regulation of neurogenesis and angiogenesis have been implicated both under normal and pathological conditions [2]. Both SVZ and SGZ niches have unique vasculature characteristics compared to non-neurogenic regions and adult NSCs extend their long processes to directly contact blood vessels, which enables the easy access of NSCs to molecules and factors in the blood [168]. Under the ischemic condition, it has been shown that angiogenic genes are upregulated rapidly after the onset of cerebral ischemia and the increased expression of angiogenic proteins can be sustained in the ischemic area for a prolonged period of time after stroke [169]. Both neurogenesis and angiogenesis have been suggested to contribute to the functional recovery after stroke [170] and the two critical biological processes might have synergistic effects and influence each other. Co-culture of ischemic neural progenitor cells with non-ischemic endothelial cells increases angiogenesis in vitro [171, 172] and co-culture of ischemic endothelial cells with non-ischemic NSCs increases progenitor cell proliferation and neuronal differentiation. On one hand, neuroblasts induced by stroke in the SVZ migrate along cerebral blood vessels to peri-infarct regions where angiogenesis occurs [96]. On the other hand, it is possible that NSC-derived progeny cells (neuroblasts and astrocytes) can regulate angiogenesis and help maintain the function and integrity of the newly generated blood vessels. Importantly, NG2-glia are also in close proximity to astrocyte, pericytes, or endothelial cells [173, 174]. It is an important component of the neurovascular unit in cerebral white matter [174]. NG2-glia and oligodendrocytes can act as a critical source of trophic factors [175, 176]. In addition, NG2-glia can support blood-brain barrier integrity by upregulating tight junction proteins via TGF-β1 signaling [177]. NG2-glia-specific TGF-β1-deficient mice exhibited cerebral hemorrhage and loss of BBB function [177]. It has been shown that signaling from NG2-glia to ECs plays an important role in angiogenesis during development. Wnt7a and Wnt7b secreted by hypoxic NG2-glia could increase the proliferation of endothelial cells and angiogenesis [178]. These data suggest close interaction and potentially synergistic effects of endogenous neurogenesis, oligodendrogenesis, and angiogenesis in stroke recovery.

5.2 Inflammation Modulation

Inflammation plays an important role in the pathogenesis of stroke, which contributes to neuronal death and impairs functional recovery. In the ischemic brain, there is activation of microglia, production of pro-inflammatory factors, and immune cell infiltration (i.e. neutrophils, monocyte/macrophages, T cells and B cells). Recent studies have shed new light on the interaction between endogenous NSCs and immune cells, such as microglia, T cells, and natural killer cells [179–182]. Both in vitro and after transplantation in vivo, NSCs can directly change inflammatory responses through releasing immunomodulatory factors [183–185]. However, it is still unknown whether endogenous NSCs in their native location have similar capacities under stroke conditions. Endocannabinoids are reported to play an important role in maintaining immune homeostatic balance within the host [186]. Anandamide, an endogenous cannabinoid, contributes to immune tolerance in the gut by promoting the presence of CX3C chemokine receptor 1 ($CX3CR1^{hi}$) macrophages, which are immunosuppressive [187]. In response to the excitotoxic damage occurring in stroke and epilepsy, SVZ NSCs can release endogenous endocannabinoids to exert a protective role for striatal neurons [188]. In the EAE model of multiple sclerosis, SVZ NSCs produce interleukin-15 and sustain functionally competent natural killer cells [180]. Studies have shown that there is an accumulation of natural killer cells in ischemic brain tissues [189–191]. These data suggest that endogenous NSCs maybe regulate stroke-induced inflammation through releasing immunomodulatory factors. In addition, NG2-glia and oligodendrocytes express a wide range of immunomodulatory molecules [192, 193], suggesting that endogenous NSCs might indirectly affect immune cell function and inflammation through regulating oligodendrogenesis. Further studies are needed to understand whether, when, and how endogenous NSCs can take over and locally manifest an immunomodulatory effect. It will help to develop novel therapies to promote functional recovery in stroke through modifying the immunomodulatory effects of endogenous NSCs.

6 Conclusion and Discussion

Brain repair processes after stroke are regulated by multiple cellular pathways, which include neurogenesis, oligodendrogenesis, angiogenesis, axonal sprouting, and synaptogenesis. The presence of endogenous NSCs in the adult brain and their capacity to generate new neurons, oligodendrocytes, and astrocytes raises hope that new therapeutic strategies can be designed based on appropriate modulation of endogenous NSCs in stroke. Over the past five decades, since its discovery, adult neurogenesis and NSCs have evolved into an established research field that has made substantial and promising progress as regenerative medicine for neurological disease. However, there are still many critical questions that need to be addressed.

The defining characteristics of stem cells are their ability to self-renew and to differentiate into various cell types. We have just started to appreciate the complexity and heterogeneity of adult NSCs. Balance and integration are important themes to consider when trying to modulate this process to improve brain recovery after injury. For example, adult NSCs have quiescent and activated states. Adult NSCs are largely quiescent in vivo, a state that recently has been recognized as not a passive state but rather maintained by active transcriptional regulation [194]. The mechanisms that trigger the activation of NSCs by entering multiple rounds of proliferation followed by potential terminal differentiation after brain injury are still unknown. Since the quiescent state is actively maintained by NSCs and might serve important roles to preserve these cells from metabolic stress and maintain genome integrity over a long lifetime, strategies that only target to enhance the activation and proliferation of NSCs might need to take cautions as these might have the risk of depleting quiescent NSCs over a prolonged period of time. In this regard, it is possible that treatment strategies that target the enhanced survival of NSCs and their progeny might be a better strategy as the majority of the newly born cells derived from NSCs fail to survive at weeks to months after stroke.

Similarly, the precise mechanisms that trigger differentiation of NSCs to different types of cells in vivo after brain injury are largely unknown. When cultured in vitro, adult NSCs are able to self-renew and differentiate into all three neuronal lineages [195]. However, under normal conditions, SVZ and SGZ cells generate different types of neurons and non-neuronal cells, suggesting that the microenvironment of the NSC niche might limit their differentiation potential. Adult NSCs are also capable of responding to a variety of brain injury by altered differentiation phenotypes as well as migration into the injured area instead of their "original path". What are the precise molecules and signals that direct the differentiation of these cells under the pathological condition? Knowledge in these areas will help us modulate the fate of these cells and help guide them to targeted areas to repair the brain. Substantial interests in the field have been focused on the neuronal differentiation of NSCs after injury; however, neuroblasts have been shown to play important roles through non-neuronal replacement mechanisms [2]. SVZ NSCs have also been reported to generate astrocytes that migrate to the injured cortex. Defects in this astrogenic process, which resulted in a shift in SVZ NSCs fate from glial cells to neuroblasts, resulted in abnormal glial scar formation and increased microvascular hemorrhage in stroke animals. In addition, although glial scar formation was previously considered as an inhibitory factor for axonal outgrowth, there is evidence indicating that the glial scar aids axonal outgrowth in spinal cord injury [196]. Therefore, strategies that aim to guide NSC differentiation towards a single cell type (neurons) might not provide desired effects in brain recovery. Considering the heterogeneity of astrocytes and their role in synapse formation and glial scar formation, whether reactive astrocyte derived from NSCs in stroke could affect axonal outgrowth and synaptogenesis also needs to be investigated. In addition, besides the role of producing new neurons and myelinating oligodendrocytes, it remains to be defined whether and how NSCs, NSC-derived neuroblasts, and NG2-glia contribute to angiogenesis and immunomodulation after stroke. Overall, understanding the

fundamental mechanisms underlying the endogenous NSC-mediated brain repair process will provide the basis for future endogenous NSC therapy for stroke. By elucidating the relationship and interactions of neurogenesis with other cellular and molecular processes such as angiogenesis, glial scar formation, and inflammation responses, it is possible that more effective therapies could be developed in the future to improve regeneration and functional recovery of the ischemic brain.

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Chapter 5
Mobilization of Endogenous Neural Stem Cells to Promote Regeneration After Stroke

Monika Rabenstein and Maria Adele Rueger

Abstract Endogenous neural stem cells (eNSC) in the adult brain mainly reside in two stem cell niches, the subventricular zone (SVZ), and the hippocampal dentate gyrus. Following cerebral insults, they are mobilized from their niches to engage in regeneration and mediate functional recovery. After cerebral ischemia, eNSC generate new neurons in a process called neurogenesis, but also indirectly mediate regeneration via pleiotropic functions including neuroprotection, reduction of neuroinflammation, revascularization, and induction of plasticity. However, the physiological capacity of the brain for self-repair after stroke is insufficient in mammals. Thus, a promising therapeutic approach in stroke constitutes the targeted activation of eNSC by pharmacological substances, e.g. osteopontin or FGL, and by non-pharmacological approaches, such as transcranial direct current stimulation (tDCS). Since treatments based on the transplantation of stem cells harbor several disadvantages including poor long-term cell survival and a lack of integration into the host circuitry, mobilizing the eNSC niche for therapeutic purposes constitutes a most promising approach in stem cell research.

Keywords Osteopontin • FGL • Ar-tumerone • Transcranial direct current stimulation (tDCS) • Recovery • Neurogenesis • Neuroprotection • Plasticity • Functional recovery

Abbreviations

BrDU	Bromodeoxyuridine
CNS	central nervous system
DCX	Doublecortin

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P.A. Lapchak, J.H. Zhang (eds.), *Cellular and Molecular Approaches to Regeneration and Repair*, Springer Series in Translational Stroke Research,
https://doi.org/10.1007/978-3-319-66679-2_5

EAE	Experimental autoimmune encephalomyelitis
EGF	Epithelial growth factor
eNSC	Endogenous neural stem cells
FGF 2	Fibroblast growth factor 2
FGL	Neural cell adhesion molecule FG Loop
NCAM	Neural cell adhesion molecule
OPN	Osteopontin
PET	Positron-Emission-Tomography
RMS	Rostral migratory stream
SGZ	Subgranular zone of the hippocampus
Shh	Sonic hedgehog
SVZ	Subventricular zone
tDCS	Transcranial direct current stimulation

1 Introduction

For a long time it has been assumed that neurogenesis does not occur in the postnatal brain. This paradigm was opposed by Altman et al. [1], who first described the ability of the postnatal mammalian brain to generate new neurons in the postnatal rat hippocampus, and by Kaplan et al., who essentially discovered neurogenesis in the adult dentate gyrus [2]. They found labeled neurons using radioactive thymidine to label all dividing cells and histological examination of postmortem brains. This proved that new neurons in the adult brain are generated following cell division. The extend of neurogenesis declines in adulthood, but remains in a significant steady-state. Subsequent studies showed that in addition to the hippocampal dentate gyrus, immature precursor cells also persist in the subventricular zone of the lateral ventricles [3, 4] and are capable of differentiating into all three cell fates of the central nervous system (CNS): neurons, astrocytes and oligodendrocytes [5–9].

2 Endogenous Neural Stem Cells Under Physiological Conditions

2.1 Neural Stem Cells in Animals Models

Endogenous neural stem cells (eNSC) in the adult brain mainly reside in two stem cell niches: the subventricular zone (SVZ) adjacent to the lateral ventricles, and the subgranular zone of the hippocampus (SGZ). These stem cell niches are part of two distinct neuronal networks: eNSC from the SVZ can migrate via the rostral migratory stream (RMS) to the olfactory bulb. ENSC from the SGZ give rise to neurons and glia cells in the hippocampal dentate gyrus (reviewed by [10, 11]).

Under physiological circumstances, eNSC are mostly in a quiescent state. Interestingly, driven by Sonic hedgehog (Shh) signalling, they are able to self-renew over the course of a year and generate multiple cell types in vivo such as (inter) neurons, astrocytes or oligodendrocytes [12]. Furthermore, neurogenesis seems not be restricted to the SVZ and SGZ. There have been several reports of adult neurogenesis in the neocortex, the striatum, the amygdala, the hypothalamus, the substantia nigra, in the brain-stem, olfactory tubercle and piriform cortex. However, data about neocortical neurogenesis remains conflicting (reviewed by [13]). Inside the stem cell niches, eNSC reside in close connection to blood vessels. Extending this anatomical connection, endothelial vascular cells stimulate the self-renewal of neural stem cells. Even activated eNSC maintain their connection to blood vessels. This microenvironment is referred to as the neurovascular niche [11, 14].

Hippocampal neurogenesis seems to play an important role in learning and memory. Exercise and an enriched environment increase hippocampal neurogenesis and thus improves learning abilities [15–17]. Importantly, while eNSC numbers and neurogenesis decline during aging [18, 19], the capacity of the remaining eNSC to respond to cerebral insults seems stable over most of the life-span [20].

Moreover, eNSCs secrete trophic factors supporting neuroprotection such as glial-derived neurotrophic factor, vascular endothelial growth factor, or Shh [21, 22]. Additionally, eNSCs promote other regenerative processes including remyelination, angiogenesis, remodeling, and immunomodulation [23, 24].

2.2 Neural Stem Cells in Humans

The knowledge of eNSC derived neurogenesis in humans is restricted by the limited amount of detection methods (reviewed by [25]). The first study reporting about neurogenesis in the human hippocampus examined human brain tissue that was obtained postmortem from patients who had been treated during a cancer treatment with the thymidine analog, bromodeoxyuridine (BrdU), that labels DNA during the S phase [26]. Another method to estimate human neurogenesis was established by Jonas Frisén's group by measuring the concentration of nuclear bomb test-derived 14C in genomic DNA [27–29]. Though, limitations of this technique are the demanding infrastructure and the natural decline of 14C levels [28].

In humans, the rostral migratory stream is organized around a tubular extension of the lateral ventricle that reaches the olfactory bulb [30]. However, neurogenesis in the olfactory bulb in humans seems not to reach relevant levels [31]. On the other hand, relevant human adult hippocampal neurogenesis with an estimated number 700 new neurons are added per day was described [27]. Moreover, integration of newborn neurons into the striatum was observed in humans [32]. Notably, in most cases, the intrinsic response of eNSCs is obviously not sufficient to lead to detectable neocortical neurogenesis after stroke [33]. Of note, all of the latter results were obtained by 14C measuring. This method can only detect larger numbers of new cells that were generated at a given time point with a detectable limit at about 1% of the total

population of neurons [29]. Therefore, neurogenesis occurring at low levels, and new neurons that are not permanently integrated into the circuitry, may not be tracked using this method. This may explain contradictory data about neocortical neurogenesis occurring after stroke, which was reported to be present in immunocytochemical analyzes of human postmortem brain slices [34]. Additionally, some reports found cortical neurogenesis in small numbers in stroke animal models [35, 36].

3 Neural Stem Cells After Ischemia

Stroke is one of the major causes of adult disability [37]. To date, re-perfusion treatment is only possible in a narrow time window, and there is no neuroprotective or even regenerative treatment for the subacute or chronic phase after stroke yet. Thus, current treatment in this phase is limited to functional treatment such as physiotherapy. From the pathophysiological point of view, after the initial ischemic damage with disruption of the blood flow that leads to necrotic cell death, brain resident immune cells such as microglia and astrocytes are rapidly activated, and blood-borne immune cells (granulocytes, T-cells, monocytes/macrophages) are recruited from the blood stream to the lesion site [38–43]. This process is called neuroinflammation. There are many beneficial effects of neuroinflammation such as containment of necrotic damage, trophic support, support of neurons and mobilization of endogenous stem cells [44]. But on the other hand, persistent neuroinflammation can also cause secondary tissue damage by excessive release of proinflammatory cytokines and reactive oxygen species [45].

The immune cells attract eNSC to the site of the lesion by secretion of various inflammatory cytokines such as stromal cell-derived factor-1, tumor necrosis factor-alpha, and interferon-γ [46–49]. This attraction of eNSC is also referred to as mobilization of eNSCs. In various models of cerebral ischemia in experimental animals, including transient global ischemia, transient focal ischemia, or permanent focal ischemia, a mobilization of eNSC was demonstrated [50–53]. However, this eNSC mobilization is not sufficient to provide functional recovery, because the majority of newly generated neuroblasts in ischemic stroke models die by the time they have reached the peri-infarct area [52]. Moreover, in humans, no relevant neocortical neurogenesis in humans was detected after stroke [33].

4 Mobilizing the Endogenous NSC Niche

Since the endogenous neural stem cell response after stroke is not strong enough for sufficient repair processes, boosting the eNSC response by pharmacological or non-pharmacological methods constitutes a promising therapeutic approach for stroke. In contrast, the transplantation approach of “exogenous” cells is associated with certain disadvantages like poor long-term cell survival, a lack of integration into the

host circuitry, immune reactions against the transplants, and limited availability of appropriate cells (reviewed by [54]). Mobilizing the endogenous neural stem cell niche overcomes those difficulties and is additionally less invasive.

4.1 Pharmacological Mobilization of Endogenous Neural Stem Cells

ENSCs can be mobilized for therapeutic purposes by different types of drugs. One group of substances consists of stem cell growth- and regulation factors that target specific intracellular signaling pathways: Early studies reported that intraventricular treatment with fibroblast growth factor 2 (FGF 2) and epithelial growth factor (EGF) stimulates the proliferation of eNSCs in vivo [55, 56]. Intraventricular co-treatment with FGF 2 and EGF increases the number of hippocampal pyramidal neurons after cerebral ischemia by enhancing eNSC proliferation, and their differentiation into neurons [57]. Likewise, augmenting long-term FGF2 expression in rats after stroke increases SVZ and cortical neurogenesis and behavioral outcome [35]. Notch signaling is an important signaling pathway in eNSC and evokes pleiotropic effects in stem cells. Notch receptor activation promotes the survival of neural stem cells. Transient administration of Notch ligands to the brain of adult rats increases the numbers of newly generated precursor cells and improves motor skills after ischemic injury [21]. Moreover, angiopoietins are significant regulators of endothelial and hematopoietic stem cells. Angiopoietin2 rescues injured dopamine neurons with motor behavioral improvement in an experimental model of neurodgeneration [58]. The neural cell adhesion molecule (NCAM) enhances neurite outgrowth, synaptogenesis, and neuronal differentiation. Its mimetic peptide FG Loop (FGL) induces NSC mobilization in vitro and in vivo, and supports oligodendroglial differentiation [59]. After focal cerebral ischemia, FGL mobilizes eNSC from the niches and enhances regeneration by amplifying remyelination and modulating neuroinflammation via affecting microglia [60]. Another important eNSC signaling pathway is initiated by the ligand sonic hedgehog. Jin et al. showed that oral administration of a sonic hedgehog agonist increased functional recovery, neurogenesis and angiogenesis after experimental stroke [61].

In a second pharmacological approach, eNSC mobilization can also be induced by certain nutrition ingredients: Curcumin and ar-turmerone are the major bioactive compounds of the herb Curcuma longa. Ar-turmerone induces NSC proliferation in vitro and promotes neuronal differentiation of eNSC. Concordantly, there was also increased proliferation and mobilization of eNSC in vivo as shown by Positron-Emission-Tomography (PET) [62].

A third group of drugs mobilizing eNSC are endogenous or exogenous factors that are involved in (neuro-)inflammation: Osteopontin (OPN) is an endogenous phosphoglycoprotein with important roles in tissue homeostasis, wound healing, immune regulation, and stress responses. OPN increases survival, proliferation, migration, and neuronal differentiation of eNSC. Increased survival and migration

are mediated via the chemokine receptor CXCR4. After cerebral ischemia, OPN increases neurogenesis [63] (Fig. 5.1). Additionally, OPN seems to polarize microglia to a neuroprotective subtype in an inflammation setting [64]. The tetracycline antibiotic minocycline is commonly used to treat bacterial infections. Additionally, it has pleotropic effects on immune processes [65, 66]. In stem cells, minocycline enhances cell survival in vitro, and increases eNSC activity in both the SVZ as well as the hippocampus in animals after experimental stroke [67]. Additionally, minocycline antagonizes the rapid glial differentiation induced by proinflammatory cytokines in vitro, and restores the neurogenic and oligodendrogenic potential [68].

4.2 Non-pharmacological Mobilization of Endogenous Neural Stem Cells

Clinical data suggest that transcranial direct current stimulation (tDCS) may facilitate rehabilitation after stroke [69, 70]. However, the neurobiological mechanisms underlying tDCS remain poorly explored. TDCS can be applied with either with an anodal or a cathodal current polarity, and with various current densities. In the healthy rat brain, certain polarities and current densities of tDCS increase neural stem cell migration and activate microglia [71, 72]. Under specific conditions, tDCS accelerates functional recovery in animals after experimental stroke. Moreover, both anodal and cathodal tDCS at different current densities induce neurogenesis (Fig. 5.2). Only cathodal tDCS recruits oligodendrocyte precursors towards the lesion, but also supports a proinflammatory M1-polarization of microglia. In contrast, anodal tDCS leads to downregulation of the constitutive expression of Iba1 by microglia [73, 74]. In conclusion, the different tDCS polarities seem to exert different effects on eNSC as well as on migroglia. TDCS acts through multifaceted mechanisms that far exceed its primary neurophysiological effects, encompassing proliferation and migration of stem cells, their neuronal differentiation, and modulation of microglia responses.

5 Future Perspectives

ENSC can be targeted by pharmacologial or non-pharmacological approaches. Thereby, enhancement of eNSC proliferation, migration and differentiation to neurons and oligodendrocytes is possible in order to promote neuroregeneration and functional recovery. As a second step, a translational approach to establish these therapies in clinical treatment for humans is needed. Such clinical trials could include osteopontin (OPN) that modulates eNSC as well as immune cells, and can be applied via a nose spray [75]. As for non-pharmacological approaches, tDCS is already applied experimentally in the clinical setting [69, 70]. With more knowledge

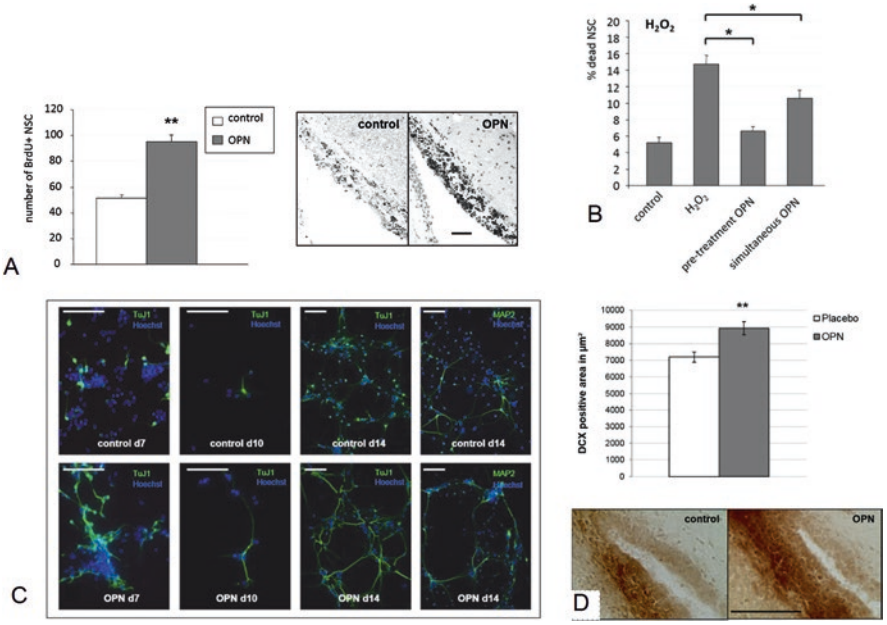

Fig. 5.1 Osteopontin (OPN) increases survival, proliferation and neurogenesis of neural stem cells (NSC). (**a**) Adult male rats injected with a single dose of 500 µg OPN i.c.v. displayed a significantly higher number of proliferating NSC in the SVZ as the major NSC niche, corroborating the effects of OPN on NSC proliferation in vivo (values displayed as means ± SEM; $^{**}p < 0.01$). Representative images from the SVZ of rats treated with either placebo (*left*) or OPN (*right*; scale bar represents 200 µm). (**b**) NSC cultures were exposed to oxidative stress by H_2O_2 (300 nM for 24 h), increasing cell death as assessed by propidium iodide staining. Pre-treatment of NSC with 6.25 µg/ml OPN 24 h prior to oxidative stress completely rescued NSC from this toxicity, while simultaneous addition of OPN and H_2O_2 prevented about half the cells from dying (values displayed as means ± SEM; $^{*}p < 0.05$). (**c**) Generation of TuJ1-positive neurons (*green*) during differentiation was increased by OPN treatment (*lower row*) as compared to control (*upper row*) at days 7, 10, and 14 after mitogen withdrawal. During that period, the axon length grew notably and neurons began to form networks; both observations were more pronounced in OPN-treated cells. By day 14, mature MAP2+-positive neurons had formed (*right column*; scale bars represent 100 µm). (**d**) Osteopontin (OPN) promoted neurogenesis after stroke in vivo. In adults rats that underwent photothrombosis, a single i.c.v. injection of 500 µg OPN significantly increased the area covered by DCX-positive neuroblasts in the SVZ (values are displayed as means ± SEM, $^{**}p < 0.01$). Representative, DCX-stained images from the SVZ of rats subjected to cerebral ischemia, treated with either placebo (*left*) or OPN (*right*). OPN treatment led to an increase of neuroblasts in the SVZ (scale bar represents 100 µm). Adapted from Rabenstein et al. [63] with permission

about the neurobiological and polarity-dependent effects, a better targeted use of tDCS in the clinic could be archived. Currently, in an animal model of experimental autoimmune encephalomyelitis (EAE), inhibition of Gli1—a transcriptional effector of the sonic hedgehood pathway—improves the functional outcome and offers neuroprotection. This inhibition can be achieved by intraventricular application of

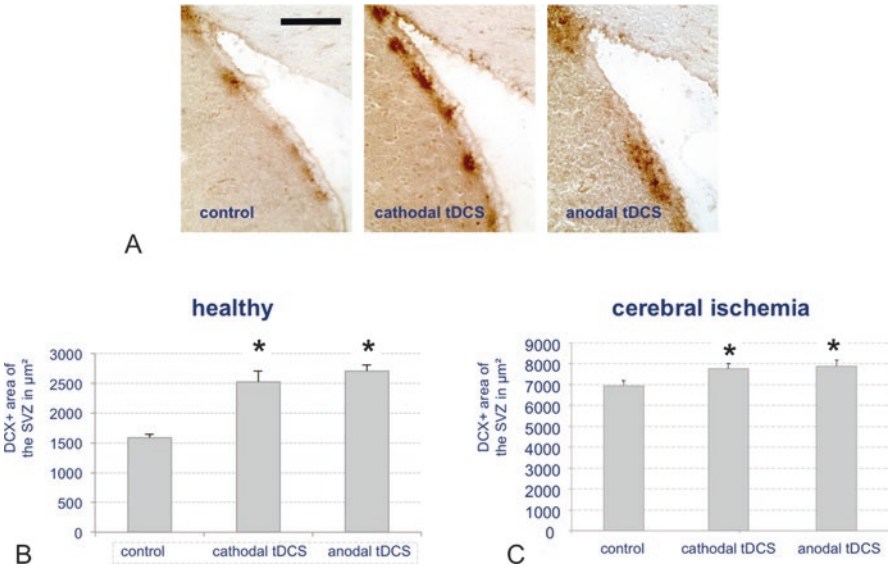

Fig. 5.2 Multisession tDCS induced neurogensis in the subventricular zone (SVZ). (**a**) Representative, DCX-stained images from the SVZ of mice, treated with either sham (*left*), cathodal tDCS (*middle*) or anodal tDCS (*right*). Multisession cathodal or anodal tDCS at 99 kC/m^2 increased DCX immunoreactivity in the SVZ (scale bar represents 100 µm). Multisession cathodal or anodal tDCS increased the number of DCX+ neuroblasts in the SVZ of control animals (**b**) and rats subjected to cerebral ischemia (**c**) (values are displayed as means ± SEM, *$p < 0.05$). Adapted from Pikhovych et al. [74] and Braun et al. [73] with permission

GANT61, a small molecule inhibitor of Gli12 [76]. This pathway might be interesting for targeted eNSC activation after stroke as well. However, for clinical application a less invasive application methods needs to be found.

Most importantly, in order to translate experimental findings into the clinical setting, translational read-outs need to be advanced to non-invasively monitor treatment effects. Moreover, it is crucial to learn more about physiological human neurogenesis, to then evaluate the treatment efficacy of eNSC mobilization in humans. In this, MRI detection methods require invasive labelling to specifically detect eNSC: This can be achieved either by direct intraventricular injection of labels or viral- or antibody-coupled labels, thus all of these methods are not applicable in humans. Another option is PET-imaging with the radiotracer 3′-deoxy-3′-[^{18}F]fluoro-L-thymidine that labels proliferating cells. This approach offers a promising method to noninvasively quantify eNSC in the live brain [77, 78].

Taken together, targeted activation of eNSC by pharmacological substances, e.g. stem cell regulating factors or osteopontin, and by non pharmacological approaches such as transcranial direct current stimulation (tDCS), constitute a promising approach to facilitate regeneration and enhance recovery after stroke.

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Chapter 6
Transcriptional and Genomic Advances on the Pathophysiology of Stem Cell Repairment After Intracerebral Hemorrhage

Sheng Zhang, Yongjie Zhou, and Yujie Chen

Abstract Intracerebral hemorrhage is a life-threatening disease characterized by a sudden rupture of cerebral blood vessels, and it is widely believed that neural cell death occurs after exposure to blood metabolites or subsequently damaged cells. Based on these disappointing results of 1026 neuroprotective agents, researchers turned their interests on neurogenesis, which is traditionally considered as an endogenous neuroprotective mechanism after acute central nervous system injuries. However, because of complexity in stem cell survival, migration, differentiation, and maturation, current strategies have either been proved unsatisfactory or resulted in serious side effects during clinical translation. It is well known that transcriptional and genomic pathways play important roles in ensuring the normal functions of stem cells, including proliferation, migration, differentiation and neural reconnection. And reprogramming technology and other non-invasive electromagnetic stimulation were recently employed and proved effective for the stem cell characteristics. Therefore, in the present chapter, we sought to summarize the advances in the pathophysiology and strategies of stem cell repairment after ICH at the level of transcription and genome, hoping to provide potential sparks for better stem cell repairment for ICH patients.

Keywords Stem cell • Intracerebral hemorrhage • Transcriptome • Genome • Bioinformatics • Reprogramming technology • Neurological recovery

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P.A. Lapchak, J.H. Zhang (eds.), *Cellular and Molecular Approaches to Regeneration and Repair*, Springer Series in Translational Stroke Research,
https://doi.org/10.1007/978-3-319-66679-2_6

Abbreviations

ICH	Intracerebral hemorrhage
LncRNA	Long non-coding RNA
miRNA	MicroRNA
NSC	Neural stem cell
SPIO	super-paramagnetic iron oxide
STICH	Surgical Trial in Intracerebral Hemorrhage
SVZ	subependymal ventricular zone
US FDA	United States Food and Drug Administration

1 Introduction

Intracerebral hemorrhage (ICH) often leads to high mortality and morbidity [1, 2]. Despite long-standing and worldwide efforts, no effective neuroprotective strategies are available to improve the neurological outcomes in patients with stroke, including ICH. Although surgical decompression for cerebral hemorrhage benefits the survival of patients, but the long-term outcome is still not improved. A famous clinical trial, Surgical Trial in Intracerebral Hemorrhage (STICH, Phase I and II) has failed to provide enough evidence to support early surgical hematoma removal comparing to conservative therapy [3, 4]. And the recombinant activated factor VII significantly reduces hematoma volume without improving survival or functional outcomes in ICH patients [5]. Therefore, potential therapeutic strategies targeting secondary brain injury are attracting a lot of attentions in translational studies of intracerebral hemorrhage [6, 7].

Secondary brain injury is triggered by blood metabolites which subsequently activate cytotoxic, excitotoxic, reactive oxygen species-related, and inflammatory-mediated pathways, and so on. Nevertheless, according to a systemic review, 1026 neuroprotective agents works for the neurological outcomes in animal stroke model, have failed to exhibit convincing clinical benefits [8, 9]. Based on these disappointing results, researchers turned their interests on neurogenesis, which is traditionally considered as an endogenous neuroprotective mechanism after acute central nervous system injuries. Previous studies indicate neurogenesis occurs after ICH to repair the brain lesions and restore brain connections [10–12]. In addition, researchers have made great efforts to transplant exogenous stem cells to the brain lesions from different sources. However, because of complexity in stem cell survival, migration, differentiation, and maturation, current strategies have either been proved unsatisfactory or resulted in serious side effects during clinical translation [13–15]. Therefore, in the present chapter, we sought to summarize the advances in the pathophysiology and strategies of neurogenesis after ICH at the level of transcription and genome, hoping to provide potential sparks for better stem cell repairment for ICH patients.

2 Current Situation of Stem Cell Repairment After Intracerebral Hemorrhage

2.1 Potential Neuroprotective Effects of Stem Cell Repairment

Back to 1998, Eriksson PS, et al. found the nascent neurons in hippocampus tissue from post mortem cancer patients [16, 17], providing the first evidence for human neurogenesis [16]. More recently, Spalding, et al. retrospectively marked the hippocampal cells in post mortem patients exposed to nuclear testing before death with the ratio of 14^C to 12^C in DNA, and found that the turnover rate of newborn neurons in the dentate gyrus could be as high as 700 per day [18]. Later, by using two-photon microscopy, Shen J, et al. obtained specimens from patients with hypertensive ICH and found that neural stem cell (NSC)-specific proteins and cell proliferation markers were localized in the perihematomal areas of basal ganglia and the parietal lobe [10]. These data suggest that ICH could induce de novo neurogenesis in the adult human brain, potentially with the ability to repair brain lesions and improve neurological outcome in patients suffered with ICH.

Similar with other acute central nervous system injuries, the preclinical studies of stem cell repairment strategy were gradually carried out to support the beneficial role of stem cell after intracerebral hemorrhage including proliferation, migration, and differentiation. Back in 2004, Tang T, et al. found Nestin-stained or BrdU-labeled cells were mainly located in the basal ganglion and nearby SVZ around hematoma and ependyma after ICH in rats. Additionally, no cells positive for these markers were found in control or sham groups or in non-lesioned parenchyma [19]. Masuda T, et al. injected BrdU for 2 weeks after ICH in rats and found that BrdU-labeled cells significantly increased in both the contralateral and ipsilateral SVZs. Meanwhile, doublecortin-positive, immature, and migratory neurons were also seen in the dorsal striatum and peri-hematoma area 2 weeks post-ICH. In addition, they also noticed clusters of doublecortin-stained cells in the striatum surrounding the hemorrhagic lesion 4 weeks post-ICH. These findings implicate that experimental ICH induces the proliferation and migration of endogenous NSCs to repair the hemorrhagic lesion [12]. In addition to endogenous NSCs, exogenous NSC transplantation also exhibits the potential to attenuate neurological deficits after hemorrhagic stroke. In 2003, Jeong SW, et al. intravenously transplanted human NSCs into experimental ICH rats. Their results indicated that NSCs can cross blood brain barrier and enter the rat brain with ICH. Interestingly, those surviving NSCs in the rat brain helped with the functional recovery [20]. Another investigation transplanted alltrans retinoic acid-induced NSCs into the contralateral ventricle up to 7 days after ICH, and found new neurons and astrocytes surrounding the hematoma lesions of the brain 4 weeks later in all rats receiving the transplantations [21]. Moreover, these results were confirmed by super-paramagnetic iron oxide (SPIO)—labeled human NSCs detected by 3T Magnetic Resonance Imaging, which indicated the presence of prominent NSCs in the periventricular

region at 4 and 6 weeks post-transplantation [22]. Most importantly, compared with the control group, the NSC-transplanted rats exhibited excellent functional performance on neurofunctional tests after 2–8 weeks, which indicates that the exogenously supplied NSCs may be used for the functional recovery after hemorrhagic stroke [23].

In addition to NSCs repairment strategies, other types of stem cell were also demonstrated positive in neuroprotective after ICH. In 2009, Liao W, et al. human umbilical cord-derived mesenchymal stem cells were tested for the ICH rat model by intracerebrally transplantation, they found the neurological recovery was enhanced, potentially due to neuroinflammation inhibition and angiogenesis [24]. Similarly, Liu, AM. et al. also demonstrated the umbilical cord-derived mesenchymal stem cells could enhance remyelination and functional recovery in ICH rat model [25]. After them, adipose-derived stem cells [26], bone marrow stromal cells [27], human bone marrow-derived mesenchymal stem cells [28–30], and bone marrow-derived mononuclear cells [31] were proved to be benefit for ICH models one and another. More recently, induced pluripotent stem cells were transplanted into ICH animal models, which could improve the neurological function as expected [32, 33]. According to a meta-analysis, 30 studies using five different type of stem cells showed consistent improvements in neurobehavioral function, but the extensive potential of stem cells repairment for ICH should be further evaluated with more high-quality preclinical studies and clinical trials [34].

2.2 Limitations of Stem Cell Therapy

Despite the potential neuroprotective effects of different stem cells, a lot of factors could influence the efficacy of stem cell repairment for ICH patients, such as intervention timepoint, administration routes, microenvironment of stem cell, the source and status of stem cells, and possible immune responses. Other factors including but not limited to metabolism regulators, epigenetic modifiers, vascular constrictors or dilators, modulators of immune response, activators or inhibitors of signal transduction pathways. Moreover, proliferation, differentiation, maintenance and self-renewal of stem cells in niche are controlled by a network of intrinsic and extrinsic regulators, such as neurotrophins, cyclins and cyclin-dependent kinases, transcription factors. These factors act in concert within their biological network during the establishment and maintenance of neural connections. Epigenetic modulations during hippocampal development can also have impacts on one's learning and memorizing abilities. Genetic polymorphism in genes involving neurogenesis may have essential roles in variations of stem cell differentiation between individuals in adult neural regeneration [35]. Thus, analysis and manipulation of favorable genetic variations for neurogenesis may have the vital practical significance for ICH therapeutic implications [35].

3 Transcriptional and Genomic Advances on the Pathophysiology of Stem Cell Repairment

3.1 Genomic Analysis for the Stimulation of Endogenous NSCs

In mammals, new neurons are constantly generated in the subependymal ventricular zone and subgranular zone of the dentate gyrus throughout developmental stage and adult life. This continuous neurogenesis after birth may be important in processing information, daily learning and memorization etc. During hippocampal neurogenesis, doublecortin-positive immature neurons and neuronal precursor cells mature into neurons. In the immature stage, cells are sensitive and susceptible to extrinsic stimuli. However, knowledge on the dynamics which lead to neuron maturation is limited. Moreover, to date, purification of NSCs in vitro proves to be a challenging task to allow for investigation of their biology and application in clinical medicine.

Due to the development of "omics" technology, emerging evidence has demonstrated that both transcriptional and genomic pathways play important roles in ensuring the normal function of stem cells. At the transcriptional level, sequence-specific transcription factors and coregulators work together to orchestrate the transcriptional landscape of stem cells, which determines the on/off state of target genes, thereby controlling the cell fate of stem cells. At the genomic level, the replication and repair machineries maintain the genomic stability of stem cells. By examining gene expression at single cell level using RNA-seq technology, Gao Y, et al. found that there existed two subgroups among immature neurons with distinct gene expression profiles and different molecular markers. Comparisons of the two subgroups indicated that Notch and Sonic Hedgehog and the Hippo pathways are all important in neuron maturation and NSC activity [36, 37].

Factors which form a regulatory network to support NSC self-renewal has not been fully elucidated up to now. Understanding of the key transcription factors, the promoter region and other non-coding regions that they bind, and the target genes that they regulate, will be essential in unleashing the full potential of these cells for therapeutic use. At the center of this regulatory network are SOX family and FOX family transcription factors, nuclear factor I, basic helix-loop-helix transcription factor family. Coordinated action of these factors to promote proliferation and at the same time prevent untimely differentiation and quiescence is crucial to NSC self-renewal [38]. By analyzing the region specific regulatory networks based on available published databases on subependymal ventricular zone and subgranular zone, Mateo JL, et al. discovered the potential microenvironment associated differences based on membrane and nuclear receptors via HIF-1α, Ar, and NR3C1. They also performed cell fate determinant test for NSCs from subependymal ventricular zone to the interneurons of olfactory bulb and NSC populations from subgranular zone to the granule cells of the granular cell layer. The existence of membrane and nuclear

receptors in this region-specific regulatory network shows the importance of niche-derived extracellular molecules and region-specific factors for the neurogenesis in subependymal ventricular zone and subgranular zone [39].

Genomic approaches in modern time have facilitated unprecedented advances in our understanding of the development, function and evolution of central nervous system. By contrast, little is recorded or published about the possible interplay between different genetic factors, epigenetic modulators, non-coding RNAs and environmental factors in causing or modulating neurological disorders in populations from under-developed countries [40]. Both pharmacological intervention and genetic manipulation of epigenetic modulators can trigger profound changes in molecular expression, neuron identity and complex behavioral and cognitive phenotypes. Apparently, epigenetics plays a non-trivial role in the pathogenesis of neurological disorders. Emerging paradigms in possible connections between epigenetics and hemorrhagic stroke include the followings: how gene mutations of epigenetic factors induce hemorrhagic stroke; how is genetic polymorphism of epigenetic factors linked to disease risk of hemorrhagic stroke; how changes in the expression, localization, or function of epigenetic factors affect hemorrhagic stroke; how epigenetic factors modulate disease-linked genomic loci, protein expression and cellular pathways; and how differential epigenetic profiles from patient-derived tissue samples affect disease outcome [41].

3.2 Microenvironment Complexity for the Exogenous Stem Cell

Since the sole neuron protective strategies could not exhibit satisfied outcome in clinical trials, neural vascular coupling that is accepted as Neural Vascular Unit and Vascular Neural Network provides crucial guiding direction for exploring latent mechanisms of other participators near neuron and their associating with numerous central nervous system diseases [42]. A complex network of elements, consisting of macromolecules of the extracellular matrix, glial cells, astrocytes, oligodendrocytes, adhesion molecules for cell-cell and cell-extracellular matrix connections, blood vessels, neurotrophins, and so on. All of them have an impact on tissue homeostasis and maintenance of a homing microenvironment for stem cells in central nervous system. Among these components, extracellular matrix derived from stem cells provides a unique and indispensable microenvironment that helps with stem cell differentiation and neural regeneration. Analysis of protein expression by two-dimensional gel electrophoresis and liquid chromatography-tandem mass spectrometry provided proteomic profiles that corresponded to unique niche properties for each group tested. Proteomic results demonstrated that NSC-derived extracellular matrix can impact the decision-making process of stem cell fate by offering microenvironment for specialized stem cell niches in the process of tissue development and regeneration [43].

Metabolites and nutrients in the bloodstream fulfil the energy demands of adult stem cells. However, more context-specific roles have been shown for several circulating factors. An array of diffusible, non-diffusible and circulating cues influences stem cell quiescence, proliferation, self-renewal and differentiation. A key question is how stem cells integrate all of these signals, particularly since they can have overlapping or antagonistic effects. Some ligands, such as PEDF and BTC, have multiple sources [44–46]—does the source influence the effect on stem cells? Most of neurovascular studies have focused on endothelial cells. The difficulty in identifying pericytes in vivo means that their contributions to NSC behavior are only just emerging [47]. How do pericytes and astrocytes interact with the endothelial cell-NSC crosstalk? Advances in single cell transcriptional profiling, as well as cell type-specific tools for manipulating cells in vivo, will no doubt shed light on these questions.

MicroRNA (miRNA) is a recently discovered group of small, genome-encoded endogenous RNAs that are transcribed but are not translated into proteins [48]. It was reported to play an key regulatory role in many cellular functions, including cell growth, proliferation, differentiation, lineage determination and metabolism [49]. In central nervous system, miRNA was well established to be vital in neurogenesis [50], neural development [51], differentiation [52] and synaptic plasticity [53].

Exosomes are types of nano extracellular vesicles, naturally released from different kinds of living cells, can be taken up by recipient cells [54]. Faure et al. described the release of exosomes by neuron in vitro [55]. And Taylor et al. reported the secretion of exosomes by astrocytes, also in vitro [56]. Furthermore, exosomes can also be found in cerebral spinal fluid in animal models [57, 58], which might be a reminder of its vital role in brain physiologies. Exosome has been recently known as intermediate link in cell-cell communication, with the capability of transferring the proteins, DNAs, microRNAs, non-coding RNAs and lipids with or without direct contact [59]. Xin et al. demonstrated exosomes secreted by mesenchymal stem cells can transfer microRNA-133b into neurons, resulting in the induction of synaptic growth [60]. Morel et al. showed that neuron secretes microRNA-124a via exosomes, which subsequently transport into astrocytes, then indirectly increasing protein expression [61]. Taken together, these finds support the hypothesis that exosome could mediate cell–cell communication in central nervous system. But, emerging evidences suggest exosome based interventions and technologies maybe promising in central nervous system disease diagnostic [62–65]. And their simple structure and ability to cross the blood-brain barrier makes exosome-based cell and gene therapies a bright prospects in the future [66–69].

Long non-coding RNA (LncRNA) is a classic non-coding RNA with transcript frame longer than 200 nucleotide, usually in cytoplasm or nuclear, being well transcripted but lack of protein-coding capability [70]. At the beginning, LncRNA was considered to be the accessory substance of RNA polymerase II transcription, with no meaningful biological function. But, growing evidences suggests LncRNA maybe prevalent in cerebral cortex, hippocampus and olfactory bulb in central nervous system, involve in cell differentiation, cellular transportation, cell-cycle

regulation, stem cell reprogramming and other molecular genetical and cellular functions [71], and play an important role in various pathologies including ICH and other central nervous system diseases [72, 73].

4 Assistant Approaches for Stem Cell Repairment of Intracerebral Hemorrhage

4.1 Reprogramming Technology

Cellular reprogramming technology has created new opportunities in understanding human disease, drug discovery, and regenerative medicine. While a combinatorial code was initially found to reprogram somatic cells to pluripotency, a "second generation" of cellular reprogramming involves lineage-restricted transcription factors and microRNAs that directly reprogram one somatic cell to another. This technology was enabled by gene networks active during development, which induce global shifts in the epigenetic landscape driving cell fate decisions. A major utility of direct reprogramming is the potential of harnessing resident support cells within damaged organs to regenerate lost tissue by converting them into the desired cell type in situ.

Reprogramming technology enables the production of neural progenitor cells from somatic cells by direct trans-differentiation. However, little is known on how neural programs in these induced neural stem cells differ from those of alternative stem cell populations in vitro and in vivo. It was reported that transcription factor-mediated reprogramming can efficiently convert differentiated cells into induced pluripotent stem cells, then theoretically possible to obtain a large number of neural stem cells in vivo or in vitro for the transplantation. However, the mechanisms related to the amenability of these cell types to be reprogrammed are still unknown, and tumors are likely to form due to the presence of residual undifferentiated cells following transplantation of the induced cells. Therefore, future clinical application of reprogramming technique for stem cell replacement after ICH are still needed to be improved with transcriptional and genomic analysis of proper artificial manipulations.

Although there were not much practices of transcriptional and genomic analysis for the reprogrammed stem cells for ICH patients. Hallmann AL, et al. performed comparison on murine brain-derived NSCs and pluripotent stem cell-derived neural progenitor cells, which revealed distinct global, neural, metabolic and cell cycle-associated marks in these two populations, with significant implications for the applications of induced NSCs [74]. And the genome-wide gene expression analyses reveal unique cellular characteristics related to the amenability of hematopoietic stem cells into high-quality induced pluripotent stem cells [75]. In addition, chromatin connectivity networks analysis revealed the pivotal genes of reprogramming functions, which linked the chromatin architecture to coordinated gene expression

in embryonic stem cells [76]. Furthermore, the network-based approaches could also be used to identify potential targets and drugs for neuroprotection and neurorepair after stroke [77].

4.2 Non-invasive Electromagnetic Field Stimulation

Despite of these novel technologies, traditionally physical methods were employed and demonstrated benefit for the neurological recovery in central nervous diseases. Francis, et al. find that adult mice exposed to electromagnetic field can produces a significant enhancement in the number of new-born neurons in dentate gyrus [78–80]. BrdU and nestin-Corporation method shows us that electromagnetic field also can promote the number of and BrdU and nestin-positive cells within the area between subependymal ventricular zone and lesion 1 week after brain injury, which indicating that electromagnetic field exert a positive effect on proliferation and migration of neural stem cell [81]. Cuccurazzu, et al. also indicated electromagnetic field stimulation could promote adult hippocampal neurogenesis [82]. Meanwhile, Arias-Carrión, et al. demonstrated that transcranial magnetic field stimulation promoted neurogenesis in cells of subependymal ventricular zone for the nigrostriatal lesions [83]. More importantly, the repeated transcranial magnetic stimulation was already approved by US FDA for the treatment of depression. It may conventionally be used for the ICH patients with enough evidences for its neuroprotective effects and underlying mechanisms.

A number of miRNAs are the key factor that involved in determination of the stem cell fate, for instance, NSCs differentiation and proliferation [84–86]. Given to the significant effects of repeated transcranial magnetic stimulation exert on the expression of gene, this technology reminds us it is of great possibility to modulate miRNA in theory. Guo, et al. found that after 10 Hz repeated transcranial magnetic stimulation, cerebral ischemia rat model exhibited a remarkably enhancement of miR-25. Meanwhile, Brett, et al. demonstrated that the miR-106b/25 cluster mainly promoted the adult NSCs proliferation [87, 88]. In addition, Liu, et al. also performed experiments in the focal cerebral ischemia rat model, which demonstrated the miR-106b/25 cluster could increase the NSC proliferation in vitro after high frequency repeated transcranial magnetic stimulation in a dose dependent manner [89].

Meanwhile, more and more proof suggests that epigenetic mechanism especially chromatin modifications may act as a critical role in the modulation of differentiation and proliferation of NSCs [90, 91]. Leone, et al. demonstrated a marked increasing of the pro-proliferative gene Hes-1 and neuronal determination genes NeuroD1 and Neurogenin1 after exposing to electromagnetic fields [92]. And Hes1 is a repressive type of bHLH transcriptional factor that sustain the stemness for NSCs by the means of repressing pro-neural gene expression [93]. In contrast, inactivation of Hes1 means the effect repression of pro-neural genes weakened and correspondingly upregulates the expression of pro-neural genes, including Mash1,

Neurogenin1, and NeuroD1, result in acceleration of neuronal differentiation [94–96].

5 Perspective and Conclusion

In the past 20 years or so, multiple technologies have been developed to utilize the regenerative potential of stem cells and the plasticity of neural cells in central nervous system to repair brain lesions and improve structural and functional recovery after acute central nervous system injuries, including ICH [97]. Based on the pathophysiology of secondary brain injury after ICH, targets regarding prediction, diagnosis, treatment strategies, and neurofunctional recovery need to be further identified and verified in large cohorts of patients, especially those controlling stem cells at both the transcription and genomic levels (Fig. 6.1). Bioinformatics methods is a new field that focuses on the acquisition, storage and analysis of physiological and other data relevant to the bedside care of patients [98]. Advanced statistical and mathematical tools are now being applied to the large volume of clinical and physiological data routinely monitored in neurocritical care with the goal of identifying better markers of secondary brain injury and providing clinicians with an improved ability to target specific parameters in the management of ICH patients [99], which may provide much more information about therapeutic strategies for endogenous neurogenesis and exogenous stem cell transplantation for ICH patients.

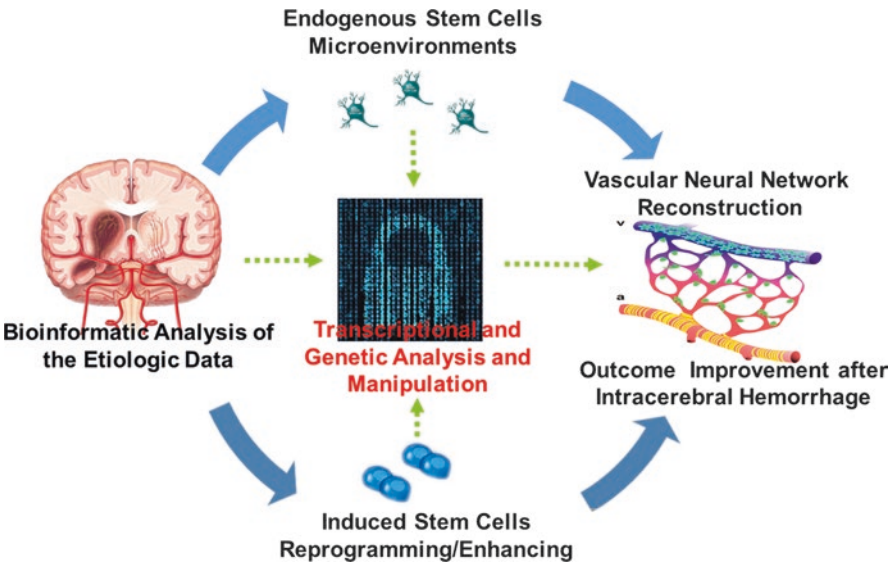

Fig. 6.1 Diagram of transcriptional and genetic analysis for applications of stem cells in intracerebral hemorrhagic

Acknowledgements This work was supported by Incubation Foundation of Interdisciplinary Laboratory of Physics and Biomedicine (Grant No. WSS-2015-08), Basic Science and Advanced Technology Research Project of Chongqing (Grant No. cstc2016jcyjA1730), National Natural Science Foundation of China (Grant No. 81501002, 81220108009) and National Basic Research Program of China (973 Program, Grant No. 2014CB541600).

Conflicts of Interest: The authors declared no potential conflicts of interest.

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Chapter 7
Modulation of Post-Stroke Plasticity and Regeneration by Stem Cell Therapy and Exogenic Factors

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Abstract Revascularization therapy in the acute post-stroke phase nowadays is reducing the grade of disability and mortality after cerebral ischemia. Post-acute to chronic therapeutic strategies in the phase of irreversible brain parenchyma damage showed until now controversial results in pre-clinical studies: currently there are no effective treatment strategies apart from neurological rehabilitation aiming at restoration of functional post-ischemic deficits.

Spontaneous functional recovery appears immediately after stroke and was proven to correlate with the endogenous regeneration potential represented by rewiring of

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P.A. Lapchak, J.H. Zhang (eds.), *Cellular and Molecular Approaches to Regeneration and Repair*, Springer Series in Translational Stroke Research,
https://doi.org/10.1007/978-3-319-66679-2_7

neuronal circuits through promotion of dendritic and axonal sprouting, improving axonal function, synaptogenesis, neurogenesis, and angiogenesis. These observations have led to numerous preclinical studies investigating a new therapeutic direction after stroke, the neurovascular restoration impacting stroke recovery potential.

This chapter summarizes achievements to date, current challenges and ongoing research in the field of regenerative processes after ischemic stroke, focusing on the formation of functional anatomical pathways responsible for enhanced recovery.

Keywords Stroke • Regeneration • Plasticity • Repair • Stem cells • Neural progenitors • Neurogenesis • Neuroprotection • Endothelial progenitors • Trophic factors

Abbreviations

AMPA	Alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
AQP	Aquaporin
ATSC	Adipose-tissue stem cell
BBB	Blood-brain barrier
BDNF	Brain-derived neurotrophic factor
BMSC	Bone-marrow derived stem cell
BrdU	5-Bromo-2′-deoxyuridine
CCL2	C-C chemokine ligand 2
CCR2	C-C chemokine receptor type 2
CM	Conditioned medium
CNS	Central nervous system
DCX	Doublecortin
EC	Endothelial cell
EPC	Endothelial progenitor cell
Epo	Erythropoietin
ERK	Extracellular signal-regulated kinase
ESC	Embryonic stem cell
GABA	Gamma aminobutyric acid
GFAP	Glial fibrillary acidic protein
HIF-1	Hypoxia-inducible factor-1
HuNu	Human nuclear antigen
IL	Interleukin
LTD	Long term depression
LTP	Long term potentiation
LV	Lateral ventricle
MAPK	Mitogen activated protein kinase
MMP	Matrix metalloproteinase
NMDA	N-methyl-D-aspartate
NPC	Neural progenitor cell

NO	Nitric oxide
NSC	Neural stem cell
PI3K	Phosphoinositide 3-kinase
Robo	Roundabout protein
SEM	Standard error of the mean
SGZ	Subgranular zone
STDP	Spike timing depending plasticity
SVZ	Subventricular zone
TPEN	*N*,*N*,*N*′,*N*′-tetrakis-(2-pyridylmethyl)ethylenediamine
TSP	Thrombospondin
TuJ1	Neuron-specific class III beta-tubulin
UCBC	Umbilical cord blood stem cell
VCAM-1	Vascular cell adhesion molecule 1
VEGF	Vascular endothelial growth factor
VLA-4	Very large antigen-4
Zn	Zinc
Zn^{2+}	Ionic zinc

1 Introduction

The controversial principle of *diaschisis* introduced 100 years ago by *Constantin von Monakow* represents the beginning of understanding the general model of plasticity underlying functional recovery after central nervous system (CNS) damage nowadays. *Diaschisis*, meaning 'shocked throughout' in Greek was defined by an 'interruption of function' in an intact brain region which will lead to a 'struggle for the preservation of the disrupted nervous function, and the CNS is always prepared for a struggle' [1, 2].

Ischemic stroke results from a sudden impairment of blood supply in specific parts of the brain, being the leading cause of long-term disability in adults in industrialized countries [3]. Sensorimotor and cognitive impairment after stroke are often severe with little chance of complete rehabilitation, which is associated with high socio-economic costs. Therefore, there is high demand for the development of new, effective treatment strategies to improve the functional outcome after ischemic brain damage.

The scientific efforts from the last decade showed that not only the brain itself has an intrinsic potential for reorganization and repair after stroke [4], but also that these processes of regeneration can be successfully stimulated by means of extrinsic factors. This plasticity potential is represented at the anatomical level by: 1.) recruitment of pathways that sustain the same function as the destroyed ones but have a different anatomical form, 2.) synaptogenesis, 3.) dendritic arborization, 4.) fortification of functionally silent synaptic connections, 5.) long distance fiber sprouting and branching, and 6.) endogenous neurogenesis [5–8]. These events take place in

the first days up to weeks after the ischemic lesion, and the susceptibility to external therapeutic influence is negatively correlated with time. However, post-stroke plasticity is challenged by unexpected and sudden onset of ischemic damage [9], and by the high complexity of the damaged neural structures [10]. Novel therapeutic approaches like transplantation of stem and progenitor cells or administration of factors influencing endogenous repair capabilities of the post-ischemic brain have been investigated in the last decade. The purpose of this chapter is to provide an overview of the current research on neuroregenerative strategies after stroke focusing on underlying mechanisms of action, therapeutic window and possible implications for targeted neurorehabilitation.

2 Clinical Aspects of Stroke Treatment Research

The human brain detains its own rescue mechanisms in case of acute ischemic injury such as: 1.) recruitment of existing collateral blood vessels and induction of angiogenesis, preparing them for takeover in case of sudden obstruction [11], 2.) glial scar formation in the close vicinity of the ischemic core with neuroprotective potential, 3.) self-regeneration by reactivation of ontogenetic repair mechanisms [12]. These three pathways observed after stroke were further investigated in preclinical and clinical studies, giving rise to three therapeutic directions: re-establishing cerebral blood flow (revascularization), neuroprotection and neuroregeneration.

2.1 Revascularization

Mechanical thrombectomy with stent retrievers after large artery occlusion has been proven in recently published randomized studies [13] to re-establish blood flow and to reduce the functional disabilities, being nowadays the gold standard of acute stroke therapy (for review see *Balami et al.* [14]). The reperfusion of the ischemic tissue in the therapeutic window is meant to save the penumbra, limiting the ischemic damage, as well as to prevent vasogenic edema. It also sets the basic conditions for regenerative processes in the peri-infarct zone after stroke.

2.2 Neuroprotection

Neuroprotection is a broad term for mechanisms and strategies aiming at preventing neuronal cell death, therefore reducing deleterious effects of ischemic injury. This terminology is being used in preclinical research with regard to treatments that

prevent or interrupt the molecular injury cascade in the penumbra and preventing secondary neuronal death [15, 16].

Neuroprotective strategies were developed on all progression pathways of ischemic injury described earlier: molecular injury, brain edema, inflammation, excitotoxicity, apoptosis, and spreading depression [17, 18].

Glutamate antagonists were studied with regard to their inhibitory effect upon peri-infarct depolarization and proved to reduce the size of ischemic lesion [19, 20]. Trying to reverse or stop the cascade of molecular injury after stroke lead to the development of different strategies like: stopping neuronal death by excitotoxicity by glutamate antagonists, using antioxidant substances to stop the formation of reactive species of oxygen or of peroxynitrite, antiapoptotic substances meant to stop the delayed neuronal death [17].

Formation of cytotoxic edema was considered as a target for aquaporin (AQP) channels, which are located in the plasma membrane and facilitate water transport. Inhibition of AQP water conductance was demonstrated to reduce the severity of ischemic brain edema [21]. Later studies proved that an intrinsic mechanism of early induction of AQPs may decrease cytotoxic edema formation after stroke but has no influence upon blood-brain barrier (BBB) disruption and therefore has a limited time effect after stroke [22].

The cellular inflammatory response after ischemia was proven to have both detrimental effects contributing to lesion expansion but also to play an important role in the orchestration of lesion repair, the outcome after stroke being seen as a result of the interaction between the injured brain and the immune system [23].

The most active inflammatory pathway after stroke is lead by cytokines and their answer after stroke. Especially the cytokine interleukin (IL)1-beta was for a long time considered a strong neuroprotective target, since administration of IL1-beta receptor antagonists reduces infarct size [24].

The translation of these therapies failed repeatedly, despite the convincing preclinical and phase IIb available data. The SAINT-II (Stroke Acute Ischemic NXY Treatment) study investigating the antioxidative agent NXY-059 as neuroprotective therapy after stroke in patients had to be stopped due to lack of efficiency in the beginning of the phase III trial [25, 26].

2.3 Neurovascular Restoration

Since the main clinical impact of stroke is due to its long time disability effect and because neuroprotective studies did not succeed in the clinical trials, the focus of stroke research changed in the last years on neuroregenerative approaches. The observation of endogenous regeneration potential after stroke, by means of neurogenesis [8, 27, 28] angiogenesis [29], axonal and dendritic sprouting potential [30] and synaptogenesis [30] started a new therapeutic direction after stroke, called neurovascular restoration.

2.3.1 Endogenous Neural Stem Cells as a Possible Pool for Regeneration After Stroke

Formation of neural stem cells (NSCs) starts in the gastrulation phase and continues throughout the embryonic brain by a continuous proliferation of NSCs and subsequent differentiation and migration of neural progenitor cells (NPCs) [31, 32]. After birth there are still neurogenic niches situated in the subventricular zone (SVZ) and the subgranular zone (SGZ) of the dentate gyrus [33]. Accordingly, proliferation of residential NSCs is observed in the adult brain in the SVZ, SGZ and the posterior periventricular area [34–37]. This represents an endogenous pool of NSCs which was proven to be activated by focal ischemia [38] both in the ipsilesional and in the contralesional hemisphere [39, 40], presenting a well determined timing following transient focal ischemia by reaching the peak point 1–2 weeks after stroke and returning to sham levels by 3–4 weeks [41, 42].

The process of neurogenesis includes three major anatomical steps: proliferation, migration and differentiation [43], which have to be followed by functional integration of the newborn neurons, including integration in the extracellular matrix environment and electrophysiological integration in neuronal circuits.

Proliferation of neurons after stroke was intensively studied until now with regards to different growth factors, some of the most promising being erythropoietin (Epo) and vascular endothelial growth factor (VEGF). Ischemia was shown to stimulate the hypoxia-inducible factor-1 (HIF-1) pathway as a main player in the signal cascade after stroke. The smallest reduction in oxygen partial pressure in the brain leads to a strong activation of HIF-1. Both VEGF and Epo are responsible for downstream effects of the transcription factor HIF-1 cascade. Epo knock-out mice have deficiencies in post-ischemic neurogenesis [44, 45] and VEGF was proven to promote neurogenesis both *in vitro* and *in vivo* [46]. Further neuroregeneration-specific aspects of these two growth factors are going to be discussed later in detail.

Migration and differentiation of endogenous NPCs in the normal brain was demonstrated to follow the route of the rostral migratory stream towards the olfactory bulb, whereas in the ischemic preconditioned brain, the NPCs migrate towards the injured areas in the brain [47, 48]. Important mediators in this process of migration and maturation are represented by matrix metalloproteinases (MMPs), especially the MMP9 molecule, which is upregulated in the infarcted cortex at 7–14 days in rats and was shown to colocalize with the NPC marker doublecortin (DCX) and proliferating 5-bromo-2′-deoxyuridine (BrdU) positive cells migrating from the SVZ [49]. *Wang et al.* proved in their studies that conditioned medium from Epo-treated epithelial cell cultures significantly promoted NPC migration, which was blocked by specific MMP inhibitors [50].

Even if there is ample evidence for migration and maturation of NPCs to the ischemic lesion, an aspect that still causes controversies involves the functionality of these neurons, their long-time survival and their integration in the peri-neural and angiogenetic milieu in order to sustain the beneficial recovery after stroke. Among other molecules, VEGF and Epo are thought to be promising candidates to facilitate this functional integration.

2.3.2 Angiogenesis and Neurovascular Remodeling After Stroke

A strong intercellular orchestration is needed to create the permissive conditions for functionally relevant axonal and dendritic sprouting after ischemic injury of the brain. The vascular and the nervous system share multiple similarities in their development, both of them using long-distance projections to reach their targets, being guided by gradients of chemokines and growth factors. Especially in the peripheral nervous tissue, the parallel tracking of blood vessels and nerves is obvious. In the CNS, neurogenesis takes place in the embryological vascular niches where endothelial cells (ECs) proliferate. This is why the two systems have to be taken into consideration as a homeostatic unit, especially in neuropathological conditions such as stroke.

Angiogenesis in the adult brain is the hypoxia-driven sprouting of new capillaries from postcapillary venules [51]. Tissue hypoxia in the adult brain stimulates the activation of HIF-1α expression, which then stimulates the transcription of VEGF, VEGF receptors flt-1 and neuropilin-1, and angiopoietin [52]. Besides the molecular aspect of angiogenesis activation, there are two further systems that are implicated immediately after stroke: loss of vascular integrity and cell matrix degradation [53]. These two processes activate growth factors, their receptors and the guidance molecules which were until then in a dormant phase by being incorporated in the cellular matrix. One of the most important activated growth factors is VEGF, which induces endothelial cell proliferation and their migration [54, 55]. The VEGF family comprises 5 related genes: VEGF-A, -B, -C, -D and PIGF (placenta induced growth factor). VEGF-A is the vascular permeability factor and is known in several isoforms (VEGF-A_{204}, -A_{189}, -A_{165}, -A_{145}, -A_{121}), with different amino acid length and has the capacity of binding to heparin sulfates. VEGF-A_{165} has some degree of heparan sulfate binding which reduces its diffusibility, but at the same time increases its ability to stimulate VEGF receptors [56].

As discussed for neurorestoration, an important aspect of angiogenesis is its functionality translated either by a significant increase in overall blood flow to the tissue that suffered an ischemic damage or by creating the foundation for late restoration processes in the ischemic tissue together with the neural network. The formation of new blood vessels after stroke seems to develop parallel to neurogenesis, being initiated rather late at 48 h after stroke [57, 58]. Because of this delay, there are no reasons to assume that angiogenesis influences brain hemodynamics during an acute ischemic stroke in a relevant way [53]. However, the observed timing hints towards a coupling of neurovascular remodeling after stroke in order to prepare the necessary background for long term restorative processes, e.g. axonal sprouting.

Another important aspect in the process of new blood vessel formation is the VEGF induced disruption of the BBB immediately after stroke, leading to edema formation. Early post-ischemic administration of VEGF in rats increased BBB leakage and infarction volume, whereas its late administration (48 h) enhanced angiogenesis and decreased BBB leakage, resulting in improved recovery volume [59].

2.3.3 Axonal Sprouting and Plasticity

An interesting general observation in the maturation of the corticospinal tract was that the early widespread distribution reaches the specific mature distribution by means of collateral selection. Neuronal cell death is not known to take place in the developing brain, so the hypothesis of postnatal reorganization could be explained just by means of collateral elimination. Using retrograde fluorescent tracer injections into the pyramidal decussation at the spinomedullary junction in adult versus postnatal rats, *Stanfield et al.* could prove that beside the frontal and parietal cortex, the occipital cortex was involved in building the corticospinal tract in postnatal rats [60]. These studies lead to the conclusion that transient pyramidal tract axons are eliminated during development, e.g. being found as projections to the superior colliculus and/or the pons [61, 62]. The understanding of developmental sculpturing of cortical efferent systems is important in further perception of remodeling processes in the adult brain after stroke or other types of injury.

It is now well accepted that the CNS has an intrinsic recovery capacity after stroke, by means of reactivating the ontogenetic machinery stimulating gene expression, protein synthesis, and cellular genesis, reconstructing the needed environment for recovery [63]. Preclinical and clinical studies on unilateral ischemic brain damage demonstrate an increased amount of corticospinal projections and shift of cortical sensorimotor functions to the intact hemisphere. Whether the intact hemisphere increases functionality after contralateral stroke just by means of increasing pyramidal corticospinal projections is not clear. A series of recent studies could identify stimulation of interhemispheric, cortico-reticular or cortico-thalamic pathways [5, 6, 64]. The involvement of the intact pyramidal tract in taking over functions of the damaged contralateral pyramidal system requires large–scale reorganization and a competition between the two cerebral hemispheres for spinal synaptic space. This implies that the degree of abnormality of these corticospinal projections following unilateral lesions might not reflect simply the extent of the initial lesion but also the consecutive competitive disadvantage of the surviving corticospinal projections. This competitive disadvantage would lead to cortico-spinal projections from the intact hemisphere progressively replacing a part of the surviving cortico-spinal projections from the damaged hemisphere and thus to a progressive degeneration.

Post-ischemic endogenous responses of the CNS go in line with an enhanced sensitivity to rehabilitative [65] and plasticity-promoting [66, 67] treatments, opening a time window in which ontogenetic brain repair mechanisms might successfully be reactivated [12, 68]. Stroke recovery is associated with reorganization of neuronal circuits both at the cortical and subcortical level. A series of events set the stage for brain reorganization in the intact hemisphere, such as increased angiogenesis [69] and axonal sprouting [66, 70].

Recruitment of contralesional brain areas correlated with a better recovery from stroke in animal studies [66, 70]. By administering anterograde tract tracers into the contralesional motor cortex, these authors suggested that contralateral projections may be recruited by plasticity-promoting therapies, underlining the relevance of contralesional reorganization for neurological recovery. However, models of permanent

focal cerebral ischemia were used in the latter studies, in which motor cortex tissue was irreversibly destroyed. Brain plasticity ipsilateral to the stroke was not systematically assessed in these studies.

The vascular system is strongly linked to the neural system due to common ontogenic developing pathways. When investigating the circulatory system in cases of neurovascular pathologies of the brain, a series of dynamic processes were identified, which modulate development, survival and differentiation of neurons, rewriting the embryologic developmental phase in a restricted time and space manner [71]. This interconnected developmental network also depends on an important common regulator: the VEGF protein family and its receptor system.

2.3.4 Dendritic Elaboration and Dendritic Spine Proliferation

Whereas in the uninjured adult brain dendritic branching and spines are considered to be stable entities [72], important changes in density of dendritic spines were observed in ischemic situations [73].

By means of two-photon imaging techniques, *Brown et al.* demonstrated an increase in dendritic spine formation with a peak around 1–2 weeks and lasting around 6 weeks in the peri-infarct region after cortical ischemic injury [74]. Until now, no direct link between the rate of dendritic spine formation and functional recovery after stroke has been shown. Due to the spatial and temporal coincidence between dendritic branching and spine reorganization and changes in functional representation of the peri-infarct region [75] and functional recovery [76], restorative therapies focused also on stimulation of dendritic branching and spine density in the early phase after stroke.

After an ischemic injury that affects the axons of pyramidal neurons in the cortex without having a direct effect upon dendrites, significant dendritic spine loss was observed, which was proven to be a result of profound deafferentation. With time, this is followed by an increase in dendritic spine densities and neuritic outgrowth. There are two main sprouting directions involved: the horizontal cortico-cortical connections [6, 77] and the vertical connections from the contralesional hemisphere that travel through the corpus callosum [78].

Large-scale dendritic plasticity was proven to depend on the balanced elongation-retraction of pyramidal dendrites in the peri-infarct cortex after a small photo-thrombotic stroke [79]. Layer V of pyramidal neurons in the cortex is considered the main excitatory zone, therefore differences in dendritic branching and connectivity in this region will be expected to have a major impact upon cortical circuits. Still the evidence that this branching takes place is controversial, and some studies done with the MCAO stroke model found no differences in dendritic branching and spine densities between lesioned and control animals [80, 81]. By repetitive imaging *in vivo* over 3 months after ischemia, another study failed to prove evidence of *de novo* branching formation in the surviving L5 pyramidal neurons in the peri-infarct cortex [82].

The failure of spontaneous regeneration to induce functional recovery after stroke was proved not to be a failure of forming new connections [83], but mainly to be dependent on the non-permissive environment. The main players in the inhibition of the sprouting are the growth-inhibitory proteins, parts of the CNS myelin [84].

2.3.5 Synaptic Plasticity

Synaptic plasticity during the developing phase experiences a burst early in the postnatal period especially in the occipital cortex, reaching a density that is approximately twice the density in the adult brain [85]. Waves of synaptic plasticity appear also in parieto-temporal and then frontal regions during development, reaching their peaks around early adolescence [86, 87].

In physiological situations in the brain, synapses are modulated (strengthened or weakened) and shaped by activity-induced mechanisms called also Hebbian plasticity. Whereas regulation of individual synapses by means of long term potentiation (LTP) or long term depression (LTD) is the main mechanism responsible for learning and memory processes. In addition, mechanisms regulating levels of activity are considered important in synaptic plasticity for network function [88]. The Hebbian plasticity describes positive-feedback mechanisms responsible either for strengthening of effective synapses or for weakening of passive synapses. Regulation of neuronal activity during synaptic modulation was theoretically and experimentally devised in three possible underlying processes: synaptic scaling, spike timing depending plasticity (STDP) and synaptic redistribution.

Synaptic scaling refers to the competitive interaction between synapses coupling on the same neuron. The biological substrates that lead to synaptic scaling are dependent of glutamate receptors number. This leads to specialization of neuronal pathways depending on their synaptic stimulus. Whereas synaptic scaling is non-Hebbian plasticity forming active neuronal pathways by means of mainly post-synaptic firing rate, STDP is thought to respect Hebbian plasticity by considering both pre- and postsynaptic activity. Synaptic redistribution was observed in some forms of cortical neurons in which short-term plasticity of synapses induced by LTD can be modified by LTP, which increases the presynaptic elimination of the neurotransmitter. The mechanism of synaptic redistribution is not completely understood. For further information please see the review of *Abbott and Nelson* [88].

In the ischemic cortex, synaptogenesis is progressively increased in the peri-infarct region together with markers of axonal sprouting such as GAP-43, underlining the importance of the penumbral region in synaptic reorganization after stroke [30].

2.3.6 Modulation of the Immune Response and Inflammation

MMPs represent a family of zinc endopeptidases with a major role during development of the CNS and distinct functions in pathological states. Recent data showed specific roles of MMPs in different time periods after stroke. Whereas acute

post-ischemic activation of MMPs leads to increased ischemic injury by enhancing the neuroinflammatory response [89], the postacute activation was shown to contribute to neurovascular remodeling and promote stroke recovery [90].

3 Cell Therapy and Brain Plasticity After Stroke

In the last decade, extensive research efforts have been carried out to establish cell-based therapies, e.g. transplantation of NSCs and/or NPCs, bone-marrow derived stem cells (BMSCs), umbilical cord blood stem cells (UCBCs), adipose-tissue derived stem cells (ATSCs), and embryonic stem cells (ESCs) as a possible experimental therapeutic avenue for ischemic stroke and other disorders of the CNS (for review see *Lindvall et al.* [91]). A series of clinical studies has already demonstrated the feasibility and safety of this approach in clinical practice (for review see *Bliss et al.* [92]).

Transplanted NPCs have been shown to survive, migrate and integrate in the post-ischemic host brain, thereby acquiring adequate neuronal and glial phenotypes and display functional electrophysiological integration into neuronal circuitry (for review see *Hermann et al.* [93]).

Investigations on the possible underlying mechanisms of cell transplantation have revealed that processes other than direct replacement of neurons and glial cells by the grafted NSCs or NPCs are involved in promoting neurological recovery after stroke [94]. The grafted cells orchestrate tissue plasticity of the host brain [95, 96]. These effects include secretion of neurotrophic factors, immunomodulation, and angiogenesis [97–102].

We have previously shown that transplantation of human NPCs improved functional outcome after experimental stroke in rats with temporal coincidence of increased dendritic arborization of layer V pyramidal neurons and promotion of cortico-cortical, cortico-striatal and cortico-spinal axonal projections [103].

Extensive migration of grafted cells towards the perilesional area was observed (Fig. 7.1). Only a very small percentage (about 6%) of the grafted NPCs showed differentiation into the neuronal lineage, most of them stayed in an undifferentiated state. At the same time, grafted animals showed robust functional recovery demonstrated with a battery of neurological tests, as compared to sham-operated controls (Fig. 7.1). Impaired axonal transport processes were partially restored in grafted animals, as demonstrated by reduced amyloid precursor protein accumulation. Using *in vitro* assays with indirect co-culture of human NPCs and cortical neurons, we demonstrated that increased dendritic and axonal plasticity depends on molecules secreted by NPCs. In a further step, some of these mediating factors were identified as VEGF, thrombospondins 1 and 2, and slit using immunodepletion assays (Fig. 7.2). Endogenous remapping of the ipsi- and contralesional hemispheres is a well-known phenomenon in recovery after ischemic damage [104–109]. It is reasonable that the above-mentioned soluble factors secreted by the grafted NPCs and their progeny, among many others, influence the host cells during this process by promoting dendritic and axonal regeneration. Other types of stem cells,

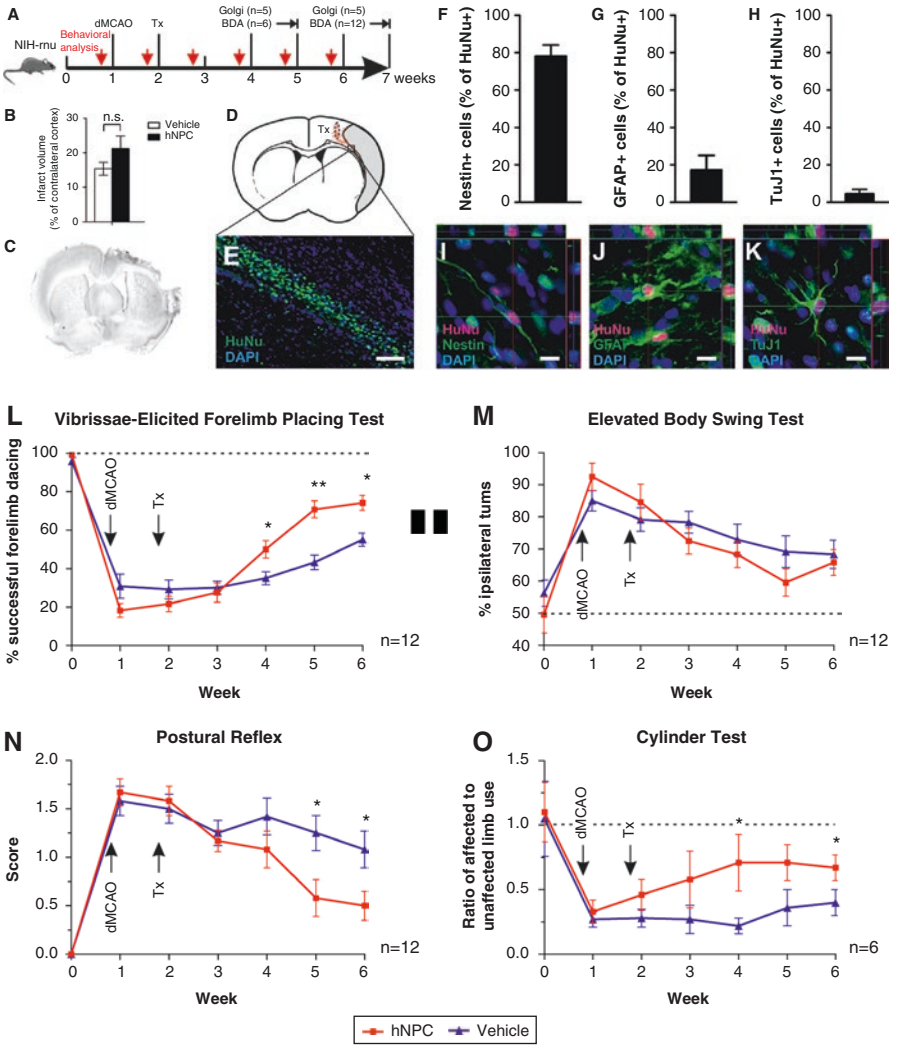

Fig. 7.1 Fate of transplanted human NPCs (hNPCs) and behavioral recovery after experimental stroke in rats. Experimental setup (**a**). Transplanted animals (hNPC) showed a tendency to smaller infarct volumes than controls (Vehicle) (**b**). Distal middle cerebral artery occlusion (dMCAO) resulted in consistent cortical infarction (**c**). 4 weeks after transplantation, the majority of human HuNu+ cells are found in the ischemic boundary zone (**d, e**). Most of the cells remain in a undifferentiated state (Nestin+; **f**), while smaller portions show astrocytic (GFAP; **g**) or neuronal (TuJ1; **h**) differentiation. Confocal immunofluorescence photomicrographs of Nestin+ (**i**), GFAP+ (**j**) and TuJ1+ (**k**) cells co-localizing with HuNu in the peri-infarct area. hNPC-grafted animals (hNPC) demonstrate improved functional recovery as compared to sham-operated controls (Vehicle) using the vibrissae-elicited forelimb placing test (**l**), the elevated body swing test (**m**), the postural reflex test (**n**), and the cylinder test (**o**). Scale bars: **e**: 50 μm, **i–k**: 10 μm. Mean ± SEM; *p < 0.05, **p < 0.01. *Data partially published in Andres et al. Brain 2011 Jun;134(Pt 6):1777-89. doi: 10.1093/brain/awr094, with kind permission from Oxford University Press*

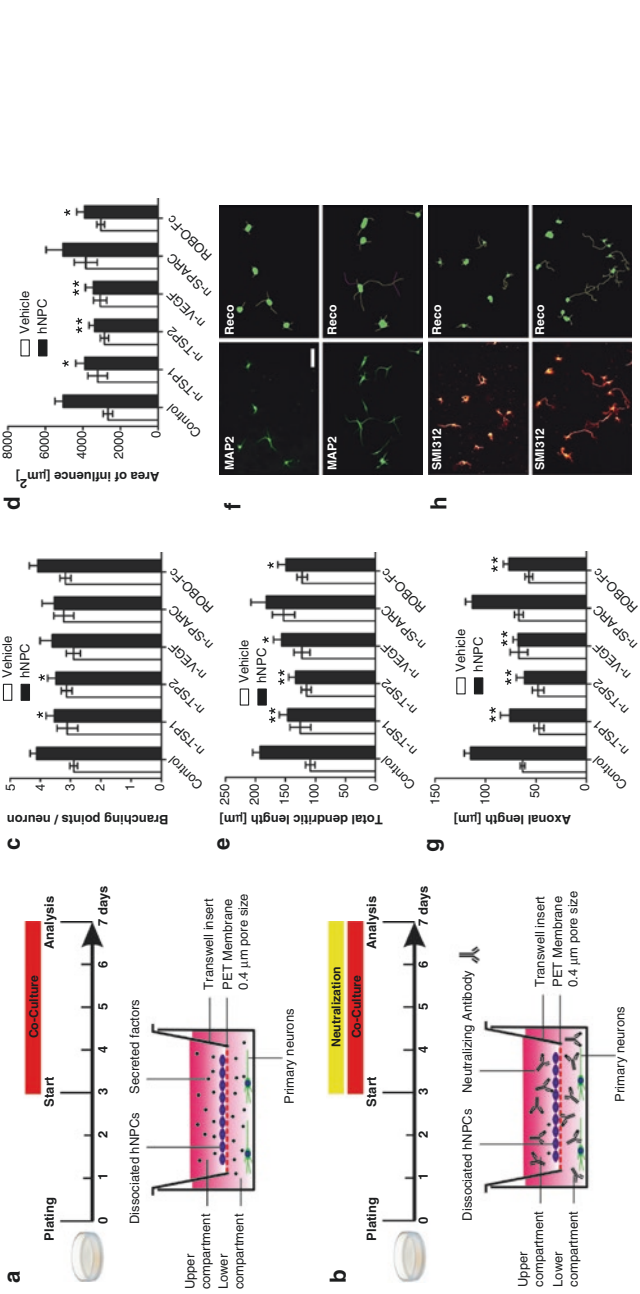

Fig. 7.2 Identification of human NPC (hNPC)-secreted factors mediating the effects on dendritic plasticity and axonal outgrowth *in vitro*. Experimental setup of co-culture experiments (**a**) and immunodepletion studies for identification of specific molecules (**b**). hNPCs were indirectly co-cultured with rat E14 primary cortical neurons for 7 days. Dendritic complexicity and axonal outgrowth were quantified using MAP2 (dendritic marker) and SMI312 (axonal marker) immunofluorescence and automated high-throughput analysis. As compared to untreated controls, the presence of hNPCs significantly promoted dendritic branching (**c**), total dendritic length (**d**), axonal outgrowth (**e**), and area of influence per individual neuron (**f**). Neutralization of factors significantly reduced the effects of hNPCs on dendritic branching (**c**; TSP1, TSP2), total dendritic length (**d**; TSP1, TSP2, VEGF, ROBO-Fc), axonal length (**e**; TSP1, TSP2, VEGF, ROBO-Fc), and area of influence (**f**; TSP1, TSP2, VEGF, ROBO-Fc). Representative photomicrographs and digital reconstructions (Reco) of neurons co-cultured with (hNPC) and without (Vehicle) hNPCs stained for MAP2 (**g**) and SMI312 (**h**). Scale bar: 50 µm, Mean ± SEM; *$p < 0.05$, **$p < 0.01$. *Data partially published in Andres et al. Brain 2011 Jun;134(Pt 6):1777-89. doi: 10.1093/brain/awr094, with kind permission from Oxford University Press*

like BMSCs, UCBCs, ATSCs, and ESCs as well, might have distinct action profiles (for review see *Andres et al*. [110]).

In another study, we investigated the effects of grafted murine NPCs on microglial activation, proliferation and phagocytosis [111]. VEGF secreted by NPCs was identified to mediate potent effects after grafting NPCs in mice. Thus, neural precursor cells are not only influenced by surrounding microglia, but also regulate microglia functions and activity. This might also play an important role in stroke and needs to be addressed in further studies.

Intravascular, i.e. intraarterial or intravenous administration of NPCs, is another feasible approach for cell transplantation in stroke. The advantage of this technique is the widespread distribution of the grafted cells in larger ischemic areas, which is not accomplishable by means of focal stereotactic transplantation. NPCs are recruited across the BBB by mechanisms similar to transendothelial homing of immune cells, including endothelial attachment and rolling along the endothelial surface. This process is facilitated by the integrin very large antigen-4 (VLA-4) expressed on immune cells as well as NPCs, which supports tethering and rolling on flow on vascular cell adhesion molecule 1 (VCAM-1). Expression of VCAM-1 is upregulated on the endothelial surface after stroke, leading to transendothelial recruitment of immune cells from the systemic circulation into the ischemic brain parenchyma. We have previously demonstrated that enrichment of NPCs by fluorescence activated cell sorting for VLA-4 and intracarotid delivery promoted cell homing to the area of stroke in mice and improved behavioral recovery [112]. In a second step, the interaction between CC-chemokine ligand-2 (CCL2) in the perivascular space and its receptor CC-chemokine ligand receptor-2 (CCR2) expressed on the plasma surface of NPCs was identified to be critical for targeted homing of intravascularly delivered NPCs [113]. Blocking CCR2 or using NPCs derived from CCR2 knock out animals led to a significant reduction of transendothelial migration as shown by bioluminescence and immunohistochemical studies. On the other hand, increasing the expression of chemokine receptors on the cellular surface by chemical pretreatment of the cells with brain derived neurotrophic factor (BDNF) augmented the transendothelial migration [114]. According to the temporal profile of adhesion molecule upregulation and chemokine expression after stroke the are ideal therapeutic windows for intravascular cell delivery. In an ischemic reperfusion rat stroke model we have demonstrated that intraarterial cell injection was most efficient 48 h after the ictus [114].

Recent clinical studies demonstrated the feasibility of transplantation of modified BMSCs (SB623) with improvement in clinical outcome 1 year following stable, chronic stroke [115].

4 Cell-Free Treatment Strategies

The demand of therapeutic options complementing thrombolysis and the better understanding of the endogenous repair mechanisms after stroke have favored the development of interventions based on cell transplantation. The observation that neurovascular plasticity is crucial in brain recovery has brought different cell types directly involved

in angiogenesis and neurogenesis in the focus. In the regenerative scenarios following the ischemic insult, in addition to NSCs and NPCs, endothelial progenitor cells (EPC) are of particular importance being both targets and effectors. This is not surprising in view of the functional and anatomical coupling of vascular and neuronal cells (which is particularly evident in the neurogenic niche). Indeed, preclinical studies have confirmed that NPCs transplanted in stroked rodents are capable to generate neurons and promote angiogenesis. Similarly, EPC not only integrate in the brain vasculature, but also display neuroprotective actions. Another feature that makes EPCs suitable for therapy is their capacity to be readily recruited to the ischemic site.

Despite the evidence that intravenously transplanted EPCs engraft in the brain capillaries of stroked rats [116], it is now clear that soluble factors released by transplanted cells promote cell viability by providing trophic support, modulating local immunity and activating tissue remodeling processes, including angiogenesis. Thus, the paracrine actions of transplanted cells are not considered any more as 'bystander effects', but present with major tissue regenerative activities that precede the eventual differentiation and replacement of injured cells (if occurring at all). This concept and the presence of limitations inherent to cell transplantation such as the poor efficacy due to extensive death of grafted cells, microemboli, and tumor formation. have inspired a new type of therapeutic strategies designed on the administration of cell-secreted factors also referred as secretome. Indeed, using a rat hindlimb ischemia model, we have demonstrated that the EPC secretome in the form of conditioned medium (EPC-CM) has the same or even superior therapeutic capacity than transplanted cells [117]. These observations have been extended in a rat model of stroke [118]. In addition, we could demonstrate that intraventricular infusion of EPC-CM significantly increased the number of DCX-positive neuronal precursors in the SVZ of adult naïve rats (Fig. 7.3).

For the translation into clinical practice, the effectors of the diverse secretomes and their mechanisms of action still need to be fully elucidated [119]. We and others have recently reported that the EPC secretome-induced effect on viability of rat brain microvascular ECs under standard culture conditions is mediated by PI3K/AKT and MAPK/ERK activation [120, 121]. Moreover, EPC-CM significantly protected rat brain microvascular ECs from an ischemic insult induced by an oxygen-glucose deprivation. Importantly, these effects seem to be mediated not only by growth factors such as BDNF [121], but also involve not yet identified lipidic factors [122].

5 Pharmacological Treatment

Small molecules, e.g. the neuroprotective and differentiation-inducing ergogenic amino compound creatine [123], and many others, can be systemically administered in order to promote endogenous repair processes after stroke or to improve the fate of grafted NPCs. However, this is usually limited by low penetration of the BBB, resulting in poor CNS bioavailability, and systemic side effects.

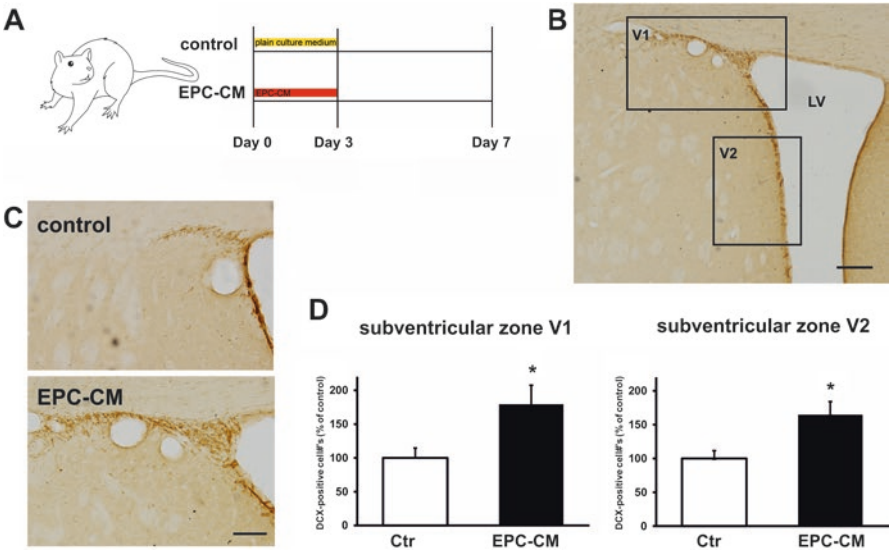

Fig. 7.3 Effects of endothelial progenitor cells-derived conditioned medium (EPC-CM) on number of doublecortin (DCX) positive neuronal precursors in the subventricular zone of adult rats. EPC-CM or control medium was infused into the right lateral ventricle (LV) by means of an Alzet pump for a period of 3 days. After 7 days, brains were removed and processed for immunohistological analyses (**a**). DCX-positive cells were examined in two regions of the subventricular zone (V1, V2) as indicated with *boxes* (**b**). The digitalized representative photomicrographs demonstrate a higher number of DCX-positive cells in V1 after EPC-CM treatment as compared to controls (**c**). Scale bars: 200 μm (**b**), 100 μm (**c**). Significantly higher DCX-positive cell numbers were observed in the EPC-CM groups (*filled bars*) as compared to controls (Ctr, *open bars*) in both the V1 and V2 regions (**d**). Values are expressed as percentage of controls and are given as mean ± SEM. *$p < 0.05$ vs. corresponding controls

Inhibition of tonic (extrasynaptic) gamma aminobutyric acid (GABA) signaling during the repair phase in the post-ischemic brain has been shown to promote functional recovery in mice, suggesting that GABA plays an important role in modulating brain repair [124]. Administration of N,N-Dimethyl-2-(6-methyl-2-p-tolylimidazo[1,2-a]pyridin-3-yl)acetamide (Zolpidem), a GABA agonist specific to the α-1 receptor subtype, was demonstrated to improve behavioral recovery [124].

Zinc (Zn) homeostasis, which is integral to normal CNS functioning, might also be involved in regenerative processes after ischemia and other CNS disorders [125]. Zn ions (Zn^{2+}) have been shown to play a crucial role in the modulation of synaptic transmission as well as in cortical plasticity [126]. Zn is required for the mammalian brain development and physiology. Under normal circumstances, Zn is tightly bound to many proteins in the CNS, whereas ionic Zn is a major etiological factor in CNS damage or diseases due to its toxicity [125, 127–129]. As thus, intracellular Zn^{2+}-concentrations are tightly regulated, as proper homeostasis is critical in the maintenance of cellular processing [130, 131]. Excessive exposure to extracellular Zn^{2+} on the other hand damages neurons of the CNS. Namely, transient forebrain

ischemia in rats leads to an accumulation of chelatable, ionic Zn in degenerating CA1 neurons of the hippocampus, as well as in the cerebral cortex, thalamus, striatum, and the amygdala [127]. Interestingly, this accumulation precedes neurodegenerative processes, which could be prevented by the intraventricular injection of a Zn chelating agent, wherefore the early occurring toxic release of Zn^{2+} may be a key mechanism underlying selective neuronal cell death after ischemia [127]. As thus, administration of Zn^{2+}-chelating agents such as N,N,N′,N′-tetrakis-(2-pyridylmethyl) ethylenediamine (TPEN) could represent a potential cell-targeted therapy. TPEN significantly suppressed cell death, apoptosis, and neuronal glutamate release in primary cultured neurons undergoing a hypoxic-ischemic insult [132]. Moreover, there is a striking feature of a delayed rise in intracellular free Zn^{2+} in CA1 neurons just before the onset of histologically detectable cell death. Intrahippocampal injection of 1-naphthyl acetyl spermine, a selective channel blocker of GluR2-lacking alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA)-type glutamate receptors at *Schaffer* collateral to CA1 synapses 9–40 h after transient ischemia greatly reduced the late rise in intracellular free Zn^{2+} in postischemic CA1 neurons and afforded partial protection against ischemia-induced cell death. This receptor subtype appears to be an important therapeutic target for prevention of ischemia-induced neuronal death in humans [133].

Beside Zn^{2+}, nitric oxide (NO) is implicated in the pathogenesis of post-ischemic neuronal damage. The addition of exogenous NO or N-methyl-D-aspartate (NMDA) in order to increase endogenous NO leads to peroxynitrite (ONOO-) formation and consecutive Zn^{2+} release from intracellular stores in cerebrocortical neurons. Free Zn^{2+} in turn induces respiratory block, mitochondrial permeability transition, cytochrome c release, generation of reactive oxygen species, and p38 MAP kinase activation. This crosstalk between NO and Zn^{2+} dependent apoptotic signal transduction pathways may contribute to the delayed loss of neurons after ischemia [134].

Furthermore, we recently showed that Zn^{2+}-dyshomeostasis represents a major suppressor of axonal regeneration in the CNS, with Zn^{2+}-chelation (i.e. with TPEN) leading to persistent survival of many damaged neurons [135]. Thus, synaptic Zn^{2+} represents a previously unknown, critical suppressor of regeneration that might become a crucial player in neuroprotective and plasticity-enhancing strategies after stroke.

A greater understanding of the role of Zn^{2+} for cellular processes following CNS injuries where aberrant metal homeostasis is implicated in disease pathogenesis may therefore allow for the development of new potentially promising therapeutic approaches.

6 Conclusions

Ischemic stroke is the leading cause of severe long-term disability in the Western population, with very few therapeutic options. After an ischemic lesion, the neurovascular units are niches for NSCs and NPCs, supporting the regeneration potential,

and the brain is being reshaped by means of neuronal sprouting or by unmasking the existing, but functionally silent connections. *Kreisel et al.* proposed in 2006 a timeline classification of recovery processes after stroke, differentiating between five distinct stages: (1) hyperacute phase from the stroke event up to 6 h after; (2) acute phase lasting up to the fourth day after stroke characterized by secondary events; (3) subacute phase from the second day up to 2–3 weeks characterized by brain remapping and functional plasticity; (4) consolidation period lasting up to several months and being characterized by functional alteration; (5) chronic phase characterized with the tendency of the events to become static [136, 137]. Despite intense research efforts during the last decade, effective therapeutic agents that promote the repair phase of recovery are still missing. There is a high heterogeneity in the preclinical data, making it unable to be synthetized and translated to the next clinical level. Elucidating underlying mechanisms of endogenous repair processes and plasticity of the brain is critical for the development of new therapeutic strategies for stroke in humans.

Acknowledgements This research was funded by the Swiss National Science Foundation (Grants No. 31-064975.01, 31-050824, 31-102075/1, 3100A0-112529, 31003A-135565, 406340-128124, 146632, PBBEB-117034, PASMP3-123221/1, PBBEB-146099 and PBBEB-155299), the Swiss Parkinson Foundation, the HANELA Foundation, a Research Grant from the University Hospital Berne, Inselspital (RGI-84800855), the Evelyn L. Neizer Fund, and by the Department of Clinical Research at the University of Berne.

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Chapter 8
Stem Cell-Paved Biobridge: A Merger of Exogenous and Endogenous Stem Cells Toward Regenerative Medicine in Stroke

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Abstract Stroke is a significant unmet clinical need with therapeutic options limited to tissue-type plasminogen activator (tPA), which has a small therapeutic window and risk for hemorrhagic transformation. Stroke is a multiphasic disease with a complex pathology. After the initial insult, a cascade of events occur causing secondary cell death and the expansion of the penumbra. The major contributing factors to this secondary cell death are depletion of growth factors, neuroinflammation, and disruption of the neurovascular unit. There is a need for more innovative and effective therapies that can target the diverse pathological consequences of stroke. To this end, stem cell therapy is a promising approach for stroke. Pre-clinical studies have demonstrated the potential of stem cells for treating neurological disorders, including stroke. Here, we discuss diverse stem cell types which have generated encouraging results for advancing to the clinic. Then, we examine the mechanisms of action of stem cells—cell replacement, by stander effect, and a novel biobridge concept advanced by our laboratory. These mechanisms work in concert to afford the neuroprotection and neuroregeneration after stroke. We envision that an in-depth understanding of the benefits and drawbacks of various stem cells and their mechanisms of action will guide the translational entry of stem cell therapy from the laboratory into the clinical setting.

Keywords Adult-derived stem cells • Ischemia pathology • Stem cell mechanisms • Stem cell migration • Neuroregeneration • Neuroprotection • Extracellular matrix remodeling • Stem cell therapies • Translational research

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P.A. Lapchak, J.H. Zhang (eds.), *Cellular and Molecular Approaches to Regeneration and Repair*, Springer Series in Translational Stroke Research,
https://doi.org/10.1007/978-3-319-66679-2_8

Abbreviations

BDNF	Brain-derived neurotrophic factor
BM-MSC	Bone marrow-derived mesenchymal stem cells
CCI	Controlled cortical impact
ECM	Extracellular matrix
EGF	Epithelial growth factor
FGF	Fibroblast growth factor
GDNF	Glial cell line-derived neurotrophic factor
hBMSCs	Human bone marrow stromal cells
IA	Intra-arterial
IC	Intracranial
IL-1β	Interleukin-1-beta
iPSCs	Induced pluripotent stem cells
IV	Intravenous
MCAO	Middle cerebral artery occlusion
MMP	Metallomatrix protein
NGF	Nerve growth factor
NSCs	Neural stem cells
SCF	Stem cell factor
SDF-1	Stromal-derived factor 1
SGZ	Subgranular zone
SVZ	Subventricular zone
TBI	Traumatic brain injury
TNF-α	Tumor necrosis-alpha
tPA	Tissue-type plasminogen activator
VEGF	Vascular endothelial growth factor

1 Introduction

Stroke continues to be a leading cause of death and disability in America, with approximately 800,000 people being affected annually [1]. Responsible for roughly 5% of American deaths—the fifth leading cause [2]—long term consequences for stroke survivors can range from mild functional impairments to severe disability [1]. Accounting for healthcare costs and loss of productivity, an economic burden of $33.6 billion is attributed to stroke, with this figure projected to increase in the future [1]. In fact, the economic burden of stroke has increased notably in recent years, largely due to improved treatment protocols and a resulting decreased mortality rate [1]. Despite posing such a prevalent medical and economic burden, therapeutic options for stroke have been limited to tissue-type plasminogen activator (tPA) and physical therapies to alleviate symptoms. Unfortunately, the clinical benefits of tPA are minimized by its narrow therapeutic window, with the risk of

hemorrhagic transformation rising sharply and its efficacy decreasing significantly over the initial 1–6 h timeframe [3–5]. As a result, the search for innovative and effective therapies which maintain their therapeutic value over the acute, sub-acute, and chronic pathological stages of stroke continues.

Stem cell therapies have been explored as a possible treatment to this unmet clinical need, having demonstrated both neuroprotective effects in the acute stage, as well as regenerative capacity in later stages of stroke [6–10]. Furthermore, stem cell therapies offer unique advantages over traditional pharmaceuticals by providing a dynamic and adaptive therapeutic profile—a likely requisite for any intervention capable of providing substantial functional recovery from the complex neurodegenerative pathology of stroke [11–15]. Apparent from the completed clinical trials of stem cell transplantation is their relative safety via both intracerebral and intravenous administration [16] (NCT01501773, NCT00535197, NCT00859014, NCT01716481). Unfortunately, clearly demonstrating their efficacy has proven more difficult due to a number of practical difficulties in outcome measurements, patient enrollment numbers, and trial design [17, 18]. As a result, basic and translational laboratories have engaged in a concerted effort to better understand the mechanisms by which stem cells offer their therapeutic effects in the hopes of inspiring more successful clinical trials. Following the recent *in vitro* and *in vivo* studies of our laboratory, we have proposed a third mechanism by which stem cells convey therapeutic effects, the *biobridge*, which works cooperatively with the two well-established mechanisms of cell replacement and bystander effects (secretion of trophic factors, cytokines, and anti-inflammatory molecules, among others) [19–21]. This novel mechanism, whereby transplanted stem cells assist the migration of endogenous stem cells from neurogenic niches in the subventricular zone (SVZ) and the subgranular zone (SGZ) to the region of damaged tissue via extracellular matrix remodeling, was demonstrated in a controlled cortical impact (CCI) model of traumatic brain injury (TBI) [21]. Here, we expand this concept by revealing preliminary data which indicate the formation of a similar structure in the middle cerebral artery occlusion (MCAO) model of ischemic stroke. When contemplating the clinical feasibility of cell-based therapies for the treatment of stroke, the biobridge concept advances the notion that transplanted stem cells can work in synchrony with endogenous stem cell repair mechanisms. This provides a clearer understanding of the mechanisms by which stem cells confer their therapeutic benefits, and also supports their safety by demonstrating that long-term effects generated by cell therapy may not require transplanted stem cell survival per se, but rather endogenous stem cells can subsequently continue the regenerative process despite non-survival fates of the grafted cells.

2 The Many Facets of Stroke Pathology

Stroke is defined as a pathological state whereby a reduction in blood flow effects one or more regions of the brain, which may be caused by an obstructed vessel resulting in ischemic stroke or a ruptured blood vessel, leading to hemorrhagic

stroke [1]. Ischemic stroke is more common and has a lower mortality rate [1]. The cells that directly lose their supply of glucose and oxygen die quickly, as neurons are exceedingly sensitive to metabolic stress. This ischemic tissue region comprises the infarct core; these cells are vulnerable to primary cell death processes and are less amenable to therapeutic intervention [22, 23]. Oxygen and nutrient deprivation causes mitochondrial damage and an increase in reactive oxygen species, both of which contribute to cell death cascades [22]. Additionally, without proper energy supply, the cell membrane is no longer able to uphold ionic homeostasis, which drives improper calcium ion concentrations within the cell, further contributing to cell death pathways [24]. The acute damage to these cells ultimately leads to cell death, with little opportunity for intervention.

Despite stroke being an acute event, the resulting pathophysiology of this event persists chronically, a product of a phenomenon known as secondary cell death [23]. The necrotic cells within the infarct core leave in their wake a toxic microenvironment. Leaked substances from these cells have the capacity to reach adjacent healthy cells and cause harm [23]. For example, following stroke, high levels of glutamate are released into the microenvironment and reach concentrations that lead to excitotoxicity in neighboring cells [23]. This region of cells susceptible to secondary cell death is referred to as the penumbra. Researchers often focus on this region of cells due to a higher likelihood of restoration and a wider therapeutic window. Unlike the infarct core, the penumbra is not fixed—this region of secondary cell death may continue to expand over weeks, months and even years [25, 26].

There are many components contributing to secondary cells death after stroke including depleted growth factors, neuroinflammation, and blood-brain barrier (BBB) breakdown [27–30]. Appropriate growth factor levels within the microenvironment must be sustained for cell survival, with loss of these factors resulting in apoptosis. Several types of growth factors contribute to neuron homeostasis including, but not limited to, glial cell line-derived neurotrophic factor (GDNF), brain-derived neurotrophic factor (BDNF), nerve growth factor (NGF), fibroblast growth factor (FGF), vascular endothelial growth factor (VEGF), stromal-derived factor 1 (SDF-1), epithelial growth factor (EGF), and stem cell factor (SCF). Highlighting their importance, preclinical studies have displayed neuroprotective benefits using GDNF, BDNF VEGF, SDF-1, and SCF treatments following cerebrovascular injury [30].

The neuroinflammatory response after stroke is a double-edged sword. While inflammation plays an important neuroprotective role in the acute phase, chronic inflammation perpetuates secondary cell death [22]. The neuroinflammatory process is triggered by damage-associated molecular patterns (DAMPs) propagated by dying and dead cells. Some of the DAMPs are high mobility group box-1 (HMGB1), heat shock proteins, and hyaluronan [31]. Once the inflammation process is initiated, the vulnerable cells within the penumbra are activated and secrete pro-inflammatory cytokines including tumor necrosis-alpha (TNF-α), interleukin-1-beta (IL-1β), and interleukin-6 (IL-6), among others [30]. This stroke-induced inflammatory response further exacerbates cell death and BBB breakdown. The BBB is a part of the dynamic neurovascular unit which is composed of vascular cells (endothelial cells, pericytes, and smooth muscle cells), supporting glial cells (astrocytes,

microglia, and oligodendrocytes), neurons, and extracellular matrix [32]. Aberrant neuroinflammation dramatically disrupts the interactions between components of the neurovascular unit. Pro-inflammatory cytokines interfere with the tight connections between the astrocytic end-feet, pericytes, and endothelial cells, causing a leaky BBB [33]. The damaged BBB permits the entry of circulating cells and substances which are typically excluded or tightly-regulated from the brain, inducing further inflammation and upsetting the homeostatic solute balance, which results in intracranial edema [33]. Also, a number of molecular factors which are upregulated following injury—such as Notch, HMBG1, and SPARC—prompt microglia toward an M1-like phenotype, favoring mobility, phagocytosis, and the section of additional pro-inflammatory cytokines [34–36]. Finally, the inflammatory cytokines promote the upregulation of adhesion molecules (i.e. ICAM1, E-selectin, P-selection) on the endothelial cells and attract peripheral immune cells to adhere and enter the brain [32, 35–38]. Altogether, this inflammation contributes to a hostile environment which, if prolonged, can cause further damage to neural cells.

The pathology of cerebrovascular diseases is not isolated within the central nervous system (Fig. 8.1). Peripheral body systems have received increasing recognition for their role in cerebrovascular disease progression. Inflammatory signals that result post-injury travel through the circulatory system and impact systemic inflammation which may propagate cerebral inflammation. This brain-to-periphery interplay is both permitted and heightened by BBB breakdown, as peripheral lymphocytes and monocytes easily pass through compromised vessels, migrating toward the inflammatory signals originating from the site of injury. Treatment options will be

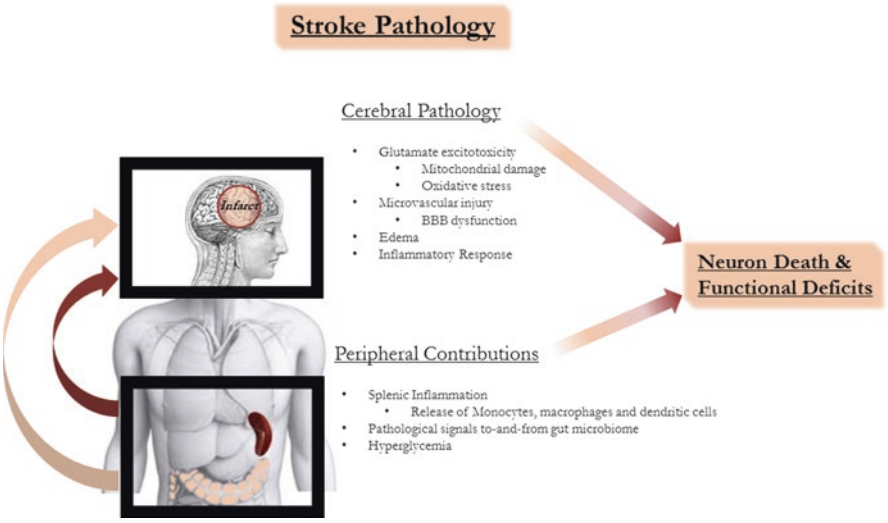

Fig. 8.1 A diagram of stroke pathology which includes both cerebral damage and peripheral contributions. Importantly, the loss of BBB fidelity permits the transfer of pathologically relevant molecules to and from the periphery. Changes in the peripheral organs—especially the spleen and gut—have been shown to accompany and contribute to worsening outcomes

rendered most effective if they consider peripheral body systems, due to their capacity to exacerbate brain injury. For example, preclinical studies suggest that mitigation of peripheral inflammation—particularly in the spleen—may be a primary mechanism of intravenous stem cell injection after stroke [9]. Indeed, the spleen is a significant contributor to systemic inflammation as a consequence of cerebral insult [9, 39, 40]. Following stroke, the physical size and function of the spleen alter, impacting brain health [41–43]. Under pathological conditions, the spleen will release splenocytes into circulation causing further neurodegeneration. Animals that receive splenectomies prior to cerebrovascular insults display improved cognitive function and decreased lesion volumes [44]. While this method is not practical for clinical use, this knowledge of the spleen-brain inflammatory axis highlights the critical role of the spleen in neuropathology. In addition to the spleen, research has also revealed that the gut microbiome plays a vital role in stroke pathology. Depletion of the proper intestinal flora leads to poorer outcomes in animal models of stroke, testifying to the significance of the microbiome to global health [45]. Our understanding of stroke as a global disease-state gives insight on how to properly assess and develop effective treatments [46, 47]. As we will discuss in great depth later, intravenous transplantation of stem cells has the unique ability to utilize trophic mechanisms to abrogate central and peripheral inflammation, in addition to forming the biobridge structure which helps facilitate repair by way of endogenous stem cell optimization, all working to reduce the pathological consequence of stroke.

Stroke pathology is complex and multiphasic. The initial metabolic restriction and glutamate toxicity are not the only factors that cause damage to the neurovascular unit. In fact, the subsequent secondary cell death in the form of growth factors deficiency, neuroinflammation, and BBB breakdown can further exacerbate the injury for an extensive period of time. Current stroke treatment is limited to restoration of the blood flow through tPA or mechanical means which is only effective when targeting the supracute stage of pathology [3]. While the body has a small capacity to repair and regenerate neural damage, these efforts are insufficient to overcome the overwhelming damage of secondary cell death. Therefore, there is a tremendous need for novel therapeutic strategies that can address this multifaceted pathology of stroke. The complexity of stroke pathology necessitates a therapy that has as an equally complex and diverse array of therapeutic mechanisms. To this end, we and others have proposed stem cell therapy as a promising therapeutic strategy. Briefly, stem cells exert their therapeutic benefits through replacing loss or damaged cells, providing trophic factors and anti-inflammatory cytokines, and via the novel concept of the stem cell-paved biobridge. These mechanisms will be expanded upon in later sections.

3 The Evolution of Stem Cell Research

Stem cells are a small population of cells which possess specific characteristics, including the ability to self-replicate, to differentiate into various cell lineages, and to express specific cell markers [48]. Self-replication gives stem cells the ability to

preserve their characteristics and maintain a reservoir population of stem cells within several niches of the body. The capability of stem cells to differentiate into different cell types is vital to their role in preserving homeostasis and to the maintenance of various body systems [49]. For example, the body continually regenerates red blood cells to replace the old by using stem cells within the bone marrow. Collectively, the capacity of stem cells to self-replicate and differentiate into various lineages is referred to as the property of *stemness* [50]. Each type of stem cell has a characteristic level of stemness which is an important factor to be considered when contemplating any potential therapeutic treatment.

There are several ways to classify stem cells. The most common type of classification is based on the origin of the harvested stem cells. For example, umbilical cord stem cells and adipose stem cells are harvested from the umbilical cord and adipose tissue, respectively. Depending on a stem cell's potency, defined as the number of cell types a stem cell can differentiate into, a stem cell can be classified as totipotent, pluripotent, or multipotent [51]. Totipotent stem cells can become all cell types including extraembryonic cells, whereas, pluripotent stem cells can develop into all cell types except for extraembryonic and placental cells. Multipotent stem cells can give rise to various cell types, yet much more limited than totipotent and pluripotent stem cells [51]. In general, the earlier the cell is harvested within the developmental process, the higher the stem cells' potency (i.e. embryonic). Additionally, stem cells can be classified molecularly based on their profile of expressed cell markers. Bone marrow-derived mesenchymal stem cells, for example, are positive for CD29, CD44, CD105, CD73, CD90, CD106, and CD166 markers, while negative for CD14, CD34 and CD45 [52, 53]. In this section, we will discuss the unique properties and pros/cons of specific stem cell types which have shown promising preclinical results, with an emphasis on the relevance and feasibility for clinical translation.

3.1 The Early Era of Stem Cell Research and Initial Cell Sources

When the stem cell research field first developed, stem cells were primarily isolated from fetal tissues. Fetal stem cells have been shown to afford therapeutic benefits in preclinical models of many neurological disorders, including stroke, and were the cornerstone of early stem cell research in the 1970s and 1980s [54, 55]. These benefits include neuroprotective and neuroregenerative effects through secreting anti-inflammation molecules, releasing growth factors and differentiating into neuronal cells [56]. Furthermore, fetal stem cells demonstrate greater graft survival and ability to hone in on sites of injury when compared to adult stem cells [57, 58]. Unfortunately, fetal stem cells have been plagued by notions of immorality since their discovery, with opponents citing a lack of respect for human life and a possible justification for abortion as grounds for restricting research efforts [55]. From 1987 to 1992, these ethical concerns manifested as a moratorium—a legislative

suspension of all funds related to fetal stem cell research—which pushed scientists to search for non-fetal stem cell sources [55].

In an attempt to avoid these ethical concerns, varying methods have been used to develop and harvest alternative stem cell sources which produce potent therapies in lieu of fetal tissue. One such effort in the neurological field involved creating neuron-like hNT by exposing NT2-N embryonic carcinoma-derived stem cells to retinoic acid. These cells terminally differentiate into post-mitotic neurons, and were shown to survive and integrate into host neural networks [59]. Despite promising preclinical data [60], this line of cells was beset by concerns of tumorigenicity [61]. In a Phase I clinical trial, 12 patients—9 male and 3 female—with an age range from 44 through 74 years old, were transplanted with hNT cells developed by Layton Bioscience Inc. [62]. The study concluded that the transplantation of the hNT cells was safe and feasible, however consensus on the efficacy could not be reached due to small sample size [62]. The first postmortem analysis of a participant was reported 27 months after implantation [63]. The analysis showed that the hNT cells survived at 27 months after implantation with no evidence of tumor, additional infarcts, or neurodegenerative diseases [63]. However, this patient did not show motor recovery after transplantation [63]. While Phase I and Phase II clinical trials ultimately revealed the safety of these cells—with no adverse cell-related serological effects [60, 63], and moderate functional improvements—the inadequate patient sample size and ongoing concerns over their cancerous origin and high proliferative capacity would severely cripple investigations into this cell line. In light of cell lines such as hNT, the genetic modification of stem cells emerged as a potential solution to a number of issues which dampened progression into the clinic, such as artificially reducing proliferation/tumorigenicity, improving graft survival, and heightening anti-inflammatory effects [64].

Cell lines such as the conditionally immortalized human neural stem cell, CTX0E03 or CTX, developed by ReNeuron aimed to maintain all facets of stem cell therapeutic efficacy while eliminating tumorigenic risks [65]. ReNeuron utilized c-mycER(TAM) technology in human first trimester fetal cortical cells to develop conditional growth control with a fusion protein containing the growth promoting gene, c-myc, and a hormone receptor regulated by the synthetic drug, 4-hydroxytamoxifen (4-OHT) in producing the CTX-DP immortalized cell line [66]. This allowed the cells to be cultured to large quantities *in vitro* with 4-OHT-containing media, yet have their growth cycle arrested upon transplantation in the absence of 4-OHT [66]. With the support of promising preclinical data, CTX cells entered a phase 1 clinical trial named PISCES in 2010 (NCT01151124) and were shown to improve primary outcome measurements in male stroke patients [67]. A narrow patient pool of 11 males aged 60+, and the open-label, single-arm study design calls into question the extent to which reliable conclusions can be made regarding the efficacy of CTX cell implantation on functional recovery (NCT01151124). Arguably, the modified nature of these CTX cells may have negative effects on their stemness and therapeutic characteristics. In particular, with the lineage commitment

of the cells artificially restricted to neuronal phenotype, the ability of these neuronal-like cells to migrate is likely reduced, thus compromising their efficacy. This underscores the important balance which must be found when genetically modifying stem cells; being that stem cells are such complex biologics, scientists must be mindful not to unintentionally diminish major therapeutic mechanisms of stem cells by modifying dynamic and far-reaching pathways. When compared to unmanipulated or minimally-manipulated cell types, CTX cells (as well as SB623, which will be discussed shortly) took significantly longer to gain clinical approval, largely due to additional regulatory obstacles including long term *in vivo* preclinical studies and safety mechanism demonstrations which were required for all modified cell types [68]. The complications and dangers of genetic modification were first made evident in clinical trials of viral vector gene therapy which displayed the risk of fatal side-effects in some patients, producing an atmosphere of fear and apprehension surrounding all forms of genetically modified therapies [69]. This had the result of dampening and greatly delaying the clinical entry of genetically engineered stem cell types, such as CTX, which faced the skepticism of a wary Food and Drug Administration with the tragic loss of life fresh in their memory, and a negative public perception of all things genetically modified [70]. These unfavorable attitudes severely crippled the clinical progress of genetically modified stem cells—which objectively possess unique therapeutic potential.

Turning to embryonic stem cells, being from an early stage of development, these cells are considered the gold standard for stemness, with intrinsically high potency and high proliferative rates. In fact, only embryonic stem cells from the first few cell divisions after fertilization have true totipotent characteristics and are free from replicative senescence. These qualities make embryonic stem cells diverse in their applications. The use of embryonic stem cells arose from scientific efforts to steer clear of fetal-derived and cancer-derived cell lines, seeing as both were fraught with public image issues. Formed from *in vitro* blastocysts fertilization [71], embryonic stem cells evaded a portion of the moral issues surrounding fetal stem cells, yet fell short of acquiring complete public acceptance. While preclinical evidence has repeatedly demonstrated the efficacy of embryonic stem cells in neurological disorders [72], their wide-scale use has been similarly hindered by ethical, moral, and tumorigenic concerns.

Pressure from politicians and public opinion concerning embryonic and fetal stem cells, as well as the failed clinical trials of gene therapy which negatively affected the view of genetically engineered stem cells, have pushed scientists in the field of adult stem cells to look for alternative sources. For the past few decades, scientists have been able to identify and isolate adult-derived stem cells from various sources. These stem cells circumvent the ethical issues faced with embryonic stem cells, however, they pose challenges of their own. Some of the adult-derived stem cells which will be discussed are bone marrow-derived mesenchymal stem cells (BM-MSCs), extraembryonic stem cells, and induced pluripotent stem cells (iPSCs). While there are many other stem cells, these cell types currently hold the most potential to advance to the clinic.

3.2 Transitioning to Adult-Sourced Stem Cells

3.2.1 Bone-Marrow Mesenchymal Stem Cells

Mesenchymal stem cells (MSCs) are a class of multipotent stem cells that can be harvested from many adult mesenchymal tissues such as bone marrow, adipose tissue, and placenta. Of these, BM-MSCs are the most common and also the most studied adult stem cells, with multiple pre-clinical studies showing their therapeutic benefits in various neurological disorders such as TBI, amyotrophic lateral sclerosis, and particularly in stroke [9, 73–75]. One of the major advantages of BM-MSCs is the ability for autologous transplantation. BM-MSCs can be harvested and amplified from a patient's own tissues, thereby eliminating the concern of post-transplant immunologic rejection. However, an argument can be made against using autologous stem cells because the patients' BM-MSCs might be less potent than healthy donors'. Recent studies have linked stroke neurological deficits with changes in the peripheral systems such as inflammation in the spleen or alterations in the gut microbiome [9, 76]. These peripheral alterations could negatively affect the health and therapeutic efficacy of the patient's BM-MSCs. In terms of tumorigenicity, some studies have reported that BM-MSCs may induce tumor formation [77–79], however, BM-MSCs have been deemed safe in both pre-clinical and clinical studies [70, 75, 79–82]. These advantages of BM-MSCs are particularly relevant in attempting to transition BM-MSCs into the clinic. Importantly, BM-MSCs can still exert their beneficial effects despite short survival time and lack of neuronal differentiation [9, 16, 81, 83]. While appearing paradoxical, BM-MSCs' mechanisms of action rely more heavily on immunomodulation, modifying the microenvironment, and secreting trophic factors rather than differentiating and integrating into neural networks [53]. This is a distinct advantage of these cells, as clinically it is more feasible to give a stem cell "booster shot" to compensate for the low survival rate rather than attempting to control the formation of tumors inherent in other cell types. However, BM-MSCs also pose challenges that must be considered. BM-MSCs may behave differently depending on their location, method of extraction, isolation, and culture [79]. Therefore, it could be difficult to have a consistent and homogenous pool of BM-MSCs in mass production. Another limitation of BM-MSCs is that it requires time to collect, isolate and amplify the autologous BM-MSCs before they can be transplanted back into the patient, limiting the accessibility of these stem cells and their availability to the population at large.

Homogenous subpopulations of BM-MSCs may offer distinctive benefits. One such cell type, multilineage-differentiating stress-enduring (Muse) stem cells can be found within bone marrow (in addition to all connective tissues), and have displayed characteristics which make them highly appealing for therapeutic exploration [84]. These pluripotent cells have shown a unique ability to remain viable within highly stressful microenvironments [84]. Furthermore, the asymmetrical divisions and low telomerase activity of Muse cells mean low tumorigenicity and minimal risk of teratoma formation. Another subpopulation of MSCs found within bone marrow is the

Very small embryonic-like (VSEL) stem cell [85]. These cells are roughly half the size of hematopoietic stem cells, and maintain the pluripotent ability to differentiate into cells from all three germ layers [86]. Additionally, these cells have been shown to form small clusters that resemble embryonic bodies *in vitro*, which could have implications in the efficiency these cells can be cultured, as well as preserving their highly potent characteristics [86].

BM-MSCs have experienced success within the clinic. A GDNF-releasing, Notch-induced human bone marrow-derived mesenchymal stem cell line—SB623—was employed in a Phase I clinical trial beginning in 2011 (NCT01287936), after displaying significant amelioration of stroke symptomology in preclinical animal models. GDNF—glial cell line-derived neurotrophic factor—confers potent pro-survival effects. SB623 stem cells undergo *ex vivo* gene delivery to heighten their neurotrophic properties via enhanced GDNF secretion. As of now, the SB623 clinical trial has demonstrated relative safety, and preliminary reports of efficacy in chronic stroke patients [80]. Similar to the CTX cells described previously, consideration must be given to the genetically modified nature of these stem cells, and how these modifications may inadvertently affect the stemness and therapeutic properties of the cells.

3.2.2 Extraembryonic Stem Cells

Extraembryonic stem cells are a collective term for the adult-derived stem cells found in the placenta, the umbilical cord, the amnion, and Wharton's jelly [83, 87–89]. Placenta-derived MSCs, umbilical cord blood-derived MSCs (UCB-MSCs), and amnion-derived MSCs are the focus of many current investigations. Considering these stem cells' common origins, they share many therapeutic properties with BM-MSCs, such as modulating neuroinflammation, stimulating endogenous neurogenesis, releasing trophic factors, and promoting functional recovery in pre-clinical animal models of stroke [87, 90–92]. However, extraembryonic stem cells can differentiate into more cell types than BM-MSCs [89]. Recent studies have demonstrated that UCB-MSCs and placenta-derived MSCs can differentiate into neuronal cells that express markers such as Nestin or β-tubulin III—important markers of neuronal identity and function [93]. Similarly, recent studies have also reported that Wharton's jelly-derived MSCs can differentiate into various cell types such as glial, neuronal, and endothelial cell [94]. There are several advantages of extraembryonic stem cells compared to embryonic, fetal, and bone marrow-derived stem cells. These tissues are currently considered waste products, thus posing no health risk to the mother or baby, and circumventing any ethical issue related to the extraction of these extraembryonic stem cells. In the case of amnion-derived MSCs, the stem cells can be collected during amniocentesis—a safe, routine procedure during pregnancy [95]. These stem cells can then be expanded and ready to treat any disease associated with childbirth, such as hypoxia, or cryogenically preserved for future catastrophic events such as stroke or TBI [95]. However, there are also downfalls associated with extraembryonic stem cells. These extraembryonic tissues contain a

variety of cells, making it difficult to isolate a homogeneous population of stem cells. Moreover, the amount of stem cells in these tissues is limited, especially in amnion fluid, requiring more time to amplify these stem cells prior to transplantation. In addition, it is expensive and unrealistic to maintain all extraembryonic tissues for every baby. Only a small portion of the population can afford to cryogenically preserve these tissues for an extended time.

3.3 Induced Pluripotent Stem Cells: A New Horizon for Stem Cell Research

Contrary to previous dogma, recent studies have demonstrated that differentiated adult cells can be reverted back to earlier stem cell states. Through molecular manipulation, these cells can regain their stemness, especially their proliferative property [89]. These cells are termed induced pluripotent stem cells (iPSCs). One of the challenges of adult stem cells is the limited number of passages before the cells stop proliferating. iPSCs are molecularly enhanced to increase the stemness (both proliferative and differentiating capacities) of the cells and can be scaled to large quantities. Furthermore, iPSCs also bypass the ethical issues associated with harvesting embryonic or fetal stem cells. In pre-clinical studies of stroke, iPSCs have shown promising results for improving neurological deficits, decreasing neuroinflammation, promoting neurogenesis and increasing angiogenesis [96–99]. Another major advantage of iPSCs is their ability to be redirected to differentiate into various cell lineages. For example, iPSCs can be induced into neural cells such as neurons, astrocytes, microglia, and vascular endothelial cells. While iPSCs have many advantages, tumorigenesis is a major concern when using iPSCs. In most cases, cancerous genes are used to induce the iPSCs, therefore it is important to control the tumorigenic property before iPSCs can advance further into the clinic.

3.4 Challenges in Translating Stem Cell Therapies to the Clinic

Finally, it is worth noting that there are many other logistic challenges that must be considered before any of the stem cells discussed can successfully advance into, through, and beyond clinical trials. These challenges include reaching a consensus on ideal cell type, dosage, number of transplants, timing, and route of administration. Indeed, the current clinical trials mentioned above (NCT01151124, NCT01287936) are being carefully analyzed and scrutinized for sub-optimal design and small patient pools.

The ideal timing and route of the administration depend on the intended purpose of the stem cell transplantation. Within the context of stroke, the distinct acute and

chronic pathological phases must be considered. Intracranial (IC) transplantation is preferable in the acute and subacute phase of stroke. In these time frames, the presence of stem cells at the penumbra dampens the hostile environment and reduces the spread of the infarct core. Conversely, in the chronic phase, the inflammation both in the brain and the periphery is the main concern. Therefore intravenous (IV) or intra-arterial (IA) injection of stem cells may pose as better alternatives. In addition, if there is a need for multiple transplantations or injections, IV and IA are much more desirable choices. Of note, during the IV and IA injection, the majority of stem cells are trapped in the peripheral organs such as lung and spleen. However, the route of administration does not have to be mutually exclusive; an appealing option may be first transplanting via IC injection followed by IV booster shots for maximizing effectiveness.

The growing number of unique stem cell types begs the question of which is the best candidate stem cell type for clinical application. As discussed previously, each of the various stem cell types has their specific strengths and weaknesses. A well-designed preclinical research effort geared toward evaluating the safety, efficacy and mechanism of action of each stem cell type may reveal the optimal transplantation regimen of cell therapy for clinical trials. In particular, determining the appropriate stem cell dosage, timing, and route of delivery in animals with direct human application will be critical in advancing cell therapy to the clinic.

Stem cell therapies for stroke are at a pivotal point currently. Preclinical evidence has continued to accumulate for the past four decades which indicates that transplantation of stem cells offers significant amelioration of stroke-induced deficits, both when delivered acutely as well as chronically. Furthermore, IV and IC administration have displayed unique benefits and practical advantages which broaden the applicability of stem cell transplantation and heighten their far-reaching potential. The issues described above, however, have crippled the advancement of this therapy, resulting in limited clinical trials with inconsistent measures of efficacy. Careful evaluation of the six most recent clinical trials of BM-derived stem cell therapies in stroke—four within the subacute phase of stroke (NCT01716481, NCT00859014, NCT01501773, NCT00535197), and two within the chronic phase (NCT01151124, NCT01287936)—confirms the disconnect between lab and clinic, and reveals the gaps which still exist in our knowledge of stem cell therapies. As additional clinical trials proceed with enlisting larger cohorts of patients, pursuing long-term follow-up, and thoroughly assessing the status of the transplanted cells, we will be able to further evaluate the safety, efficacy, and mechanisms of action of stem cell therapy for stroke. Indeed, the mechanisms of action by which stem cell confer their therapeutic benefits in stroke are yet to be fully understood. How stem cells achieve this regenerative process stands as the primary challenge for stem cell researchers within the field, and is a vital step in designing more successful clinical trials [68, 100, 101]. The following section will discuss the canonical mechanisms of action for stem cells, as well as explore the concept of the biobridge and how it advances our understanding of the host-transplant interactions which mediate stem cells' therapeutic effects.

4 Stem Cell Therapy: Moving Beyond the Cell Replacement Paradigm

Given the multifaceted pathology of stroke, therapies targeting only a single pathology are unlikely to resurrect the motor and cognitive deficits caused by stroke, particularly at the chronic stages. Stem cell therapy is unique in its potential to be beneficial over a wide therapeutic window and its capacity to mitigate the diverse pathological processes observed after stroke [102]. The two known and widely-accepted mechanisms by which stem cells elicit neuroprotective and neuroregenerative effects after stroke are cell replacement and bystander effects [103, 104].

Initially, it was proposed that transplanted stem cells would serve the same function as they do within the body—generating new cells and replacing dead or damaged tissue. Transplanted stem cells were predicted to differentiate and directly replace loss cells, however, studies have demonstrated that within the injured brain, this notion is at best partially correct due to various factors [105, 106]. First, the majority of transplanted stem cells do not survive even when immunogenicity is accounted for through autologous transplant or Immunosuppressants [107]. Second, while many stem cells have demonstrated that they can differentiate into neuronal cells *in vitro* under highly-controlled conditions, they failed to do so in large numbers within *in vivo* model [108, 109]. One explanation for both issues is that transplanted stem cells enter a hostile microenvironment which is not conducive to long-term survival, differentiation and maturation. Thus, merely increasing the number of transplanted cells would not solve the problem. Furthermore, even with the small number of differentiated and living cells, there is little evidence to support that these cells integrate into neural networks to a significant extent, hence cell replacement is not considered a primary mechanism of action of stem cells.

Instead, evidence supports that the therapeutic capacity of stem cells lies largely within its bystander effects in which the stem cells secrete trophic factors and anti-inflammatory cytokines [110]. Stem cells secrete a cocktail of vital growth factors and, as mentioned previously, a reduction in growth factors is a key player in secondary cell death [111, 112]. For example, in animal studies, BM-MSCs secrete a variety of trophic factors which stimulate the neuroregeneration process [113]. Some of the notable trophic factors are VEGF, BDNF, NGF, insulin growth factor-1, and hepatocyte growth factor [113]. Similarly, several growth factors such as VEGF and BDNF were elevated after the transplantation of UBC-MSCs or placenta derived-MSCs [114]. In addition to growth factors, stem cells secrete anti-inflammatory molecules that mitigate neuroinflammation [115]. Stem cells secrete microvesicles and exosomes known to contain growth factors, proteins, anti-inflammatory cytokines such as IL-10 and IL-4 [9, 74, 116, 117], microRNA and lncRNA such as nuclear enriched abundant transcript 1 (NEAT1) and metastasis associated adenocarcinoma transcript 1 (MALAT1) which play key roles in inflammation, gene expression, and cell survival [74]. When transplanted after stroke, not only do stem cells have the capacity to sequester inflammation at the ischemic source, but also throughout the periphery. Intravenous administration of human

bone marrow stromal cells (hBMSCs) in rats following stroke resulted in the preferential migration of stem cells to the spleen compared to the brain [9]. Treated animals presented with lower infarct volumes, and reduced cerebral and splenic inflammation [9]. Interestingly, this study reported that a greater number of hBMSCs observed in the spleen correlated to decreased infarct and peri-infarct volume, as well as lower TNF-α density in the spleen [9]. Viewed holistically, these results indicate that peripheral implantation of stem cells may afford neuroprotection indirectly by moderating the overactive and global inflammatory response following stroke by similar anti-inflammatory mechanisms as observed in IC injection.

Mounting evidence has shifted the consensus respecting the primary mechanism of action from cell replacement paradigm toward bystander effect [118]. Indeed, stem cells are now well known for their therapeutic trophic mechanisms that contribute to neuroprotection. However, even combined, both mechanisms do not fully explain the endogenous recovery effect observed after transplantation. While the trophic factors can stimulate endogenous stem cells to proliferate and differentiate, it is unclear how these endogenous stem cells can then migrate to the injured brain regions [119]. Migration is a challenging and complex process, especially in a mature adult brain. Without external support and guidance, inflammatory cytokines are not enough to attract the endogenous stem cells over long distances. To this end, we propose a third mechanism of action for stem cell transplants that our lab has revealed—the formation of a stem cell-paved biobridge—which furthered our understanding of how endogenous stem cells achieve migration from deep neurogenic niches to distal injured regions of the brain.

5 The Biobridge: Exogenous Stem Cells Guide Endogenous NSCs Towards Repair

For the past five decades, the scientific community has been aware of the neurogenic capacity of the adult mammalian brain [120], however, the precise role and regulation of neural stem cells (NSCs) remains an active area of research. Evidence contradicts the original assumption that the primary role of endogenous NSCs is to regenerate damaged tissue after brain injury. Instead, NSCs take part in brain plasticity by both direct and indirect mechanisms which are crucial for certain types of hippocampal and/or olfactory bulb-dependent learning and memory [121]. Unfortunately, NSCs' capacity for tissue regeneration after brain injury is extremely limited despite an increase in activation following such injurious events. Poor cell survival and proliferation, lack of commitment to neuronal lineage, and limited migration are all challenges that prevent these endogenous NSCs from facilitating significant regeneration after brain injury [21].

Much like peripheral inflammatory cells, transplanted stem cells are drawn towards molecular signals from the peri-infarct area. Extracellular matrix (ECM) remodeling allows these cells to move through the brain parenchyma. Interestingly,

the process of migrating exogenous stem cells benefits endogenous neural stem cells (NSCs) as well [21]. NSCs are not ubiquitous throughout the brain, but are instead restricted to neurogenic niches in two brain regions—the SVZ of the lateral ventricles and the SGZ of the dentate gyrus of the hippocampus (although quiescent NSCs have been identified in other brain regions) [121]. When brain injury occurs at sites distal to these locations, the potential for robust repair or neuroprotection afforded by endogenous NSCs is diminished due to their limited capacity for migration. In a previous study, our lab discovered that this shortcoming of NSCs may be compensated for by additional mechanisms of transplanted stem cells [21]. In this investigation, a controlled cortical impact was delivered to the frontal cortex of Sprague-Dawley (SD) rats, a common model for TBI. Intracerebral injection of Notch-induced hBMSCs (referred to as SB623, supplied by SanBio Inc.—see Sect. 3.2.1) [8, 122] was performed 7 days post TBI. Locomotor and neurological tests were completed prior to TBI, pre-transplantation, and monthly following transplantation for up to 3 months. As expected, at 1, 2 and 3 months post TBI, treated animals displayed significant improvements in motor and neurological tasks. Histological analysis at both 1 and 3 month time-points also showed reduced lesion size and improved cell survival in the peri-impact area. Notably, the engraftment rate for the transplantation was minimal, at only 0.60% at 1 month post-transplantation and 0.16% at 3 months.

While these findings were similar to other reports of stem cell transplantation after TBI, immunohistochemistry and laser capture revealed a previously unreported phenomenon in which exogenous stem cells form a cellular bridge between the neurogenic SVZ and the lesion within cortex. With the formation of this biobridge came successful endogenous stem cell migration; a pathway was observed alongside the same trajectory of the migrating injected stem cells. The pattern of endogenous stem cell migration was remarkably different between treated and untreated animals. In vehicle injected animals, endogenous cells were sparse throughout peri-impact cortical regions and newly formed neural cells within the SVZ were nearly absent. Additionally, cell proliferation and neural differentiation was stunted in non-treated animals. By contrast, in animals that received the SB623 cells, at 1 month post-transplantation, robust endogenous cell proliferation (Ki67) and immature neural differentiation (nestin) was observed in peri-impact cortical regions and the SVZ, with migrating cells (DCX) along the corpus callosum. Immunohistochemistry revealed hBMSCs localized within the impacted region, down into the cortex, across the corpus callosum and along the ventricles to the location of neurogenic niche. At 3 months post transplantation, $DCX^+/HuNu^+$ (human nuclei) cells were identified alongside the hBMSCs transplanted cells indicating that non-transplanted cells were able to navigate through the ECM that was likely recently remodeled by the migrating hBMSCs. It is important to note that the transplanted stem cells survival was largely diminished by 3 months, suggesting that even though these cells did not persist, endogenous cells were still able to utilize the same route through the ECM where they could continue to migrate through and thrive, sustaining endogenous recovery efforts despite the absence of transplanted stem cells.

To better understand the mechanism of biobridge formation, we explored metallomatrix protein (MMP) expression, specifically, MMP-9. Molecular analysis via

laser-capture revealed increased MMP-9 expression along the migratory pathway [21]. Notably, TBI-vehicle animals also displayed an increased MMP-9 expression following stroke, however, this upregulation reverted to levels comparable to sham animals at 3-months post-injury. In SB623 transplant animals, MMP-9 expression doubled that of TBI-vehicle animals at 1 month post-TBI and expression increased ninefold by month 3. This data suggest the importance of this neurovascular proteinase in the long-term neural regenerative efforts of transplanted stem cells. While these results indicate that endogenous cells alone increase MMP-9 expression after brain injury, stem cell transplantation promotes a more robust mechanism for ECM remodeling than unaided endogenous stem cells by leaving a direct pathway for the endogenous stem cells to utilize.

Complementary to these *in vivo* results, an *in vitro* study presented SB623-promoted cell migration via an ECM-mediated mechanism [21]. Primary rat cortical cells were grown by themselves or co-cultured with SB623 cells in two different conditions—with or without Cyclosporin-A, an MMP-9 inhibitor. Co-culture of SB623 cells without the presence of MMP-9 inhibitor significantly enhanced the migration of primary cortical rat cells. The migration of primary cortical rat cells into the chamber containing the SB623 cells was significantly reduced when treated with Cyclosporin-A, with no significant difference compared to the cultures without stem cells. This study further supports that stem cells, particularly SB623 cells, promote cell migration mediated largely via MMP-induced ECM remodeling.

Moreover, it is believed that migratory trophic factors released by the exogenous stem cells such as cysteine-x-cysteine motif chemokine ligand 14 (CXCL14) and monocyte chemoattractant protein 1 (MCP1) further promote endogenous stem cell migration from the neurogenic niche. It is important to note that the transplanted MSC's long-term survival was not necessary for functional improvements in this study. Instead, the therapeutic benefit was attributed to their ability to manipulate the microenvironment and stimulate endogenous stem cell migration, proliferation, and differentiation. These findings positively address some of the tumorigenic concerns mentioned in previous sections, as eventual death of transplanted stem cells and loss of stemness characteristics are increasingly regarded as important in preventing tumorigenesis.

To further investigate this novel stem cell mechanism of action, we designed a pilot study to investigate if a similar biobridge formation occurs after stem cell transplantation in the MCAO stroke model. We would like to share our promising ongoing study. Normal male SD rats (n = 10, average weight = 200 g) were subjected to MCAO surgery. Three days post stroke, the animals were split into two groups that received a one-time transplantation of human BM-MSCs by either IC (n = 5) with 1.0×10^6 cells or IV (n = 5) with 4.0×10^6 cells. The animals were sacrificed and processed for immunohistological staining at day 7 post-stroke. Similar to previous reports, we observed MSCs in the cortex (Fig. 8.2) and striatum in both IC and IV groups (Fig. 8.3), showing that the MSCs can infiltrate the brain either through IC or IV transplantation. Interestingly, the transplanted MSCs from the IC injection group mainly traveled along the corpus callosum, while the MSCs from the IV group disperse throughout the striatum and cortex. DCX^+ stain-

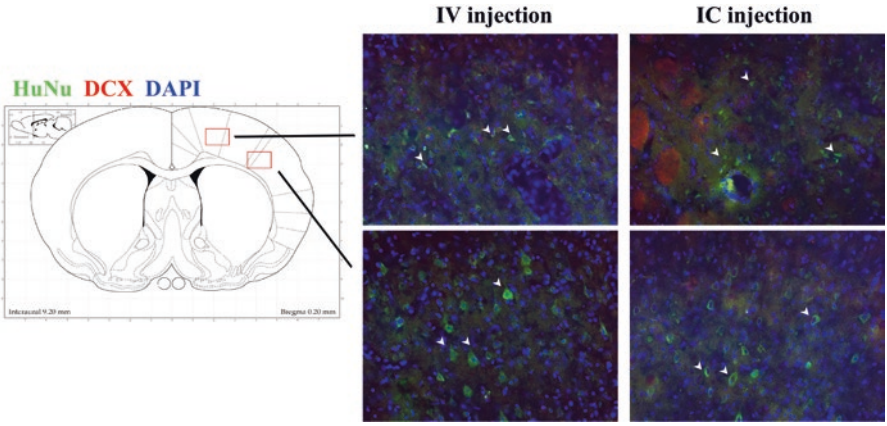

Fig. 8.2 Distribution of human BM-MSCs (HuNu$^+$) in the cortex. After transplantation, BM-MSCs (HuNu$^+$) succesfully infiltrated the ischemic brains in both IC and IV route of administration. HuNu$^+$ cells were detected, however DCX$^+$ cells were not found in the cortex

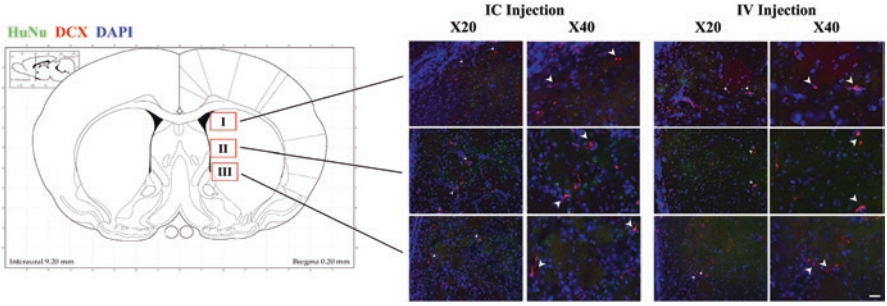

Fig. 8.3 Distribution of human BM-MSCs (HuNu$^+$) and immature neurons (DCX$^+$) in the striatum. HuNu$^+$ cells from the IC group traveled along the corpus callosum, whereas HuNu$^+$ cells from the IV group dispersed throughout. Transplanted human BM-MSCs were found around DCX$^+$ cells, paving the way for these immature neurons to migrate toward the penumbra

ing, a marker for cell migration, revealed that DCX$^+$ cells from the IV group traveled along the corpus callosum and the ventricle wall (Fig. 8.3). The DCX$^+$ cells found in the IC group traveled more laterally. However, it is worth noting that the DCX$^+$ cells from the IV group traveled further into the striatum compared to the IC group. The migration pattern of the immature neurons is summarized in Fig. 8.4. To further validate our findings, we performed another set of staining for proliferating neuronal cells (Ki67$^+$/Nestin$^+$). Similar to the DCX staining results, Ki67$^+$/Nestin$^+$ cells were found along the corpus callosum and ventricle wall. In addition, fewer Ki67$^+$/Nestin$^+$ cells were found further into the striatum (Fig. 8.5). In conclusion, we have demonstrated in this pilot study that a similar phenomenon

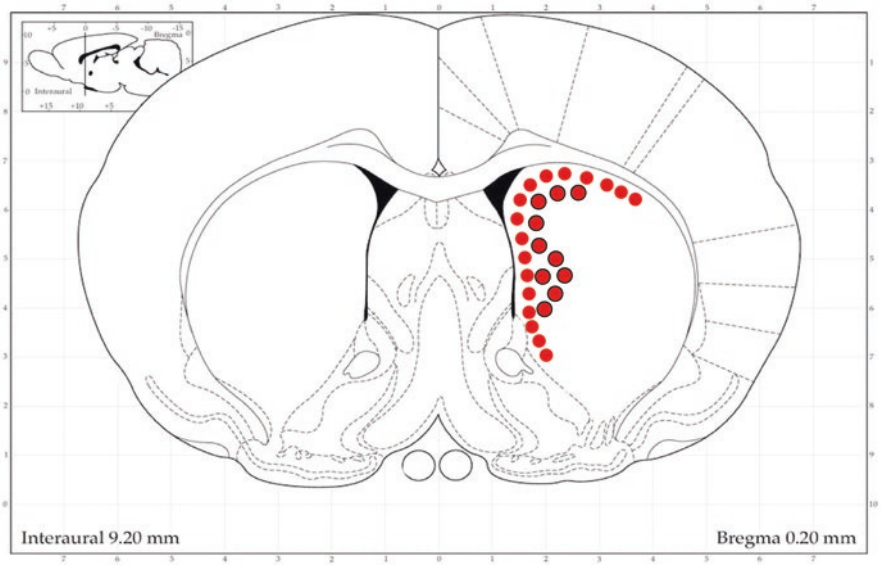

Fig. 8.4 Schematic of the distribution of DCX$^+$ cells between the IV and IC group. DCX$^+$ cells from the IV group travel along the corpus callosum and the ventricle wall. DCX$^+$ cells from the IC group travel more horizontally compared to IV group. However, more DCX$^+$ cells from IV group travel further into the striatum compared to IC group

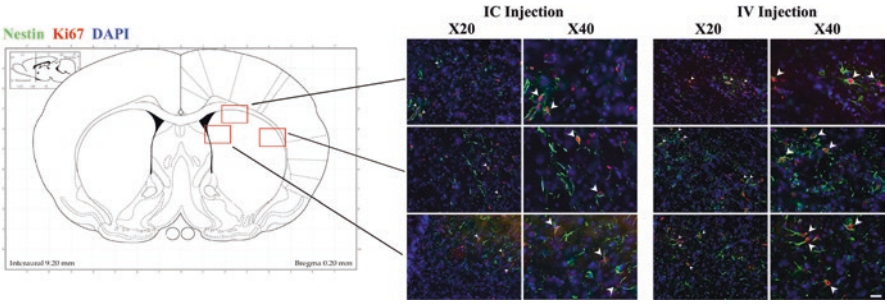

Fig. 8.5 Distribution of proliferating neuronal cells (Nestin$^+$Ki67$^+$) in the striatum. Nestin$^+$Ki67$^+$ cells have similar migration pattern compared to the DCX$^+$ immature neurons. In both IC and IV groups, Nestin$^+$Ki67$^+$ cells were found along the wall of the ventricle and the corpus callosum

reported in TBI, whereby transplanted BM-MSCs modify the environment to facilitate the migration of proliferating and immature neurons, also occurs in stroke. Interestingly, with the help of transplanted stem cells, both DCX$^+$ and Nestin$^+$Ki67$^+$ cells utilized the corpus callosum as a highway to travel further into the penumbra.

6 Future Directions for Advancing the Biobridge Concept

Although the biobridge concept has now been demonstrated in TBI and preliminarily in stroke, barriers still exist to translating these findings into being clinically relevant. A more complete understanding of the cellular and molecular processes which define the biobridge formation and how the assisted migration of endogenous stem cells can be optimized by exogenous transplantation must be unveiled before patients can benefit from these findings. Future studies should aim to more fully characterize the underlying molecular changes that produce the biobridge. Our group has revealed the role of MMP-9 in extracellular matrix remodeling in vitro, yet this single protein is unlikely to account for the totality of the extensive remodeling seen within the biobridge region. Conditional MMP-9 knock-out animals could be valuable in further illustrating the role this protein has in vivo [123–125]. Moreover, transplanted stem cells modified to overexpress MMP-9 and other remodeling factors may reveal a target for heightening the graft-host cell interactions, providing an avenue by which this new mechanism could be utilized to improve clinical outcomes. Importantly, data on the global effects of MMP-9 after stroke are inconclusive, and thus exploring the biobridge formation in MMP-9 knockout mice could help characterize the complex roles which MMP-9 has after brain injury, perhaps playing protective and detrimental roles in different capacities.

Future research efforts should investigate the molecular interactions and crosstalk of the transplant and host stem cells. Here, we describe the remodeling processes observed in brain regions where host stem cells overlap with transplant stem cells. Importantly, transplanted MSCs have been shown to secrete factors which not only promote the survival of host neurons, but are also likely to promote survival of the endogenous stem cells which they come into close contact with. The vast prosurvival secretion profile of transplanted hMSCs, such as wnt3a, VEGF, and BDNF, among others [110], could mean that endogenous stem cells are both guided, and nurtured, by transplanted cells, thereby heightening their regenerative capacity upon arrival to the peri-injured regions. Additionally, factors such as wtn3a and VEGF have been shown to inhibit the quiescent state of host stem cells, wherein their migratory and regenerative properties are stagnated [121]. Beyond the ECM remodeling discussed extensively above, exploring how transplanted MSCs enhance the therapeutic capabilities of host stem cells through cell-to-cell interactions will further enhance our understanding of the robust benefits offered by stem cell transplantation.

The chronological characteristics of the biobridge also deserve additional evaluation—both with regards to its structure and composition over time, as well as how its development varies with different transplant time points. To date, our group has only investigated the progress of the biobridge formation out to 3 days in stroke, making it imperative for additional studies which investigate the biobridge structure and formation through the sub-acute and chronic phases. Understanding how ongoing molecular changes encourage the migration of endogenous stem cells could

provide indications for the effects which acute biobridge formation, and sub-acute progression, have in ameliorating chronic deficits. Discrepancies in the ideal time point for stem cell transplantation post-injury already exist, so careful consideration must be given in determining the transplant time which not only augments the biobridge formation but also gives equal consideration to the various other therapeutic mechanisms occurring concurrently.

Finally, the prevalence of the biobridge concept in other neurological disorders should also be explored. That this process has been demonstrated in two different disease models indicates that this graft-host cell interaction is a more general mechanism of stem cell therapies, and not specific to the pathologies of a single disease. Indeed, this therapeutic mechanism may have far-reaching implications in other neurological diseases amenable to cell transplantation, although the intricacies of its formation may vary greatly between diseases with and without focal damage. This was partly demonstrated in our TBI versus stroke comparison, with TBI brains showing a more unidirectional biobridge and stroke brains displaying a three-dimensional, multi-directional biobridge. How this biobridge concept manifests in neurological disease without focal lesions—i.e. amyotrophic lateral sclerosis, multiple sclerosis, Alzheimer's disease, transient global ischemic events, and neonatal hypoxic—will need to be further explored.

7 Conclusion

Tailoring the use of stem cell therapies in stroke, TBI, and other neurological disorders is an ongoing scientific effort. The unique pathology of neurodegenerative diseases poses a challenge seemingly too large for traditional pharmaceuticals to compensate for, and thus alternative therapeutic options—namely stem cells and regenerative medicine—have received increased attention. Stroke, in particular, has received significant attention as a possible beneficiary of stem cell transplantations. The various pathological processes which accompany stroke appear highly compatible with the dynamic therapeutic profile of stem cells. Transplanted stem cells' ability to secrete anti-inflammatory factors, pro-survival/anti-apoptotic molecules, and to integrate into the host parenchyma contribute to the benefits which they confer. The therapeutic capacity of stem cells in stroke has been demonstrated repeatedly in pre-clinical investigations, yet translating this promise into widely-available clinical treatment options has been slow. This is in no small part to the inherent complications which accompany non-traditional pharmaceuticals, including issues of dose, timing, route of administration, and stem cell source.

The shortcomings of clinical trials of cell transplantation have resulted in a renewed effort to explore the basic science mechanisms of stem cell therapies. The path to successful clinical trials will likely be paved by basic science discoveries concerning the complex therapeutic mechanisms of stem cells. Here, we described a novel therapeutic mechanism of stem cells, the biobridge, which works in conjunction with the

established mechanisms to produce the functional improvements observed following stroke, as well as TBI. The discovery of this mechanism has both basic science, as well as translational, Implications; that exogenous stem cells interact with and encourage the movement of endogenous host stem cells to regions of damage aids in explaining the seemingly paradoxically-robust functional recovery seen in stem cell transplantations despite minimal graft survival rates. Moreover, understanding the extracellular matrix remodeling capacity of transplanted stem cells provides a novel bioengineering target for genetically enhancing stem cells. These findings, in the context of the larger scientific effort to better understand the details of stem cell therapeutic modalities, assist in providing the preclinical basis for more effective clinical trials, bringing stem cell therapies closer to positively impacting stroke patient recovery.

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Chapter 9
Bone-Marrow-Derived Cell Therapies in Stroke: Immunomodulatory Effects

Laith Maali and David C. Hess

Abstract Cell therapies have attracted significant attention in treating multiple neurological disorders including stroke. The preclinical studies have paved the road in understanding the potential clinical applications of cell therapies in stroke recovery. Cells can be obtained from multiple sources and transplanted through different routes. Animal and human studies suggest that cell therapies exert their effect via paracrine and immunomodulatory effects rather than physically replacing the damaged cells. Clinical studies are still in the early phases but show safety and feasibility and some hints at efficacy.

Keywords Cell therapies • Stem cells • Bone marrow derived • Mesenchymal stem cells • Multipotent adult progenitor cells • Stroke • Phase 2 clinical trial • Immune modulation • Immunomodulation • Neuroprotection • MultiStem

Abbreviations

ACTH	Adrenocorticotropic hormone
APCs	Antigen presenting cells
DAMPs	Danger-associated molecular pattern molecules
GABA	gamma-Aminobutyric acid
IA	Intra-arterial
IL	Interleukin
INF	Interferon
IP	IFN-γ-inducible protein
IV	Intravenous
MAPC	Multipotent adult progenitor cells

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P.A. Lapchak, J.H. Zhang (eds.), *Cellular and Molecular Approaches to Regeneration and Repair*, Springer Series in Translational Stroke Research,
https://doi.org/10.1007/978-3-319-66679-2_9

MCP	Monocyte chemoattractant protein
MHC	Major histocompatibility complex
MIP	Macrophage inflammatory protein
miRNA	microRNA
MMP	Matrix metalloproteinases
MNC	Mononuclear cells
MSC	Mesenchymal stem cells
NIHSS	National Institute of Health Stroke Scale
RANTES	Regulated on activation normal T cell expressed and secreted
RNA	Ribonucleic acid
r-tPA	Recombinant tissue plasminogen activator
SCID	Severe combined immunodeficiency
TLRs	Toll-like receptors
TNF	Tumor necrosis factor

1 What Are Cell Therapies?

Stroke is the second cause of death and the most common cause of adult neurological disability worldwide [1]. The main goal of current stroke management is to achieve a rapid recanalization to limit brain tissue damage. Currently, the only approved drug to be used in such a treatment is recombinant tissue plasminogen activator (r-tPA) [2]. On the other hand, this drug can only be used in the first 4.5 h of symptoms onset and only about 5% of acute ischemic stroke patients receive this therapy and despite that about half of them will end up with significant long term disability [3]. Even with the improved functional recovery with endovascular thrombectomy, the therapeutic window for such a treatment remains under 6 h according to current guidelines, although this time window will likely expand [3].

Cellular therapies emerge as a promising additional treatment to help limit the brain damage and improve functional outcome after acute stroke beyond the limited therapeutic window of r-tPA and endovascular thrombectomy. Cell therapy refers to cellular material that is able to exert a desired biological effect. In the 1990s, cell therapies were for the first time shown to be a potential treatment in patients with Parkinson's disease, as studies showed a positive effect of fetal striatal graft in increasing GABA release and reorganizing GABA receptors that led to physical and intellectual benefits [4]. Since that time, cell therapies evolved and other cell types were identified to reduce neurological injury, including positive studies in animal models of spinal cord injury [5] and traumatic brain injury [6] and stroke [7–11].

It is now believed that cell therapies do not mainly act by replacing injured cells but rather they exert their benefit by the activation of injured tissue to remodel and prevent further injury [12]. Many types of cell therapies release growth factors and cytokines that lead to immunomodulation, brain repair and cell survival [13].

2 Cell Therapy Types

There are multiple cell types involved in cell therapies, but they can be classified according to their original source: embryonic, induced pluripotent stem cells and adult cells. Adult derived cells are usually taken either from the bone marrow, adipose tissue or dental pulp cells. Another source is from the umbilical cord, the amniotic fluid or the placenta at the time of birth. We will focus in this chapter on the adult bone marrow derived cell as it is type furthest along in the translation from the bench to the bedside.

The bone marrow contains multiple types of cells, and based on the culturing techniques, we can generate mononuclear cells (MNC), mesenchymal stem cells (MSC) or multipotent adult progenitor cells (MAPC). MNC contains mature and immature cells of lymphoid, myeloid and erythroid origin. They do not require culture and can be quickly isolated which make them easy to use in acute to subacute phases of stroke as an autologous transplant. MNC have be shown to improve neurological outcomes in animal model, they reduced stroke lesion size, promoted angiogenesis and suppressed lymphocytic infiltration [7–9, 14]. Based on this, human MNC therapies were initiated and they are still in the early phases, but so far showing feasibility and safety with different routes of administration [15–17].

MSC are plastic adherent cells and require longer times to expand in culture than MNC. They can be easily derived from multiple sources including the bone marrow, adipose tissue and umbilical cord, but the majority of animal studies of MSC focused on the bone marrow derived cells. Bone marrow-derived MSC reside in the stromal part of bone marrow and sometimes termed marrow stromal cells [10], they have low immunogenicity and have strong immunomodulation capabilities. Autologous transplantation usually requires weeks to months to culture which limit its use in the acute phase of stroke [18], but their low immunogenicity suggest their safety and benefit for allogeneic and autologous transplantation in human [18–20] and animal studies [21].

MAPC are more primitive cells compared to MSC they have a broad differentiation capability. As MSC they have low immunogenicity and strong immunomodulation capabilities. They require long term culture so their use in acute stroke is limited unless as an allogeneic transplant [22]. Compared to MSC they have distinct phenotype protein and gene expression pattern [23]. They showed benefit in a stroke animal model [24] and safety in human studies [25].

3 Route of Administration

There are three main routes for cell administration: Intravenous (IV), intra-arterial and intracerebral. Intravenous route is the preferred method given the ease of use and especially if the main goal from the cells is to exert their effect through a systemic process. With IV administration the majority of MSC, for example, get entrapped

passively in the lung vasculature given their relative large size, but some cells are able to migrate to the ischemic area and the peri-infarct zone [26–28]. Based on that, the possibility that MSC benefit patients with stroke by becoming brain cells is unlikely [12]. The first reported IV MSC transplantation in human was performed by Bang et al. [29]; in their study they showed significant improvement in the modified Rankin score and Barthel index up to 6 months after IV MSC transplantation compared to control group.

Intra-arterial (IA) route provides some benefits with selective delivery of cells to the area of injury, deliver a higher number of cells and bypass the peripheral systemic entrapment. On the other hand, IA route carries some clear risks, like microvascular plugging with large sized cell delivery [30–32].

Intracerebral route using stereotactic injection places the cells in a specific area, helps control the dose and leads to a better survival for stem cells [33], and is the preferred route if migration outside the brain is not wanted. This route showed benefit when neural stem cells were used in an animal model [34], also was feasible and was tolerated well in humans without significant serious adverse events [33].

The best route of cell delivery is not yet clear and depends on the cell type. Multiple animal model studies showed clear benefit with IV route and when compared to IA and intracerebral routes the benefits were similar or greater [31, 35]. In general, bone marrow derived cells may be optimized for an intravenous route while neural stem cells are more optimal if delivered intracerebrally.

4 Stroke Affects the Immune System

Stroke is a multiorgan systemic disease and not solely a brain lesion. Stroke induces immune changes and systemic inflammation in both animal and human models [36–38]. The inflammatory process in stroke begins immediately after the vascular occlusion with activation of complement, platelets and endothelial cells [39, 40].

Shortly after ischemia, a variety of signals are released from dying cells and cells under stress, called danger-associated molecular pattern molecules (DAMPs) [41]. These signals which include purines, cytokines, and chemokines activate the innate immune system via the activation of the toll-like receptors (TLRs) and scavenger receptors on microglia, astrocytes, endothelial cells and perivascular macrophages [42, 43]. The activated microglia initiate the inflammatory response by the release of inflammatory cytokines (like IL-1β, TNF-α), chemokines to recruit macrophages and leukocytes infiltration [44]. The activated perivascular macrophages also release pro-inflammatory cytokines and chemokines [45]. Adhesion molecules are up-regulated shortly after stroke, they appear to be involved in the migration of neutrophils into the stroke area [46]. Neutrophils release pro-inflammatory molecules upon activation, these molecules, including MMP-9, contribute to blood brain barrier breakdown a leukocytes activation and infiltration [47].

The adaptive immune system requires longer time to get activated. B lymphocytes secret antibodies against the brain-derived antigens as part of the humoral

immune response [48]. Antigen presenting cells (APCs) including dendritic cells, increase in number in the ischemic brain while decrease in the periphery [49, 50]. These APCs process brain antigens and present them with MHC molecule that get recognized by T lymphocyte receptors and that leads to their recruitment into the ischemic brain [41, 50].

Multiple studies favor the cellular adaptive immunity as a mechanism of damage in stroke [49–52]; this cell mediated damage was found to be non-antigen or cell receptor driven [53]. In animal model studies, focal ischemia led to rapid splenic activation of T lymphocytes and the release of local cytokines in the acute phase which was accompanied by a reduction in the number of immune cells in the peripheral lymphoid organs (spleen and thymus) and eventually decreased levels of tumor necrotic factor-α (TNF-α) and interferon-γ (INF-γ) that contributed to immunosuppression and infections after stroke [37, 54].

Offner et al. studied the effect of the loss of T and B lymphocytes in animal stroke model, based on their previous work [54] which indicated a detrimental effect of the immune system in stroke. They used mice with severe combined immunodeficiency (SCID) and found that in the absence of T and B cells, post-stroke inflammatory mediators were largely suppressed in the acute phase both in the brain and spleen of SCID mice, with improvement in early post-ischemic histological damage. Also, the post-stoke intra-splenic cytokines and chemokines expression were reduced as well as the loss of splenocytes compared to their control mice. Yilmaz et al. also showed smaller infarct volumes in lymphocyte deficient mice [55]. The role of T regulatory cells in stoke is controversial; Liesz et al. showed that T-regulatory cell depletion increased brain damage and deteriorated functional outcome [52], while Kleinschnitz et al. showed that T-regulatory cell depletion dramatically reduced stroke size [56].

Early after stroke, ischemic cortex shows a significant increase in the expression of inflammatory cytokines (TNF-α, IL-1β, IL-6) and chemokines (RANTES, IP-10, MIP-2) [57], plasma levels of IL-6 also increases [57, 58]. In the spleen, inflammatory factors (TNF-α, INF-γ, IL-6, MCP-1, IL-2) levels significantly increase along with anti-inflammatory factor IL-10 [57]. These studies confirm that stroke is not a local brain disease but it also affects the spleen and the immune system response.

Another study showing the instrumental role of spleen in mediating immune response after stroke; performing splenectomy in their animal model resulted in reduction in the neurodegeneration and the immune response in the splenectomized animal after middle cerebral artery occlusion [59].

Infection after stroke is another major problem, and is seen more often with major strokes [60]. There is an accumulating clinical evidence suggesting that acute stroke induces immunological changes that facilitate the appearance of infections. Acute stroke leads to the release of inflammatory cytokines, which in turn induce the pituitary gland to secrete ACTH which induces glucocorticoids secretion from the adrenal cortex [61], which has a strong antiproliferative and apoptotic properties on the immune system and at the same time suppress further cytokine synthesis [62].

5 How Do Cell Therapies Work?

Cell therapies are not believed to work through replacing injured cells, but rather through the release of biological factors that promote recovery and suppress further damage [10, 12, 13]. Different cell types have different ways to exert their beneficial effects. These mechanisms include: neuroprotection, promoting angiogenesis, promoting neurogenesis, glial scar prevention, local and systemic anti-inflammatory effects [10, 12, 13, 63–65]. For example, bone marrow derived hematopoietic stem were able to counter-regulate the up-regulation of proinflammatory cytokines and chemokines receptor gene transcripts in the spleen, thus preventing the activation of the immune gene transcripts in the splenocytes which result in a reduction of the number of cells entering the blood circulation and brain tissue [66]. Neural stem cells were found to produce a broad spectrum of trophic factors (Nerve growth factor, brain-derived neurotrophic factor, glia-derived neurotrophic factor) [67] which play an important role in neuroprotection by promoting cell survival [68], they also promote angiogenesis and restoration of blood-brain barrier integrity via the production of endothelial growth factors [69]. MSC was found to induce cell proliferation in the subventricular and subgranular zones which suggest their neurogenesis benefits [65, 70, 71], also they release bioactive substances that promote the proliferation of glial cells [72, 73]. Cell therapies modulate the immune system rather than suppressing it, they down-regulate pro-inflammatory, pro-apoptotic cytokines like TNF-α and INF-γ [74], secrete biological factors that suppress immune cell migration and infiltration in to the brain [75], and overall suppress subtypes of T-cells [76–79] and modulate B-cells function [80].

The effect of cell therapies on the immune system, as mentioned, is selective. They were shown to improve mortality, reduce inflammation and enhance bacterial clearance in sepsis animal model [81]. The selective suppression effect and inflammation reduction is potentially the reason that cell therapies might reduce infection rates seen in stroke. In the MASTERS trail, there was a signal for lower infection rates in the therapy group compared to placebo [25].

The mechanisms that cell therapies interact and communicate with the injured brain cells are still not fully understood, but there is an increasing evidence to support the paracrine effect by stem cells via the release of extracellular vesicles able to exert a biological activity [82–85]. An important type of these vesicles are exosomes which can be released by different types of cells [86] and especially produced in large amounts from MSCs [87]. Most exosomes contain several proteins, cell surface receptors, trophic factors, RNAs and Micro-RNAs (miRNAs) [84, 86, 88–91]. The content and the amount of exosomes released can be altered by cellular damage and stress like hypoxia [92–94]. miRNAs are a small non-coding sequences of RNA that have the capability to regulate genes, pathways and biological activities within cells [95–97]. Multiple studies have shown that MSC exosomes modify recipient cell characteristics and regulate their protein expression through miRNA transfer [98–100] and thus participating in stroke recovery [101]. Xin et al. were able to demonstrate for the first time a systemic treatment of cell-free exosomes derived from MSCs and showed an increase in neural plasticity and recovery after stroke in their animal model [82, 102].

6 Clinical Trials in Cell Therapies

Clinical trials in cell therapies still for the most part in early phases, mainly focusing on bone marrow derived cells with autologous transplantation but recently allogeneic transplantation.

Based on the animal studies, Savitz et al. showed the safety of IV autologous MNC infusion given 24–72 h after stroke [15]. Friedrich et al. also showed the safety of intra-arterial infusion of autologous MNC 3–7 days after stroke [103]. Other studies also have shown the good safety profile of MNC infusion in humans [104].

Most of MSC human studies were done on patients with chronic stroke, given the relative longer culture time required to obtain these cells. The first reported MSC transplantation in human was performed by Bang et al., they used IV route with autologous cells infused 4–9 weeks after stroke symptoms, they reported improvement in both modified Rankin score and Barthel index up to 6 months, also no adverse events were reported up to 1 year later [29], a 5-year follow study showed sustained improvement in functional outcome without significant side effects or a change in mortality compared to the control group [105]. Other studies also reported the safety of IV MSC transplantation in humans [18, 20, 104].

MAPC have been shown to enhance recovery in animal model through modulating the immune response and targeting the spleen inflammatory response [11, 106] (see Fig. 9.1). MultiStem® a proprietary cell therapy from Athersys is a MAPC derived from the bone marrow, and has shown promising potential to treat other conditions other than stroke. Based on the promising results of the preclinical studies, Hess et al. conducted a phase II, multicenter, double-blinded, randomized, placebo-control study; in the MultiStem in Acute Stroke Treatment to Enhance Recovery Study (MASTERS), they aimed to establish the highest safe, well tolerated IV dose of MultiStem® while assessing for any efficacy on stroke recovery. They enrolled patients aged 18–83 years with moderate to severe acute ischemic stroke and a National Institute of Health Stroke Scale (NIHSS) score of 8–20 within 24–48 h after stroke onset. This was a dose escalation trial where patients were first randomized in Group 1 to a receive a low dose (400 million cells) or placebo and once this was determined to be safe to be randomized to a high dose (1200 million cells) or placebo in Group 2. The high dose was determined to be without safety issues, so patients were randomized in Group 3 to 1200 million cells or placebo. Groups 2 and 3 were combined for the primary safety and efficacy analyses. There were no dose limiting toxicity, difference in adverse events or difference in stroke recovery at 90 days [25]. On the other hand, an exploratory analysis of their data suggested an increase in excellent outcome (mRS $\leq$ 1, NIHSS $\leq$ 1 and Barthel index $\geq$95) at 1 year follow up. Multiple biomarkers were also measured at 2, 7 and 30 days post treatment and their analysis showed that MultiStem® reduced T lymphocytes (CD3+ and FoxP3+ T-cells) at 2 days and inflammatory cytokines (TNF-α, IL-1β, IL-6) at 7 days. This is the first data to show that cell therapies modulate the immune system after acute stroke in humans [25].

Neural stem cells were studied in the human neural stem cells in patients with chronic ischemic stroke (PISCES), a phase I, single arm, non randomized, dose

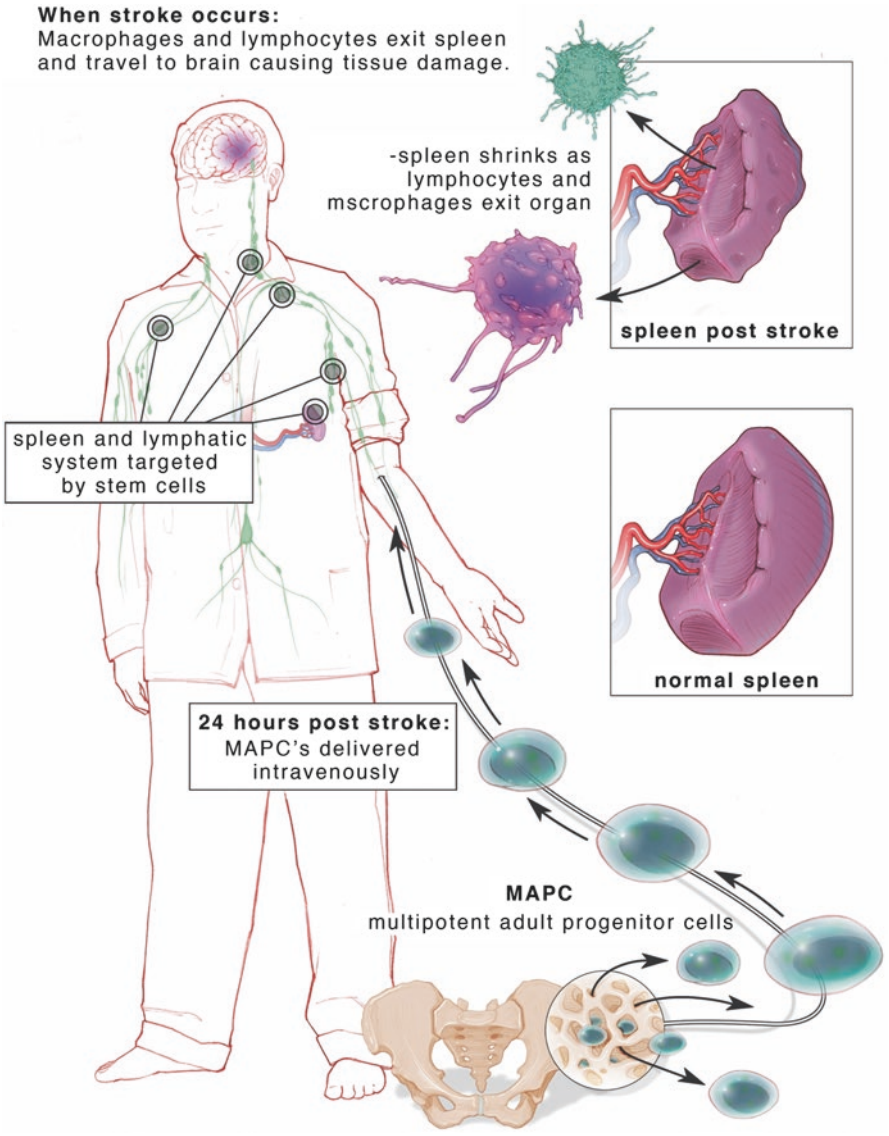

Fig. 9.1 Cell therapies—modulating the immune system. After stroke, proinflammatory signals leads to splenic lymphocytes activation and release. These lymphocytes target the ischemic brain and cause further damage. Cell therapies, such as MAPC, act by targeting the spleen and preventing immune system activation and later immunodepression

escalation clinical trial of 11 subjects to assess neural stem cell transplantation safety and feasibility via intracerebral stereotactic implantation 6–60 months after ischemic stroke. There were no significant adverse events related to the cells and there was improvement in neurological outcome at 12 months follow up [33].

Currently, PISCES II, a phase II trial is ongoing. Steinberg et al. reported an interim results from their 2 year phase 1/2a study single arm, non randomized trial of stereotactic administration of modified bone marrow-derived MSC in patients with chronic stroke; their analysis showed safety and feasibility, along with improvement in neurological function at 12 months [107]. However, the lack of a control group makes activity of the therapy difficult to evaluate.

7 Cell Therapies Direction and Development

The Stem Cell Therapies as an Emerging Paradigm for Stroke (STEPS) committee has published a series of recommendation for both preclinical and clinical research in stem cell therapy to help guide stem cell research. STEPS I publication focused on: the validation of animal studies, build robust safety studies in humans, and selecting the right time and patients for cell therapies [108]. STEPS II focused on providing guidelines for human safety trails and the need to obtain a signal for efficacy [109]. STEPS III focused on the design of clinical trials in phase IIb and III, and covered topics on patient selection, timing of therapy, desired endpoints, assessing cell therapies activity through biomarkers, concomitant rehabilitation therapy and their potential application in chronic stroke [110].

It is still too early to know whether cell therapy in stroke will be successful. To date, cell therapy trials have shown safety and feasibility. This is crucial as it is important to avoid the mistakes of gene therapy trials. The MASTERS trial suggests that an earlier time window of 18–36 h may be optimal and planned clinical trials in Japan, North American and Europe will enroll patients in the 18–36 h time window.

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Chapter 10
The Combination of Stem Cell Factor (SCF) and Granulocyte-Colony Stimulating Factor (G-CSF) in Repairing the Brain Post-acute Stroke

Li-Ru Zhao, Suning Ping, and Fei Hao

Abstract Stroke represents the leading cause of long-term disability in adults worldwide. Most stroke survivors suffer from lifelong neurological deficits. Developing a pharmaceutical approach to enhance brain repair and improve functional outcomes post-acute stroke is a very important but less investigated area in stroke research. Stem cell factor (SCF) and granulocyte-colony stimulating factor (G-CSF) are the well-characterized vital hematopoietic growth factors for regulating hematopoiesis. Increasing evidence supports that SCF and G-CSF also play roles in the nervous system. Over the past decade, preclinical studies have demonstrated that SCF in combination with G-CSF synergistically enhances stroke recovery in the subacute or chronic phase. In this chapter, we have reviewed the biological function of SCF and G-CSF in hematopoiesis, neural plasticity, and neurogenesis, and summarized the preclinical studies illustrating the neurorestorative effects of SCF and G-CSF post-acute stroke.

Keywords Stem cell factor • Granulocyte-colony stimulating factor • Brain repair • Stroke • Subacute phase • Chronic phase

Abbreviations

AD	Alzheimer's disease
BBB	Blood-brain barrier
BDA	Biotinylated dextran amine

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P.A. Lapchak, J.H. Zhang (eds.), *Cellular and Molecular Approaches to Regeneration and Repair*, Springer Series in Translational Stroke Research,
https://doi.org/10.1007/978-3-319-66679-2_10

BDNF	Brain-derived neurotrophic factor
CADASIL	Cerebral autosomal dominant arteriopathy with subcortical infarcts and leucoencephalopathy
CNS	Central nervous system
CSF	Colony stimulating factor
CXCR4	C-X-C chemokine receptor type 4
DRGs	Dorsal root ganglia neurons
ECs	Endothelial cells
G-CSF	Granulocyte-colony stimulating factor
GCSFR	G-CSF receptor
GM-CSF	Granulocyte macrophage-colony stimulating factor
HPCs	Hematopoietic progenitor cells
HSCs	Hematopoietic stem cells
LTP	Long-term potential
NSCs/NPCs	Neural stem/progenitor cells
PPF	Paired-pulse facilitation
SCF	Stem cell factor
SDF-1	Stromal cell-derived factor 1
SGZ	Subgranular zone
SHRs	Spontaneous hypertensive rats
Sl	Steel gene
SVZ	Subventricular zone
tPA	Tissue plasminogen activator
U-type spines	Uncertain type spines
W	White-spotting gene
YFP	Yellow fluorescent protein

1 Introduction

Stroke remains the leading cause of long-term disability in adults worldwide [1, 2]. Stroke not only represents a serious medical condition but it also causes huge medical and financial burdens throughout the world [1–3].

A stroke has three clinical phases: the acute phase, subacute phase, and chronic phase. The exact time frame of these three phases varies among individuals as the duration of the three phases is dependent upon the size and location of the infarcts, the responsive capacity of cerebrovascular collateral circulation, the metabolic state of brain tissue, and patient's age and medical comorbidities. In general, the acute phase is the first 48 h after stroke symptom onset, the subacute phase represents the period from 48 h up to 3 or 6 months post-stroke, whereas the chronic phase starts 3 or 6 months after stroke [4–10].

Currently, there are only two therapeutic approaches available for stroke patients. The *first one* is the thrombolytic/thrombectomy treatment for ischemic stroke

patients in the acute phase. The therapeutic time windows for thrombolytic/thrombectomy approach are limited up to 4.5 h post-stroke for thrombolysis by tissue plasminogen activator (tPA) [11, 12] and within 6–8 h post-stroke onset for the thrombectomy [13–15]. The *other* treatment for stroke patients is physical therapy. The therapeutic window for physical therapy is restricted to the first 6 months post-stroke [16–18]. Due to the narrow time window and intracerebral hemorrhage risk of the thrombolytic/thrombectomy treatment [11, 13, 19], the majority of stroke patients are not able to receive this treatment in the acute phase [20]. In addition, many stroke survivors do not receive or complete the physical therapy post-acute stroke because of financial or family-related issues. Developing new therapeutic strategies, therefore, is highly important to reduce stroke-induced disability and enhance stroke recovery.

Over the past two decades, the vast majority of stroke research has targeted the neuroprotection in the acute phase, and little attention has been paid to enhancing stroke recovery in the subacute or chronic phase of stroke. In fact, the neuroprotective agents have all failed in clinical trials [21]. Searching for therapeutic approaches to improve stroke recovery post-acute phase becomes highly recognized in the stroke research field today.

Stem cell factor (SCF) and granulocyte-colony stimulating factor (G-CSF) are the essential hematopoietic growth factors that critically regulate hematopoiesis [22–24]. Beside their roles in the hematopoietic systems, a large body of evidence shows that SCF and G-CSF also play roles in the nervous system. SCF and G-CSF do not only promote neural plasticity [25–30] and neurogenesis [31–33], but they can also enhance brain repair in both the subacute [34] and chronic phases [35–41].

In this chapter, we have reviewed the discovery of SCF and G-CSF in the hematopoietic system and current knowledge concerning the biological function of SCF and G-CSF in hematopoiesis, highlighted the studies demonstrating the effects of SCF and G-CSF in promoting neural plasticity and neurogenesis, and summarized up-to-date research progress regarding the effective and mechanistic determinations of SCF and G-CSF on brain repair in the subacute and chronic phases of experimental stroke.

2 The Discovery and Essential Role of SCF and G-CSF in the Hematopoietic System

SCF and G-CSF are the hematopoietic growth factors that are critically involved in regulation of blood cell production and mobilization of bone marrow stem cells. Since the discovery of SCF and G-CSF, great effort has been made to elucidate their biological function. Over the past six decades, there have been many breakthroughs in understanding the mechanisms underlying SCF- and G-CSF-regulated hematopoiesis and in developing potential therapies for using SCF and G-CSF in clinical trials. In this section, we have summarized the current understanding of the essential role of SCF and G-CSF in the hematopoietic system.

The discovery of SCF (also known as kit ligand, steel factors and mast cell growth factor) and its receptor c-kit took place in 1990s [42]. Observations of white spots on a few mice among thousands of laboratory mice lead to the identification for the loci of steel (Sl) and white-spotting (W), which encode SCF and c-kit receptor, respectively [43]. Mutations at either of these two loci result in similar phenotypes with coat color alterations, anemia, and lack of mast cells in the tissue and neonatal mortality [44]. These findings offer critical information concerning the *in vivo* function of SCF and c-kit, and highlighting its important roles in hematopoiesis, melanogenesis and fertility. In addition, it has been demonstrated that the W mutation-induced c-kit dysfunction affects hematopoietic stem cells and hematopoietic progenitor cells (HSCs/HPCs), while the Sl mutation impairs stromal cell function. These findings are in accordance with the *in vitro* study that was reported in 1977 [45]. In this *in vitro* study, Dexter and Moore demonstrated the stromal-dependent hematopoietic cells culture, and proposed that Sl and W encoded a ligand-receptor pair.

Many studies have revealed that there are two forms of natural SCF due to alternative splicing of the DNA transcripts. A shorter form consists of 220 amino acids and produces a membrane-bound form of SCF. The soluble SCF, which consists of 165 amino acids, is derived from a full length 248 amino acids cleaved in the extracellular domain [46]. Both the soluble and membrane-bound forms of SCF are biologically active. However, the two forms of SCF have distinct but overlapping roles [47]. Membrane-bound SCF is expressed on stromal cells, endothelial cells (ECs) and fibroblasts in the bone marrow and induces more persistent tyrosine kinase activation than soluble SCF [48]. In 1991, Brannan and colleagues reported that Steel-Dickie mice exhibited anemia, pigmentation and germ cell defects as these mice only produced soluble SCF due to genome deletion affecting the transmembrane and cytoplasmic domain [49]. These research findings suggest that membrane-bound SCF plays a unique biological role in the stromal cells, ECs and fibroblasts in the bone marrow. C-kit is expressed on normal hematopoietic cells and several other cell types, including mast cells [50], melanocytes [51] and a wide range of non-hematopoietic cell types as ECs [52], interstitial cells [53] and astrocytes [43]. Interaction between SCF and c-kit is the initial and key step for triggering the downstream signaling. It has been shown that the SCF/c-kit system has an important function not only in mouse but also in humans and other primates due to its pleiotropic effects on hematopoietic cell survival, proliferation, differentiation and mobilization [54]. SCF acts directly on HSCs/HPCs, promotes HSC/HPC entry to the cell cycle, and facilitates HSC/HPC proliferation [55]. SCF enhances the primitive HSC survival by suppression of apoptosis [56, 57]. In 1992, Valent and colleagues reported that SCF induced mast cell development from immature hematopoietic cells in human bone marrow [58], suggesting the effect of SCF on cell differentiation. This study was also confirmed by Irani and colleagues, who observed similar results in human fetal liver [59]. In addition, SCF is also a potent agent for mobilization of murine and human HSCs from bone marrow to peripheral blood [43], which shares a similar role as G-CSF.

The discovery of G-CSF occurred much earlier than SCF. The identification of G-CSF happened in the 1960s by *in vitro* assays measuring the ability of the growth factors to stimulate colony formation of bone marrow cells (see review by [60]). During the 1970s, a detailed category of colony stimulating factor (CSF) had been published, and G-CSF had been defined as a stimulator specific for colonies containing predominantly neutrophils [61]. G-CSF is produced by a variety of cells, of which, monocyte/macrophage lineage cells are the most prominent source [60]. G-CSF is also produced by normal mesothelial cells [62], fibroblasts [63] and ECs [64]. G-CSF, like other growth factors, exerts its biological functions by binding to the G-CSF specific receptor, G-CSFR. G-CSFR expression has been found on a variety of hematopoietic cells, including myeloid progenitors, mature neutrophils, monocytes, myeloid cells, lymphoid leukemia cells, and normal B and T cells [65].

Generally, G-CSF is known to have multiple functions in regulation of HSC/HPC proliferation, differentiation and mobilization, neutrophil production and mobilization from the bone marrow, neutrophil progenitor cell proliferation and differentiation, and the state of functional activation of neutrophils. In 1987, Tamura and colleagues reported that G-CSF mobilized large numbers of hematopoietic cells from the bone marrow into the circulation [66]. In addition, increased progenitor cells of all lineages were detected in the spleen of G-CSF-treated mice. These results were further confirmed by Duhrsen and colleagues in cancer patients with G-CSF treatment [67]. The administration of G-CSF to the patients showed significant increases of circulating HSCs, followed by a slight reduction in the frequency of bone marrow progenitor cells. However, the absolute number of the progenitor cells in the bone marrow was still increased. Together, all these data support the efficacy of G-CSF on HSC/HPC mobilization and proliferation. As peripheral blood is one of the important sources for stem cell transplantation, the biological effects of G-CSF in HSC/HPC mobilization and proliferation therefore allow autologous and allogeneic HSC transplantation in the clinical setting [68]. G-CSF is also a strong stimulator for neutrophil activation. Masja and colleagues reported that G-CSF increased the release of inflammatory granules [69]. In addition, G-CSF stimulates the survival and primitive proliferation of progenitor cells *in vitro* by combination with other factors. McNiece and colleagues observed more numerous and larger colonies of progenitor cells after combination treatment of G-CSF and GM-CSF as compared to either single factor [70].

G-CSF also shows a synergistic effect with SCF in regulating many important biological responses. As stated earlier, both SCF and G-CSF have effects on regulating survival, proliferation, differentiation, and mobilization of HSCs/HPCs and hematopoietic lineage; the combination of SCF and G-CSF shows an enhanced effect. SCF in combination with G-CSF increases more progenitor cell mobilization in peripheral blood than SCF or G-CSF alone [71]. Many clinical trials have further confirmed this finding. Combined treatment of SCF and G-CSF show beneficial effects on peripheral blood progenitor cell mobilization with an increased number of CD34+ cells/kg in circulating system in patients who have received high dose chemotherapy for lymphoma [72, 73], breast cancer [74] and multiple myeloma

[75]. The synergistic effect of SCF + G-CSF on HSC/HPC proliferation has also been illustrated. The synergistic effect of combined treatment of SCF and G-CSF in HSC/HPC proliferation is not only because of the enhanced ligand/receptor interaction [76], but it also due to the marked shortening of the duration of G0/G1 phase [76]. A direct effect of SCF and G-CSF on cell cycle distribution has been identified, and this effect is mainly induced by the regulation of cyclin-dependent kinase inhibitor p27kip1 [77]. Besides, the combination of SCF and G-CSF also shows a synergistic enhancement of STAT3 and MAPK signaling [76], which is involved in promoting the cell proliferation.

3 The Role of SCF and G-CSF in the Central Nervous System: Neural Plasticity and Neurogenesis

In addition to the effects of SCF and G-CSF in the hematopoietic system, increasing evidence shows that SCF and G-CSF also play a role in the central nervous system (CNS). Receptors for SCF and G-CSF have been found to express in the brain [78, 79], particularly in the neural stem cells/neural progenitor cells (NSCs/NPCs) [31–33, 80], and in cerebral neurons [32, 80] of adult mice and rats. It has been demonstrated that both SCF and G-CSF can pass through the blood-brain barrier [32, 81]. These findings suggest that hematopoietic growth factors, SCF and G-CSF, may have biological function in the CNS.

Numerous *in vitro* and *in vivo* studies have examined the contribution of SCF and G-CSF in the neuronal plasticity. SCF and G-CSF have been shown to play a key role in regulation of the neural plasticity in both the developing and adult brains. *In vitro* studies have shown that SCF supports the survival of c-kit-positive dorsal root ganglia neurons (DRGs) and promotes the neurite outgrowth of mouse embryonic DRGs through the c-kit receptor tyrosine kinase activity [25]. In addition, SCF has also shown to increase the neurite outgrowth of cultured cortical neurons [30]. In cultured brain slices, SCF selectively promotes outgrowth of commissural axons, which highly express SCF receptor [82]. *In vivo* studies, commissural axons fail to exit the floor plate in SCF and c-kit mutant mice [82]. In addition, c-kit conditional knockout mice show delayed extension of callosal fibers within the contralateral cortex and fail to innervate their target area [83]. At the functional level, SCF mutant mice exhibit a reduction of baseline synaptic transmission between dentate gyrus and hippocampal CA3 pathway and show deficits in spatial learning and memory [27]. C-kit mutant rats and mice both display impairments of paired-pulse facilitation (PPF) and long-term potential (LTP) in the hippocampal mossy fiber-CA3 pathway and a deficit in performance in Morris water maze task [28, 84]. In mouse brain slices, SCF binding to c-kit receptor activates PI3K/PLA_2 intracellular pathway, modulates PPF and LTP, and regulates synaptic transmission in the hippocampus [84]. These studies suggest that SCF/c-kit signaling is involved in the structural and functional regulation of synaptic plasticity. In addition to SCF, G-CSF also participates in neural plasticity.

G-CSF knockout mice show impaired LTP, reduced densities of NMDA receptors and dendritic complexity of hippocampal neurons in the dentate gyrus and the CA1 region, and deficiency in spatial learning and memory [29]. G-CSF treatment restores impaired long-term depression (LTD) in a mouse model of Alzheimer's disease (AD) [85]. In addition, subcutaneous injection of G-CSF increases the dendritic length and complexity of pyramidal neurons in the peri-infarct cortex in the cerebral ischemia rats [86]. Furthermore, it has been demonstrated that G-CSF in combination with SCF synergistically promotes neurite outgrowth and network formation of cultured cortical neurons through the PI3K/AKT/NF-kB/BDNF pathway [30]. Collectively, these research data suggest that SCF and G-CSF, the two hematopoietic growth factors, act as neurotrophic factors to regulate the neural plasticity during development and maturity.

The role of SCF and G-CSF in promoting neurogenesis and directing NSCs/NPCs to give rise to neurons has been illustrated in both *in vitro* and *in vivo* studies. There are two neurogenic regions in the adult mammalian brain, including the subventricular zone (SVZ) surrounding the anterior part of lateral ventricles and subgranular zone (SGZ) of the hippocampal dentate gyrus. NSCs/NPCs in these regions have regenerative potential, which has been postulated as a likely source for neural repair. Infusing SCF into the cerebrolateral ventricle has been shown to increase the number of newborn neurons in the SVZ [31]. Injection of anti-c-kit antibody into the cisterna magnum increases the number of cell death and results in thinning of the cerebral cortex, suggesting essential role of SCF/c-kit for cortical progenitor cell survival [87]. In cultured NSCs/NPCs, G-CSF is shown to promote the differentiation of NSCs/NPCs into neurons in a dose dependent manner [32, 88]. In G-CSF knockout mice, hippocampal neurogenesis is strongly diminished, and the mice show deficits in behavioral plasticity [29]. Peripheral or intraventricular administration of G-CSF has been demonstrated to increase the neurogenesis and promote the proliferation and differentiation of NSCs/NPCs, not only in the intact mice and rats [32, 88], but also in the animal models of neurological disorders, including the cerebral ischemia, perinatal hypoxia, irradiation-induced brain injury, traumatic brain injury, AD, cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy (CADASIL) and bacterial meningitis [32, 34, 89–98]. G-CSF-induced neurogenesis is probably associated with G-CSF receptor-mediated phosphorylation of transcription factor STAT3/5 [88]. Besides, combination of SCF and G-CSF has been reported to have a synergistic effect in facilitating the proliferation of intrinsic NSCs/NPCs in a mouse model of cerebral ischemia [34]. When adding SCF and G-CSF into the culture medium during the proliferating stage of NSCs/NPCs, SCF in combination with G-CSF (SCF + G-CSF) shows a dual function in directing cell cycle arrest and promoting neuronal fate commitment through the regulation of neurogenin 1 [33]. Together, these studies reveal that SCF and G-CSF are involved in the regulation of NSC/NPC proliferation and neurogenesis.

In addition to promoting the proliferation and differentiation of intrinsic NSCs/NPCs, the combination of SCF and G-CSF also mobilizes bone marrow-derived cells, causing them to migrate into the brain and differentiate into various types of

cells, including neurogenesis. The fate of bone marrow-derived cells in the brain is dependent upon the microenvironment of the brain. In the subacute and chronic stroke brain, SCF + G-CSF treatment augments bone marrow-derived endothelial cells and neurons [34, 36]. In the brains of CADASIL mice, SCF + G-CSF selectively directs bone marrow-derived cells toward neuronal fate commitment [99]. In the APP/PS1 transgenic mice, bone marrow-derived microglial cells are significantly increased in the brain following SCF + G-CSF treatment, suggesting that SCF + G-CSF treatment leads to an enhancement in microglial fate commitment of bone marrow-derived cells in the brain with β-amyloid deposits [92, 100, 101]. G-CSF treatment has also been shown to mobilize bone marrow-derived mesenchymal stem cells, promote the migration and differentiation of mesenchymal stem cells into the neurons, and contribute to neurogenesis in the brains of AD mice [102]. The C-X-C chemokine receptor type 4 (CXCR4)/stromal cell-derived factor 1 (SDF-1) has been shown to be a key mediator in G-CSF-based recruitment of bone marrow-derived cells [102]. Together, these studies suggest that although bone marrow-derived cells possess different phenotypes in various brain conditions, these cells may participate in the neurogenesis and brain repair.

4 SCF and G-CSF Combination in Brain Repair Post-acute Stroke: Effective and Mechanistic Determinations

As stated in the previous section, substantial evidence has revealed the capacity of SCF and G-CSF in promoting neural plasticity and neurogenesis, and the permeability of the BBB to SCF and G-CSF. These discoveries provide a scientific base for seeking to determine the therapeutic effectiveness of SCF and G-CSF in enhancing brain repair and stroke recovery in the subacute phase and/or the chronic phase of stroke.

Here we highlight the preclinical studies demonstrating the efficacy and possible mechanisms of SCF and G-CSF in brain repair during subacute or chronic phases of experimental stroke.

4.1 The Effects of SCF and G-CSF on Brain Repair in the Subacute Phase of Stroke

There are a few preclinical studies demonstrating the therapeutic efficacy of SCF and G-CSF in the subacute phase of stroke. Using a transient focal ischemia model in Sprague-Dawley rats, Lee and co-workers [103] reported that intraperitoneal injections of G-CSF for 3 days beginning at 4 or 7 days post-ischemia led to motor function improvement, infarction size reduction, and increased angiogenesis. Kawada and colleagues [34] injected SCF and G-CSF subcutaneously during the period of 11–20 days after induction of focal cerebral ischemia in C57BL mice, and

observed that the SCF and G-CSF treatment increased the number of bone marrow-derived neuronal cells in the ipsilesional hemisphere and promoted the proliferation of intrinsic NSCs/NPCs in the SVZ. In addition, they also found that the SCF + G-CSF synergistically enhanced NSC/NPC proliferation in the SVZ when compared with treatment of SCF or G-CSF alone [34]. How SCF + G-CSF optimally repairs the brain in the subacute phase of stroke has not been clarified. Using the same treatment paradigm as reported by Kawada and colleagues [34], SCF + G-CSF treatment was found to upregulate IL-10, an anti-inflammatory cytokine, and to reduce infiltration of microglial/macrophages in the infarcted brain [104]. Although inhibiting inflammation by SCF + G-CSF may provide a favorable microenvironment for neurogenesis in the subacute phase of stroke, the causal link among the SCF + G-CSF-induced neurogenesis, anti-inflammation, and motor function improvement remains to be elucidated.

4.2 The Effects of SCF and G-CSF on Brain Repair in the Chronic Phase of Stroke

Most stroke patients still carry different degrees of disability when they enter into the chronic phase of stroke although many of them have received thrombolytic therapy in the acute phase [19] and physical therapy during the subacute phase [16]. However, in the chronic phase, there has been no therapy available for enhancing stroke recovery as it has been believed that the opportunity for obtaining recovery is largely ended by the time stroke patients enter the chronic phase [17, 105].

Brain plasticity is an intrinsic ability of the brain to reorganize its function and modify its structure in response to stimuli and injuries from both internal and external sources. Accumulating evidence supports that brain plasticity exists throughout a person's lifespan [106–111]. Accordingly, there is a possibility that a stroke-damaged brain may still be reparable during the chronic phase.

Over the past decade, our research team has demonstrated the safety, efficacy and possible mechanisms of SCF and G-CSF on stroke recovery in the chronic phase of stroke using rat and mouse models of cerebral cortical ischemia.

4.2.1 The Efficacy, Safety and Effective Dosage of SCF and G-CSF on Stroke Recovery in the Chronic Phase of Stroke

Systemic administration of SCF and G-CSF during the period of 3.5–6 months after cerebral cortical ischemia has been demonstrated and validated to be effective for brain repair in spontaneously hypertensive rats (SHRs), C57BL mice, or transgenic mice with C57BL genetic background [35–39, 41]. SHRs are used for making a stroke model because hypertension is the most important risk factor for stroke in humans [112]. Chronic hypertension leads to extensive pathological changes in the cerebrovasculature [113, 114]. Numerous studies have illustrated that the cerebral

cortical ischemia model in SHRs shows a more consistent and larger infarction in the cortex than in normotensive rats due to poor collateral circulation [35, 80, 114–121]. This model also causes permanent deficits in somatosensorimotor function that last up to the chronic phase of stroke [35, 80, 118–122]. In addition to the cortical infarct model in SHRs, we also use C57BL mice or transgenic mice with C57BL genetic background to make the cerebral cortical infarct model for exploring the mechanisms behind the SCF and G-CSF-enhanced brain repair in chronic stroke.

First of all, the therapeutic efficacy of SCF and G-CSF on stroke recovery in the chronic phase has been examined using the cerebral cortical ischemia model in SHRs. SCF (200 μg/kg), G-CSF (50 μg/kg), or SCF + G-CSF was subcutaneously injected daily for 7 days beginning at 3.5 months post-ischemic stroke. Among the treatment groups, only the SCF + G-CSF treatment led to a stable and long-term (17 weeks) improvement in somatosensory motor function. SCF alone treatment improved functional outcomes but the improvement did not present as stable as the SCF + G-CSF combination treatment. G-CSF alone treatment, however, did not result in functional benefits. The research data of field-evoked potentials lent further support to the neurobehavioral findings and revealed a reestablished normal pattern of somatosensory pathways by SCF + G-CSF treatment [35]. These findings provide first evidence that SCF + G-CSF combination treatment in the chronic phase of stroke can enhance stroke recovery.

Given the fact that stroke has the highest incidence in the elderly [2], the safety, efficacy, and optimal dosage of SCF + G-CSF combination treatment on chronic stroke recovery have been assessed in experimental stroke using aged SHRs and C57BL mice [37]. Six dosages of SCF + G-CSF ranging from 5 μg/kg (SCF) and 2.5 μg/kg (G-CSF) to 200 μg/kg (SCF) and 50 μg/kg (G-CSF) have been examined [37]. The treatment was initiated at 3–4 months post-experimental stroke. All the tested dosages did not show either acute or chronic toxicity to the livers and kidneys, demonstrating the safety of SCF + G-CSF treatment for chronic stroke in the aged population. The higher dosages (SCF/G-CSF: 200/50,100/25, and 50/25 μg/kg) showed the most effective outcomes in mobilizing circulating stem cells and in stably improving functional recovery. The intermediate dose of SCF + G-CSF (20/10 μg/kg) displayed a short-term improvement, whereas the dosages less than 20/10 μg/kg did not lead to functional improvement in chronic stroke in aged SHRs. These findings demonstrate that SCF + G-CSF treatment for chronic stroke recovery is a safe and effective therapeutic approach for the aged population and acts in a dose dependent manner.

4.2.2 The Possible Mechanisms Underlying the SCF + G-CSF-Enhanced Recovery in the Chronic Phase of Stroke

We have employed the approaches of bone marrow-derived cell tracking, molecular manipulation, live brain imaging, whole brain imaging, axon tracking, immunohistochemistry, confocal imaging, and neurobehavioral testing to determine how SCF + G-CSF repairs a stroke-damaged brain in the chronic phase.

By tracking bone marrow-derived cells through bone marrow transplantation, our study has revealed that increased bone marrow-derived endothelial cells and bone marrow-derived neurons are involved in SCF + G-CSF-enhanced angiogenesis and neurogenesis in the brain of chronic stroke [36].

Previous studies have shown that the receptors for SCF and G-CSF are expressed in cerebral neurons [32, 80] and cerebral endothelial cells [81] of adult mice and rats, and that both the SCF and G-CSF can pass through the blood-brain barrier of the adult rodent brain [32, 81]. Can SCF + G-CSF treatment in chronic stroke remodel the neural networks in an aged brain? To address this question, we used 2-photon microscopy to scan the brain area adjacent to the infarct cavity before and after SCF + G-CSF treatment in aged Thy-1-YFPH mice (C57BL background) [38]. In the brains of Thy-1-YFPH mice, the yellow fluorescent protein (YFP) is exclusively expressed in the layer V pyramidal neurons [123]. The mushroom spines with large heads on the dendrites are unique spines forming functioning synapses [124, 125]. Before SCF + G-CSF treatment, the mushroom spines of layer V pyramidal neurons were decreased, and the uncertain type (U-type) spines, which cannot build synapses with other neurons, were increased in the chronic stroke brain. This observation indicates that reduced synaptic circuits occur in the peri-infarct cavity cortex in the chronic stroke brain. However, 6 weeks after treatment, increased mushroom spines with decreased U-type spines were found in the brains of SCF + G-CSF-treated stroke mice. In addition, the densities of dendrites and PSD-95 were also increased in the ipsilesional cortex by SCF + G-CSF treatment. These findings demonstrate that SCF + G-CSF intervention in the chronic phase of stroke enhances synaptic network regeneration in the ipsilesional cortex of aged brains.

To distinguish whether SCF + G-CSF can directly modulate neural network formation, we carried out an *in vitro* study by determining neurite outgrowth of primary cortical neurons [30]. We found the expression of SCF and G-CSF receptors on the neurite growth cones. SCF + G-CSF showed synergistic effects in promoting neurite extension, activating NF-kB, and upregulating brain-derived neurotrophic factor (BDNF). Blockage of NF-kB activation eliminated the SCF + G-CSF-increased neurite outgrowth and BDNF production [30]. These data demonstrate the direct and synergistic efficacy of SCF + G-CSF in promoting neurite outgrowth, which is the initial step for generating neural networks. SCF + G-CSF enhances neurite extension through the NF-kB signaling.

Based on the *in vitro* findings, we then sought to use NF-kB inhibitor for blocking SCF + G-CSF-promoted neural network regeneration and to elucidate whether there is a dependent link between the SCF + G-CSF-enhanced neural network remodeling in the ipsilesional cortex and the SCF + G-CSF-improved motor function in chronic stroke. In an *in vivo* study [39], the NF-kB inhibitor was infused into the lateral ventricle through an osmotic pump for 7 days beginning at 1 h before a 7 day treatment (s.c.) of SCF + G-CSF, which was initiated 4 months after cortical ischemia. To track axons projecting from the contralesional hemisphere, an anterograde neuronal tracer, biotinylated dextran amine (BDA), was injected into the somatosensorimotor cortex in the contralesional hemisphere. After motor function

testing 2 and 6 weeks after treatment, mice were sacrificed at 10 weeks post-treatment. Our findings have revealed that SCF + G-CSF-increased BDA-labeled axons, PSD-95 accumulation, and blood vessel density in the peri-infarct cavity is eliminated by NF-kB inhibitor. In addition, the SCF + G-CSF-induced motor functional improvement is also prevented by NF-kB inhibitor. These data suggest that the SCF + G-CSF-improved functional outcome in chronic stroke may depend on the regeneration of neural networks and vasculature in the peri-infarct cavity cortex. However, this terminal determination study is limited to clarify the dynamically causal link between the SCF + G-CSF-promoted neural network rewiring and functional improvement in chronic stroke.

To over come this limitation, we conducted a unique study combining live brain imaging and motor function evaluation to simultaneously examine the dependent relationship between the SCF + G-CSF-enhanced synaptic network remodeling and motor function improvement in the chronic phase of experimental stroke [40]. To prevent the influence of behavioral testing-induced neural network remodeling, the following two sets of experiments were carried out simultaneously: (1) Thy1-YFPH mice with cortical infarction for live brain imaging at 2 and 6 weeks post-SCF + G-CSF treatment, and (2) Thy1-YFPH mice with cortical infarction for motor function assessment at 2 and 6 weeks post-SCF + G-CSF treatment. The SCF + G-CSF treatment was initiated at 6 months post-experimental stroke. We observed that once the SCF + G-CSF-increased mushroom spines in the ipsilesional motor cortex were eliminated by NF-kB inhibitor, the SCF + G-CSF-improved motor function was simultaneously prevented. This observation provides solid evidence validating a clear causal link between SCF + G-CSF-promoted neural network remodeling and motor functional improvement and strongly supporting that the SCF + G-CSF-enhanced neural network rewiring in the ipsilesional motor cortex is required for SCF + G-CSF-improved motor function in the chronic stroke.

It is worth noting that the SCF + G-CSF-increased dendritic spine head size, PSD-95 accumulation, and blood vessel density in the peri-infarct cortex are much greater than in the contralesional hemisphere of chronic stroke brain and in the intact control mouse brain [39, 40]. Blocking the SCF + G-CSF-induced "over growth" of synaptic networks and vasculature in the peri-infarct cortex by NF-kB inhibitor leads to abolition of the SCF + G-CSF-improved motor function in chronic stroke [39, 40]. These findings reveal that SCF + G-CSF-strengthened synaptic function in the peri-infarct motor cortex plays a vital role in motor functional improvement in chronic stroke.

5 Concluding Remarks

SCF and G-CSF were initially discovered as critical hematopoietic growth factors to regulate hematopoiesis. SCF in combination with G-CSF has been demonstrated to have synergistic effects in promoting the proliferation, differentiation and survival of HSCs/HPCs, and in mobilization of HSCs/HPCs into the blood.

Numerous studies have demonstrated that both SCF and G-CSF are crucially involved in neural plasticity and neurogenesis. These findings significantly advance our knowledge of these two hematopoietic growth factors: the biological function of SCF and G-CSF is not only limited in the hematopoietic system but it also acts in the CNS. In addition to the synergistic effects of SCF + G-CSF in the hematopoietic system, our research team has, for the first time, illustrated that the combination of SCF and G-CSF also synergistically promote neurite outgrowth of primary cortical neurons.

Over the past decade, the contribution of SCF and G-CSF in brain repair post-acute stroke has been determined. Importantly, it has been demonstrated that SCF in combination with G-CSF synergistically enhances brain repair in the subacute phase (by Kawada's group) and chronic phase (by our research team) of experimental stroke. These findings extend current understanding concerning the neurorestorative efficacy of SCF + G-CSF in brain repair post-acute stroke and provide a new approach for enhancing stroke recovery.

Acknowledgments The authors would like to thank Sandy McGillis for her assistance in editing the manuscript. This work was partially supported by the National Institutes of Health, National Institute of Neurological Disorders and Stroke (R01 NS060911), National Institute on Aging (1R01AG051674), and Department of Veterans Affairs (I01 RX002125) in the United States.

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Chapter 11
Mesenchymal Stromal Cell Therapy of Stroke

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Abstract Stroke is a major cause of high mortality, morbidity and long-term disability worldwide. Development of neuroprotective and neurorestorative therapies for stroke has been a target of intense research. Accumulating preclinical literature has identified that bone marrow mesenchymal stromal cell (MSC) treatment of stroke improves neurological functional outcome after stroke. This chapter focuses on the therapeutic effects and molecular mechanisms underlying MSC treatment of stroke, such as angiogenesis, arteriogenesis, neurogenesis and white matter remodeling, as well as a discussion on the interaction/coupling among these restorative events. In addition, the role of microRNAs (miRNAs) and MSC secreted exosomes in mediating intercellular communication between MSCs and parenchymal cells of the brain, and their effects on the regulation of neurovascular remodeling and white matter remodeling after stroke are discussed.

Keywords Mesenchymal stromal cell • Stroke • Angiogenesis • Neurogenesis • White matter remodeling • MicroRNA • Exosome • Neurorestoration

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P.A. Lapchak, J.H. Zhang (eds.), *Cellular and Molecular Approaches to Regeneration and Repair*, Springer Series in Translational Stroke Research,
https://doi.org/10.1007/978-3-319-66679-2_11

Abbreviations

BBB	Brain-blood barrier
BDNF	Brain-derived neurotrophic factor
BMMNC	Autologous bone marrow mononuclear cell
CRT	Corticorubral tract
CST	Corticospinal tract
DM	Diabetes mellitus
FGF-2	Fibroblast growth factor-2
GDNF	Glial cell line-derived neurotrophic factor
HGF	Hepatocyte growth factor
HLA-DR	Human leukocyte antigen-antigen D related
IA	Intra-arterial
IC	Intracerebral
ICH	Intracerebral hemorrhage
ICV	Intracerebro ventricular
IGF	Insulin-like growth factor
IN	Intranasal
IV	Intravenous
miRNA	MicroRNA
MSC	Mesenchymal stromal cell
MSC-Exo	Exosome derived from MSCs
mTOR	Mammalian target of rapamycin
NGF	Nerve growth factor
NPCs	Neural progenitor cells
NSCs	Neural stem cells
OPC	Oligodendrocyte progenitor cell
PGF	Placental growth factor
PTEN	Phosphatase and tensin homologue
SHRSP	Spontaneously hypertensive stroke prone
T1DM	Type 1 diabetic
tPA	Tissue plasminogen activator
VEGF	Vascular endothelial growth factor

1 Introduction

Stroke remains one of most common causes of death and major cause of disability all over the world [1]. Tissue plasminogen activator (tPA) remains the only pharmacological agent approved by the US Food and Drug Administration (FDA) for the treatment of ischemic stroke. There is a compelling need to develop novel therapies specifically designed to reduce neurological functional deficits after stroke. Decades of preclinical research and the failure of several clinical trials have drawn attention to the limitations

of acute neuroprotection [2, 3]. Therapeutic and repair potential of cell-based therapies, including bone marrow mesenchymal stromal cells (MSC) have been investigated in stroke and multiple neurodegenerative disease models [4]. MSCs are being evaluated in human clinical trials for efficacy in treating genetic diseases of bone, and in reducing the severity of graft versus host disease [5–7]. The therapeutic effects of MSCs have been measured in myocardial, limb and brain ischemia [8–12]. In this chapter, we discuss the therapeutic effects and mechanisms of MSC treatment of stroke.

1.1 Mesenchymal Stromal Cells

Mesenchymal stromal cells were first isolated from the bone marrow of adult guinea-pigs in the 1960s [13, 14]. They were described as cells which could rapidly adhere to the plastic of culture flasks and were characterized by their fibroblast-like morphology [13–15]. Now MSCs are recognized as a heterogeneous subset of multipotent precursors residing in the stromal fraction of many adult tissues [16]. To discriminate MSCs from other stem cells, the International Society for Cell Therapy has provided three criteria: (a) adherence to plastic of culture flasks; (b) specific surface antigen expression; (c) multipotent differentiation potential [17].

MSCs can be isolated from a variety of adult rodents tissue such as the bone marrow, adipose tissue, cord blood cells, skin, lung, muscle, etc. [14, 16, 18–20]. The bone marrow is a major source of MSCs and has been widely studied as a treatment option for stroke [17–19, 21]. MSCs have great potential as therapeutic agents, since they are easy to harvest and can be expanded from patient's own bone marrow without serious ethical or technical problems [22]. MSCs also represent a promising source for autologous cell transplantation therapies [22].

MSCs have been distinguished from other hematopoietic stem cells according to the expression of surface molecules CD73, CD90, and CD105 but in the absence of CD34, CD45, Human Leukocyte Antigen-antigen D Related (HLA-DR), CD14 or CD11b, CD79a, or CD19 [14, 17, 23, 24]. MSCs can differentiate into osteoblasts, adipocytes and chondroblasts [16, 25]. MSCs can also differentiate into cerebral parenchymal cells such as neurons and astrocytes in animals subject to experimental ischemic stroke model [26–28]. Autologous MSC treatment has been employed in patients with cerebral infarction and spinal cord injury and has the advantage of a lower risk of graft-versus-host disease [4].These advantages make MSCs therapy a new avenue for stroke treatment.

1.2 MSC Treatment Improves Stroke Outcome in Preclinical Studies

During the past two decades, numerous studies have employed MSC treatment for ischemic stroke in rodents and demonstrated that MSC administration in the acute phase (1–3 day) of ischemic stroke improves sensorimotor function, and in the

ischemic penumbra promotes arteriogenesis, angiogenesis, reduces apoptosis, promotes endogenous cell proliferation, augments synaptogenesis, increases nerve regeneration and regulates inflammatory and immune responses in the ischemic brain [2, 24, 29–39]. MSC therapy initiated at a delayed time point can also reduce neurological functional deficits when administered intravenously at 7 days after stroke and even at 1 month after stroke by promoting neurorestorative effects in the (IBZ) [29, 36, 40].

Apart from ischemic stroke, several pre-clinical and clinical studies have demonstrated the safety and efficacy of MSC treatment for hemorrhagic stroke [41, 42]. In intracerebral hemorrhage (ICH) and subarachnoid hemorrhage, MSC treatment attenuates neurological deficits, decreases brain-blood barrier (BBB) leakage, reduces apoptosis, activates axonal remodeling, enhances endogenous neurogenesis and promotes anti-inflammatory effects [43–48]. While there are only a few studies that investigated the effects of MSC treatment in the sub-acute and chronic phase of ICH; MSC treatment has been reported to enhance neural progenitor cells (NPCs) proliferation from 48 h to 14 days after ICH [45]. Rehabilitation of patients is a vital component of ICH treatment and further studies are required to investigate neurorestorative therapy and long term functional outcome using MSCs for ICH.

Poor clinical translation of successful experimental therapies for stroke in part has in part been attributed to the widespread use of healthy, male, young animals in pre-clinical experiments [49, 50]. In reality, stroke patients suffer from one or more co-morbidities such as diabetes mellitus (DM), old age or hypertension [51, 52]. Co-morbidities aggravate stroke outcome and result in poor long term recovery after ischemic stroke. For example, diabetes mellitus is associated with microvascular and macrovascular disease and is a predisposing risk factor for stroke [53–56]. Neurovascular alterations induced by DM also influence the safety and efficacy of stroke therapies [54, 56, 57]. Stroke in type 1 diabetic rats (T1DM) increases mortality and BBB leakage resulting in reduced functional recovery compared to non-diabetic stroke rats [58]. MSC therapy administered in the acute phase (1 day after stroke) of stroke in T1DM rats induced worse functional outcome and adverse effects such as increased mortality, aggravated BBB permeability, higher risk of brain hemorrhage, and more macrophage infiltration [59]. However, MSC therapy initiated at a delayed time point (3 day after stroke) has been demonstrated to reduce vascular damage, promote endogenous neurorestorative effects and improve the functional outcomes post stroke in type two diabetes rats [57, 60, 61]. Therefore, MSC treatment initiation time point may be crucial in determining safety and efficacy of MSC therapy in the diabetic stroke population. Advancing age and hypertension are other major risk factors for stroke [62, 63]. In aged rats subject to stroke, MSC treatment decreased neurological impairment, reduced ischemic lesion volume, and promoted brain remodeling processes including angiogenesis, neurogenesis, and synaptogenesis [62].

Hypertension leads to worse functional outcome and increases ischemic lesion burden after stroke [46, 64]. Treatment of stroke with MSCs in spontaneously hypertensive stroke prone (SHRSP) rats decreased neurological deficits and improved BBB integrity [46]. Overall, preclinical findings indicate that MSC treatment

improves recovery both in the acute and chronic periods of the stroke. Furthermore, when administered with specific protocol, MSC induced therapeutic effects persist in the setting of risk factors of stroke.

1.3 Gene Modification of MSCs to Enhance Therapeutic Benefits

To amplify the efficacy of MSC therapy for stroke, several gene modifications have been investigated. MSCs can be easily transfected via vectors such as lentivirus, adenovirus or adeno-associated virus carrying target genes [4, 65, 66]. A wide variety of genes have been incorporated into the MSCs and reported to induce greater functional recovery including microRNA-145 [61], nerve growth factor (NGF) or Noggin (an antagonist of bone morphogenetic protein) [65, 67], microRNA-133 [68], hepatocyte growth factor (HGF) [66], fibroblast growth factor-2 [69], brain-derived neurotrophic factor (BDNF) [70], vascular endothelial growth factor (VEGF) [71] in ischemic stroke; and glial cell line-derived neurotrophic factor (GDNF) in hemorrhagic stroke [72]. Collectively, these studies indicate that gene modification of the MSCs can enhance its therapeutic effects, but additional preclinical and clinical trials are needed to investigate the safety, efficacy, risk of genotoxicity and other potential adverse effects of such modification.

1.4 Comparison of Administration Routes of MSCs for Stroke Treatment

MSC administration after stroke via different routes such as intracerebral (IC), intracerebro ventricular (ICV), intravenous (IV), intra-arterial (IA) and intranasal (IN) have been reported to promote functional outcome after stroke [4, 24, 30, 73–76]. Among these cell delivery routes, which is the optimal delivery mode for MSCs? Currently, there is no definitive answer and in this section we discuss the pros and cons of various delivery routes.

1.4.1 Intracerebral (IC) and Intracerebro Ventricular (ICV) Injections

IC injection is an invasive and clinically challenging method although it can directly deliver cells to the ischemic brain tissue. IC administration facilitates the trafficking of transplanted cells to the ischemic region, but can also lead to secondary brain trauma and poor distribution of cells within the target lesion [76]. Many experimental stroke studies have shown that IC injection is a reliable route for delivery of MSCs and may provide strong therapeutic effects for stroke recovery [11, 77, 78]. For instance, IC delivery of ~4×10^5 MSCs cultured with NGF at 24 h after stroke

in rats significantly improved neurological functional outcome [11]. Steinberg GK et al. found that IC injection of MSCs in 18 patients with chronic stroke is safe and associated with improvement in clinical neurological outcome end points at 12 months [79]. However, several clinical trials report that IC injections may lead to several adverse events such as seizures, syncope, asymptomatic subdural hematoma and so on [80].Therefore, IC route is limited for use in animal experiments. ICV injections are less invasive, can deliver more cells, and may produce more extensive cell seeding than IC route [81]. In a clinical trial involving ten chronic stroke patients (seven ischemic and three hemorrhagic), researchers investigating ICV delivery route found that some patients developed fever and meningeal signs 48 h after delivery of cells via ICV injection [76, 82].

1.4.2 Intravascular Injections

A majority of studies employ IV or IA routes for delivery of stem cells. Intravascular administrations are less invasive and MSCs systemically infused into ischemic rats have been observed to migrate to the injured brain tissue [83, 84]. Mounting evidence indicates that IV injections of MSCs can successfully traffic cells to the ischemic zone which plays a vital role in recovery and stroke outcome in preclinical and clinical trials [29, 61, 65, 85, 86]. Chen et al. was the first to demonstrate that IV administration of MSCs in rats subject to stroke resulted in significant improvement in neurological outcome [29]. IV administration of autologous MSCs has already been demonstrated to be safe and efficacious in small clinical trials [87–90]. However, intravascular modes of cell therapy also face some challenges. For example, a large fraction of IV transplanted MSCs home to peripheral organs such as lungs, liver, and spleen, thereby limiting potential engraftment in the ischemic lesion in the brain [91, 92]. In 2001, Li et al. first demonstrated that intra-artery injection of MSCs significantly increases MSC migration into brain and improves functional outcome in stroke rats [93]. Selective IA administration of human MSCs have substantially increased migration of transplanted hBM-MSCs in the target brain than IV administration [94]. Although cells IA administered pass the BBB and traffic to ischemic brain, IV and IA achieve similar structural and functional outcomes after stroke at low and high doses of autologous bone marrow mononuclear cell (BMMNC) treatment [95]. One preclinical study comparing IV and IA BMMNC delivery found significant reduction in infarct volume, greater cell engraftment, and improved motor function with IV administration than with IA delivery [96].

1.4.3 Intranasal (IN) Delivery

In recent years, IN delivery has emerged as a novel method to transplant MSCs. IN administered MSCs pass through the BBB, and facilitates cell migration from the nasal mucosa to the central nervous system through the cribriform plate and movea into the brain parenchyma along the olfactory neural pathways, corpus callosum,

and blood vessels [4, 97, 98]. IN delivery is non-invasive, and it decreases any adverse effects associated with intravascular administration. Transplantation of hypoxic preconditioned MSCs reached the injured cortex and were deposited outside of blood vessels 1.5 h after administration, and decreased ischemic lesion volume, and improved motor function in experimental stroke [73, 99]. These therapeutic effects were also observed in hemorrhagic stroke and ICH [100].

1.5 Stroke Clinical Trial for MSCs Therapy for Stroke

In a recent meta-analysis of stem cell therapies for patients with brain ischemia, MSC therapy was found to significantly enhance neurological function and quality of life, but additional investigations may be required to further support the safety and efficacy of stem cell transplantation in stroke patients. A number of clinical trials have investigated the optimal time point, dose and delivery mode for cell therapy for stroke, and are listed in Table 11.1.

2 Mechanisms of the Action of MSCs Treatment for Stroke

The mechanisms underlying neuroprotective and neurorestorative effects of MSCs are still unclear. Currently, it is widely accepted that MSCs can induce brain protection and remodeling primarily by secreting factors that enhance brain tissue repair by promoting vascular remodeling and white matter remodeling and neurogenesis [2–4, 24, 45, 86, 101]. Recently, researchers have found that exosomes derived from MSCs and the exosome cargo microRNA may mediate MSCs induced restorative effects after stroke [16, 102].

2.1 MSC Induced Therapeutic Effects via Cell Replacement

Originally, the mechanism of action of MSC induced therapeutics in stroke was thought to be cell replacement. When progenitor and stem cells are placed in the injured brain, they can differentiate into brain cells [103]. MSCs transplanted after stroke can migrate to the ischemic brain, and since MSCs are multipotent [104–108], they can differentiate into various tissue lineages, such as neurons, astrocytes, and endothelial cells [67, 109, 110]. However, preclinical studies have also shown that upon intravascular delivery, only a small fraction (1–10%) of MSCs migrate to the ischemic lesion [11, 12, 29, 93, 111, 112], and only 2–20% MSCs possibly differentiate into brain cells [11, 29, 111], and approximately only 2% of transplanted MSCs differentiate into endothelial cells in the ischemic brain, 14 days after

Table 11.1 Summary of recent and ongoing clinical trials for stroke treatment using MSCs

Study phase	Mode of delivery	Cell type	Time from onset	Primary outcome measure	Secondary outcome measure	Clinical identifier
I	IV; IT	Umbilical cord, mesenchymal	IV, 7–14 days; IT, 1 week after IV	Function outcome	Function outcome; MRI changes	NCT01389453
I/IIa	IV	Autologous bone marrow mononuclear	24–72 h	Safety; feasibility	Function outcome	NCT00859014
I/IIa	IV	Autologous mesenchymal stem	Within 6 weeks	Safety; feasibility	Function outcome	NCT00875654
I/II	IA	Autologous bone marrow CD 34+ stem	7 days	Safety	Function outcome	NCT00535197
I	IV	Autologous mesenchymal stem	7–30 days	Safety; feasibility	Function outcome	NCT01501773

administration [12]. This shows that at most, only a minor subpopulation of MSCs assume a parenchymal brain cell phenotype, and probably do not contribute to functional recovery [12].

2.2 MSCs Induced Therapeutic Effects via Secretion of Trophic Factors

MSCs secrete large amounts of angiogenic, anti-apoptotic, and mitogenic factors [113]. MSCs do not incorporate into the adult growing vasculature; but may function as supporting parenchymal cells and microglia [114, 115]. MSCs express mRNAs for a wide spectrum of angiogenic/arteriogenic cytokines including VEGF, BFGF2, Angiopoietin-1 (Ang1) and placental growth factor (PGF), insulin-like growth factor (IGF) and SDF [116–120].These growth factors play a crucial role in maintaining and augmenting brain plasticity process such as neurovascular remodeling and white matter remodeling and synaptic plasticity. BMSCs also stimulate brain parenchymal cell production of trophic factors [24, 121, 122]. These cytokines and trophic factors have both paracrine and autocrine activities [121]. MSCs behave as small biochemical and molecular "factories", producing many cytokines [123–125] and trophic factors that may affect the compromised brain tissue neurovascular and white matter remodeling in the ischemic border zone [24].

2.3 MSCs Treatment Promotes Endogenous Brain Plasticity

2.3.1 Vascular Remodeling

The stimulation of vascular remodeling is an important therapeutic target for recovery after stroke [126–128]. Stroke patients with a higher cerebral blood vessel density appear to make better progress and survive longer than patients with lower vascular density [129]. Vascular remodeling includes angiogenesis and arteriogenesis. Arteriolar collateral growth (Arteriogenesis) and new capillaries (Angiogenesis) support restored perfusion in the ischemic border after stroke, and increase long-term neurological functional recovery [130]. Numerous angiogenic factors, growth factors and cytokines have been discovered in the MSC secretome, all of which alter endothelial cell behavior and promote capillary tube formation in vitro and increase angiogenesis in vivo [131]. Increasing VEGF/Flk1 and Ang1/Tie2 signaling pathways plays an important role in MSC treatment induced angiogenesis and vascular stabilization [132]. Very early transplantation of human MSCs (1 h after MCAO) produced increased neurological recovery and decreased infarction volume as well as promoted angiogenesis [133]. Therefore, MSCs have the ability to effectively recruit and participate in vascular remodeling [11, 12, 24, 29, 33, 36, 38, 134].

2.3.2 White Matter Remodeling

Promoting functional outcome after stroke is not only related with vascular remodeling, but also involves white matter remodeling and rewiring of neuronal circuits [3, 135]. White matter remodeling includes axonal remodeling, oligodendrogenesis and remyelination. Oligodendrogenesis involves oligodendrocyte progenitor cell (OPC) proliferation and differentiation into mature oligodendrocytes (OLs) which form myelin sheath around axons. Since injured OLs do not form new myelin and mature OLs do not proliferate, oligodendrogenesis is essential to form myelin sheaths around the new sprouting axons after stroke [136]. MSC treatment stimulates oligodendrogenesis identified by increasing OPC proliferation and differentiation into OL as well as promotes myelination and axonal outgrowth post stroke [3, 61, 99, 110, 137, 138]. Functional recovery post stroke is positively correlated with enhanced white matter integrity in the ischemic brain [138].

In addition, the corticospinal tract (CST) neuronal pathway is also required for motor functional recovery. The severity of motor impairment after stroke is correlated with the extent of ischemic injury to the CST in all phases of stroke [139, 140]. After stroke, bilateral innervations occur through axonal sprouting of the uninjured corticorubral tract (CRT) and CST [141]. CST axonal remodeling in the spinal cord and pyramidal neuronal reorganization in the bilateral cortices promotes neuronal communication and partially contributes to spontaneous functional recovery in the chronic phase of stroke [142]. MSC treatment significantly increases synaptic proteins in the denervated motoneurons and increases axonal restructuring on the de-afferented red nucleus and the denervated spinal motoneurons [141]. MSCs significantly promote

neuronal remodeling of the CST originating from the contralesional cortex in mice subjected to unilateral pyramidotomy after ischemic stroke which may contribute to motor recovery after stroke [143]. Axonal sprouting and rewiring is highly correlated with improved functional outcome after stroke [141, 144].

2.3.3 Neurogenesis

Neurogenesis is defined as the proliferation and differentiation of neural stem cells (NSCs) and neural progenitor cells (NPCs) into neurons. In response to stroke or brain injury, NSCs increase in the subventricular zone (SVZ) and subsequently increase neuroblasts [145]. The newly generated neuroblasts can migrate from the SVZ to peri-infarct regions [145–147]. Therapies that increase post stroke neurogenesis have been associated with improved neurological recovery in rodents [2, 101, 148]. MSC treatment increases NPC proliferation and migration from the SVZ to the ischemic boundary zone where they differentiate into parenchymal cells [149–151]. IV or IC delivery of MSCs in adult or neonatal stroke rats promotes neurogenesis and synaptogenesis, which are associated with functional recovery after stroke [12, 36, 152]. Enhanced neurogenesis and axonal remodeling likely underlie the improved functional outcome following MSC treatment after ischemic brain injury [153].

2.4 Exosome and microRNA Mediates MSC Induced Therapeutic Effects

Stem-like cells, such as MSCs, have been shown to directly secrete paracrine factors, but also membrane vesicles including exosomes [154]. Exosomes are a subpopulation of cellular secreted vesicles (also referred to as extracellular vesicles) that range ~30–100 nm in diameter. Compared to other cell types, the MSC is the most prolific exosome producer [155]. Most exosomes contain conserved proteins such as CD81, CD63, and CD9, Alix and Tsg101, as well as the unique tissue/cell type specific proteins that reflect their cellular source [156]. The exosome membranes are enriched with cholesterol, sphingomyelin, and ceramide [157]. Exosomes contain a variety of biologically active molecules, such as proteins, messenger RNAs (mRNAs), and microRNAs, and these bioactive molecules can mediate exosomal intercellular communication. Using proteomics analysis of exosome derived from MSCs (MSC-Exo), Otero-Ortega et al. identified more than 2000 proteins in MSC-Exo that could be implicated in brain repair [158]. MSCs induce neurological recovery post stroke and neural injury primarily by paracrine via exosomes produced by these cells (MSC-Exo), which mediate restorative actions of MSCs [68, 159–161]. Administration of MSC-Exo significantly improves stroke outcome by promoting neurovascular remodeling [162, 163]. Similarly, systemically injected

human MSC-Exo improved long-term neuroprotection, promoted neuroregeneration, enhanced neurological recovery, and modulated peripheral post-stroke immune responses in mice [164]. MSC-Exo treatment significantly decreases lesion size, increases white matter remodeling identified by fiber tract integrity, axonal sprouting and white matter repair as well as improves functional recovery compared with the control group at 28 days after ICH [158]. Treatment of stroke with MSC-Exo improves functional outcome, as well as enhances angiogenesis, neurogenesis and neurite remodeling [160, 165, 166]. Improved neurological outcome after MSC-Exo therapy for stroke is comparable to the therapeutic effect observed with MSC therapy, suggesting that MSC-Exo-mediated cell-cell communication may contribute to the therapeutic effect of the MSC therapy.

Exosomes play an important role in intercellular communication by transferring exosomal protein and RNA cargo between source and target cells in the brain [167–169]. Many studies have identified that various miRNAs are present in MSC-Exos, and MSCs release functional small RNAs via exosomes that seem to convey the essential features of MSCs [68, 159–161]. MicroRNAs are short sequences of non-coding RNA (containing about 22 nucleotides) found in plants, animals and some viruses, that function in RNA silencing and post-transcriptional regulation of gene expression [170]. Among its myriad of functional properties, microRNAs regulate angiogenesis, neurogenesis, inflammation and stem cell biology [171]. Cell-based therapy, by IV injection release exosomes that contain enriched microRNAs that also stimulate endogenous brain cells to subsequently release microRNAs or genes, ultimately promoting brain plasticity after stroke [172]. MSCs treated with ischemic brain extract produced exosomes with neuroprotective effects in a stroke model in rats, inducing functional recovery mediated by transfer of microRNA-133b in exosomes [68]. MSC-Exo increase neuron and astrocyte miR-133b expression and MSCs communicate with brain parenchymal cells and regulate neurite outgrowth by transfer of miR-133b to neural cells via exosomes [166]. Using primary cortical neuron culture, Zhang et al. found that MSC-released exosomes promoted axonal growth, and inhibition of argonaut 2 protein (a primary microRNA machinery protein) significantly abolished MSC-exosomes induced axonal growth [165]. In addition, MSCs inhibit macrophage activation by shedding miRNA-containing exosomes [173]. Our previous study also shows that diabetic MSCs (DM-MSCs) and exosomes derived from DM-MSCs have decreased microRNA-145 expression [174]. DM-MSC treatment of stroke in DM-stroke improved functional outcome and vascular and white matter remodeling, as well as decreased serum microRNA-145 expression compared with vehicle treatment [174]. These data demonstrate that MSCs mediate their functional benefit in stroke at least partially by the transfer of exosomes with active microRNAs to parenchymal cells.

MSCs-Exo can be manipulated to deliver microRNAs to enhance recovery of injured tissues [167, 175]. Treatment of stroke with tailored exosomes enriched with the miR-17-92 cluster significantly increases neural plasticity and functional recovery after stroke [176]. Tailored exosomes derived from MSC further enhance neurite growth via the Phosphatase and tensin homologue/Mammalian target of rapamycin (PTEN/mTOR) signals by increasing the microRNA-17-92 cluster

[177]. Exosomes derived from microRNA-133b-overexpressed MSCs also improved neurite remodeling/brain plasticity and neurological functional recovery after stroke compared to MSC-Exo treatment group [178]. Therapeutic modulation of microRNAs generated by MSCs, and either mimicking or antagonizing microRNA actions, may enhance MSC induced therapeutic efficacy. Hence, the use of microRNAs as novel regulators and therapeutic modulators of individual microRNAs of MSCs have been proposed to improve therapeutic efficiency [179].

3 Conclusions

Early or delayed MSCs treatment of stroke (ischemic or hemorrhage stroke) significantly improves functional outcome in neonatal, adult and aging populations as well as in diabetic and hypertension stroke populations. The mechanisms underlying neuroprotective and neurorestorative effects of MSCs are still unclear. However, MSCs secrete trophic factors, exosomes with their cargo microRNAs, that may modulate brain tissue repair and restorative mechanisms such as neurovascular remodeling and white matter remodeling. We thus have major insight into the restorative mechanism by which MSCs promote neurological recovery. Preclinical studies in many models of neurological injury and disease have demonstrated therapeutic efficacy of MSC treatments, and there have been clinical trials that support the therapeutic approach of MSC therapy for stroke and other forms of neural injury. Thus, in concert, this compendium of studies and data, support the further development and clinical application of this promising therapy for the treatment of stroke, and other forms of neural injury.

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Chapter 12
Combination Treatment of Mesenchymal Stem Cells (MSCs) and Angelica sinensis' Active Ingredients for Ischemic Stroke

Qian Zhang and Yonghua Zhao

Abstract At present, mesenchymal stem cells (MSCs) are regarded as a candidate for neovascularization and tissue regeneration after ischemic stroke. Numerous studies reported that Angelica (also called Dong quai, a well-known Chinese herbal medicine) extracts and its active ingredients such as ligustilide, n-Butylphthalide and sodium ferulate had significant effects of anti-inflammatory, anti-activation of oxygen free radicals, angiogenesis, anti-platelet aggregation, neuroprotection and so on. Angelica' active compositions facilitated MSCs to migrate into infarcted zone and differentiation. Moreover, MSCs combined with angelica' active components improved neurological function and decreased infarcted volume, advanced neovascularization and neurogenesis, regulated astrocytes characteristics, enhanced regional cerebral blood flow and glucose metabolism, as well as reduced brain-blood barrier permeability in infarction. Consequently, the structure and function of neurovascular unit in infarct region partly obtained recovery. Therefore, the combination treatment was a valuable therapy aimed at improving post-stroke restoration.

Keywords Angelica sinensis • Combination treatment • Ischemic stroke • Mesenchymal stem cells

Abbreviations

BBB	Blood-brain barrier
BDNF	Brain-derived neurotrophic factor
BMP	Bone morphogenetic proteins
BP	n-Butylidenephthalide

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P.A. Lapchak, J.H. Zhang (eds.), *Cellular and Molecular Approaches to Regeneration and Repair*, Springer Series in Translational Stroke Research,
https://doi.org/10.1007/978-3-319-66679-2_12

BrdU	5-Bromo-2′-deoxyuridine
CBF	Cerebral blood flow
CXCR4	Chemokine (CXC motif) receptor-4
DCX	Doublecortin
DG	Dentate gyrus
EC	Endothelial cell
ERK	Extracellular signal-regulated kinases
FA	Ferulic acid
FDA	Food and Drug Administration
FDG	^{18}F-2-deoxy-glucose
GDNF	Glial cell line-derived neurotrophic factor
GFAP	Glial fibrillary acidic protein
HIF	Hypoxia-inducible factors
HUVEC	Human umbilical vein endothelial cell
MCAo	Middle cerebral artery occlusion
MRI	Magnetic resonance imaging
MSC	Mesenchymal stem cell
mTOR	Mammalian target of rapamycin
NBP	n-Butylphthalide
PET/CT	Positron emission tomography–computed tomography
PWI	Perfusion-weighted imaging
SDF-1	Stromal cell-derived factor-1
SF	Sodium ferulate
STEPS	Stem Cells as an Emerging Paradigm in Stroke
SVZ	Subventricular zone
TTC	2,3,5-Triphenyltetrazolium chloride
Tuj-1	Neuron-specific class III beta-tubulin
VEGF	Vascular endothelial growth factor
vWF	Von Willebrand factor

1 Introduction

Ischemic stroke is the most common cerebralvascular disease. Due to blood flow blockage by arterial thrombus, amounts of neurons in ischemic central and penumbral regions occur to necrosis and apoptosis, which resulted in attenuation of neurological function. Evidence indicated that stroke was the second leading cause of death and the major cause of disability globally, especially in developing countries [1]. There are 15 million individuals suffer from stroke in every year worldwide, and in the United States, among 800,000 stroke patients, 75% of them have never experienced stroke before, and 25% undergo recurrent attack [2]. Moreover, mortality of ischemic stroke is predicted to nearly double by 2032 [3]. Although stroke has been major threat to life expectancy and quality, there are relatively few treatment

options available to ameliorate neurological function due to complicated etiological and pathophysiological evolutions after ischemic stroke [4]. At present, recombinant tissue plasminogen activator (rt-PA) is still approved by United States Food and Drug Administration (FDA) for dissolution of thrombus and improvement of cerebral flow, but narrow therapeutic time window (3–6 h) and multiplicative individual exclusion criteria limit it to be widely applied [5, 6].

In recent years, cell therapy is regarded as a promising approach. Bone marrow-derived mesenchymal stem cells (MSCs) have been demonstrated to be able to differentiate into neuronal cells and replace injured neurons after cerebral ischemia, as well as activate endogenous restorative responses (e.g. neurogenesis, angiogenesis and synaptogenesis) against injured brain, so autologous MSCs were transplanted into stroke patients in 2005 [7–11]. Evidence indicated that five stroke patients accepted stereotactically transplanted MSCs treatment, and the therapeutic results showed that their neurological functions were improved and no complications happened after 1 year's observation [12]. In 2007, the National Institutes of Health and FDA issued consensus-based guidelines on the development of cell therapies for stroke, entitled "Stem Cells as an Emerging Paradigm in Stroke" (STEPS). Current STEPS 3 had discussed how to successful complete translation from animal models to patients and optimize clinical trial designs for acute and chronic stroke [13].

Angelica (Angelica sinensis (Oliv.) Diels), commonly called Dong quai in Chinese, is a dried root derived from an herb in the family Apiaceae, which is used over thousands of years as a well-known Chinese medicine. Since 1980s, angelica extract and its active ingredients began to be used to treat ischemic cerebrovascular disease. Liu and colleagues observed 1404 patients of acute cerebral infarction, and 692 of them treated with angelica injection, 390 of them used Danshen (Salvia miltiorrhiza) injection, 322 of them treated with low molecular dextran. Consequently, the total effective rate was 78.7%, 63.6% and 59.3% respectively after treatment, suggesting neurological functional recovery in angelica injection group was better than those in other two groups [14]. Study indicated that Z-Ligustilide, a main component of volatile oil in angelica, could reduce infarct volume and cerebral edema in a dose-dependent way, and ameliorate injured neurological function after 2 h in middle cerebral artery occlusion (MCAo) rats, suggesting it had obvious neuroprotective effect [15]. n-Butylphthalide (NBP) derived from phthalides compounds in angelica has been identified as a new drug for the treatment of ischemic cerebrovascular disease by China FDA. Previous study indicated that it could improve cognitive deficits in rats with chronic cerebral ischemia [16]. Moreover, in a randomized, double-blind and multi-center research on 535 stroke patients, it showed that patients' neurological functional scores in NBP group were obviously higher than those in ozagrel group [17]. The extract and active ingredients in angelica exert multi-efficacy in the treatment of ischemic stroke.

It has been demonstrated that the effect of combining MSCs with pharmacological agents on stroke is superior to MSCs treatment alone or pharmacological agents. For example, combined treatment of BMSCs with simvastatin could further facilitate MSCs' migration and differentiation, as well as enhance arteriogenesis and angiogenesis and reduce infarction volume, which contributed to the amelioration

of functional outcome after cerebral ischemia [18–20]. Our previous study also evidenced that simvastatin combined with MSCs could obviously activate astrocytes and increase astrocyte-derived stromal cell-derived factor-1α (SDF-1α), vascular endothelial growth factor (VEGF), brain-derived neurotrophic factor (BDNF) and glial cell line-derived neurotrophic factor (GDNF) expressions post-stroke, as well as up-regulate Akt/mammalian target of rapamycin (mTOR) signaling pathway in oxygen glucose deprived astrocytes [21]. Recently, the optimization of pharmacological agents in combination treatment began to transfer to Chinese medicines. Astragaloside IV, Naomai Yihao capsules, Buyang huanwu tang and Tongxinluo combined with MSCs had been demonstrated to notably promote angiogenesis and attenuate ischemic injury [22–25]. Based on angelica extract and active ingredients' multiple efficacy on ischemic stroke, our research group devotes the investigation of combination treatment of angelica and MSCs and finds some synergic functions and mechanisms.

2 Amelioration of Neurological Outcome and Reduction of Infarcted Volume

According to the evaluation of Garcia JH neurological score which included six sections: (1) evaluating animals' spontaneous activity; (2) symmetry of four limbs' movements when rat was held suspended by the tail and symmetric forepaws were assessed, (3) climbing wall of wire cage; (4) body proprioception; (5) reaction to touch on either side of the trunk; (6) response to vibrissa touch [26], we observed that derived from angelica's active ingredients, sodium ferulate (SF), as well as SF and n-Butylidenephthalide (BP) combined with MSCs began to improve neurological functional outcomes from day 3, and the increased trend always kept to day 7 after ischemia, suggesting the amelioration of neurological outcome was obviously superior to MSCs alone [27, 28]. Additionally, Bederson scale was administrated for neurological assessment following stroke, which included forelimb flexion, resistance to lateral push and circling behavior [29]. The scores are as below when SF (60 mg/kg) and BP (10 mg/kg) combined with MSCs (2×10^6 cells/ml, intravenous injection) were applied for the treatment of MCAo model in rats.

The scoring scale indicated that ischemic animals would have more significant neurological deficits than non-ischemic animals, resulting in a higher score. It indicated that neurological functional scores in SF + BP + MSC group began to reduce at day 3, showing neurological deficit had been attenuated (Table 12.1).

Magnetic resonance imaging (MRI) scanning and 2,3,5-triphenyltetrazolium chloride (TTC) staining showed whatever SF or SF and BP combined MSCs, both of therapeutic methods notably reduce infarcted volume post-stroke [30, 31].

Table 12.1 Bederson scale for neurological functional assessment (n = 20, means ± SD)

Group	3 h	1 day	3 days	7 days
Sham	0.02 ± 0.01	0.03 ± 0.03	0.02 ± 0.02	0.05 ± 0.02
MCAo	2.50 ± 0.21	2.56 ± 0.02	2.26 ± 0.14	2.08 ± 0.13
MSC	2.50 ± 0.12	2.64 ± 0.05	2.14 ± 0.12	1.45 ± 0.14*
SF + BP + MSC	2.50 ± 0.21	2.43 ± 0.17	1.35 ± 0.15*	1.09 ± 0.02*

*P < 0.05, vs. MCAo group

3 Acceleration of MSCs Differentiation and Migration

As a main organic acid in angelica, ferulic acid (FA) had been demonstrated that it could decrease infarction size and improve neurological function in MCAo rats through anti-oxidative and anti-inflammatory actions [32]. SF is the sodium salt of FA, which been used as an important agent for cardiovascular and cerebrovascular diseases. In 2005, Wang and colleagues firstly found that SF could induce Human MSCs to express neural proteins, such as nestin, neuron specific enolase and glial fibrillary acidic protein (GFAP), as well as advance MSCs to differentiate into neural-like cells in vitro [33]. Our previous study also indicated that SF could enhance 5-bromo-2′-deoxyuridine (BrdU)-labeled bone-derived MSCs to express nestin, GFAP and neuron-specific class III beta-tubulin (Tuj-1) in ischemic rat stroke model, suggesting it facilitated the differentiations of MSCs into astrocytic- and neuronal-like cells. Another experiment showed that adipose-derived MSCs were incubated with SF (5 μg/ml) and BP (0.75 μg/ml) for 3 and 7 days. Cultured MSCs in SF + BP group obviously presented neuronal morphology at day 3, and some cells even possessed long neuronal-like synapses under light microscopic observations. At day 7, fluorescence staining results also showed that combination of SF and BP could noticeable advance MSCs to express Tuj-1 (Fig. 12.1).

In order to illustrate the differentiated mechanisms, we investigated bone morphogenetic proteins (BMP) 2/4 and Notch-1 signaling pathways. As a member in the transforming growth factor beta superfamily, BMPs and their signaling systems play important roles in regulation of neural activity and rescue of injured neurons, and they could selectively and dose-dependently increase multipotent progenitor cells in murine embryonic subventricular zone (SVZ) to differentiate into astroglial lineage [34, 35]. It is reported that BMP2 and BMP7 levels began to enhance from 1 to 4 weeks in mice ischemic brain, which were associated with astrogliosis [36]. In our study, we found that SF combined with MSCs up-regulated BMP2/4 pathway post-stroke, which might be related to the differentiation of MSCs into astrocytic-like cells [30]. The mammalian family of Notch receptors consists of four members, Notch-1 through Notch-4, all of which are single pass transmembrane proteins. Study evidenced that activated Notch receptor promoted the survival and numbers of murine somatic and human embryonic stem cells by induced the expression of the specific target genes hairy and enhancer of split 3 (Hes3) and Sonic hedgehog, resulting in amelioration of motor skills after ischemic injury [37]. Not only Notch signaling plays a role in keeping the progenitors from differentiating into neurons,

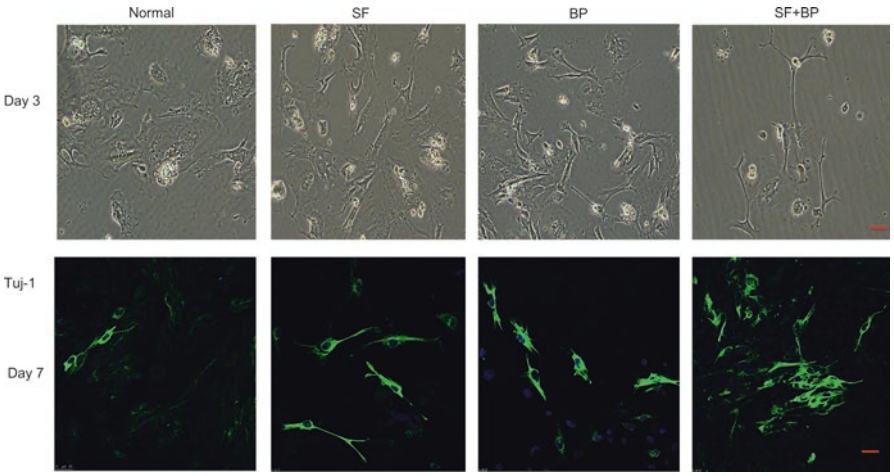

Fig. 12.1 Morphology and differentiation of cultured MSCs incubated with SF and BP. *First panel*: morphology of cultured adipose-derived MSCs incubated with SF and BP for 3 days under optical microscope; *Second panel*: immunofluorescence staining of Tuj-1 in MSCs after 7 days (scale bar: 20 μm)

but also down-regulated Notch1 signaling accelerated striatal astrocytes to carry a latent neurogenic program after stroke [38]. Moreover, there was cross-talk between Notch and BMP signaling pathways, which embodied that BMP2 enhanced Notch-induced transcriptional activation of Hes-5 and Hes-1 in mouse neuroepithelial cells [39]. In our study, it showed that the expressions of Notch-1, Hes1 and Hes5 in combination treatment of SF and MSCs group decreased, which might contribute to the differentiation of MSCs into neural-like cells [30].

Due to low migration efficiency of the transplanted BMSCs into the lesion area, MSCs treatment is limited. Wang and colleagues reported that SDF-1α and chemokine (CXC motif) receptor-4 (CXCR4) could systemically regulate transplanted MSCs towards ischemic zone in the MCAo rat model [40]. In the bone marrow, CXCR4 on endothelial cells and MSCs recruited peripheral blood SDF-1 to translocate into bone marrow, subsequently resulting in the homing of transplanted human $CD34^+$ hematopoietic progenitors to the bone marrow, and the effect was crucial related to SDF-1 gradient [41, 42]. Evidence indicated that up-regulated SDF-1/CXCR4 axis increased SVZ neuroblast cell migration after stroke [43]. Therefore, improving SDF-1α gradient in cerebral damaged tissue might contribute to MSCs recruitment into ischemic zone. Through Western blot and RT-PCR assay, it suggested that SF combined MSCs significantly up-regulate SDF-1/CXCR4 axis, which was beneficial to recruit more stem/progenitor cells to migrate into infarcted lesion [27]. Additionally, luciferase labeled adipose-derived MSCs was injected into the margin of laser illuminated area in photochemically induced stroke model, and it showed that SF and BP could obviously promote MSCs' abilities of survival and migration (Fig. 12.2).

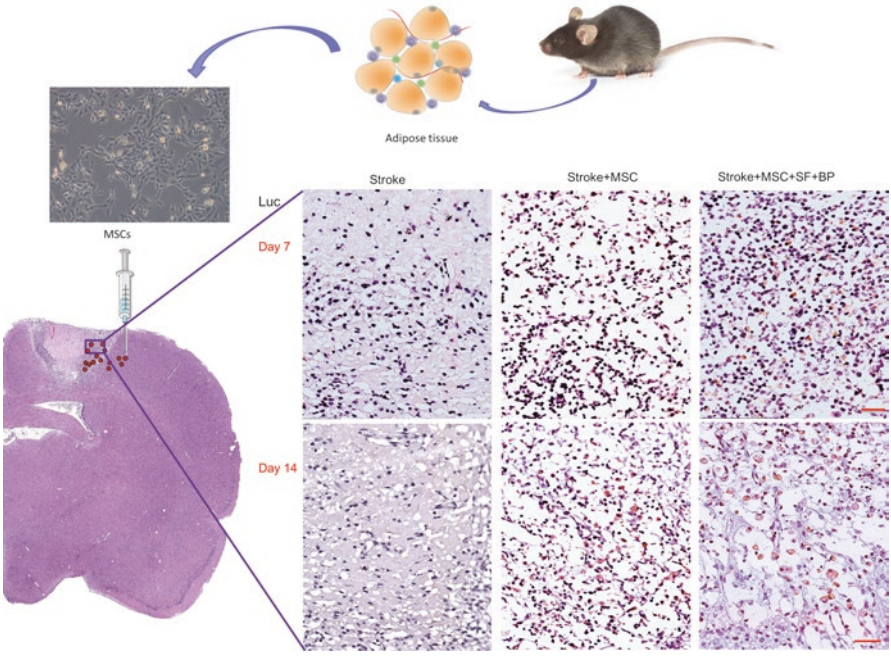

Fig. 12.2 Migration of Luciferase labeled adipose-derived MSCs at day 7 and 14 post-stroke. Luciferase immunohistochemistry staining images suggested that SF and BP could advance migration of adipose-derived MSCs into infarcted zone (scale bar: 50 μm)

4 Enhancement of Angiogenesis and Neurogenesis

Being a potential therapeutic candidate in the treatment of ischemic stroke, MSCs are capable of promoting angiogenesis and neurogenesis after cerebral ischemia [11, 44, 45]. Exogenous transplanted MSCs not only directly differentiated vascular endothelial cells (ECs), but also induced endogenous angiogenic responses to amplify angiogenesis and vascular stabilization after stroke [11, 46]. In addition, angiogenetic gene-modified MSCs, e.g. MSCs transfected with the angiopoietin-1, placental growth factor, VEGF and Flk-1 gene showed the greatest structural-functional recovery and notably angiogenesis and neurogenesis post-stroke [47–49]. Komatsu and colleagues thought that angiogenesis accounted for the main therapeutic effects, although there were several hypotheses in the treatment of MSCs [50]. Our previous review summarized the actions of new vessel formation after ischemic stroke, which included the improvement of cerebral blood flow (CBF) and metabolism in infarction lesion, removal of necrotic debris, enhancement of neurotrophic components for neuronal remodeling and endogenous stem/progenitor cells migration [51]. Based on the efficacy of angiogenesis, it has been recognized to be the basis and prerequisite for neurogenesis. However, not all angiogenesis is advantageous for stroke by MSCs transplantation. In Type 1 diabetic MCAo rats, angiogenesis by grafted MSCs deteriorated internal carotid artery neointimal formation and blood-brain barrier (BBB)

leakage, which possibly was due to increased expression of angiogenin. The adverse effects promoted mortality and the risk of brain hemorrhage [52]. Thereby, the homeostasis of angiogenesis should be taken into account after cerebral ischemic stroke.

More and more evidences indicated that the occurrences of angiogenesis and neurogenesis were coupled processes rather than separate after stroke [53, 54]. It suggested that coculture of neural progenitor cells from SVZ with cerebral ECs from the stroke boundary notably increased neural progenitor cell proliferation and neuronal differentiation, and inhibition of VEGF receptor 2 decreased these beneficial effects on neurogenesis and angiogenesis [53]. Sun and colleagues also demonstrated that VEGF exerted primary role in neuroprotection, survival of new neurons and angiogenesis after cerebral ischemia [55]. In addition to promoting synaptic and axonal plasticity and advancing neurogenesis of MSCs, BDNF also involved in angiogenesis after stroke [56–58]. Phosphorylated AKT could activate mTOR in sequent up-regulate Hypoxia-inducible factors (HIF)-1α expression, consequently improve VEGF expression which contributed to angiogenesis post-stroke, another hand AKT/mTOR had been demonstrated as a crucial target to regulate new neuron development and was essential to maintain endogenous neuronal progenitor pool [59, 60]. Additionally, reports also showed that BDNF could bind to Tropomyosin receptor kinase B (TrkB) receptor and then activate AKT/mTOR signaling resulted in neuroprotective actions in stroke [61, 62]. AKT/mTOR pathway is a central regulated approach of angiogenesis.

Evidence showed that angelica extract and active ingredients were able to improve angiogenesis. Lam and colleagues demonstrated that human umbilical vein endothelial cells (HUVECs) and zebra fish intestine capillaries incubated with angelica extract presented obvious angiogenic abilities, whose mechanism was mainly related to p38 and Jun N-terminal protein kinase 1/2 phosphorylation [63]. FA could advance HUVECs to secret VEGF, platelet-derived growth factor and HIF-1α, consequently, promote the ability of angiogenesis via activation of PI3K signaling pathway [64]. As a new drug for cerebralvascular disease approved by China FDA, NBP was capable of increasing brain microvessels density against stroke, whose mechanisms were associated with enhanced expressions of VEGF, VEGFR and HIF-1α as well as activation of extracellular signal-regulated kinases (ERK)1/2 and PI3K/Akt-endothelial nitric oxide synthase (eNOS) signal pathways [65–68].

It has been observed that angiogenesis and neurogenesis simultaneously taken place in the penumbra, and newly born, immature neurons derived from neural stem cells (NSCs) in SVZ and dentate gyrus (DG) closely associate with the remodeling vasculature in this neurovascular niche [69]. In rat permanent bilateral common carotid artery occlusion model, angelica extract that contained the component Z-ligustilide improved neurogenesis in the hippocampus and cognitive decline due to hypoperfusion though enhanced expressions of BDNF and phosphorylated cyclic adenosine monophosphate-responsive element binding protein and γ-aminobutyric acid [70]. In vitro, FA promoted proliferated ability of cultured neural stem/progenitor cells derived from embryonic telencephalon and the number and size of secondary formed neurospheres; in vivo, it increased the number of newly generated cells in the hippocampal DG of corticosterone-treated mice [71]. Previous study suggest that BP, a kind of alkylphthalide derived from the volatile oil of angelica, had ECs protective, vasorelaxing, antiplatelet and antianginal effects, as well as maintained stem

cells pluripotency [72–76]. Based on the characteristics of SF and BP, we chose SF and BP as representative constituents of angelica and discuss the effects and mechanisms combined with BMSC on angiogenesis and neurogenesis after ischemic stroke. In our study, we think that SF and BP was a "Trigger point" which embodied that they advanced MSCs to synthesize VEGF and BDNF, subsequently AKT/mTOR cascade was activated in cerebral parenchymal cells, consequently the combination treatment improved angiogenesis and neurogenesis. In order to define the role of astrocytes on angiogenesis in the treatment of MSCs combined with SF and BP, we investigated astrocyte-derived neurovascular trophic factors and found that combined treatment could obviously increase the expressions of astrocyte-derived VEGF and BDNF via activation of astrocytic AKT/mTOR signaling, resulting in migration and tube formation of HUVECs [28, 31]. Additionally, Immunohistochemistry staining images indicated that adipose-derived MSCs combined SF and BP promoted Von Willebrand factor $(vWF)^+$ capillary density compared with SF + BP group and MSC group and Western blotting showed combination treatment notably enhance Doublecortin (DCX) expression in ischemic boundary zone, suggesting the enhancements of angiogenesis and neurogenesis (Fig. 12.3).

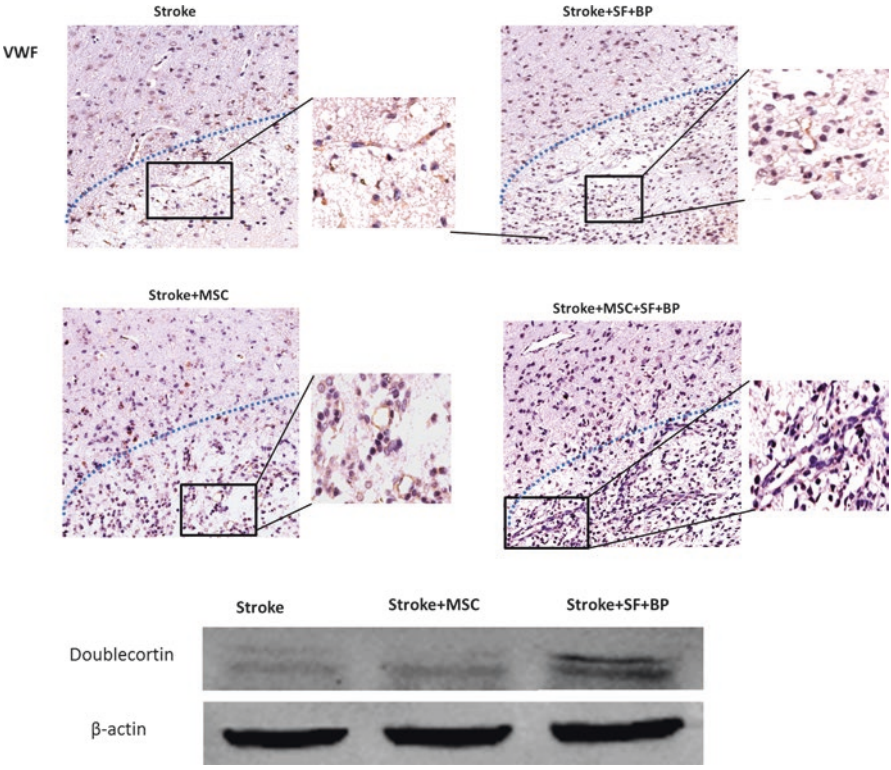

Fig. 12.3 vWF positive capillary and DCX expression in ischemic boundary zone after ischemia. Immunohistochemistry staining images and western blotting showed SF and BP combined with MSCs significantly improve angiogenesis and neurogenesis

5 Regulation of Astrocytes, Activation or Inhibition?

Evidences indicate reactive astrocytes play an important neuroprotective role through enhanced number of mitochondria and antioxidant enzyme activity, reabsorption of glutamate, antitoxic action of free radicals and anti-apoptosis, and regulating immunological response against ischemic brain injury [77]. In the study of cerebral energy metabolism, Kajihara and colleagues demonstrated that astrocytes increased cytoplasmic storage capacity of glycogen granules in the ischemic penumbra after ischemic stroke, and protoplasmic astrocytes gradually became into fibrous astrocytes as ischemic time went by [78]. Previous evidence showed that MSCs treatment could reduce thickness of glial scar formation by reactive astrocytes, consequently decrease inhibition of axonal and synaptic growth, as well as neuronal functional regeneration in the later stage of cerebral ischemia [79, 80]. However, the newest report suggested that scar formation by regulation of astrocyte had advantageous action for axonal regeneration in severe spinal cord injury [81]. On the other hand, in the maintenance of BBB integrity, reactive astrocytes are traditionally thought as detrimental actions which present promoted endothelial permeability and VEGF secretion, as well as decreased occludin and claudin-5 proteins expressions [82–85]. Therefore, it should be compromised evaluation between the beneficial and adverse effects of reactive astrocytes post-stroke.

Present study showed that NBP reduced GFAP-positive astrocytes induced by chronic cerebral ischemia, and inhibited the amyloid β (Aβ)-induced astrocyte activation and pro-inflammatory molecules, which contributed to against ischemic stroke and Alzheimer's disease [16, 86]. Our previous study indicated that SF and bone-derived MSCs respectively activate astrocytes at day 3, and SF combined with MSCs more significantly promoted GFAP expression in ischemic penumbra. Interestingly, when SF and BP combined with adipose-derived MSCs were used to treat photothrombotic stroke, we found SF and BP notably inhibited activation of astrocytes in ischemic boundary zone, but adipose-derived MSCs activated astrocytes at day 7 after ischemia. Simultaneously, the combination treatment also suppressed GFAP expression to a certain extent (Fig. 12.4). The results suggested that different composition of angelica might exert differential actions on astrocytes post-stroke.

The detrimental effect of reactive astrocytes on BBB permeability mainly attributed to its VEGF secretion. Study indicated that knockdown of VEGF no longer damaged endothelial barrier, so astrocyte-derived VEGF has been described as a key mechanism in BBB breakdown [84]. In addition to reactive astrocytes, adult human dental pulp stem cells also were found to secret VEGF-A resulted in enhancement of permeability across an in vitro model of BBB [87]. Shimotake and colleagues demonstrated that VEGF receptor-2 inhibition advanced ischemic injury and reduced endothelial cell proliferation in neonatal rats, whereas it attenuated BBB permeability in diabetic mice after stroke, whose action was associated with enhanced endothelial transcytosis rather than tight junctions [88, 89]. Recent report evidenced that astrocyte-derived Pentraxin 3 bound to VEGF, subsequently notably decreased VEGF-induced endothelial permeability in vitro, and astrocytes existed at least two subclasses by different migratory abilities after

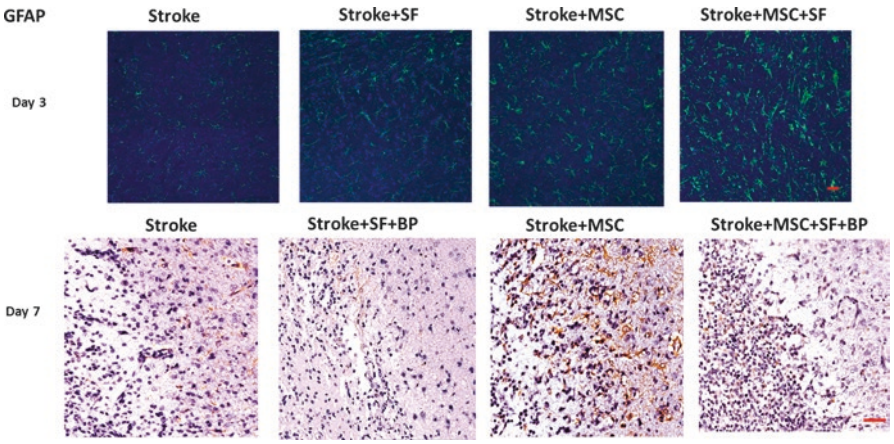

Fig. 12.4 Angelica' active ingredients combined with MSCs regulated GFAP expression in penumbra post-stroke. *First panel*: Immunofluorescence staining indicated that combining SF and bone-derived MSCs could activate astrocyte after 3 days in MCAo model; *Second panel*: SF and BP combined with adipose-derived MSCs inhibited reactive astrocytes after 7 days in photothrombotic stroke model (scale bar: 50 μm)

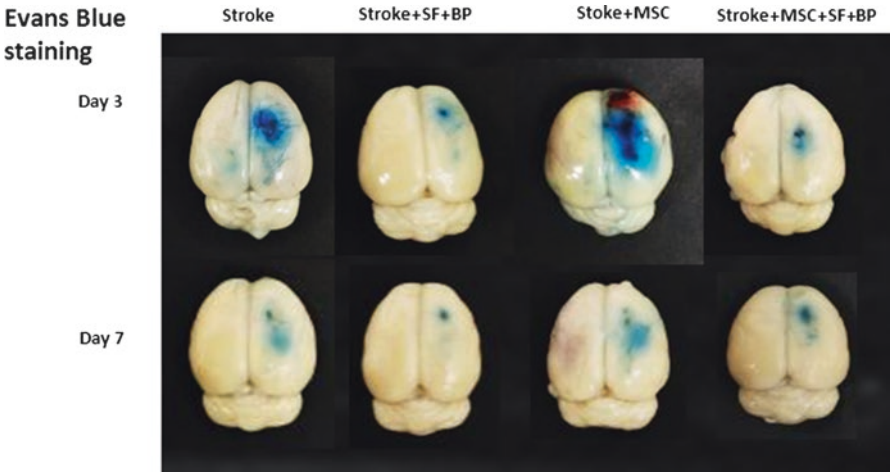

Fig. 12.5 Evaluation of blood-brain-barrier integrity after photothrombotic stroke. Representative images of brain EB staining in rat at day 3 and 7 were presented

cerebral stoke [90, 91]. Therefore, the results reflect the multifaceted actions of VEGF and astrocytes on BBB integrity and endothelial function. In our study, Evans blue staining showed that SF + BP group distinctly reduced BBB leakage compared with other three groups whatever at day 3 or 7 after stroke, which might attribute to inhibitions of astrocytes activation, and SF and BP combined with MSCs presented the effect of maintenance of BBB integrity which was notably superior to MSC alone treatment, suggesting that SF and BP could be against side effect of increased BBB leakage of adipose-derived MSCs (Fig. 12.5). But VEGF

expression in combination treatment was the highest (Data no show), whether the therapy influenced subtypes of astrocytes, astrocyte-derived Pentraxin 3, endothelial function or regulation of VEGF receptors, exact mechanisms need to be further illustrated in future experiment.

6 Improvement of Cerebral Blood Flow and Glucose Metabolism

Neurovascular coupling is responsible for controlling regional CBF by neurons directly or astrocytes indirectly secreting vasodilator factors targeting on vascular cells under physiological condition [92]. Moreover, it reported that reactive astrocytes exerted key effect on rCBF regulation, and they could modulate rCBF longer than neurons under pathological conditions [93]. We examined perfusion-weighted imaging (PWI) by MRI to evaluate CBF after photothrombotic stroke. It showed that SF and BP combined with adipose-derived MSCs could significantly ameliorate CBF in the infarction zone (Fig. 12.6a). Previous study had demonstrated that the combination treatment could significantly enhance angiogenesis and neurogenesis, as well as regulate astrocytes, so we thought the interactions of newly neuron, astrocyte and neovascularization contributed to enhancement of CBF in infarction. Additionally, ^{18}F-2-deoxy-glucose (FDG)-positron emission tomography–computed tomography (PET/CT) was administrated to assess glucose metabolism. As shown in Fig. 12.6b, the cortical metabolic defect partially recovered with the time prolongation, and it seemed to more obvious amelioration in MSC + SF + BP groups at day 14, suggesting that combination treatment could enhance glucose metabolism. We also observed that combing SF and MSCs promoted Glucose transporter 1 expression in ischemic boundary zone [30], which might be a mechanism of glucose metabolism.

In summary, these studies uncovered that angelica' active ingredients could advance MSCs migration and differentiation, and combining MSCs with angelica' active ingredients ameliorated neurological function and reduced infarction volume, improved neovascularization and neurogenesis, regulate astrocyte characteristics, enhanced CBF and glucose metabolism, and decrease BBB permeability, which restitute the structure and function of neurovascular unit in infarction zone after stroke. Therefore, the combination treatment should be a more effective therapy due to synergic functions of supplementary approaches.

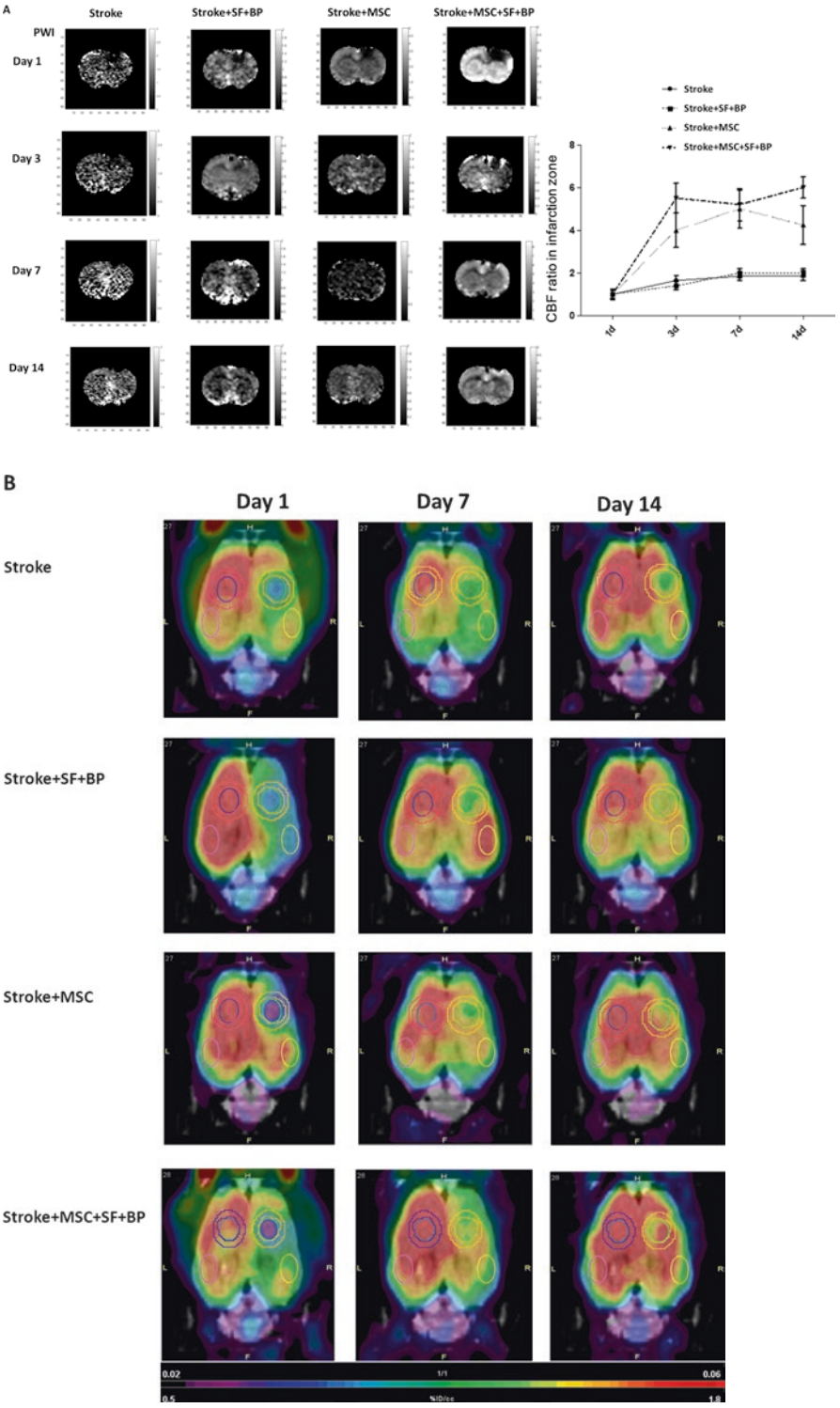

Fig. 12.6 Evolution of cerebral blood flow and glucose metabolism in infarct lesion. (**a**) Images of PWI at day 1, 3, 7 and 14 after ischemia were presented and quantitative analysis of CBF. (**b**) ^{18}F-FDG PET imaging at day 1, 7 and 14 was showed

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Chapter 13
Gene Therapy for Cognitive Recovering After Ischemic Stroke

Johanna Gutierrez-Vargas, Rafael Posada-Duque, and Gloria Patricia Cardona-Gómez

Abstract Cerebrovascular accident (CVA) is the second leading cause of death in the world and the first cause of disability in adults, being a 34% of affected people younger than 65 years old. Which is an important consequence by sedentary lifestyle and a high intake of fats and sugars. One of the major shortcomings of current therapeutical approach is the lack of comorbidity studies, intervention time (less than 4.5 h) and the short time of protection or follow-up study, which unprotect for long-term sequelae in the patients. Gene therapy has been shown to be a very useful tool for the treatment of neurodegenerative diseases; specifically in cerebral ischemia there are few experimental studies, which are mentioned in this chapter. The most of them have a pretreatment approach, which does not facilitate the clinical translation, therefore, a major challenge of gene therapy is that it to be implemented as post-injury therapy, which is supported by our results using shRNAmiR carried out in adeno associated viral vector, preventing and reversing neurodegeneration, neurovascular unit uncoupling and cognitive impairment, which could be relevant in the field of translational medicine.

Keywords Stroke • Neurodegeneration • Cognitive impairment • Dementia • Gene therapy • RNA interference • Translational medicine

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P.A. Lapchak, J.H. Zhang (eds.), *Cellular and Molecular Approaches to Regeneration and Repair*, Springer Series in Translational Stroke Research,
https://doi.org/10.1007/978-3-319-66679-2_13

Abbreviations

AAV	Adeno associated viral vectors
AD	Alzheimer's disease
ALS	Amyotrophic lateral sclerosis
BDNF	Brain-derived neurotrophic factor
CDK5	Cyclin-dependent kinase 5
CVA	Cerebrovascular accident
Glu	Glutamate
HMG-CoA	3-Hydroxy-3-methylglutaryl coenzyme A
LTD	Long term depression
LTP	Long term potentiation
MCAO	Middle cerebral artery occlusion
NFTs	Neurofibrillary tangles
NMDA	N-Methyl-D-aspartic acid
PD	Parkinson's disease
RNAi	RNA interference
Rosc	Roscovitine
rt-PA	Recombinant tissue plasminogen activator
shRNAs	Short hairpin RNAs
TEER	Transendothelial resistance
WHO	World Health Organization

1 Background and Significance of Cerebral Ischemia

Cerebrovascular accident (CVA) is the second leading cause of death in the world and the first cause of disability in adults [1] According to the World Health Organization (WHO), 15 million people suffer a stroke each year; of them 5.5 million die (10% of all deaths) and another 5 million are permanently disabled [2]. Being a 34% of affected people younger than 65 years old [2]. In terms of disability, CVA are among the top five contributors, which entails huge economic burdens on health systems because of the resources needed to care for acute patients and the long-term care of survivors [1, 2].

The two main mechanisms that cause brain damage in stroke are ischemia and hemorrhage [3]. Ischemic stroke accounts 87% of strokes [2], because a thrombus or plunger lead to the cerebral artery occlusion producing anoxia in the affected cerebral parenchyma [4, 5], which causes a decrease of brain metabolism due to the non-availability of the necessary substrates. The ischemia effects are rapid since the brain does not store the necessary glucose as the main energy substrate, making it unable to perform anaerobic metabolism [6]. Most severe degree of blood hypoperfusion progresses rapidly towards irreversible damage due to necrotic death, which represents the *ischemic nucleus* (Fig. 13.1). This zone has low cerebral blood flow

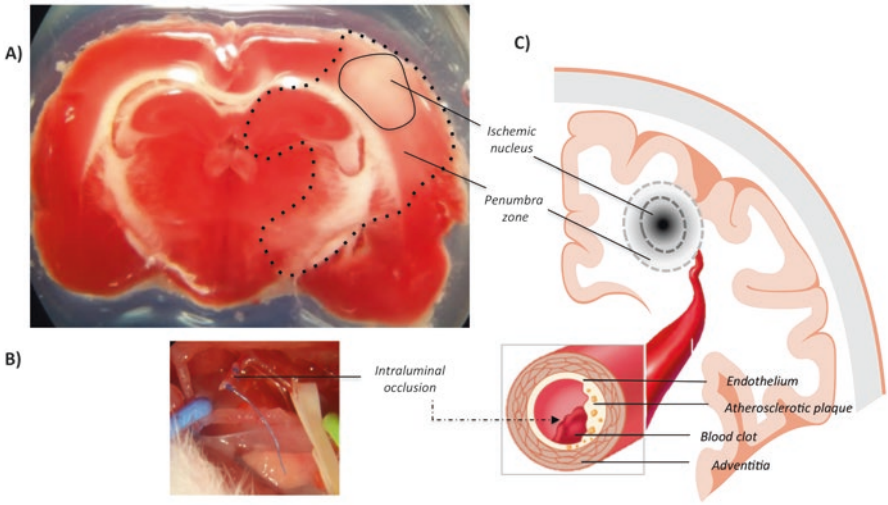

Fig. 13.1 Stroke after focal ischemia. This is mainly divided into the ischemic nucleus and the penumbra zone (**a, c**). (**a**) Infarct area produced by (**b**) intraluminal occlusion of middle cerebral artery during 60 minutes using a 4.0 nylon monofilament recovered with poly-L-lisine and 24 hours of survival in a tMCAO in vivo model in rats. (**c**) Stroke scheme, which usually occurs due to the formation of an atherosclerotic plaque or a blood clot at the level of the middle cerebral artery in humans. -.-.- analogue situation

(<10% of baseline) and irreversible damage of the energy metabolism. The remaining hypoperfused tissue surrounding the ischemic nucleus has an imbalance in the mechanisms of self-regulation of blood flow and is known as the *penumbra zone* [7] (Fig. 13.1). In this region, neurovascular unit show dysfunction, retaining a minimal metabolic activity and altered structural integrity following a pattern of cellular uncoupling and apoptotic death [8–10]. The penumbra is potentially recoverable and represents a key zone for therapeutic intervention in cerebral ischemia. This critical period of viability that this volume of brain tissue is at risk, is known as the "window of opportunity", since the deficit created by ischemia can be partially or completely reversed within a critical period of 2–4.5 h. However, unless perfusion improves, cells in the penumbra zone are at risk of dying within a few hours by necrosis [11].

At the molecular level, the ischemic neuronal injury development is largely due to an increase in the release of excitatory neurotransmitters into the synaptic space, mainly glutamate. This process called "excitotoxicity" is a canonical response as a consequence of ion pump failure and energy deficit, as well as the neurotransmitter uptake mechanism insufficiency by astrocytic cells [12]. So, the glutamate overload leads to prolonged stimulation of ionotropic glutamate receptors of AMP (α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid) and NMDA (N-methyl-D-aspartic acid) receptors, increasing drastically the calcium (Ca^{2+}), sodium (Na^{+}), potassium

(K^+) and water influx in neurons. Excessive ion accumulation and simultaneous deregulation of several signaling pathways mediated by proteases, lipases, and nucleases, alter neuronal function and lead to cell death [13].

2 Short-Term and Long-Term Disabilities After Cerebral Ischemia

The total physical disability in the survivors of a cerebral infarction is simultaneous or secondary to the disease, temporarily or permanently, causes damages in the activities of the daily life, like feeding, dressing, care of personal hygiene, use of transport, among others [14]. However, cognitive impairment following ischemic event is almost always ignored in patients, due precisely to the severity of the physical disability that most of them present. These patients have a degree of repercussion that can be divided into mild cognitive impairment and dementia [15]. According to Nys et al., a high survivors proportion of cerebral infarction have cognitive impairment after 3 months and sometimes dementia is only detected after several years of injury [16]. The prevalence of cognitive impairment ranges from 20 to 80% is defined according to each country's case series, diagnostic criteria and post-injury time [15] although post-ischemia cognitive impairment is underestimated in survivors yet. According to WHO, latinamerican people with dementia will increase by 368% in 2050, higher than USA and Europe [17–19]; because sedentary lifestyle and a high fats and sugars intake.

There is evidence to suggest that hippocampal deterioration is associated with post-ischemia dementia. A study by Szabo et al. suggests that lesion in the hippocampus could lead to persistent memory deterioration, considered to be the usual consequence of occlusion of the posterior cerebral artery [20]. In recent studies, Gemmell et al., when analyzing hippocampal volume in postmortem samples of patients with dementia, suggested that altered brain volume was between 10 and 20% in the CA1 and CA2 regions and 20% in the CA3 and CA4 regions of the hippocampus [21, 22]. However, the mechanism of post-ischemia hippocampal injury related to cognitive impairment is still uncertain. Some studies, including ours, have shown that Tau hyperphosphorylation, neurodegeneration hallmark, is closely correlated with cognitive disorder and dementia after cerebral ischemia [23–25].

So far there is no effective treatment to treat cognitive impairment post-ischemia. Some drugs used for the treatment of dementia in Alzheimer's disease have shown some positive effects on cognitive impairment post-ischemia [26]: for example, anti-inflammatory agents, blood-brain barrier modulators, endothelin antagonist, flavonoids, immunosuppressive agents, antidepressants, neurotrophic agents, among others. However, although none of them has been established as an effective treatment, somes strategies have been defined for prevention of cognitive impairment, which focus on acute treatment (to limit damage from the onset of injury and prevent early recurrence) and the prevention of long-term recurrence, but does not exist a standard gold for post-ischemia treatment yet.

3 Neuroprotective Therapies in Cerebral Ischemia: Translational Medicine Perspective

Over the past two decades, neuroprotective agents designed to block cell death have been investigated in animal models of cerebral ischemia. Numerous drugs have been found that reduce the size of infarction in ischemia models in rodents, rabbits and primates. However, neuroprotection as a strategy for the treatment of stroke, which have been effective in experimental models have failed in phase III clinical trials [27]. Currently, the only approved therapy for cerebral infarction is limited to the treatment with recombinant tissue plasminogen activator (rt-PA), which although improving functional prognosis, is used in less than 3% of patients [28].

Several difficulties have been identified in establishing effective therapy that limits the devastating consequences of reduced blood flow in the penumbra zone and prevents secondary insults induced by reperfusion [8]. For example, the focus on a single event of the ischemic cascade may not be sufficient to decrease the consequences of a multifactorial condition such as cerebral ischemia [29]. In this context, a treatment for one specific target over time under a controlled physiological condition may be ineffective. However, if we used stem events in the ischemia/reperfusion phenomenon, such as glutamate excitoxicity and avoided its propagation wave by local neural circuits, we could block several associated downstream pathophysiological phenomena, such as the activation of calpains, caspases, enzymatic activity, substrate dysregulation, activation of death pathways, depolymerization of the microtubule and actin cytoskeleton, among others.

For example, the use of statins has shown beneficial effects following an ischemic event [9, 30]. Statins, competitive inhibitors of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase, are cholesterol-lowering drugs and with multiple pleiotropic effects in cerebral ischemia, such as enhancement of endothelial function, have potential anti-oxidant, anti-thrombotic and anti-inflammatory, among others [6]. Our studies have shown that post-ischemic statin therapy recovers from neurological deficit, memory deficit and learning, as well as active survival pathways and synaptic plasticity associated with neuronal recovery [31]. These drugs also regulated NMDA-like glutamate receptors, which play a crucial role in glutamate excitotoxicity, as a pathogenic primary event. Also, another alternative has been proposed, where the combination of two or more potential neuroprotective agents, each one against different targets respect to the neurodegenerative cascade, achieve synergistic protective action on ischemic injury, such as statins and rt-PA [32]. However, minimum adverse reactions should be demonstrated in combinated therapies [27].

Another, large discrepancy between the results obtained in the laboratory and the clinical trials makes difficult to translate many therapies, because is not considered the morbidity condition of the patients [27]. For example, in stroke models, researchers often choose young and healthy animals; however, patients with cerebral infarction are often elderly and suffer multiple chronic diseases, such as arteriosclerosis, hypertension, diabetes and hyperlipidemia [33]. In addition, the most studies in

ischemia models have been performed at short term, based on the fact that treatments established in the first few hours of symptom onset are more likely to become effective therapeutics. However, many agents considered effective in acute phase are not effective in the sub-acute or chronic phase. Therefore, long-term protection studies and to follow-up the health progress of the treated patients for longer time is completely necessary for post-ischemic stroke patient care protocols and to propose a post-ischemia gold standard treatment.

4 Gene Therapy as Treatment for Neurodegeneration

Gene therapy is an alternative treatment to pharmacological, surgical and conventional approaches that are being developed both experimentally and clinically. Basically, it consists of the specific modification of a gene to prevent or remedy a pathological condition for the organism. This technique is based on interfering, correcting or replacing the defective gene within cells expressing the pathology by the normal gene and its correct functional protein to slow down, stabilize or reverse the course of the disease [34].

Depending on the type of disorder, gene therapy is performed through one of the following approaches [35]: Gene addition: consists of introducing a correct copy of the functional gene so that the corresponding protein in the tissue is produced in adequate quantities to be treated. It is the most used procedure. Gene Correction: we seek to correct the altered gene by replacing it with the correct gene by homologous recombination, although for the moment this method is inefficient. Instead, it is possible to exchange a specific mutated nucleotide, which is useful for treating diseases with point mutations. Gene suppression: consists in reducing the expression of a particular gene. This is achieved using RNA interference (RNAi), including short hairpin RNAs (shRNAs), that induce the degradation or silencing of target messenger RNA (mRNA) [36].

In the framework of therapy based on gene interference, shRNA administration has been tested in experimental models and clinical trials using various vehicles including viral vectors, liposomes and nanoparticles [37]. Viral vectors have a greater efficiency in DNA-RNA transfer, but are more likely to generate an undesired immune response in the patient. Integration of the gene of interest into the vector requires removal of the genes conferring its virulence by incorporating in its place the desired sequence. Among the viral vectors, the most used are retroviruses, adenoviruses, lentiviruses, herpes simplex virus and adeno-associated viruses [38, 39]. The latter are those with less inflammatory response. Non-viral vectors are less effective in transference, but safer and simpler to elaborate, characterize and manipulate [36].

Neurodegenerative diseases have been one of most evaluated in gene therapy, after cancer and genetic and metabolic diseases. Over the past decade, scientists have made great strides in evaluating shRNA-based therapies for diseases affecting the central nervous system (CNS) [37].

For a number of neurodegenerative disorders (e.g., Alzheimer's disease (AD), Parkinson's disease (PD) and post-stroke infarction) abnormal accumulation of proteins seems to play a central role in the onset of disease and/or in progression [40]. Thus, a modest reduction in neurotoxic protein levels is expected to provide significant therapeutic relief. Using a shRNA-based approach, researchers have successfully inhibited the expression of disease-causing proteins in animal models, where in most cases this inhibition correlates with improved neuropathological and behavioral phenotypes. For example, AD is the leading cause of dementia worldwide and is characterized by the presence of amyloid plaques and neurofibrillary tangles (NFTs) in the brain. In our previous research the silencing of CKD5, an enzyme responsible of tau hyperphosphorylation in dementia, reduced the NFTs production in a triple transgenic AD mice model [41]. Also, in PD the second most common neurodegenerative disease and histopathologically characterized by the formation of Lewy bodies in the brain, composed mainly of alpha-synuclein (α-syn) protein [42]. A single mutation in α-syn as well as genetic duplication or triplication of the gene are linked to hereditary parkinsonism, which was blocked using shRNA in PD model in rats [43].

4.1 Preclinical Studies of Gene Therapy in Cerebral Infarction

shRNA is shown as a potential therapeutic tool in animal models of acute cerebral injuries [44] with satisfactory results (Table 13.1). Initially, researchers focused on neuroprotective strategies to limit the spread of apoptosis. For example, shRNAs against Beclin1, a protein responsible for cellular autophagy and apoptosis, against caspase-3 and for kinase regulator of apoptotic kinase 1 (ASK1) showed decreased infarct volume and improved neurological outcomes respectively [45–47]. In an effort to target vascular compartments was reduced protease-1 activated receptor (PAR-1) in a mouse t-MCAO model. PAR-1 is involved in blood clotting and serves as another viable option for thrombolytic strategy along with rt-PA. shRNA against PAR-1 7 days before the injury resulted in a decrease in infarct volume and lower neurological deficit at 24 and 72 h post-injury [48]. However, it is important to emphasize in those studies, shRNA administration occurred before the ischemic event, which limits clinical translational approaches. But, the hypoxia-inducible factor 1 (HIF-1), implicated in neurovascular unit dysfunction, was decreased by shRNA one hour after t-MCAO in rats, avoiding blood-brain barrier disruption and improving behavior skills, in addition to a lower expression of the p53 protein and less activity of caspase-3 [49]. This study brings high hopes in the field of translational therapy, because shRNA was administered after ischemic injury and the results were satisfactory.

Another important mechanism of injury is neuroinflammation. Previous studies have shown the G protein coupled to receptor 17 (GPR17) is involving in neuroinflammation; particularly, GPR17 inhibited by an antisense oligonucleotide showed beneficial effects after ischemic stroke [50]. Zhao et al. specifically examined the

Table 13.1 *In vivo* studies using shRNA in Cerebral ischemia Models

Therapeutic target	Patho-physiological event	Ischemia model	shRNA administration method	Intervention time	Findings
Beclin 1 [45]	Apoptosis and autophagy	t-MCAO in rats	Intraventricular injection	7 days before	Reduction of infarct volume and improvement in functional tests
Caspase 3 [46]	Apoptosis	Entotelin-1 injection in rats	Cortical injection	24 h before 24 h after	Reduced behavioral deficit (significantly 24 h before ischemia)
Ask1 [47]	Apoptosis	t-MCAO in mice	Intraventricular osmotic mini-pump	3 days	Reduction of infarct volume
PARK1 [48]	Coagulation cascade	t-MCAO in mice	Intraventricular injection	7 days before	Reduction of infarct volume and neurological deficit
HIF1 α [49]	Hypoxia	t-MCAO in rats	Intraparenchymal injection	<1 h after	Reduction in mortality, neurological deficit, blood-brain barrier injury, and cell death
HMGB1 [50]	Neuro-inflammation	t-MCAO in rats	Intranasal	1 h before	Reduction of infarct volume and behavioral improvement
GPR17 [51]	Microgliosis neuro-inflammation	t-MCAO in rats	Intraventricular injection	2 days before until 7 days after	Reduction in neuronal loss, neurological deficit, and infarct volume

effect of shRNA against GPR17 on microglial activation in the acute and chronic stages, finding a microgliosis reduction after 14 days, but not 24 h after injury [51]. Another important protein during the post-ischemic neuroinflammatory phase is the high mobility group Box 1 protein (HMGB1), secreted by necrotic cells for the recruitment of pro-inflammatory cells [52]. An intracortical injection of shRNA against HMGB1 was found to have a neuroprotective effect after ischemic stroke through reduction of microglial activation and neuronal apoptosis [53]. In subsequent experiments, intranasal administration of this shRNA in a rat model of t-MCAO resulted in a significant reduction of HMGB1 in various regions of the brain but not in the liver, lung, kidney, or heart. In addition, this effective reduction

correlated with improvements in behavioral tests [54]. It is important to highlight that intranasal administration is one of the most promising tools for a clinical translational perspective, not only for the delivery of shRNA in the brain, but also for the delivery of other types of drugs [55, 56].

4.2 CDK5 as a Potential Therapeutic Target for Cerebral Ischemia

Cyclin-5 kinase (CDK5) is a serine/threonine kinase, a member of the cyclin-dependent kinase family. Similar to other members of this kinase group, CDK5 binds to p35 and p39 regulatory subunits to be activated, similar to cyclins [57]. However, CDK5 is not directly involved in cell cycle progression and its activity predominates in postmitotic neurons [58], since their p35 and p39 activators are expressed mainly in the central nervous system (CNS) [59]. However, although CDK5 activity is vital for the proper development of the CNS, and synaptic plasticity [60], but its deregulation has been shown to play a critical role in the chronic neurodegenerative events of several diseases, including Alzheimer's disease (AD), Parkinson's disease (PD) and Amyotrophic lateral sclerosis (ALS).

4.2.1 CDK5 at the Synapse: Functions and Dysfunctions

CDK5 plays an important role in a variety of physiological and pathological processes. This multifunctionality includes participation in the development of the nervous system, synaptic plasticity and neurodegeneration [57]. CNS requires programmed migration, differentiation and neurons connection to form functional circuits capable of expressing synaptic plasticity. Studies in several lines of mutant mice have shown that CDK5 is critical for all these stages of CNS development [60]. Phenotypically null mutant mice for CDK5, as well as double mutants for p35/p39 exhibit an alteration of cortical laminar architecture, as well as cytoarchitecture disorders in the cerebellum, brainstem and hippocampus [61].

Molecular mechanisms of synaptic plasticity occur at the pre and post-synaptic levels and involve the regulation of vesicle release, changes in the conductance of ion channels and modulation of kinase and phosphatase activities [62]. Various studies have shown that CDK5 has an important role in synaptic plasticity directing phosphorylation of key substrates for synaptic plasticity, e.g. ionotropic glutamate receptors, cell adhesion proteins, and cytoskeletal proteins [57, 63]. Within the cytoskeleton proteins that are a direct substrate of CDK5, is the Tau protein, which participates in the assembly and disassembly of microtubules through the dynamic incorporation of tubulin monomers to form neuronal axons; which contribute to the maintenance of cellular form and transport. Tau also establishes

links between microtubules and other cytoskeletal elements, such as neurofilaments, spectrin and actin filaments. In the normal brain, the balance between phosphorylation as well as the dephosphorylation of tau by CDK5, other kinases and phosphatases, leads to structural and conformational changes that regulate cytoskeletal stability and axonal morphology [64]. Another of the great functions of CDK5 is in learning and memory [60]. A positive role of CDK5 was identified in $p35^{-/-}$ mice, which show depotentiation, LTP reduction and defective induction of long-term depression (LTD), electrophysiological mechanisms crucial for memory formation [65]. Similarly, $Cdk5^{flox/flox}$ T29 mice in which there is no CDK5 expression in hippocampal CA1 pyramidal neurons, revealed memory impairment and synaptic plasticity [66]. However, Hawasli et al. showed that the initial suppression of 50% of CDK5 protein in a mouse knockout conditional model enhances LTP and NMDAR-mediated synaptic plasticity and improves learning and memory skills [67].

In spite of the functions that CDK5 fulfills in plasticity, neuronal development and memory, its over-activation triggers neurodegenerative events ranging from cell death, microtubule destabilization, alteration of the actin cytoskeleton, loss of cellular adhesion and alterations of memory and learning. Deregulation of CDK5 activity begins with the cleavage of its p35 and p39 activators through the calpain, which are a group of cytosolic proteases activated by intracellular calcium [68]. After an ischemic event, the overactivation of NMDA and AMPA-type glutamate receptors leads to an increase of intracellular calcium, activating the calpain, which cleaves the p35 and p39 activators, generating p25 and p29 respectively (Fig. 13.2). The half-life of p25 and p29 is significantly longer (about threefold greater than p35 and p39), as well as the binding to the kinase of these activators is much stronger than p35 and p39 binding [69, 70], which results in higher p25/CDK5 (or p29) activity, compared to p35/CDK5 (or p39). On another hand, p25 and p29 lack an amino-terminal myristoylation site, a necessary modification to maintain the protein at the plasma membrane [71]. Thus, the interaction of p25 with CDK5 not only leads to an activation of the kinase in a sustained manner, but also modifies its cellular distribution, concentrating on the cytoplasm and nucleus, altering its substrate specificity and triggering cell death [71–73]. Also, CDK5 expression as well as p35 cleavage has been found to increase in penumbra region after middle cerebral artery occlusion (MCAO) in rats [74] and an increase in expression of CDK5 and cleaved fragment p25 has been associated with neuronal damage in brains of patients affected by cerebral ischemia [75].

Additionally, *in vivo* models indicate that the sustained increase of CDK5 activity in Alzheimer's disease correlates with aberrant tau hyperphosphorylation, forming aggregates of this protein in the cell body [41] and consequently induce cellular death and learning and memory decline [63]. Mice overexpressing long-term p25 levels have been shown to undergo LTP impairment in the hippocampus and memory deficits along with significant neuronal loss. However, when p25 is expressed transiently, LTP is enhanced, the number of dendritic spines and synapses increases, without observing neurodegeneration [76].

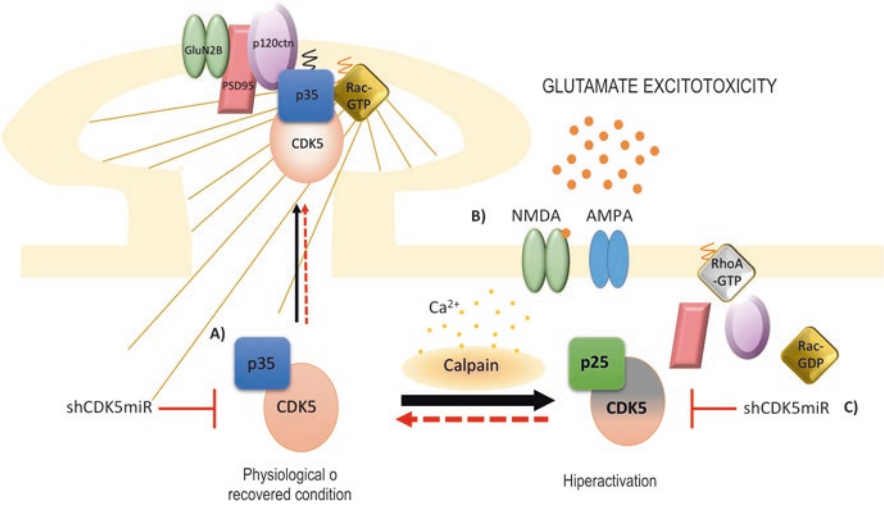

Fig. 13.2 CDK5 regulation in mature neurons. (**a**) CDK5 alone is an inactive catalytic subunit. This kinase is activated by p35 and then recruited to the membrane through myristoylation of its N-terminal region. p35 is a protein with a short life span and is degraded by the proteosome. (**b**) When a glutamate excitotoxicity condition occurs as in cerebral ischemia, calpain is activated by increasing intracellular calcium concentrations and cuts to p35 on a C-terminal fragment generating p25 fragment. p25 has a longer half-life and dissociates from membranes, where it is able to sustained CDK5 hyperactivation. (**c**) However, when is reduced the CDK5 overactivation by shCDK5miR in adult brain or mature neurons, p35 may to be associated to p120ctn, PSD95 and NR2B subunit in a Rac1-active dependent mode in the cell membrane and promote or strengthen the synapses

4.2.2 Strategies to Control the Over-Activation of CDK5

Because of the importance of CDK5 in neurodegeneration, it is presumed that this kinase is a good therapeutic target to prevent or even stop pathologies associated with Tau hyperphosphorylation, as we demonstrated in an aged triple transgenic Alzheimer's disease mice model already [25, 41]. In fact, various *in vitro* and *in vivo* studies have shown that blocking over-activity of CDK5 may have a beneficial effect and generates neuroprotection. Two main strategies have been used for these purposes: direct inhibition with the use of CDK5 inhibitors and indirect action by preventing the excessive generation of the associated p25 activator through the use of calpain inhibitors [57]. So, roscovitine, a pharmacological inhibitor of CDK5, exerts a neuroprotective effect *in vivo*, following systemic pre and post-ischemia administration in experimental models of stroke [77]. Roscovitine acts on different cell types (neurons and glial cells) and through various mechanisms: anti-apoptotic, anti-excitotoxicity and possibly anti-inflammatory pathways. Thus, even when roscovitine is administered 2 h post-ischemia reduces the volume of infarction in rats subjected to transient focal ischemia [57]. On another hand, the best-known

calpain inhibitor, MDL 28170, causes reduction in infarct volume when was gave 30 min after occlusion of the middle cerebral artery. In addition, in this same study, several therapeutic windows were evaluated to determine the maximum delay between the onset of ischemia and the efficacy of the therapy, finding that MDL 28170 reduced the infarct volume after 0.5, 3, 4 and 6 h onset of ischemia, but not after 8-h delayed [78]. However, the main difficulties of the pharmacological inhibitors is the lack of specificity or off-targeting [79, 80].

On another side, a peptide was designed to inhibit CDK5, which protected in various *in vivo* models of neurodegeneration, such as Alzheimer [81], Parkinson [82] and cerebral infarction [83]. This peptide of 24 amino acids, can cross the blood-brain barrier, *in vitro* inhibit the activity of p35/CDK5 and p25/CDK5, whereas in rodent cortical neurons, inhibits p25/CDK5 without affecting the endogenous p35/CDK5 activity. In a model of cerebral ischemia, a p5-TAT peptide does not alter the levels of p35, p39, although it reduces the phosphorylation of Tau through the inhibition of p25/CDK5 activity. In addition, p5-TAT reduces levels of caspase-3 and the cerebral infarction volume; even when this was administered up to 24 h after ischemic injury promote long-term functional recovery [83]. Also, in our investigations, using a CDK5 shRNAmiR carried on adeno associated viral vectors (AAV) injected into the hippocampus of a triple transgenic mice for Alzheimer's disease, reduced tau hyperphosphorylation and amyloid beta plaques, improved learning and spatial memory to short and long-term of treatment [25, 41, 84], analog effects were obtained in post-ischemic treatment in rats [85, 86], whose studies will be detailed below.

5 Silencing of CDK5 as Post-ischemia Therapy

The above background shows that the reduction of CDK5 may be a key tool in reversing damage following cerebral ischemia. This is based on the fact that blocking CDK5 can prevent several pathological events since first hours to even months post-injury. For example, neuronal death after cerebral ischemia occurs as an early event of pathophysiological cascade, which is primarily associated with p25-mediated hyperactivation of CDK5. Because, the silencing of CDK5 may be playing a critical role at early times regulating the rate of degradation and internalization of NMDA receptors, which are essential for the excitotoxic pathway that occurs after ischemic damage, avoiding the consequent increase of calcium influx in cell, calpain and cell death pathways activation, because GluN2B (subunit of the NMDA receptor), calcium ionotropic receptor, is activated by CDK5, and its phosphorylation rate determines the dynamics of receptor internalization [87].

However, we have suggest that the silencing of CDK5 to long term post-ischemia represents an advantage to the control of CDK5 over-activation because of its high specificity and the long-term expression in the tissue preventing pathophysiological events after several post-ischemia months.

The CDK5 silencing strategy is based on the use of an endogenous microRNA (miR30) that carries CDK5 (exogenous) shRNA, which is transduced into the cells using an AAV 2.5 adeno-associated viral vector. This strategy has several advantage points because the miR-30 skeleton confers low toxicity, high processing and expression in the cell compared to the use of only the CDK5 shRNA [36]. In addition, the use of AAV, specifically serotype 2.5, confers: (1) tropism to neural cells, astrocytes and neurons; (2) decreased inflammatory response or interferon response, (3) low insertion of exogenous DNA into the host cell DNA, which decreases the probability of a tumor focus, (4) stable expression of CDK5 RNAi up to 1 year in mice and (5) ease of expression monitoring with the enhanced green fluorescent protein reporter [25, 41, 63].

Those is based in our recent research which show that the gene therapy directed to silence CDK5 in the hippocampus of ischemic rats by the middle cerebral artery occlusion (tMCAO), did not generate changes in the physiological parameters (blood pressure, pH, pO_2, pCO_2), decreased CDK5 protein levels resulting in a neurological and motor improvement during the first week after the ischemia. It also CDK5 shRNAmiR prevented dysfunctions in learning, memory and reversal learning at 1 month [85]. This effect was maintained at 4 months, preventing neuronal loss, tauopathy, microglial hyperreactivity and generating branched astrocytes [86]. CDK5 silencing increased the expression of brain-derived neurotrophic factor (BDNF) and activated the TRKB/CREB/CaMKII pathway in the hippocampus, involving calcium modulation in the spines and induction of LTP in a TRKB receptor-dependent mode in neurons [86, 88]. Also, CDK5 RNAi protected against glutamate-mediated excitotoxicity (major excitatory neurotransmitter and uncontrollably upregulated in a cerebral stroke) in primary neuronal cultures. This protection was dependent on a concomitant increase of p35, since it was blocked by the use of an RNAi against p35, which affected downstream activity of the RhoGTPases (proteins responsible of cytoskeletal remodeling and dendritic spine formation). In addition, overexpression of p35 and constitutively active Rac1 mimicked the neuroprotection exerted by the silencing of CDK5 [63]. Also, another synaptic proteins also participated in neuroprotection and synaptic plasticity induced by the CDK5 shRNAmiR, such as: PSD95, NR2B, p120ctn, N-cadherin and β-catenin. Inhibition or knockdown of CDK5 resulted in the increase of p120ctn and the neuroprotection induced on depended the expression of p120ctn in a model of glutamate-induced excitotoxicity. Thus the p35/p120ctn/PSD95/NR2B complex is involved in the synaptic recovery induced by the silencing of CDK5 [89] (Fig. 13.2).

On another hand, astrocytes perform metabolic and structural support functions in the brain and contribute to the integrity of the blood-brain barrier. Astrocytes influence neuronal survival and prevent gliotoxicity by uptaking glutamate (Glu), reactive oxygen species and nutrients. CDK5 may have a double effect on the endothelium and astrocytes as it is involved in migration, senescence and angiogenesis, and its hyperactivity is associated with dysfunction of glutamate recapture and hypoxia. Therefore, treating a possible deregulation of CDK5 with RNAi is other strategy that we have proposed as a treatment for neurodegeneration. In the C6 cell

line astroglioma and primary astrocytes, CDK5 RNAi prevented the activation of cells generated by glutamate-induced gliotoxicity, and this finding was corroborated by CDK5 pharmacological inhibition with roscovitine (Rosc). This effect was associated with the occurrence of lamellipodia, and ramifications, the activation of Rac1, the release of BDNF by astrocytes, which correlated with the protection of neurons exposed to glutamate excitotoxicity. Interestingly, inhibition of Rac1 in astrocytes blocked the release of BDNF and astrocyte-mediated neuroprotection [90]. Thus, the remodeling of the actin cytoskeleton in the hyperreactive astrocytes suggests a functional phenotype for the release of BDNF, which promotes neuroprotection.

Complementarily, in the bEnd.3 cell line from mice cerebral microvasculature, Rosc was found to recover endothelial adhesion altered by glutamate toxicity; this effect was corroborated by the increase of TEER (transendothelial resistance), p120ctn levels in membrane and decreased intercellular gaps. In addition, CDK5 RNAi increased the primary processes in bEnd.3 cells. Also, endothelial cells that were co-cultured with CDK5-knock down (KD) astrocytes showed a recovery in cell viability, an increased adhesion proteins (p120ctn and PECAM-1) and BDNF release. These findings suggest that inhibition of CDK5 or its silencing in astrocytes protects the endothelium, which promotes the BDNF release and endothelial adhesion (Posada-Duque et al. unpublished data). Taking together these findings suggests CDK5-knockdown astrocytes as a cell-gene therapy to favor the protection of neurovascular unit. Therefore, we transplanted astrocytes-CDK5 KD in the somatosensory cortex after cerebral ischemia in rats. In these experiments we obtained a general prevention of neuronal loss by transplantation of CDK5-KD astrocytes, which induced a significant stimulation in the arborization of endogenous astrocytes, involving blood vessels, accompanied by increased immunoreactivity of PECAM-1 in the motor and somatosensory areas, as well as an increased Ki67 immunostaining (proliferation marker) in the lateral ventricles, partially associated with BDNF production, which suggest us also morphological and physiological protection benefits by cell therapy base in CDK5-KD astrocyte [91], stable to 4 months post-ischemia with side effects (Becerra-Calixto et al. unpublished data).

Overall, silencing of CDK5 protects neurons, astrocytes and endothelium from adult brain suggesting neurovascular unit has a critical role in the functional and morphological recovery after a cerebral ischemia and [10, 92]. In addition, our results suggest that CDK5-KD astrocytes are a paracrine source of BDNF production, which generates neuronal and endovascular protection that would be a novel strategy to protect BBB integrity after stroke.

Therefore, CDK5 silencing could be an ideal gene therapy strategy because block stem events by the ischemia/reperfusion phenomenon, as glutamate excitoxicity associated to downstream pathophysiological phenomena, decreased calpain, sustained enzymatic activation, prevented the tau hyperphosphorylation and cell death (Fig. 13.3), generating protection of synapses, short and long term morphological and physiological recovery in the ischemic brain.

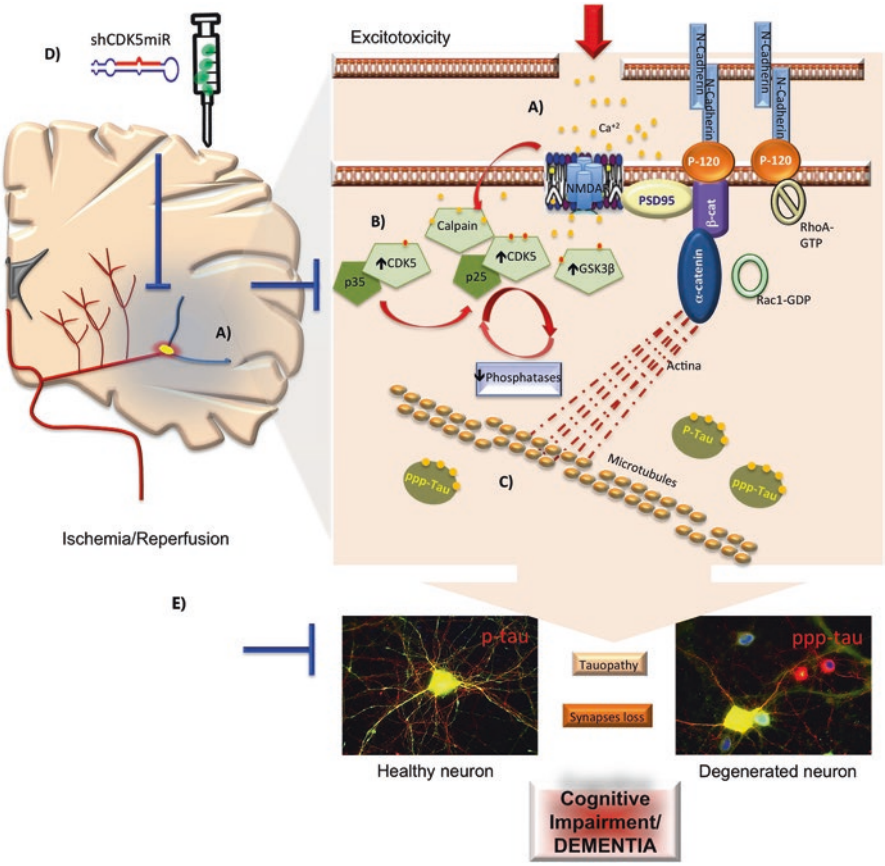

Fig. 13.3 Hypothetic model of post-ischemia gene therapy based on the silencing of CDK5 blocking excitotoxicity spreading and preventing cognitive impairment. (**a**) Anoxia / Reperfusion, and excitoxicity spreading, (**b**) Activation of CDK5, disbalance kinases / phosphatases, tau hyperphosphorylation, (**c**) Actin and microtubule cytoskeleton disassembly, loss of synapses, cognitive impairment and dementia. (**d**) Gene therapy using CDK5 RNAi blocks **a–c** events. (**e**) Recovering synapses and cognitive function. pTau: Tau phosphorylation, pppTau: tau hyperphosphorylation

6 Current and Future Challenges of Gene Therapy in Ischemic Stroke for Translational Medicine

Therefore, despite identifying potential molecular targets, including our own studies, there are considerable limitations inherent in the nervous system, such as crossing the blood-brain barrier and the difficulty of targeting specific neuronal populations. The presence of these obstacles has led to the search for new strategies for the treatment of neurodegenerative diseases, the use of viral vectors, the design of nanoparticles to improve the distribution, and future approaches for systemic

administration or functionalised nutrition, including safety and effectiveness studies, which together represent a current challenge.

An important limitation of gene therapy studies in cerebral infarction is translation to patient. There are two crucial aspects bringing results from preclinical studies to patient: one is therapeutical intervention time and the second one is shRNA administration route to the infarcted area [44]. Some studies report diverse system administration as internal carotid artery [45, 47], injection in the tail with wide distribution in the mice body [93] and by intranasal method without significant side effects [54, 94–97]. However, pharmacokinetic studies are necessaries and will available to provide assurances of the effective delivery of shRNA in the brain [98].

On another hand, inherent difficulties of central nervous system procedures, translational medicine from the discovery of useful drugs for the clinic needs to overcome several limitations as sensitivity and specificity of drugs, and patients must be classified with clear inclusion criteria [99]. Promoting the translation of therapeutic alternatives that impact morbidity and mortality is an urgent need worldwide and in Latin America. Specifically in Colombia, we need to take a step forward in translational medicine policies, although our developing countries need to first improve emergency medical care for acute injuries [100]. We also need to update norms and protocols to develop clinical trials with original candidates or strategies proposed by our Latin American countries, and gather efforts of scientists, medical specialists, pharmaceutical industry and government, accompanied by economic and social support. We must offer experimental treatments, without false expectations to the patient/family, based on the rigor of preclinical scientific evidence, in direct dialogue with the medical team, peer monitoring of phases I, II and III, and strict long-term monitoring of potential unwanted side effects [101], which could progressively strengthen the contribution to solving mental health problems [19].

7 Conclusion and Perspective

Cerebral ischemia has become a health problem worldwide due to its high mortality rate and disability in patients suffering from it. Many therapeutic strategies have been evaluated both in experimental models and in clinical trials without obtaining good results. One of the major shortcomings of these therapeutic investigations is the limitation in intervention time (less than 4.5 h) and the short time of protection or follow-up of therapy, which generates long-term sequelae in patients.

Gene therapy has been shown to be a very useful tool for the treatment of neurodegenerative diseases. However, specifically in cerebral ischemia, there are few studies at the experimental level that show neuroprotective effect, reduction in infarct volume and functional improvement, since reference is made to a pretreatment which does not facilitate an approach to the clinical problem. Therefore, a major challenge of gene therapy is that it to be implemented as post-injury therapy, which is supported by our results, as this situation recreates the problem at the clinical level and the experimental results are more relevant in the field of translational medicine.

The silencing of CDK5 has become a highly important therapeutic strategy to reduce cell death in several neurodegenerative diseases, mainly in those where the tauopathy as main hallmark of cognitive deterioration and dementia, as in cerebral ischemia (Fig. 13.3). However, although pharmacological inhibitors have been tried for this kinase, it is necessary to use methodologies that allow the directed and efficient reduction of CDK5, avoiding side effects generated by nonspecific pharmacological blocks towards other proteins and signaling pathways. It is here that gene therapy becomes very important, being a tool aimed at controlling the kinase overactivation, not only in times immediate to the ischemic lesion where cell death responses are given, but, to the long-term, where events such as the loss of spines and dendrites are key pieces in the alteration of function and appearance of delayed post-ischemia sequelae, which can be avoided. However, it is necessary to align the scientific interests of the government and the company to support the development of therapies proposed from our own country and that would help to avoid or improve the quality of life of patients with physical and mental disability post-cerebral stroke.

Acknowledgement We are grateful to the Cellular and Molecular Neurobiology Area, Antioquia Neuroscience Group (GNA), University of Antioquia for constructive academic discussion. This research was supported by projects of Colciencias (code # 111551928905, # 111554531400) (GPC-G) and CODI # 2014-970 of the University of Antioquia, Republic Bank # 3494.

Statement of Conflict of Interest: The authors declare no conflict of interest.

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Chapter 14
SB623 Preclinical and Clinical Trial Experience

Eric S. Sussman and Gary K. Steinberg

Abstract Stroke affects more than 15 million individuals each year, and is the second leading cause of mortality worldwide [1]. In the United States alone, there are nearly 800,000 strokes annually, and over seven million individuals (approximately 3% of the adult population) live with the sequelae of a prior stroke [2]. More than half of these individuals suffer from long-term limitation of functional mobility [3]. From an economic perspective, the direct and indirect costs related to stroke are as high as $65 billion per year in the United States [4].

Keywords Stroke • Neuroregeneration • Cell-based therapy • Stem cells • Mesenchymal stem cells • SB623 cells

Abbreviations

AE	Adverse event
BDNF	Brain-derived neurotrophic factor
bFGF	Basic fibroblast growth factor
DWI	Diffusion-weighted imaging
ECM	Extracellular matrix
EGF	Epidermal growth factor
ESC	Embryonic stem cells
ESS	European Stroke Scale
FDA	Food and Drug Administration
FMA	Fugl-Meyer Assessment
GDNF	Glial cell line-derived neurotrophic factor
IGF-1	Insulin-like growth factor 1
MRI	Magnetic resonance imaging

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P.A. Lapchak, J.H. Zhang (eds.), *Cellular and Molecular Approaches to Regeneration and Repair*, Springer Series in Translational Stroke Research,
https://doi.org/10.1007/978-3-319-66679-2_14

mRS	Modified Rankin Scale
MSC	Mesenchymal stem cells
NIHSS	National Institute of Health Stroke Scale
NSC	Neural stem cells
RCT	Randomized controlled trial MCA, middle cerebral artery
T2 FLAIR	T2-weighted-fluid-attenuated inversion recovery
TEAE	Treatment emergent adverse event
VEGF	Vascular endothelial growth factor

1 Introduction

Stroke affects more than 15 million individuals each year, and is the second leading cause of mortality worldwide [1]. In the United States alone, there are nearly 800,000 strokes annually, and over seven million individuals (approximately 3% of the adult population) live with the sequelae of a prior stroke [2]. More than half of these individuals suffer from long-term limitation of functional mobility [3]. From an economic perspective, the direct and indirect costs related to stroke are as high as $65 billion per year in the United States [4].

Current management of acute stroke is focused on early recognition and attempted rapid restoration of cerebral perfusion. This has traditionally been accomplished with intravenous or intra-arterial thrombolytic therapy [5]. Recently, endovascular thrombectomy has become a new standard of care in a carefully selected subgroup of patients [6, 7]. Despite these recent advances, the vast majority of stroke patients are not eligible for acute stroke interventions due to factors such as delayed presentation to a healthcare setting, lack of access, or presence of exclusion criteria. Beyond the acute period, physical rehabilitation is the cornerstone of stroke management however the benefits of physical rehabilitation taper over time, and existing functional deficits typically plateau by 6 months after stroke onset [8, 9]. At the present time, there are no FDA-approved neuro-restorative treatments for chronic stroke.

Cell-based therapy is a promising neuro-restorative option for chronic neurologic disability associated with stroke. Preclinical and early clinical studies have reported improvements in functional outcome in subjects treated with experimental stem cell-based therapies.

2 Cell-Based Therapies for Stroke

Broadly speaking, cell-based therapies involve the implantation of cellular material into a recipient to effect beneficial changes in patient sequelae. Multiple stem cell types have been studied as cell-based therapies for ischemic stroke these are broadly

classified into neural and non-neural stem cell subgroups. Neural stem cells (NSCs) have the inherent capacity to differentiate into neuronal and glial cells, and different NSC preparations give rise to varying proportions of neurons and glia. The specific types of glial cells that arise (astrocytes, oligodendrocytes and ependymal cells) also vary between NSC preparations [10]. NSCs have the theoretical advantage over non-NSCs, in that they can directly replace damaged neural and glial tissue, and several preclinical studies have demonstrated differentiation and engraftment of transplanted NSC-derived neurons and glia in stroke-damaged brain parenchyma. Kelly and colleagues demonstrated in 2004 that human fetal CNS-derived cells implanted into the ischemic rat brain survive and differentiate into site-appropriate neurons and glial cells, and that these implanted cells are capable of migrating long distances towards an ischemic lesion [11]. Similarly, Bühnemann and colleagues showed in 2006 that embryonic stem cell (ESC)-derived neural precursors implanted into infarcted territory in an experimental rat model of stroke can differentiate into mature glial cells, as well as diverse neuronal cell populations that form functional synaptic connectivity with host neurons [12]. In 2009, Daadi and colleagues further utilized MRI, bioluminescence, electrophysiology and immuno-electron microscopy to evaluate the survival and integration of ESC-derived human neural stem cells following experimental stroke in rats they demonstrated differentiation into functionally mature neurons (that expressed synaptic proteins, made synapses with endogenous neurons, and generated voltage dependent responses as well as spontaneous EPSCs), oligodendrocytes and astrocytes with prolonged survival of up to 2 months following implantation [13].

Multiple studies have also demonstrated improved neurologic function in experimental stroke models implanted with human NSCs [14, 15]. Interestingly, the studies have not consistently demonstrated an association between NSC engraftment/survival and the degree of functional recovery. For instance, Bliss and colleagues demonstrated in 2006 the robust survival and neurite extension from human neural precursor cells in animal models of stroke. This did not, however, correspond with an improvement in functional outcome [16]. This has led to the hypothesis that alternative mechanisms underlie functional improvements with cell-based therapies beyond the *direct* replacement of injured neural and glial tissue by the transplanted cells.

A variety of alternative mechanisms of therapeutic benefits derived from stem cell-based therapies have been reported. Intracarotid injection of bone marrow stromal cells (containing both mesenchymal stem and progenitor cells) in experimental stroke leads to axonal remodeling and remyelination in infarcted tissue [17]. Mesenchymal stem cell (MSC) treatments have also been shown to increase the levels of various neurotrophic factors (EGF, VEGF, bFGF, IGF-1, BDNF, GDNF) in animal stroke model brains [18, 19], and elevated levels of neurotrophic factors have been associated with increased cell proliferation and decreased apoptosis in the subventricular and subgranular zones [20]. Interestingly, axonal sprouting of neural tissue cultures in response to stem cell-based therapies can be halted by various neurotrophic factor-specific inhibitors [21]. These results suggest that enhanced neural plasticity, neurotrophic support, and endogenous neurogenesis all may underlie the therapeutic benefits observed with stem cell-based therapies. Numerous

studies have also identified increased vascularity and neovascularization in and around the ischemic territory in stem cell-treated experimental stroke [17, 22, 23]. Stem cells have been shown to elicit an immunosuppressive effect on host tissue, thereby enhancing blood-brain barrier integrity and attenuating the host immune response to xenotransplants [24–26]. Thus the functional benefits of stem cell-based therapies in stroke are likely multi-modal and potentially involve changes in neural plasticity, neovascularization, neurotrophic support, endogenous neurogenesis, and immunomodulation the precise interplay of these mechanisms has not yet been fully elucidated. Nonetheless, these purported mechanisms provide the rationale for non-NSC transplantation.

A wide range of non-NSCs have been studied in experimental stroke including cells derived from bone marrow, umbilical cord blood, peripheral blood, and mesenchymal tissue. Bone marrow-derived MSCs are multipotent stem cells that are particularly suitable as cell-based therapies for stroke due to their: (1) ease of isolation, (2) immunomodulatory properties [27–29], (3) ability to selectively target infarcted brain parenchyma [30, 31], (4) well-documented safety profile in the clinical setting [32], and (5) avoidance of the ethical concerns associated with using embryonic and fetal stem cells. In a meta-analysis of experimental stroke studies, MSC therapy was consistently associated with improvements in multiple measures of behavioral function [33].

Mesenchymal stem cells have been evaluated in several clinical trials. A 2005 Phase 1/2 randomized controlled trial (RCT) of intravenously-administered autologous MSCs in subacute middle cerebral artery (MCA) infarcts revealed a statistically significant improvement in Barthel Index at 3 and 6 (but not at 12) months in addition there was a non-significant trend towards improved modified Rankin Scale (mRS) scores that decreased in magnitude at each successive time point. No adverse effects occurred in the MSC-treated cohort [34]. This same research group also evaluated the long-term (5-year follow up) safety and efficacy of intravenously-administered MSCs in a larger cohort of patients. They reported sustained safety, as well as a statistically significant improvement in mRS and a non-significant trend towards reduced mortality in the MSC-treated cohort compared with controls [35]. Another Phase 1/2 non-randomized trial of intra-arterially administered autologous MSCs in subacute stroke patients revealed a modest but non-significant trend towards improved mRS scores in cell-treated subjects when compared with controls. Notably, two of ten MSC-treated patients (20%) in this study experienced isolated simple partial seizures at 3 months post-transplantation [36]. Another group administered autologous MSCs to chronic stroke patients in two separate non-randomized observational trials. One of these trials noted a significant improvement in Barthel Index at 2 and 6 month follow ups, however the remainder of clinical outcome assessments were not significantly different between MSC-treated patients and controls during the course of the 6 month follow up. Of note, MSC-treated patients in both studies were reported to have increased activation of primary and supplementary motor cortex on post-transplantation functional MR imaging. No treatment-related adverse events occurred [37, 38]. In 2014, Aldagen Inc. announced the results of the small Phase 2 RECOVER-Stroke Trial (not published), in which a

proprietary MSC (ALD-401) was administered intra-arterially to subacute stroke patients. There were no serious adverse events associated with this cell therapy, however the trial failed to demonstrate any significant change in the primary endpoint (mean mRS), nor any secondary endpoints, at 90-day follow up. Another Phase 2 RCT of intravenously-administered autologous MSCs in subacute stroke also failed to identify an improvement in any outcome measure including Barthel Index, mRS, NIHSS, or change in infarct volume at 180 days, however there were again, no adverse events associated with cell therapy in this trial [39]. In contrast, a 2015 Phase 1/2a non-randomized trial by another group also administered autologous MSCs intravenously to subacute stroke patients and noted statistically significant improvements in mRS, and a trend towards improved NIHSS and Barthel Index at discharge, as compared with a cohort of historical controls. Notably, this benefit was more pronounced in the cohort of patients treated with high-dose cell infusions, as compared with those treated with low-dose infusions. No adverse events occurred in MSC-treated patients [40]. The recently published MASTERS trial was a Phase 2 RCT in which allogeneic bone marrow-derived multipotent adult progenitor cells were intravenously-administered within 24–48 h of stroke onset. There was no difference in the primary safety endpoint (dose-limiting toxic events at 7 days after administration) or in the primary efficacy endpoint (global stroke recovery) between patients treated with 1.2×10^9 cells versus those treated with placebo. Notably, a post-hoc analysis identified improvements in multiple outcome measures among the subset of patients treated within 24–36 h [41]. A Phase 3 RCT is now being planned with a 24–36 h treatment window.

In summary, clinical trials of MSCs in ischemic stroke have consistently verified the safety and feasibility of MSC therapy in ischemic stroke however efficacy has not yet been reliably demonstrated [42]. It is important to note that each of the trials discussed here are early phase clinical trials designed to assess primarily safety and feasibility and not efficacy, which has typically been evaluated as a secondary outcome measure.

3 Optimizing Cell-Based Therapies for Stroke

Despite promising preclinical and early stage clinical trial data, numerous questions remain unanswered regarding the optimization of cell-based therapies for stroke patients. Existing preclinical and clinical investigations of cell-based stroke therapies have varied extensively with regard to cell type, cell dose, and timing and route of cell administration, and the ideal combination of these variables remains largely unknown. The variety of NSCs and non-NSCs that have been investigated in experimental and clinical stroke was described above. There have been no comparative studies to suggest superiority of any particular cell type to date.

When designing any cell based stroke therapy, a critical component to optimize is the cell dose itself. A recent preclinical study noted significantly reduced efficacy with low dose transplantation (5×10^5 cells), and a significantly higher incidence of

adverse outcomes and mortality with high dose transplantation (5×10^7 cells). Based on this, an intermediate dose of 5×10^6 cells was selected to optimize efficacy while minimizing cell-related complications [43]. Another preclinical study identified a positive correlation between cell dose and efficacy, with improved behavioral outcomes noted with doses of 1×10^6–10^7 cells, but not with 1×10^4–10^5 cells [44]. In contrast, a meta-analysis of experimental stroke studies noted an inverse correlation between MSC dose and behavioral outcome [33]. As noted previously, a Phase 1/2a clinical trial identified a dose-dependent effect of intravenously-administered autologous MSCs in patients with subacute ischemic stroke [40] however, this was a small non-randomized open-label study that used a matched cohort of historical controls.

Timing of cell dose administration is also an important variable for optimizing cell-based therapies for stroke. Several preclinical studies have noted superior results on a range of outcome assessments when cell therapy is administered early after stroke onset. For instance in one preclinical study, cell administration 48 h after stroke onset was associated with a significant improvement in cell survival compared with treatments administered at 6 weeks. Notably, there was no significant difference in the extent of neuronal differentiation or distance of grafted cell migration noted between the two therapeutic windows [45]. Another preclinical study identified marked reductions in infarct volume and behavioral deficits when MSCs were administered 3 h after stroke onset, and this remained statistically significant when cells were administered 24 h post-stroke. These benefits were not observed when MSCs were administered 7 days after stroke onset [44]. A meta-analysis of preclinical studies identified a larger effect size on behavioral outcomes when MSCs were administered early (i.e. 0–8 h) after stroke onset [33]. Importantly however, no head-to-head clinical studies have been conducted that directly compare the range of possible therapeutic time windows. It is important to note that cell therapy at different time points following stroke (e.g. acute vs. subacute vs. chronic) may have fundamentally different therapeutic benefits. It is possible that administration during the acute period may serve to minimize primary and secondary injury from stroke, for example, whereas subacute or chronic delivery of cell-based therapeutics is more likely to provide a neuro-restorative benefit.

In the clinical stroke setting, the most commonly studied routes of cell-based therapy delivery are intravenous, intra-arterial, and intracerebral [26]. Direct intracerebral administration implants cells directly into the desired target, and thereby theoretically maximizes the therapeutic benefit while potentially minimizing untoward systemic effects. There is conflicting data as to whether intracerebral administration offers additional therapeutic benefit over intravenous or intra-arterial systemic delivery [46, 47]. Notably, a meta-analysis of preclinical studies demonstrated the greatest effect size with intracerebral implantation of cell-based therapies [33]. It should be noted, however, that significant improvements in behavioral outcomes were noted with all routes of delivery, and that intracerebral administration techniques are inherently invasive.

4 Using Modified Mesenchymal Stem Cells for Stroke: The SB623 Experience

SB623 cells originate from bone marrow-derived MSCs developed by SanBio, Inc. (Mountain View, CA, USA) as an allogeneic cell therapy for stroke. These cells are modified by transfection with a plasmid vector containing the human *Notch1*-intracellular domain, followed by the administration of specific trophic factors. This process induces MSCs to differentiate in a highly specific manner into post-mitotic neurons [48]. *In vitro* studies have demonstrated distinct differences of SB623 cells over unmodified MSCs, which may have important implications for SB623 cells as a cell-based therapy for stroke. For instance, embryonic rat brain neurons exhibit a substantial increase in metabolic activity when grown on SB623-cell derived extracellular matrix (ECM), as compared with cells grown on unmodified MSC-derived ECM [49]. SB623 cells have also been shown to induce more potent expression of neural and glial markers than parental MSCs [50]. *In vitro* assays of angiogenesis demonstrate enhanced vasculogenesis and a significant increase in vessel branching in the presence of SB623-derived conditioned medium, as compared with unmodified MSC-derived medium [51]. In addition, *in vitro* studies have demonstrated that SB623 cells have immunosuppressive and neurotrophic supportive properties that are equal or superior to unmodified MSCs [52, 53]. In a preclinical study, implantation of SB623 cells into the striatum of experimental stroke rats one month post-injury resulted in significant improvements in locomotor and neurologic function, and significant reductions in peri-infarct cell loss [54].

Steinberg and colleagues [55] recently published the 12-month interim data from a 2-year, open-label single-arm study (ClinicalTrials.gov Identifier: NCT01287936) designed to evaluate the safety, feasibility, and clinical outcomes of the stereotactic intracerebral implantation of SB623 cells in patients with stable, chronic stroke-related motor deficits. These results demonstrated that SB623 cell therapy is generally safe and well-tolerated, and associated with significant improvements in chronic neurologic deficits at both 6 months and 1 year. With regard to the primary outcome measure (European Stroke Scale, ESS), treated patients in this study demonstrated a statistically significant improvement from baseline at 6 months ($P < 0.01$), and this improvement remained significant at 12-month follow up ($P < 0.001$). Treated patients also had improvements in various secondary outcome measures, including NIHSS score at 12 months ($P < 0.001$) and Fugl-Meyer Assessment (FMA) FMA motor score at 12 months ($P < 0.001$). Notably, mRS was not significantly improved at any follow up time point. On radiographic follow up, 72% of patients demonstrated new T2-weighted-fluid-attenuated inversion recovery (T2 FLAIR) hyperintensity on MRI 1 week after cell transplantation, which was primarily in or adjacent to the premotor cortex and along the cannula tract. The size of this hyperintense signal ranged from 0.5–9.2 cm^2 to 0.6–3.5 cm^2 in maximal diameter, and was notably diffusion-weighted imaging (DWI)-negative in all patients. This FLAIR signal was not present on the day 1 post-transplant MRI, and resolved in all patients by the month 1 or 2 MRI. There were significant Pearson correlations between the size of

the FLAIR signal hyperintensity and the change from baseline on all clinical outcome measures (ESS +0.82, $P < 0.001$ NIHSS −0.69, $P < 0.01$ FMA total score +0.71, $P < 0.01$ FMA motor score +0.67, $P < 0.01$).

With regard to safety, all patients experienced at least one treatment-emergent adverse event (TEAE), however these were typically only mild to moderate in severity the vast majority were determined by study personnel to be related to the surgical transplantation of the cell-based treatment, rather than to the actual SB623 cells. There was no significant correlation between SB623 cell dose and the incidence of any individual TEAE. Post-operative headache was the most frequent procedure-associated TEAE, occurring in 78% of patients. There were six serious AEs (defined as requiring additional hospitalization) in six patients. Specifically, one patient was noted to have an asymptomatic subdural fluid collection during the post-procedure period, which was effectively treated by burr-hole drainage. One patient had a seizure on day 70, which was determined to be unrelated to the cell treatment, but probably related to the procedure. One patient underwent stenting of asymptomatic carotid artery stenosis on day 291, which was unrelated to either the cell treatment or the procedure. One patient suffered a transient ischemic attack on day 334, which was also determined to be unrelated to either the cell treatment or the surgical procedure on the basis that it occurred 11 months after treatment administration. Finally, one patient developed a urinary tract infection and another developed pneumonia during the peri-operative period. All serious AEs were determined to be recovered or resolved by the time of the 12-month interim analysis. There were no clinically relevant changes in an extensive range of hematologic, biochemical or metabolic laboratory parameters that were monitored throughout the 12-month follow up course, nor were there any clinically significant changes in vital signs during this period. No humoral sensitization to SB623 cells was identified in any patient. It is important to recognize that this was a small, open-label, single arm Phase I/IIa trial with several limitations these included a small sample size, non-blinded non-randomized design, and highly selective eligibility criteria. Thus, the results of this trial should be interpreted in context, and as such are not necessarily applicable to the general stroke population. Nonetheless, the safety and feasibility of intracerebral implantation of SB623 cells in this trial, in combination with the neurologic improvement seen on multiple well-validated stroke outcome scales, have laid the foundation for a larger multicenter, randomized, controlled Phase 2b clinical trial of SB623 cell therapy for stroke patients that is currently underway.

5 Future Directions

Tremendous progress has been made over the past decade with regard to designing effective cell-based therapies for stroke. At this time, the results of early-stage clinical trials are just becoming available. The results to date have consistently demonstrated the safety and feasibility of utilizing stem cells in stroke patients, and preliminary data suggests that such cell-based therapies could be most promising

for improving neurologic function in this patient population. Nevertheless, there is still much to be learned from further preclinical *in vivo* and *in vitro* investigation, as well as further early stage clinical evaluation of cell-based therapies for stroke. Future studies should be aimed at defining the mechanisms of therapeutic benefit of cell-based therapies for stroke. Defining the optimal combination of cell type, dose, timing and route of delivery is also critical to provide maximum therapeutic benefit. These data will be essential for informing the design of later stage clinical trials.

Acknowledgments We thank Christine Plant for assistance with the manuscript.

Funding: This work was supported in part by funding from Bernard and Ronni Lacroute, the William Randolph Hearst Foundation and Marc Paskin.

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Chapter 15
Preparing for Future Stem Cell Clinical Trials

Keith W. Muir

Abstract Clinical trials in stem cell therapy for stroke have predominantly been small, single-centre and safety focused studies, few with blinding or concurrent control groups, and typically with wide treatment time windows and clinical entry criteria. Only recently have trials begun to consider the evolving preclinical evidence base and strategies that might translate this successfully into clinical use. The next few years will witness clinical trials that are likely to establish whether or not there is worthwhile therapeutic potential.

The accumulated experimental evidence has led to two distinct paradigms for cell therapy in stroke. In the first, systemically administered cells are delivered in the acute or early subacute phase, with a mechanism of action that is likely to be predominantly reliant on anti-inflammatory and trophic effects. With intravascular delivery, cells do not enter the central nervous system (CNS) in any significant numbers, if at all, and neither CNS nor systemic engraftment has been established. This approach reflects the great majority of experimental studies. Its likely translational route replicates established acute stroke trial paradigms. Trial designs in this area have had the advantage of evolution since the 1990s such that inclusion and exclusion criteria are well understood, as are trial endpoints.

The second paradigm is of later stage cell delivery to enhance recovery in subacute or chronic stroke. The experimental support is thinner, there being few animal models of this scenario, and there are fewer clinical trials in this time frame from which to draw designs. On the other hand, this represents a huge area of unmet clinical need lacking any very effective intervention.

Both paradigms are being addressed by currently planned or ongoing clinical trials of cell therapy. This chapter will review the main issues that require to be considered.

Keywords Clinical trials • Stem cells • Trial design • Placebo

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P.A. Lapchak, J.H. Zhang (eds.), *Cellular and Molecular Approaches to Regeneration and Repair*, Springer Series in Translational Stroke Research,
https://doi.org/10.1007/978-3-319-66679-2_15

Abbreviations

ARAT	Action research arm test
BI	Barthel index
BMMCs	Bone marrow-derived mononuclear cells
CNS	Central nervous system
MCAO	Middle cerebral artery occlusion
mRS	Modified Rankin Scale
NIHSS	National Institutes of Health Stroke Scale
NINDS	National Institutes of Neurological Disorders
PISCES-1	Preliminary investigation of stem cell effects in stroke
STEPS	Stem cells as an emerging paradigm in stroke
TMS	Transcortical magnetic stimulation
VISTA	Virtual international stroke trials archive

1 Introduction

Clinical trial design for stem cell therapy evaluation has been considered in a series of workshops involving academia, industry and regulators to produce recommendations for the translation of this potential treatment modality, the Stem Cells as an Emerging Paradigm in Stroke (STEPS) meetings [1–3]. The content and concerns of the four STEPS meetings (three reported and published, the report of a fourth being prepared at the time of writing) reflect the evolution of the field from a focus on animal models through to early phase and now later phase clinical research, and the developing experimental data in the field. Issues relating to clinical trial design and progress in the cell therapy field have additionally exercised the thinking of many investigators [4–8]. In particular, thinking has evolved from the initial concept of cell or tissue replacement as the dominant biological effect mediator, to an appreciation that much (or indeed all) of the therapeutic benefits from cell therapy might be mediated by indirect effects [6], and most recently that only certain components of cells might be necessary for a therapeutic action [9].

2 Acute Cell Therapy

It is recognised that cells delivered within the first hours after induction of cerebral ischaemia in animal models limit the extent of brain injury, and are associated with improved functional outcome [10–12]. Cells of various types and delivered by various routes have been reported to be effective, but some concerns regarding methodological quality and reporting bias have been identified [10]. More specific focus on studies of intravenous delivery of bone marrow-derived cells in rodent middle

cerebral artery occlusion (MCAO) has yielded a more consistent evidence base [12]. Typical time to administration has been prior to induction of ischaemia or within a few hours of ischaemia.

Biodistribution studies in mouse hypoxia and focal ischaemia indicated limited distribution of mouse neural stem cells to the brain [13]: intravenously delivered cells were cleared almost entirely within 1 week, while intra-arterial delivery produced higher concentrations in brain, but almost complete clearance within 2 weeks. In rat MCAO, intravenous bone marrow stromal cells were distributed predominantly to the lungs, with transient distribution to the brain, and negligible cell persistence even by 8 days [14]. In another rat MCAO study, no preferential brain uptake in MCAO rats compared to controls was seen, and again distribution was predominantly to lung [15]. In human studies, intravenously delivered bone marrow-derived mononuclear cells (BMMCs) were distributed predominantly to lungs and spleen by 2 and 24 h after injection [16]. It therefore seems unlikely that there will be significant distribution of cells to the CNS after intravascular administration, and any engraftment is likely to be very limited. With respect to outcomes, differences between intravenous and intra-arterial routes have not been observed consistently in animal studies [17]. Therapeutic effects have nonetheless been seen with most cell types in animal models of stroke, therefore it is assumed that "bystander" effects reliant on release of trophic factors and immune modulation underpin the action of stem cells delivered in this manner, and there is experimental evidence of multiple systemic effects of potential relevance. Systemic engraftment of some cell types in the lung may allow more sustained action.

Within the first hours after ischaemic stroke, intravascular delivery of a therapeutic agent is the most feasible option: intravenous delivery is straightforward, and intra-arterial delivery is increasingly an option with the wider use of endovascular thrombectomy. Intravenous delivery is clearly more practical since almost universally available, and possible at any stage after stroke.

Intra-arterial delivery requires appropriately skilled personnel and facilities, and while thrombectomy is becoming more widely available, endovascular treatment is indicated and possible in only a minority of patients, and speed of intervention is critical. It is logistically challenging to add intra-arterial cell delivery to an emergency procedure such as thrombectomy, and it would be entirely impractical to infuse autologous cells due to the requirements for cell harvest and preparation. In addition, animal studies of intra-arterial cell delivery have been complicated by cell clumping and downstream arterial occlusion [18] that produced significant complication rates potentially outweighing any advantage of improved CNS retention of cells, and requiring modification of injection rates to overcome [19, 20]. The practicality of intra-arterial delivery in the later subacute period is also questionable—the logistics and safety of intra-arterial procedures potentially requiring anaesthesia outside the acute time window for thrombectomy are challenging, and targeted delivery may be impossible due to persistent occlusion of the relevant artery. Very slow recruitment was evident in one pilot clinical study of subacute intra-arterial cell delivery, with only 6% of screened patients proving to be suitable and many exclusions due to medical instability and major arterial occlusion

precluding vascular access [21]. Whether such an approach is advantageous is unclear: while the evidence above suggests that this may allow some degree of cell delivery to the brain, it is uncertain whether this leads to long term engraftment, or whether this is necessary for a therapeutic effect [22].

Several small clinical studies using intravenously delivered cells have reported findings, but with broad patient inclusion criteria and a wide time window [23–27]. The acute use of autologous cells is extremely challenging since invasive harvest procedures are likely to be more hazardous in the face of recent thrombolytic and antithrombotic drug treatments, and neurological impairments will impair cooperation by many patients with significant stroke deficits. Two moderately large controlled clinical trials have reported early subacute use, one using autologous bone marrow cells and the other allogeneic cells [28, 29].

The phase 2 Indian multi-centre trial of Prasad and colleagues [28] had a time window of 7–30 days, treating patients with autologous bone marrow mononuclear cells infused intravenously. Randomisation to cell infusion or control was undertaken, and assessors for the outcome measures were blinded to treatment allocation. The control arm patients underwent no invasive procedures—neither cell harvest from bone marrow, nor intravenous infusion.

The MASTERS trial [29] of the Athersys "multistem" allogeneic bone marrow-derived multipotent adult progenitor cells aimed to treat patients 24–36 h after stroke onset, but requirements for cell processing in specialist facilities caused the trial to expand the time window to 48 h for practical reasons. Eligible patients were randomised to either cell infusion (400 or 1200 million cells) or placebo. The global results showed no difference in outcomes compared with control, although subgroup analysis of those treated within 36 h of onset was interpreted as supporting this earlier time window.

No safety issues were identified in these studies, and similar paradigms of intravenous infusion of cells have been deployed in other therapeutic areas including myocardial ischaemia and multiple sclerosis.

As outcome measures, these trials used well-characterised clinical scales that are familiar from other therapeutic modalities in the acute stroke setting: general measures of neurological function (the National Institutes of Health Stroke Scale [NIHSS]) [30, 31], of activities of daily living (the Barthel Index [BI]) [32] and of disability (the modified Rankin Scale [mRS]) [33, 34]. These scales are advantageous in being well understood, widely used in routine practice as well as trial settings, acceptable to regulators, and being applicable to all potential patients irrespective of stroke mechanism, location or size. For an acutely delivered therapy, especially within the first 36 h, detailed patient selection is not practical. An inclusive approach to trial design also improves generalisability of results and would allow widespread adoption of treatments if efficacy is established. The properties of the scales are well understood, and specific training is available for NIHSS and mRS to minimise inter-observer variability. Standardised structured interviews and centralised video interpretation can be used to further reduce mRS variability, with the additional possibility of ensuring blinded independent outcome scoring.

Dosing can be reasonably controlled for allogeneic cell therapies, but is highly variable in autologous cell delivery [25, 27, 28]. The population of cells present in bone marrow mononuclear cell aspirates is also mixed, with a range of cell types including haematopoietic progenitor cells and mesenchymal stem cells, and unless additional ex-vivo culture expansion is undertaken (with the additional time incurred in this step delaying any potential therapeutic use), it is extremely difficult to characterise the cells that are actually delivered.

3 Chronic Stroke Cell Therapy

Early investigation of teratocarcinoma-derived neural cells [35–37] or porcine xenografts [37] established the feasibility and basic methodological approach for delivery of cells by direct cerebral implantation. Subsequent trials have adapted these methods for cell delivery [38]. Chronic stroke—arbitrarily proposed to be 6 months or more after the ictus—is a huge therapeutic need, several million people worldwide living with long-term neurological disability as a consequence of stroke and having high costs of social and medical care [39]. Current medical interventions are limited to secondary prevention of further events, prevention or treatment of physical complications such as spasticity or mechanical joint disruption, and behavioural adaptations to deficits. The challenge is uncertainty about whether there is useful plasticity in the injured brain at late stages after stroke [40], with very limited animal model data of uncertain relevance to this time point in human stroke.

Chronic, stable patients allow trials to adopt more careful selection, planning of procedures, and targeting of cell delivery. Intracerebral implantation of cells ensures that cells can be delivered in a defined dose to a specific location. While the mechanism of cell action in chronic stroke is not fully characterised, it more plausibly includes engraftment and integration than does intravascular administration, in addition to local anti-inflammatory, immunomodulatory and trophic effects, including stimulation of endogenous neurogenesis and angiogenesis [41–43]. What proportion of cells survive and engraft long-term is unknown, as is the differentiation fate of these cells.

Two trials using human stem cells have reported recently, the Preliminary Investigation of Stem Cell Effects in Stroke (PISCES-1) trial, and the SanBio phase 1 trial. Each included small numbers of patients with no control group, in order to establish safety and tolerability of different doses.

PISCES-1 [44] used ReNeuron's human foetal cortical neural stem cell line genetically modified with the c-myc$^{ER\text{-}TAM}$ transgene to allow large-scale cell production for allogeneic cell therapy, CTX0E03 [45–47]. The study followed a similar paradigm to that studied in rodent MCAO models of "chronic" stroke, with intraputaminal implantation of doses of up to 20 million cells. In PISCES-1, 11 patients received doses of between 2 and 20 million cells, and no cell-related safety issues were identified over a 2 year follow-up period.

The SanBio trial [48] included 18 subjects administered up to ten million cells placed stereotactically in small deposits around the margin of the infarct, using genetically modified human bone marrow-derived mesenchymal stem cells. Both studies included patients 6–60 months after ischaemic stroke, with a median time to treatment of around 2.5 years. Some improvements in neurological and functional scales over the first 3 months after implantation were described, with static function thereafter. The time scale for neurological change was unexpected and not clearly consistent with cell differentiation and replacement as the major mode of action.

For phase 2 studies, trials of these agents have opted to target patients with motor deficits specifically, and employed more specific neurological scales focusing on motor function as their primary end-points. The PISCES-2 study completed recruitment in summer 2016 and reported preliminary favourable early functional improvements sufficient to justify further clinical trial development (http://4965zs3ha2l125fk78zkozo3.wpengine.netdna-cdn.com/wp-content/uploads/ReNeuron-PISCES-II-data.pdf, accessed 7 July 2017). PISCES-2 selected a population of subacute stroke patients 3–12 months after onset, with major upper limb dysfunction, and its primary endpoint was recovery of useful upper limb function defined on the Action Research Arm Test (ARAT) [49].

4 Trial Design

Rigorous standards of trial design and interpretation are key to the credibility and ultimate adoption into practice of clinical trial results.

4.1 Controls

Clinical investigations of stem cells have hitherto justified small studies without concurrent controls or blinding on grounds of practicality and patient acceptability, but controlled trials will be necessary to advance clinical practice. Randomisation, placebo controls, and blinding are critical to the integrity of trials but the invasive nature of cell harvest for autologous cell preparation and of several delivery approaches means that some compromise may be required. As noted above in relation to the experience of acute intravenous cell delivery trials, optimal design is feasible for an allogeneic cell approach such as that of the Athersys multipotent adult progenitor cell studies, but blinding becomes problematic when autologous cell harvest is required such as in the Prasad trials. For more invasive intracerebral approaches, ethical issues become significant. The acceptability of placebo surgical procedures is debated. While investigators and regulators encourage this approach in recognition of the importance of placebo effects, [50, 51] patient groups have questioned it [52]. Opinions derived from patient experience in other diseases may

not be applicable in stroke: in a neurodegenerative process such as Parkinson's Disease, deferred cell implantation has been considered to be an acceptable offer for those trial participants randomised to the control arm (assuming that test implantation is established to be effective). In stroke, however, the injury is acute and the mechanisms by which cell therapy might be effective are heavily weighted towards the early subacute period, so deferred treatment cannot be reasonably assumed to have the same potential value as early treatment.

Placebo surgery is deemed to be acceptable and is preferred by some regulators. What constitutes an appropriate balance between placebo procedures and reasonable risk is a grey area. The phase 2 SanBio trial uses a partial thickness Burr hole under local anaesthesia, without dural incision or any intraparenchymal injection, allowing a similar procedural duration and blinding the participant and trial team (outside the operating theatre environment). Other trials are likely to adopt the same approach. This has potential advantages, but two alternatives merit consideration. First, the potential harmful effect of surgery cannot be assessed by this approach. Surgery may have negative consequences, including local infection, pain or bleeding, adverse effects of sedative medication for the procedure, and of temporary cessation of preventative antithrombotic medication, as well as many more if procedures are undertaken under general anaesthesia (as was the case in the PISCES trials, for example). An alternative design would be randomisation to surgical implantation or to a non-surgical control group, allowing evaluation of the net effect (both benefit and potential harm) of the procedure, but with the disadvantage of lacking control for a placebo effect.

A second alternative would be a more invasive approach, where the control group undergoes identical intraparenchymal injection of vehicle solution. This would offer additional blinding (except possibly for the surgeon), would also control for potential non-specific (positive) effects of injecting a volume of fluid to introduce a focal lesion in the brain, and allows investigators to distinguish specifically cell-related adverse effects from those of the procedure: for example, do the T2 hyperintensities seen around needle tracts in both the SanBio and PISCES trials represent a specific tissue reaction to cell implantation, or a non-specific reaction to vehicle, or some other aspect of the process? This more invasive approach has scientific merit but is likely to be deemed to expose the control group to unacceptable risk.

It is important to frame discussions with patients in terms that recognise the experimental nature of stem cell administration, since there is a widespread assumption that benefit is expected, or indeed inevitable. Uncritical reporting of early phase clinical trial findings (for example http://www.dailymail.co.uk/health/article-3622589/Major-breakthrough-doctors-REVERSE-symptoms-stroke-Patients-walk-talk-live-normal-life-stem-cell-treatment-3-YEARS-later.html, accessed 7 July 2017) and the widespread unregulated online advertising of supposed "stem cell therapy" clinics contribute to this environment. The potential for harm (including very limited long-term safety data for most cell types) is one component of a complex discussion [53].

4.2 Sample Size Estimation and Endpoints

In the acute setting, clinical trial design is informed by a large body of experience that will allow realistic estimates of credible effect sizes using the same general outcome scales as have been deployed in other trials. The mRS benefits from development over many years to minimise subjective inter-observer variability in scoring, including rater training, independent video assessment [54] and structured interviews [55–58]. Large databases such as the Virtual International Stroke Trials Archive (VISTA) can be interrogated to model the impact of inclusion and exclusion criteria on expected outcomes in the control population [59]. Statistical methods have been developed to maximise study power (or reduce sample size), particularly through analysis of the entire distribution of the mRS rather than arbitrary dichotomous outcomes [60, 61]. The use of a broad disability scale as a primary endpoint has the advantage of applicability to all types of stroke deficit: inclusion and exclusion criteria can therefore be less restrictive, with advantages for recruitment rates and generalisability.

Sample size is likely to be moderate or large for a credible range of treatment effects, particularly with subacute interventions. While there is an analogy with acute trials in terms of design, a less appealing analogy is the failure of all clinical trials in acute stroke other than those involving reperfusion, a source of much soul-searching in the neuroprotectant field, among other notable translational failures [62, 63]. Many of the deficiencies that were postulated to underpin the failure of neuroprotectant drug trials might also apply to cell therapies: overestimates of effect size leading to trials that were too small, insufficient phenotypic detail to select relevant patients with biological targets, and a tendency to seek "responder populations" by tortured analysis of small phase 2 trials and their subgroups with consequent restrictive and (with hindsight) misdirected patient selection criteria in repeat phase 2b/3 trials. Nonetheless, there is now a track record of success for these general approaches in acute stroke through both thrombolytic drugs and thrombectomy, and the design features are recognised by clinicians and regulators.

Trials in chronic stroke face potentially greater challenges as the methodology is less standardised, largely a reflection of the absence of positive clinical trials in the rehabilitation and regeneration field [64]. Trials have elected to target what is effectively a human model system, motor deficits of upper, or both upper and lower, limb. Similar approaches have been taken in trials of rehabilitation strategies such as constraint-induced motor therapy [65]. While there is logic in the approach, since motor deficits are common after stroke and scales for motor function assessment are available, potential difficulties are illustrated by previous motor rehabilitation studies, which have been characterised by slow recruitment through the need to target patient populations with very specific deficits, and high drop-out rates. Despite enthusiasm for specific motor function scales such as the Fugl-Meyer scale among specialists in rehabilitation [66, 67], the scale is less widely understood among stroke physicians and its acceptability to regulators is unclear. Familiarity with more specialised scales such as the ARAT is less still, and specific equipment and training are required to perform the assessment. There is also uncertainty over the extent of change on ARAT that is meaningful to patients [68].

Motor function change represents only one component of a complex multi-dimensional deficit. Whilst emphasis has been placed on inclusion of adjunctive physical therapy to minimise between-site variation in multicentre trials, defining the minimum necessary duration and intensity of therapy input has proved to be challenging [69], and the content of therapy programmes may vary widely. Physical therapy represents only one of several therapy inputs that a patient is likely to receive, and even description of rehabilitative inputs has proved challenging, let alone quantification.

Combination of several different outcome scales has been proposed in order to better capture the multi-dimensional nature of stroke recovery [70], and has been an effective strategy in some acute trials such as the National Institutes of Neurological Disorders and Stroke (NINDS) trial of thrombolysis [71]. This statistical approach may strengthen findings when there is a common direction of effect, but may weaken study power if effects diverge.

Sample size ultimately depends on the variance of the outcome measure and the magnitude of the effect of the intervention. An uncomfortable reality for the field is that the costs, invasive nature of the interventions, and complexity of cell supply, are all likely to place practical constraints on trial size in cell therapies. The magnitude of the effect is difficult to estimate, and cannot be extrapolated reliably from animal studies where cell delivery has typically been much earlier than has been attempted (or is likely achievable) in the clinic. The schematic representation of different processes contributing to brain injury after stroke as a series of waves of differing size, latency and duration [72] is a useful conceptual framework, which recognises the dominant effect of very early cell necrosis due to severe ischaemia. Imaging studies support the very short time window during which the greatest part of an ischaemic lesion becomes damaged irreversibly. Thereafter there may be smaller contributions to the final infarct from late processes such as inflammation and apoptosis, but these appear to be minor contributors to the physical extent of an infarct. Clinically it has been difficult to demonstrate either delayed infarct growth (other than oedema), or any measurable neurological consequence of this, although there may be a relationship [73]. Interventions delivered at 36 h after stroke onset and likely to have a predominantly anti-inflammatory action may thus be anticipated to have a small treatment effect and to require very large sample sizes for convincing demonstration of any therapeutic action. The failure of other anti-inflammatory treatment strategies [74–78] may reflect ineffective drugs, late delivery, or trials that were too small, but equally it may signify that this mechanism has insufficient impact on tissue fate at late stage to be detected with anything other than an extremely large trial.

4.3 Patient Selection

Sample size is greatly inflated if trials include patients with no relevant biological target for a therapeutic intervention [79]. Recent acute trials have highlighted that modest sample sizes can produce highly significant and persuasive results when the relevant target population is selected—for example with selection of MCA

occlusion and appropriate volumes of viable tissue on perfusion imaging, for both intravenous thrombolysis and for thrombectomy [80–82]. The relevant clinical phenotype has not been clearly defined for acute systemic cell therapy, but demonstration of a worthwhile clinical effect is likely to follow similar principles to other acute stroke trials. In chronic stroke trials based on motor recovery paradigms, variable outcomes are well recognised and are not reliably predictable from clinical scales alone [83], especially for the more severe deficits that are likely to be overrepresented in any clinical trial of an invasive therapy. Selection of patients with potential motor response might be possible using combinations of clinical scales, brain imaging (for example to define the integrity of the corticospinal tract) and excitability of the motor system using transcortical magnetic stimulation (TMS) or similar [84–87]. Refining practical methods for patient selection that could be applied consistently across multiple clinical centres in a trial is a challenge: very complex and time-consuming imaging analysis may be difficult and may not be feasible for all scanners [88], and methods such as TMS are poorly standardised and not widely available. The adverse consequences of insufficiently informed patient selection are, however, clear, and can be documented both by head-counts of neutral and failed trials, and by the huge accompanying cost to the academic and pharmaceutical industry communities [89]. In addition, insufficient levels of phenotypic detail will impede any attempt to better define a potential "responder" population for future studies. Inclusion of imaging at least sufficient to offer an opportunity to enhance mechanistic understanding would be advantageous both for individual trials and for the field as a whole. In an analogous setting where clinical scales offered limited insight, multiple sclerosis, the identification of an imaging biomarker was the key that unlocked the door to disease modifying therapy development, by enabling clinical trials of reasonable size to be undertaken. Stem cell therapies would benefit from the same approach.

4.4 Time Windows

The nature of the intervention places constraints on cell supply for trials, as noted above, and this introduces additional limits on time windows. Experimental models of stroke indicate a complex and dynamic environment after ischaemia, and biological targets change both over time and anatomically. Underlying disease states may modify cell therapy effects and are rarely investigated in model systems. Patients undergo complex rehabilitation interventions, receive a variety of secondary preventative medications, and are prone to complications that reflect physical disabilities as well as systemic effects of stroke such as immunodepression, all of which might influence recovery patterns. To deviate far from the experimental evidence underpinning a particular cell therapy adds another confounding factor, and it would appear logical to limit at least this element that can be under the control of the trial investigators. Acute intervention in animal models has been overwhelmingly delivered in early acute stages; "chronic" intervention at most a few weeks after stroke in

rodents. Thrombolytic therapy for stroke could not show benefit when delivered an average of 4.5 h after onset [90]. It was only when the NINDS trial adhered rigidly to the narrower time window of 3 h based on animal model data (and insisted on even earlier treatment in 50% of participants by design) that benefit was eventually evident [91]. We may ignore the lessons of animal model time windows at our peril.

5 Summary and Conclusions

Two distinct paradigms for cell therapy in stroke are under investigation.

Acute systemic administration of cells follows a pathway reasonably well informed by other acute trials, but has to consider what might be a credible effect size and an appropriate time window for delivery. Allogeneic cells appear to have an advantage over autologous cells in this setting. Only limited patient selection is feasible, but generalisability of results is straightforward.

Chronic stroke allows for more targeted intracerebral administration of cells, but the invasive nature of the procedure places constraints on trial design and size, and the human model of motor system recovery is less standardised in the absence of successful clinical trial interventions. Patient selection, employing complex imaging and other advanced methods, and development of biomarkers will likely be critical to success.

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Part II
Exosomes-miRNA

Chapter 16
Extra-Cellular Vesicles: A Promising Approach for Translating Cell-Based Therapy

Benjamin Buller, Michael Chopp, and Zheng Gang Zhang

Abstract As cell-based therapies have demonstrated efficacy in the treatment of experimental and clinical stroke, their mechanisms of action warrant intense investigation and are being investigated in greater depth. It is becoming increasingly clear that one of the main ways that cell therapies based on mesenchymal stem cells (MSC) and other cells impart functional benefits to animals is through release of exosomes and other extracellular vesicles *in vivo*. Mounting evidence shows that MSCs release exosomes, and that these exosomes induce predictable and impactful changes in recipient cells. These exosome-induced cellular changes are likely mediated through the content of the exosomes, which comprise mRNA, miRNA, proteins, and other macromolecules. Many studies that have been published in the last several years have shown that treatment of animals with exosomes, harvested from MSCs and other cells, after stroke and traumatic brain injury (TBI) recapitulate the effect of the parent cells. Exosomes lack the safety and manufacturability issues that plague cell therapy, and they therefore may represent the next generation of cell-free therapies. Their biology and potential use as therapies for CNS injuries are discussed herein.

Keywords MSC • Stroke • TBI • Exosome • Microvesicle • Extracellular vesicle • Neuroresoration

Abbreviations

Ago2	Argonaute-2
Ang1	Angiopoietin-1
BBB	Blood-brain barrier

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P.A. Lapchak, J.H. Zhang (eds.), *Cellular and Molecular Approaches to Regeneration and Repair*, Springer Series in Translational Stroke Research,
https://doi.org/10.1007/978-3-319-66679-2_16

CSF	Cerebral-spinal fluid
dll4	Delta-like 4
EV	Extracellular vesicle
lncRNA	Long non-coding RNA
MCAo	Middle cerebral artery occlusion
miRNA	MicroRNA
MSC	Mesenchymal stem/stromal cell
MVB	Multivesicular body
RISC	RNA-induced silencing complex
TBI	Traumatic brain injury

1 Introduction

Cell-based therapy may be the most promising approach to achieving a clinically viable restorative therapy for stroke and other neurologic diseases. In a series of preclinical studies first published by our laboratory beginning in 2000, we showed that administration of bone marrow mesenchymal stromal cells (MSC), leads to enhanced neurological recovery in rats that are subjected to middle cerebral artery occlusion (MCAo) [1–4]. This work has been repeated and reproduced by many laboratories around the world [5–8], and other cell types have also been shown to be effective in aiding recovery from stroke [9–12]. Despite the acceptance of cell therapy as an effective treatment for animal models of stroke, the mechanisms by which it imparts functional recovery have remained elusive.

It was first hypothesized that MSC transdifferentiate into neural cells and thereby regrow brain tissue. This hypothesis was abandoned relatively quickly in favor of a paracrine hypothesis—that the cells secrete factors that stimulate growth [13]. This hypothesis too evoked skepticism, because treating animals with any number of secreted growth factors from cells has never reproduced the effect of treating with cells directly.

Extracellular vesicles (EV), including exosomes and microvesicles, are small, membrane bound spheroids of approximately 30–200 nm in diameter. They contain macromolecular cargo that includes receptors, ligands, and nucleic acids. The nucleic acid cargo of EVs comprises a mixture of mRNA, tRNA, vault RNA, microRNAs (miRNA), and long non-coding RNA (lncRNA). While the biologic functions of the majority of the contents of EVs remain unclear, their miRNA contents have been shown to be functional *in vivo*, which is particularly important given that many roles have been described for miRNAs in both the pathogenesis of, and recovery from, stroke (for review of the miRNA and stroke, see e.g. [14]). The role of exosomes and other EVs in mediating cell therapy repair, with particular attention paid to MSCs, and the putative mechanism by which they operate are discussed below.

2 EV Biogenesis Pathways

EVs comprise several subtypes, and they are secreted by virtually all cells [15]. They exist in all body fluids, including blood, cerebrospinal fluid (CSF), urine, ascites, and saliva [16–20]. The two most well studied types of EVs are exosomes and microvesicles. Each of these two types has unique attributes and a distinctive biogenesis pathway. Exosomes are spheroids that have an approximate size of 30–100 nm [21] that are generated by the endosomal pathway [22]. In this pathway, the cell membrane invaginates to form an endosome, and then successive invaginations of the endosome create a multivesicular body (MVB). Fusion of the MVB with the cell membrane releases exosomes to the extracellular space where they may dock locally or distally with other cells, or perhaps be taken up by endocytosis or macropinocytosis [23]. By contrast, microvesicles are thought to bud directly from the membrane; they have more amorphous shapes than exosomes, and they have a much larger average diameter, perhaps any size up to 2000 nm [24]. Figure 16.1 describes these separate biogenesis pathways.

Exosomes and microvesicles can be distinguished by their surface markers. Exosomes, uniform lipid bilayer spheroids, are generally marked by tetraspanin proteins including CD63, CD81, and CD9, as well as flotillin and Alix [25, 26]. Microvesicles, in contrast, generally lack tetraspanins and are of varying size, shape, and density [27]. Separating exosomes from microvesicles is difficult practically, as

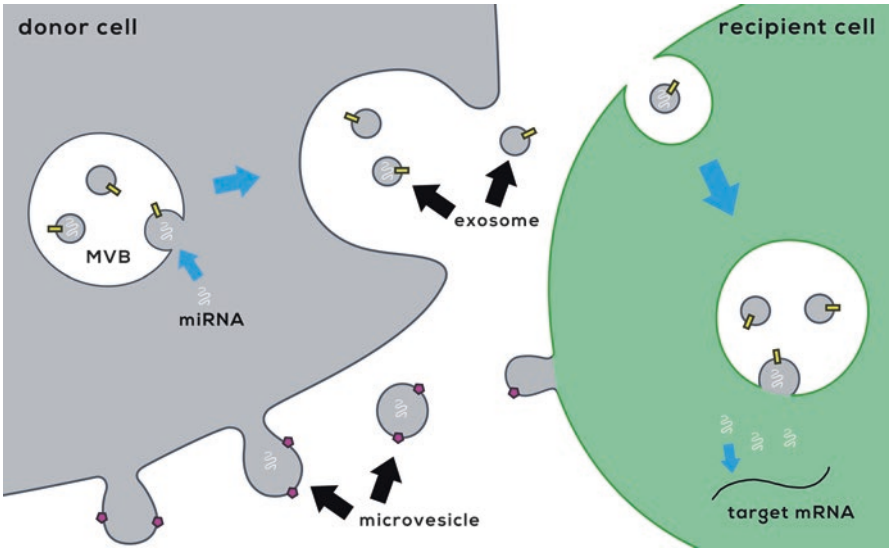

Fig. 16.1 Extracellular vesicles are shed by two primary mechanisms. In the endosomal pathway, exosomes are created by successive invaginations of the plasma membrane to create an MVB. The MVB then fuses with the plasma membrane to release the exosomes into the extracellular space. Microvesicles are created by direct budding of the membrane. EVs can be taken up by the recipient cell by direct membrane fusion or by endocytosis/macropinocytosis

their densities and sizes have a significant amount of overlap. However, separation of subpopulations of EVs may be more important from a technical scientific standpoint than a medical one. It is unclear which EV subtype, if any, contributes more to their therapeutic effects. Some studies have implicated exosomes [28], while others have implicated microvesicles [29] as the more important vesicle type in mediating the effects of parent cells. This debate may not be settled soon; however, exosomes are generally thought to be the more biologically relevant EV subtype, and the majority of the literature therefore focuses on exosomes, although it should be noted that much of the early work in EVs was clouded by a lack of consistent nomenclature to distinguish between the various subtypes of EVs.

3 Potential Therapeutic Applications

EVs have been shown to be therapeutic for many of the same neurologic diseases and injuries as have been demonstrated for their parent cells, including traumatic brain injuries and stroke [30, 31], and they are the only cell product that has been shown to recapitulate the therapeutic effect of the parent cells. EVs have only begun to be deployed clinically, so it is not entirely clear what their eventual impact may be; however, we can speculate that their adoption will be swift should they be shown to be as efficacious as parent cells in treatment of injury and disease. They have several inherent advantages over cells that make them ideal replacements for or adjuvants to cell-based therapy.

Foremost and most obvious, EVs do not divide. One of the biggest safety concerns with cell therapy is the risk of teratoma formation. Although rare, teratoma formation or other uncontrolled cell division is a real concern. Cultured cells often are observed to have genomic aberrations, and the risk of tumor transformation has tempered enthusiasm for their use, especially from regulatory agencies. Therefore, completely mitigating the risks posed by dividing cells can only be counted as a positive development. EVs are not cells, so their immediate effects are transient, and they cannot form tumors.

Second, exosomes appear to not cause microvascular embolization, nor do they induce formation of thrombi. MSC and other cells can lead to vascular occlusions in some circumstances, which can cause significant complications to the patient. By contrast, exosomes, perhaps owing to their small size, have never been reported to cause thrombosis or otherwise occlude vessels. This fact may make swifter clinical adoption more likely by further reducing risk.

Third, manufacture and delivery of EVs may prove to be simpler and produce a more reliable supply chain than cells. Cell therapy requires that cells be grown for each patient, and that they then be delivered intact and sterilely at the point of care. This requires the infrastructure to thaw and formulate the cells on site, or else a manufacturing facility in very close proximity. By contrast, EVs are stable at 4 °C for relatively long periods of time, with little detectable difference in the cargo of

exosomes that were collected fresh or stored for several weeks [32]. This remarkable property makes central manufacturing and formulation much nearer to reality than could ever be possible with parent cells. For example, EVs could potentially be loaded into premade IV bags that could be stored on site at hospitals that serve stroke and TBI patients. Because of their relative stability, the product could be on site for immediate use, with a time buffer of potentially many weeks. Pre-formulation of a hypothetical exosome product obviates the need for experienced technicians to prepare treatments on site on a patient-by-patient basis, and allows for central quality control in a way that cell therapy does not.

The most pressing barrier to quick clinical adoption of EVs for treatment of stroke is their relatively short history compared to cell therapy. Some of the earliest investigations of EVs for treatment of any neurologic disease were published by our lab in 2013 [30]. These first reports demonstrated that MSC derived EVs could impart therapeutic benefits to rats after stroke when delivered at 24 h after MCAo, and that the functional recovery of these animals is caused by enhanced white matter remodeling, including new axon growth and myelination, as well as angiogenesis. MSCs have long been known to cause remodeling of neurites and angiogenesis [33–36], further evidence that MSCs enhance functional outcomes via release of exosomes and other EVs. This finding that MSC exosomes promote recovery after stroke has been reproduced and verified by several independent labs in rats and in mice [37, 38]. Furthermore, using human cells to generate exosomes does not impact their ability to enhance neurologic recovery in rats subjected to TBI [39]. The above renders it likely that exosome-induced functional recovery after neurologic injury is generalizable across multiple species, and thus also likely applicable to human disease.

To date, the therapeutic potential of EVs derived from MSCs has been investigated most extensively preclinically. MSCs are a robust source of exosomes and other EVs, producing an abundance of them compared to other cell types [40]. However, the majority of cell types produce exosomes and microvesicles, and several of these cell types have been explored as potential sources of therapeutic EVs. For example, exosomal miR-126 is pro-angiogenic [41], and may underlie human cord blood cell mediated recovery from stroke in diabetic animals [42]; endothelial cell derived exosomes have been used to treat hindlimb ischemia [43]; and dendritic and other immune cell derived exosomes are being explored extensively as a therapy for cancer [44–48].

Most of the clinical work focused on exosomes has been dedicated to their potential as biomarkers (for review see e.g., [49, 50]). Despite their short history as a therapeutic agent, exosomes have begun to appear in clinical trials. Table 16.1 is a list of all current registered trials on clinicaltrials.gov for which ‘exosome’ is a keyword and that are targeting therapy and not biomarkers. The range of diseases is diverse, and only one so far uses MSC as a source. However, this is likely to change rapidly in the coming years.

Table 16.1 List of trials using exosomes as a therapeutic

Identifier	Institution	Disease	Source	Phase
NCT02565264	Kumamoto University	Cutaneous wound healing/ulcers	Plasma	I
NCT02138331	General Committee of Teaching Hospitals and Institutes, Egypt	Type I diabetes mellitus	MSC	II/III
NCT01159288	Gustave Roussy, Cancer Campus, Grand Paris	Non-small cell lung cancer	Dendritic cells	II
NCT01668849	James Graham Brown Cancer Center, University of Louisville	Chemoradiation-induced oral mucositis	Grape	I
NCT01294072	James Graham Brown Cancer Center, University of Louisville	Colon cancer	Curcumin-loaded plant	I

3.1 Mechanism

It is apparent that among the most important cargo that exosomes carry are miRNAs. miRNAs are often highly conserved across disparate organisms, and although they are frequently gained during evolution, they are rarely lost [51]. The number of miRNA that a species possesses correlates well with morphologic complexity [52], and any given miRNA may target many genes in a single gene network, thereby possessing the ability to efficiently shut down redundant systems [53]. More than 700 miRNAs can be detected in exosomes and other EVs [54], and they are mostly bound to Argonaute 2 (Ago2) [55, 56], a major constituent of the RNA-induced silencing complex (RISC). Silencing of targets by miRNA is RISC-dependent, so the fact that miRNA in exosomes are bound to Ago2 suggests that they are destined to bind to mRNAs in recipient cells (i.e. be functional). All this points to exosomes being a potent system to pass "information" from cell to cell in a manner that other macromolecules cannot.

It has been shown that miRNA expressed in one cell can suppress protein expression in another cell through innate mechanisms [57]. Although more than one pathway for targeted inhibition of translation from one cell to another may exist, exosomes represent a major mechanism by which this information transfer occurs. It has been shown in many studies across multiple independent labs that specific proteins can be suppressed in cells in a predictable way when the cells are incubated with exosomes containing targeting miRNA [57–60]. Therefore, the likeliest way that exosomes function is to release their miRNA contents into target cells upon being internalized, thereby affecting gene networks in the recipient cells. This hypothesis is supported by a number of studies in which the miRNA cargo of exosomes was altered to target specific genes. Xin et al. showed that over-expressing miR-133b in MSC exosomes enhances functional recovery after MCAo to an even greater extent that naïve exosomes [61, 62]. Additionally, miR-17-92 cluster

expression can target neurons and promote axonal growth via suppression of PTEN [63], and exosomes enriched in miR-17~92 constituents promote functional recovery and axonal growth more efficiently than naïve exosomes [64, 65]. It is likely that in the future, better methods of expression and more predictive targeting algorithms will allow for even more refined tuning of the therapeutic properties of exosomes.

3.2 Neurovascular Niche

Recovery from stroke is dependent on remodeling of the neurovascular niche [66, 67]. Exosomes have been shown to affect multiple aspects of the neurovascular unit during recovery from stroke and brain injury, and in *in vitro* injury models. For example, when exposed to MSC exosomes, astrocytes are stimulated to release exosomes of their own, which in turn induce downstream remodeling of axons [68]. Indeed, exosome treatment after stroke is associated with improved axonal growth and myelination [30, 64, 68]. In an apparent feedback loop, neuronal exosomes also contain biomolecules that target astrocytes, including PTEN and miR-124, which limit astrocyte proliferation and increase expression of the amino acid transporter GLT-1, respectively [69]. Furthermore, oligodendrocytes secrete exosomes that impact neuronal behavior, helping to coordinate myelination [70, 71] and supplying protective molecules in stress conditions [72].

As the name suggests, the other half of the neurovascular unit comprises cerebral blood vessels, whose function after stroke is coupled to recovery of brain parenchyma, and exosomes from MSCs promote angiogenesis [30, 37, 73]. Endothelial cells communicate with each other via exosome secretion. For example, endothelial cell exosomes contain miR-214 and miR-126, both of which are pro-angiogenic miRNAs, and they also contain angiopoietin-1 (Ang1), the primary ligand of Tie2 receptor and a potent inducer of angiogenesis [43, 74]. Endothelial exosomes also contain delta-like 4 (dll4), a notch ligand that maintains endothelial stasis [75]. The exosome system may represent a way for a large and distributed tissue such as the endothelium to maintain homeostasis over a large surface area and long distances. Treatment of hindlimb ischemia with endothelial derived exosomes significantly improves recovery of function and angiogenic sprouting [76], suggesting that supporting the natural cell communication system in the endothelium could be a strategy for treating cardiovascular disease. Dysfunction of the natural endothelial cell exosome axis may lead to pathologic conditions that are prevalent in stroke and other cardiovascular disease, such as atherosclerosis. Endothelial cells from sclerotic vessels secrete exosomes with cargo that is distinct from healthy cells [77], which may trigger damage and recruitment of inflammatory cells.

The other relevant question in cell therapy with respect to the cerebral endothelium is whether MSC exosomes can cross the blood-brain barrier (BBB). Several lines of evidence suggest that they can. First, brain tumor exosomes can readily be detected in blood, which suggests crossage of the BBB [78]. Second, *in vitro* evidence shows that endothelial cells can actively transport exosomes across the BBB [79]. Although circumstantial, these reports provide clear evidence of instances in which exosomes can in

fact cross the BBB intact. Due to their heterogeneous nature as aggregates of biomolecules that can be disaggregated *in vivo*, directly observing exosomes *in vivo* after injection is difficult, but it seems likely that therapeutic exosomes can enter the brain [80].

3.3 Inflammatory System

The inflammatory system, both in the brain and in the periphery, may mediate cell therapy after stroke [81]. When introduced IV, exosomes encounter macrophages and other immune cells of the periphery almost immediately, and macrophage depleted animals clear exosomes much slower than wild-type animals [82]. The half-life of injected exosomes in wild type rats may be as little as 2 min, with total clearance happening by 4 h [83]. The exact role of the peripheral immune system in mediating cell therapy has not been fully described, but some evidence suggests that its presence is necessary for enhancing recovery [84, 85]. Additionally, the majority of injected exosomes lodge in the peripheral organs, including the lungs, liver, and spleen [82, 83, 86], although these studies do not agree as to which organ is the primary point of exosome uptake, which may be cell source dependent. An open question for scientists who are developing exosome therapies is whether the interaction of exosomes with peripheral organs contributes to or inhibits their effectiveness as therapeutic agents. It may be that one of the ways in which exosomes from MSCs and other cells impart functional benefits is by "reprogramming" the immune system to behave in a way that supports recovery. Secreted vesicles from MSCs suppress secretion of pro-inflammatory cytokines from stimulated microglia *in vitro* [87]. In turn, secreted inflammatory factors from microglia, such as IL-1α and TNFα, stimulate astrocytes to suppress synapse formation [88], which may have serious deleterious effects on recovery. Indeed, microglia help coordinate tissue remodeling after injury, and can encourage oligodendrocyte differentiation and myelination during recovery [89–91]. Conversely, neuronal secreted exosomes can recruit microglia to prune synapses [92], which may be an innate mechanism for normal function, but also could potentially contribute to dysfunction in degenerative disease states, as aberrant synapse pruning is a hallmark of early Alzheimer's disease and other forms of dementia, for example [93, 94]. Therefore, the potential of MSC exosomes to reprogram microglia to adopt a pro-recovery phenotype is perhaps one of their greatest assets.

4 Summary and Conclusion

The use of cell therapies for recovery from stroke has gained prominence and traction in recent years due to their effectiveness in treating animal models of brain injury. Their use in clinical trials, of which hundreds now are registered, is therefore warranted, as no other regenerative or restorative treatment is available to patients.

However, exosomes are only now beginning to be investigated as a potential next generation replacement for or adjuvant to cell therapy, but awareness of them is rising quickly. Several clinical trials have been registered to investigate the use of exosomes for diseases such as cancer, wound healing, and diabetes. Should they prove to be safe and effective, exosomes will become one of regenerative medicine's best hopes for treating patients with stroke and other debilitating CNS diseases. In animal models of stroke, TBI, cognitive decline, and other CNS diseases, they have been shown to have a great impact at lessening the disease severity and quickening and deepening recovery.

The therapeutic impact of exosomes is multifactorial, but is certainly dependent on three identifiable factors: (a) the surface proteins that determine the targeted cell type; (b) the miRNA cargo that determines their function in target cells; and (c) secondary release of exosomes and paracrine factors from target cells. Deeper understanding of each of these factors will doubtlessly affect our ability to design custom treatments for stroke and other CNS diseases that are currently untreatable. Exosomes represent a unique opportunity to advance cell therapy to a place of safety, efficacy, and manufacturability that currently does not exist.

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Chapter 17
Exosome and MiRNA in Stroke

Ji Bihl, Jinju Wang, Xiaotang Ma, Yi Yang, Bin Zhao, and Yanfang Chen

Abstract Stroke is one of the leading causes of death and disability worldwide. Various types of stem cells have been applied to treat stroke and have been shown promising potential. The principal mechanism of therapeutic action has been partially ascribed to their strong paracrine capacity. Exosomes are small vesicles released from all kinds of cells and mediate intercellular communication by transferring exosomal protein and microRNA (miRNA) cargoes between cells in the brain. Among these cargoes, miRNAs play a key role in mediating biological function due to their prominent roles in gene regulation. Emerging data suggest that stem cell-released exosomes have advantages over stem cells to treat stroke, because exosomes could cross the blood bran barrier and easily to be modified and handled. Here, we first review the biogenesis, cargoes, and detection of exosomes. Then, we discussed the role of miRNAs in stroke. At last, we highlight the use of stem cell-released exosomes as biomarkers and therapeutic avenues in stroke. Perspectives on the developing role of stem cell-released exosomes mediated transfer of miRNAs as a therapeutic approach will also be discussed.

Keywords Stroke • Exosomes • miRNAs • Brain microenvironment • Biomarker • Therapy

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P.A. Lapchak, J.H. Zhang (eds.), *Cellular and Molecular Approaches to Regeneration and Repair*, Springer Series in Translational Stroke Research,
https://doi.org/10.1007/978-3-319-66679-2_17

Abbreviations

Ago2	Argonaute 2
AMPA	Alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
BBB	Blood-brain barrier
BDNF	Brain-derived neurotrophic factor
CD	Cluster of differentiation
CNS	Central nervous system
CSF	Cerebrospinal fluid
CSPGs	Chondroitin sulfate proteoglycans
CTGF	Connective tissue growth factor
DCs	Dendritic cells
Dll4	Delta-like 4
ECs	Endothelial cells
EpCAM	Epithelial cell adhesion molecule
EPCs	Endothelial progenitor cells
EPC-EXs	EPC-released exosomes
ESCART	Endosomal-sorting complex responsible for transport
EVs	Extracellular vesicles
EXs	Exosomes
FGF2	Fibroblast growth factor 2
GFAP	Glial fibrillary acidic protein
GFP	Green fluorescence protein
GluR2/3	Glutamate receptor AMPA R2/3
H/R	Hypoxia/reoxygenenation
HMGA2	High mobility group AT-hook 2
HSCs	Hematopoietic stem cells
IFN-γ	The interferon gamma
IGF	The insulin-like growth factor
L1CAM	Neuronal-specific protein L1 cell adhesion molecule
Lamp-2	Lysosomal-associated membrane protein 2
MAP 1b	Microtubule associated protein 1b
MCAO	Middle cerebral artery occlusion
miR-126-EPC-EXs	Exosomes released from miR-126 primed EPCs
miRNA	MicroRNA
MOR	Opioid receptor mu
mRNA	Messenger RNA
MSCs	Mesenchymal stromal cells
MVB	Multivesicular bodies
MVs	Microvesicles
NPCs	Neural progenitor cells
NPC-EXs	NPCs-released exosomes
NTA	Nanoparticle tracking analysis

PEG	Polyethyleneglycol
PTEN	Phosphatase and tensin homolog
Rab5	Ras-related protein
RARβ	Retinoic acid receptor β2
RhoA	ras Homolog family member A
RISC	RNA-induced silencing complex
RVG	Rabies virus glycoprotein
SGZ	Subgranular zone
Shh	Sonic hedgehog
STAT1	Signal transducer and activator of transcription 1
SVZ	Subventricular zone
TNFα	Tumor necrosis factor-α
VEGF	Vascular endothelial growth factor
VEGFR2	Vascular endothelial growth factor receptor 2
VPS4	Vacuolar protein sorting 4

1 Introduction of Exosomes

Exosomes are extracellular nanozided (30–100 nm) vesicles generated from endosomal membranes. Exosomes are generated inside cells from multivesicular bodies (MVB) and released into extracellular space via exocytosis. MVB are endosomal organelles that are characterized by internal membrane-enveloped vesicles, which were described for the first time, in neurons, by Palay and Palade in 1955 [1]. Originally, these vesicles were regarded to be pre-lysosomal structures involved in protein degradation [2]; however, recent evidence indicates that MVB mediate diverse intra- and intercellular trafficking of molecules [3].

Nearly three decades after the discovery of intracellular MVB, Trams et al. analyzed cell-free supernatants collected from human neuronal neoplastic cell lines. The supernatants were span at the speed of 100,000 × *g* for 90 min, and the pellets of these supernatants were then studied by electron microscope [4], which resulted in the discovery of extracellular membrane-enveloped vesicles ranging in size from 40 to 1000 nm. The supernatant pellets possessed enzymatic activities, including 5-nucleotidase and ATPase, suggesting that the vesicles could play some physiological roles rather than just be cellular waste products [4]. Figure 17.1 shows the process of isolating microvesicles and exosomes from cell-free supernatants.

The extracellular vesicles (EVs) found by these researchers that are today known as exosomes (<100 nm), as well as larger ectosomes (also known as microvesicles or microparticles) that typically range in the size 0.1–1.0 μm (Table 17.1). While exosomes have been shown to be derived from MVB, ectosomes shad from cellular membranes [5–9] and differ in their content, implying that exosomes and ectosomes may have distinct functions [10, 11]. In this chapter, we focus on exosomes.

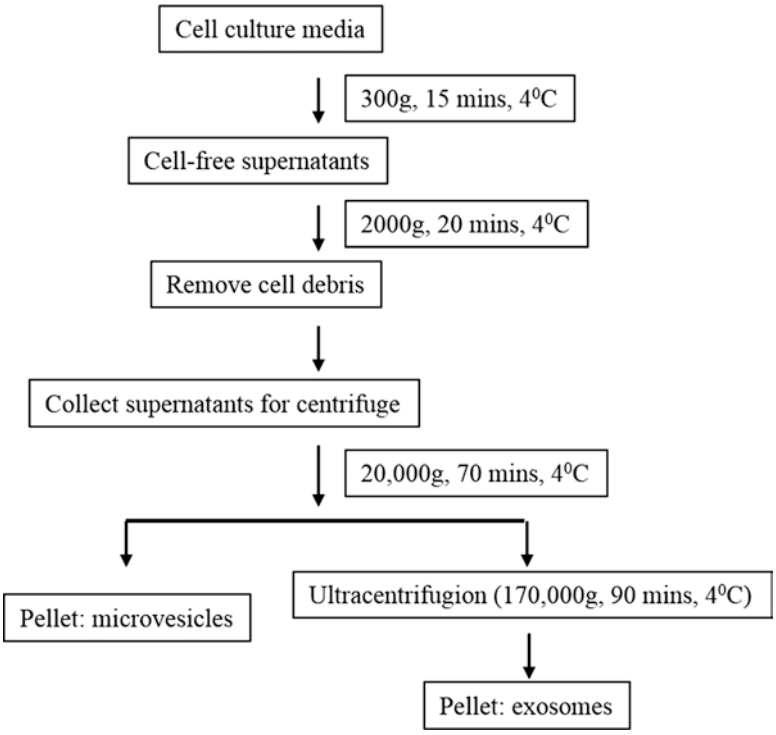

Fig. 17.1 Exosome isolation from cell culture media. The cell culture medium was collected and centrifuged at 300 × *g* for 15 min, followed by centrifugation at 2000 × *g* for 20 min to remove cells and cell debris. The cell-free culture medium was centrifuged at 20,000 × *g* for 70 min and then ultracentrifuged at 170,000 × *g* for 90 min to pellet exosomes

Table 17.1 Key features of microvesicles and exosomes

	Microvesicles	Exosomes
Size range	0.1–1 μm in diameter	30–100 nm in diameter
Biogenesis	Budding from plasma membrane	Exocytosis of MVBs
Markers	Annexin V binding, tissue factor and cell-specific markers	CD63, CD81, CD9, and Tsg101

2 Biogenesis of Exosomes

Exosomes are formed by inward budding. Therefore, the orientation of proteins and lipids of the exosome membrane is equivalent to the plasma membrane of the parental cell [12]. MVBs are late endosomes, which are refined by early endosome maturation. This maturation results in gradual changes in content and composition of the

membrane [13]. It is suggested that phosphatidylinositol-3 kinase activity is required for both the formation of MVBs and the formation of exosomes [12, 14]. A previous study showed that the inhibition of phosphatidylinositol-3 kinase results in swelling of several endocytic compartments and inhibition of MVB biogenesis [14].

The machinery involved in the formation of MVBs is relevant to exosome production and function. There are two possible fates for the MVBs: fuse with the plasma membrane, or fuse with a lysosome followed by digestion of the cargo [15]. It was reported that cholesterol rich MVBs are prone to release exosome and cholesterol-poor MVBs are targeted to lysosomal digestion [16]. Another study showed that incorporation of membrane proteins such as growth factor receptors designates MVB to lysosomal degradation [12]. The molecular machinery involved in the biogenesis of exosomes can be dependent on or independent from endosomal-sorting complex responsible for transport (ESCART). The ESCART-dependent system is associated with accessory proteins such as programmed cell death 6 interacting protein and vacuolar protein sorting 4 (VPS4), which are used as exosome markers in many studies [15]. Alternatively, in the presence of sphingomyelinase, the exosome biogenesis might be independent from ESCART. Trajkovic et al. showed that inhibition of sphingomyelinase significantly decreases the release of exosomes [17]. Simons et al. showed that MVB formation can even be independent from ESCART and sphingomyelinase. By their results, tetraspanin proteins enriched in MVBs can play a major role in the formation of exosomes [18].

MVBs designated to exocytosis release the exosomes into the extracellular space by fusion with the plasma membrane. The release of exosomes can be constitutive or inducible depending on the cell type and the state of cell activation [19, 20]. For instance, immature dendritic cells (DCs) and epithelial cells release exosomes in a constitutive manner [21]. Another study has indicated that members of Rab family are involved in classical intracellular trafficking and in fusion of cellular compartment. It was also observed that a subset of this family, such as Rab27a and Rab27b, is involved in the secretion of exosomes [22].

3 Isolation and Characterization of Exosomes

Exosomes can be isolated from cell culture supernatant and plasma using several different techniques. The most common isolation technique is differential centrifugation, whereby large particles and cell debris in the culture medium/plasma are separated using centrifugal force between 200–100,000 × g and the exosomes are separated from supernatant by centrifuging at 100,000 × g [12, 23, 24]. Exosome purity can be improved by centrifuging the samples using flotation density gradient centrifugation with sucrose or Optiprep, which results in highly purified and enriched exosomes [25, 26]. Another common technique for exosome isolation is

the monoclonal antibody based method. Antibodies against exosome-associated antigens—such as cluster of differentiation (CD) molecules CD63, CD81, CD82, CD9, epithelial cell adhesion molecule (EpCAM), and Ras-related protein (Rab5)—are used for separation [25, 26]. The antibodies can be immobilized in different media conditions and combined with magnetic beads, chromatographic matrix, plates, and microfluidic devices for separation [26]. A draw back to this technique is that non-exosomes vesicles that carry the antigens also bind to the antibody, reducing the purity of the extracted exosomes [25]. Ultrafiltration is another way to isolate exosomes based on their size differences. This method is less time consuming compared to ultracentrifugation and does not require special equipment [27]. One alternative method for isolating exosomes based on size is using high performance liquid chromatography, which provides highly purified exosomes [25, 27].

Exosomes can be characterized based on their size, protein content, and lipid content. Several methods have been used to characterize exosomes, including flow cytometry, nanoparticle tracking analysis, dynamic light scattering, western blot, mass spectrometry, and microscopy techniques [28]. Exosomes can also be characterized and marked based on their protein compositions. There are a number of proposed exosome reference markers such as lysosomal-associated membrane protein 2 (Lamp-2) and Rab5B, which is a member of the RAS oncogene family [27]. Also, proteins such as Pdcd6ip, TSG101, tetraspanin proteins resulting from exosome formation in MVBs (CD9, CD63, CD81 and CD82) and proteins enabling intracellular membrane fusion and transportation [21] are often used as markers to detect and identify exosomes [29]. There are kits to detect exosomes in vivo such as Exosomal Cyto-Tracer, the lentivector based Cyto-Tracer (Biocat GmbH) expresses the tetraspanin CD63, CD9 or CD8, which are fused to GFP or RFP. In this way, exosomes are marked and long-term and in-depth experimentation is enabled. However, these proposed markers are just general markers for total exosomes or even microvesicles and apoptotic bodies [30].

In future studies, a method to isolate/detect a pure specific population of exosomes is needed and it is important for exosome research. Related, the authors recently published a protocol to combine microbeads and Qdot to detect endothelial cells (ECs) and endothelial progenitor cells (EPCs) specific exosomes [31, 32]. In this protocol (Fig. 17.2), we used ECs or EPCs specific makers: CD105/CD144 or CD34/vascular endothelial growth factor receptor 2 (VEGFR2) to detect the EC-exosomes and EPC-exosomes. After selected with microbeads conjugated first antibodies (CD105 or CD34), the concentration of EC-exosomes and EPC-exosomes from cell culture and plasm were recognized by Qdot-labelled second antibodies (CD144 or VEGFR2) and measured by Nanoparticle Tracking Analysis system (NTA) (NS300, Malvern Instruments). Compared to the transitional flow cytometry method, the NTA has better sensitivity and specificity to detect the total exosomes, EC-exosomes and EPC-exosomes.

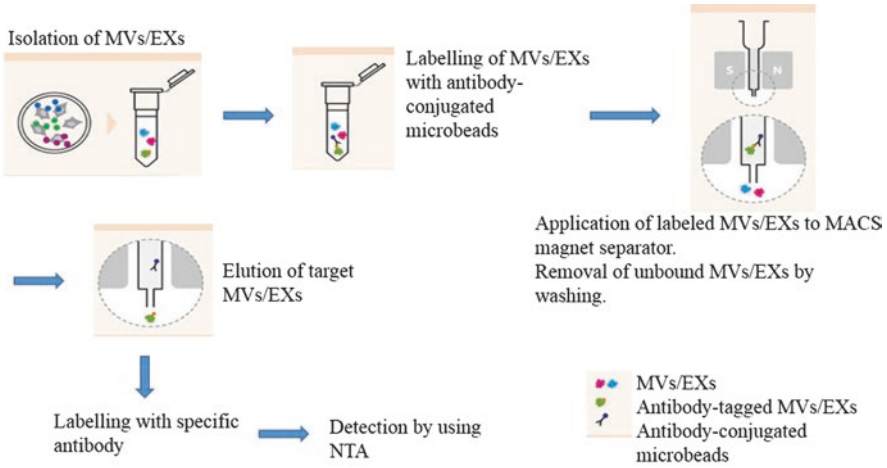

Fig. 17.2 Application of microbeads and Qdot for detection of specific MVs and EXs. The pelleted EXs were incubated with 10 μL of Biotin-conjugated specific antibodies (Miltenyi Biotec), such as anti-CD105 for ECs or anti-CD34 for EPCs, in a 100 μL reaction volume for 2 h, followed by adding 10 μL of anti-Biotin microbeads (Miltenyi Biotec). Then, a magnet module was applied to separate microbeads-labeled EXs from the total EXs. After an overnight separation, the fluid was gently removed from the magnet. The microbeads bound EXs were resuspended with 100 μL filtered PBS and added with 10 μL of multisort release reagent (Miltenyi Biotec) for 10 min to cleave off the microbeads from EXs. Afterwards, the second antibodies, such as anti-CD144 for ECs or anti-VEGFR2 for EPCs, were added to the selected EXs. After overnight labeling, the fluid was collected and enumerated by using the NTA NS300 system (Malvern Instruments). *MVs* microvesicles, *EXs* exosomes, *MACS* magnetic-activated cell sorting, *NTA* nanosight tracking analysis

4 Cargoes of Exosomes

At the beginning, it was assumed that the content of exosomes is random due to the engulfed part of the cytoplasm packaged by the membrane blebbing [33]. Later, it was observed that the content of exosomes released by mesenchymal stromal cells (MSCs) differs from their parental cells, probably caused by selective packaging. Although the sorting mechanisms of nucleic acids and proteins are poorly understood, there are some suggestions for sorting proteins inside exosomes, such as sorting via ESCART, via lipid and/or protein affinity or via sorting by protein incorporation into detergent-resistant protein complexes [19]. It is mentioned that exosomes contain some common and also cell-type-specific proteins [13].

Thery et al. reported that exosomes do not contain proteins that originated from the nucleus, mitochondrion, endoplasmic reticulum or Golgi apparatus. Instead, proteins identified in exosomes were observed in the cytosol and plasma membrane [21]. In contrast, a subsequent study by Record et al. reported that exosomal proteins could also originate from the endocytotic compartment, Golgi and nucleus, but rarely from the endoplasmic reticulum or mitochondria [19]. RNAs detected in exo-

somes of MSCs consist of mainly messenger RNA (mRNA) and microRNA (miRNA). No track of 18S or 28S ribosomal RNA was detected. Indeed, in these exosomes, the portion of mRNAs is relatively small, while majority of small RNAs as well as miRNAs were observed in precursor form [34]. Furthermore, there are essential differences between mRNA transcripts in parental cells compared to mRNA transcripts detected in exosomes [35]. Such a selection was also observed for a number of detected miRNAs, which are assumed to be exclusively packed into exosomes [36]. Baglio et al. have observed that some miRNAs are present in both exosomes and parental cells [37]. Our research group further found that the miR-126, miR-210 and miR-18a are present in both exosomes and parental cells, with higher level in exosomes.

This observation supports the existence of control mechanisms for selective packaging of miRNAs for at least MSCs [36]. Likewise, in AZ-P7a cells (a metastatic gastric cancer cell line) an enrichment of let-7 miRNA family members in exosomes was observed. The exosomal release of let-7 miRNAs into the extracellular environment maintained the oncogenesis and invasiveness of AZ-P7a cells by at least partially neutralizing the inhibitory effects of let-7 miRNAs on their targeting oncogenes such as RAS and high mobility group AT-hook 2 (HMGA2) [35, 38, 39]. In another study, it was shown that the enrichment of miRNAs in the exosomes derived from DCs is selective as some miRNAs are detected in parental cells but not in the exosomes and vice versa [40].

As a matter of fact, miRNAs are delivered to distant cells by either miRNA containing exosomes or via free miRNA molecules bound to Argonaute 2 (Ago2). It was shown that Ago2 is not only bound to free extracellular miRNAs but also to miRNAs within exosomes and other membrane-derived vesicles [38]. In most cases, miRNA was found single stranded in exosomes, yet precursor hairpin miRNAs were also detected [34, 38]. It is not clear if Ago2 is necessary for miRNA export [38]. The regulatory functions of miRNAs are accomplished through the RNA-induced silencing complex (RISC) [41]. It is believed that the mature miRNA not connected to RISC is not functional. Pre-miRNA can be loaded with RISC followed by cleavage into functionally mature RISC-loaded miRNA. The favored secretion of pre-miRNA into exosomes suggests an important physiological role after being taken up by target cells and also supports the conception that the content of exosomes is not just random [34]. Notwithstanding, miRNAs isolated from plasma exosomes, which are mostly platelet derived, has a significant different composition compared to platelets and peripheral blood mononuclear cells. As a consequence, many miRNAs are uniquely present in exosomes isolated from plasma [42].

The number and content of exosomes consistently vary based on the microenvironmental conditions of the cells and, particularly, if cells are subjected to stress factors [36]. It is shown that the miRNA content of exosomes in the plasma is different between normal and tumor-induced tissues [42]. Breast cancer cells produce exosomes with a changed pattern of miRNA [43]. Another example is that the stimulation of increased intracellular Ca^{2+} in neutrophils affect the content of exosomes released from that neutrophils [44, 45]. We also demonstrated that the miR-126 and

caspase 3 levels of microvesicles released from EPCs under tumor necrosis factor-α (TNFα) stimulation are different from the microvesicles released from EPCs under serum free condition [46]. It is important to mention that apoptotic bodies, microvesicles and exosomes contain fundamentally different RNA profiles. For instance, microvesicles isolated from cell culture often do not contain a considerable amount of miRNA. Ribosomal RNA is primarily found in apoptotic bodies [30].

Finally, the miRNA content of exosomes also depends on the maturation state of the parental cell. The miRNA content of exosomes derived from mature DCs, which promote immunity, and the miRNA content of exosomes derived from immature DCs, which downregulate T cell responses, were observed to be significantly different. However, there is no significant difference in the amount of miRNA between exosomes derived from mature DC and immature DC [40]. A selective loading is not only observed for protein and RNA but also for other types of molecules packed into exosomes. Regarding DNA as a content of exosomes, there exists a disagreement. One study announced that exosomes contain no DNA [35]; later, it was observed that astrocyte-derived exosomes might contain mitochondrial DNA [47, 48]. This discrepancy in the reported data demonstrates the need of further investigations in this field.

5 Exosomes as an Important Player in the Brain Microenvironment

The brain is composed of different cell types, including neurons, astrocytes, oligodendrocytes and microglia. Specific cell-cell contacts and particular extracellular cues originated both locally and distantly. Cells communicate reciprocally with other cells by (1) intercellular contacts, and (2) secreted molecules, such as growth factors, cytokines, hormones, etc. (paracrine or endocrine communication). A novel way of cell-to-cell communication mediated by exosomes, which carry a specific cargo of proteins, lipids and nucleic acids and are currently considered one of the most complex and physiologically relevant messengers in brain microenvironment. These extracellular vesicles are secreted by neural cells under both normal and pathological conditions and have been isolated not just from the cerebrospinal fluid [49] but also from adult human brain [50]. The suggested roles of exosomes in brain microenvironment as messengers for communication between neural and vascular cells. They are important by secreting and transporting multilevel information, including signaling, factors, and regulatory molecules. Initially thought to have a function merely in waste disposal, the involvement of exosomes in neuronal and vascular development, maintenance, and regeneration through its paracrine and endocrine signaling functions has drawn particular attention in recent years. The physiological function of brain is supported by different cellular and molecular components in the brain, balancing quiescence with proliferation, and regulating cell differentiation.

5.1 Exosomes in Neuron–Neuron Communication

Classical inter-neuronal communication involves synaptic transmission, a dynamic and plastic process that is tightly regulated by neuronal activity [51]. Exosome-mediated communication between pre and post synaptic cells participates in synaptic plasticity, as it has been shown in the Drosophila neuromuscular junction [52]. Using cultures of mixed hippocampal cells with exosomes derived from the neuroblastoma cell line N2a and labeled with green fluorescence protein (GFP)-CD63 and GFP-TTC, it was found that they interact either with neurons, astrocytes or oligodendrocytes. On the other hand, exosomes released by cortical neurons upon synaptic activation interact with neurons but not with glial fibrillary acidic protein (GFAP) + astrocytes. Furthermore, some exosomes co-localize with synaptophysin indicating that they bind to pre-synaptic sites [53].

5.2 Exosomes in Neuron–Glia Communication

The communication between neurons and glia is important for brain physiology during both development and adulthood. The different glial cell types help to maintain neuronal activity. Oligodendrocytes protect axons with the myelins heath and also provide trophic support to neurons [54]. To maintain these functions over time there is a constant communication between neurons and oligodendrocytes, but the mechanisms underlying this phenomenon are not well understood. Frühbeis et al. demonstrated that upon glutamate stimulation, oligodendrocytes secrete exosomes, which are endocytosed by neurons. Furthermore, exosomal cargoes improve neuronal metabolism and viability in situations of nutrient deprivation or oxidative stress exposure [55]. It is also noteworthy that this work demonstrated that the internalization of exosomes by neurons occurs through aclathrin and dynamin-dependent mechanism, shedding light on the mechanisms that may be involved in exosome internalization. On the other hand, the selective elimination of synaptic connections comprises the engulfment of neurites. In a recent study, it was shown that neuron-derived exosomes stimulate microglial phagocytosis of neurites via upregulation of complement factors [56].

5.3 Exosomes in Glia–Glia Communication

The communication between glial cells through exosomes has been studied to a lesser extent. Exosomes secreted by oligodendrocytes are selectively internalized through macropinocytosis by microglia, both in vitro and in vivo [57]. Remarkably, only those microglial cells that do not show antigen-presenting capacity endocytose exosomes, thus supporting the idea that different types of microglial cells co-exist and are differentially involved in immune functions.

5.4 Exosomes in Neuronal-Vascular Cell Communication

Cross talk among different classes of brain cells is also essential to generate the blood-brain barrier (BBB), which then maintains the brain internal milieu, by controlling trafficking of molecules and ions between the brain and the blood [58]. For example, in a transwell coculture system, containing both rat cortical astrocytes and neurons, brain capillary ECs were found to form over time a functional barrier layer, even in the absence of cell-to-cell contacts [59, 60]. EVs released from astrocytes/neurons have been found in ECs. Astrocytes, neurons, and ECs, metabolically labeled with 35S-methionine, as well as from unlabeled ECs, incubated for 24 h with microvesicles shed from labeled astrocytes or neurons. The results indicate the presence of metabolic protein in both labeled neurons and ECs incubated with EVs from neurons. This provides evidence to support the possibility that central nervous system (CNS) derived exosomes may potentially interact with ECs within the brain, and that they may potentially find their way to the bloodstream, where they could interact with ECs and with cells of the immune system.

5.5 Exosomes as Novel Regulators of Adult Neurogenic Niches

Niches are defined by their ability to anatomically house stem cells and functionally control their development in vivo [61]. In the mammalian brain, there are defined regions termed neurogenic niches, areas with the proper environment that are able to support and modulate neurogenesis during adulthood [62]. The first validated and most studied neurogenic niches of the brain are the subventricular zone (SVZ) of the lateral ventricles and the subgranular zone (SGZ) of the dentate gyrus in the hippocampus [63]. Nevertheless, other brain regions have been proposed as having putative neurogenic niches (e.g., substantia nigra, cerebellum, and amygdala) though the extent at which this happens in vivo and in humans remains controversial for some of them. Neurogenesis has been shown to occur in the brain after stroke [64], and recent studies have shown that adult neurogenesis is active in the hippocampus [65] and in the striatum [66, 67].

An efficient and well-regulated communication between cells is vital to ensure brain homeostasis and plasticity throughout life, particularly in the adult neurogenic niches. Thus, exosomes can serve as physiological or pathological messengers between cellular components of the neurogenic niche and coordinate the function of the adult neurogenic niches. First, neural stem/progenitor cells and most of the cell types present in the CNS, including those cells that constitute and regulate the neurogenic niche, secrete and/or are target of exosomes. Furthermore, some of the biomolecules expressed (and secreted) by niche cells have been reported to be present in exosomes under physiological or pathological conditions. Interestingly, several examples highlight the role of exosomes as (1) messengers between neural

cells (neurons and glial cells) either locally or distantly [via cerebrospinal fluid (CSF) or volume transmission]; (2) blood-CNS communicators (including their potential as therapeutic vehicles); and (3) modulators of several stem cell niches [68].

5.6 Exosomes in the CSF as Volume Transmission Vehicles

It has been proposed that the CSF compartment plays an essential role in volume transmission within the central CNS; thus, molecules or messengers secreted in one brain region may reach the CSF and exert their function in sites located far from its secretion site [69, 70]. Given the close contact between the CSF and the interstitial fluid of several brain areas, including the SVZ, it is conceivable that exosomes originated in the brain parenchyma can be found in the CSF and vice versa. Actually, isolation of membrane vesicle-enriched fractions and further proteomic studies have demonstrated the presence of exosomes in the human CSF [49, 71, 72]. Furthermore, the exosome content of the CSF is supposed to reflect ongoing brain processes, and especially those related to plasticity, disease or repair. Proteins related to the onset or progression of some CNS diseases such as Alzheimer's disease, prion disease, and Parkinson's disease, among others, have been found in the exosomal fraction of CSF-samples [73]. Exosomes in the CSF decrease with age while those derived from the embryonic CSF positively act on the stem cell niche [49, 74], revealing their influence on recipient cells. A clear demonstration of exosomal secretion into the CSF has been recently obtained in epithelial cells. Using cell-culture assays, human CSF analyses and in vivo tracing experiments, the authors describe a novel pathway of exosome-mediated folate delivery into the CSF and subsequently, into the brain parenchyma [72].

6 MiRNAs in Stroke

Stroke is one of the leading causes of death and disability worldwide. Stroke is classified into ischemic, which accounts for around 85% of cases, and hemorrhagic, which accounts for the remaining 15% [75]. Ischemic stroke occurs as a result of an obstruction within a blood vessel supplying blood to the brain, while the hemorrhagic stroke is the rupture of a blood vessel in the brain.

Ischemic stroke is a sudden loss of neurologic function resulting from a focal disturbance of cerebral blood flow due to the occluding of blood vessels. The goal of acute stroke therapies is to normalize perfusion and to preserve the maximal amount of penumbral tissue ischemia. Because it is a multifactorial disease with a short therapeutic window many clinical stroke trials have failed and the only currently approved therapy is thrombolysis.

Primary hemorrhagic stroke is caused by small arteriole ruptures, which stem from vascular pathological changes [76, 77]. The vascular pathologies include

intimal thickening, plaque formation, vascular remodeling, atherosclerosis and aneurysms, which are vulnerable to vessel ruptures. After vessel rupture, the hemoglobin enters brain tissue and induces a series of consequential pathological changes, such as edema, cell damage/death and inflammation, which ultimately cause neurological deficit.

MiRNAs are a novel and abundant class of 19- to 22-nucleotide (nt) noncoding RNAs that control gene expression at the post-transcriptional level. They play a role in the regulation of gene expression at the post-transcriptional level, via degradation or translational inhibition of their target mRNAs. miRNAs are especially important candidates for stroke therapeutics because of their ability to simultaneously regulate many target genes and since to date targeting single genes for therapeutic intervention has not yet succeeded in the clinic.

6.1 The Role of miRNAs in Ischemic Stroke

Several studies have demonstrated alterations in the cerebral "miRNA-ome" following ischemia/reperfusion [78–80] suggesting that miRNA may be an important factor in modulating the gene expression cascade that occurs in response to ischemia/reperfusion. Changes in miRNAs with ischemic brain injury have been identified using miRNA profiling techniques in a rat middle cerebral artery occlusion (MCAO) model [78, 79, 81] and in forebrain ischemia [82] as well as in stroke patients [80]. An acute alteration of the miRNA profile following cerebral ischemia would suggest that miRNAs play a role in the early stress response to ischemia in the brain, as either a negative or positive regulator of cell survival. Dharap et al. [81] demonstrated in a rat model of MCAO that while the expression of several miRNAs was altered up to 3 days post-ischemia/reperfusion, a progressive increase in miR-140, miR-145 and miR-331 was observed as early as 3 h following reperfusion.

Recently a few studies have evaluated the significance of individual miRNAs in ischemic brain damage [82–85]. miR-15a has been shown to contribute to the pathogenesis of ischemic vascular injury. Gain or loss of miR-15a significantly reduced or increased oxygen–glucose deprivation induced cerebral vascular EC death, respectively [84]. In vivo repression of miR-497 using antagomirs was found to effectively lower miR-497 levels, reduce MCAO induced infarct, and improve neurological deficits [85]. Transfection of miR-200 b and miR-200 c into Neuro-2a cells increased neural cell survival when subjected to oxygen glucose deprivation [86]. Additionally, miR-210 is positively correlated with better prognosis in stroke patients [87]. A key finding demonstrated by Buller et al. was regional expression of miR-121, primarily in the ischemic penumbra [88]. Such regional specificity of expression adds another layer of complexity to miRNA expression profiles, and may explain seemingly conflicting results in the literature. For example, in a rat model of global cerebral ischemia, Yuan et al. [82] reported that hippocampal miR-181a was upregulated following 30 min of reperfusion, however no change in brain miR-181 was observed either following permanent focal ischemia [79] or following

transient focal ischemia [81]. Ouyang et al. demonstrated [83] in a mouse model of MCAO that regional expression of miR-181 differed according to the distribution of blood flow and that anti-miR-181 can protect the brain from ischemia.

6.2 The Role of miRNAs in Hemorrhagic Stroke

The miRNA expression of a hemorrhagic insult to the brain have been investigated [80]. Total of 381 miRNAs were screened with TaqMan miRNA arrays 24 h following intraventricular injection of fresh blood, lysed blood or thrombin. Different patterns of miRNA expression were observed in both blood and brain following hemorrhage. Two miRNAs (miR-498 and miR-200b) were upregulated and one (miR-155) downregulated in both brain and blood in at least two paradigms. However, none of these were significantly different from controls in both brain and blood in at least two paradigms. Following injection of lysed blood, 17 miRNAs were upregulated and 12 downregulated more than twofold in brain, while 21 miRNAs were upregulated and 20 downregulated more than twofold in blood. A report has examined circulating miRNA expression profile in patients with intracerebral hemorrhage, focusing on discriminating intracerebral hemorrhage with hematoma enlargement from intracerebral hemorrhage without hematoma enlargement [89]. In total, 866 miRNAs were screened with microarray analysis, 30 of which were differentially expressed between intracerebral hemorrhage patients with hematoma enlargement and intracerebral hemorrhage patients without hematoma enlargement (Table 17.2). At present, there are no human studies investigating in detail the differential expression of circulating miRNAs in intracerebral hemorrhage compared to normal controls. The ability to predict in the clinic which patients is likely to develop hemorrhage would be more helpful. Furthermore, the authors found that the biological processes implicated by the differentially expressed miRNAs include apoptosis, inflammation, coagulation and collagen biosynthesis [89].

6.3 The Potential Role of miRNAs in Stroke Therapy

There are two major approaches to develop miRNA-based therapeutics: mimics to increase effective levels of a miRNA, and inhibitors or antagomirs to reduce them. miRNA mimics are small, chemically modified, double stranded RNA molecules that load the active strand into the RISC which then binds the target mRNA to induce translational silencing. miRNA mimics can be used to restore a loss of function of beneficial miRNAs. miRNA inhibitors and antagomirs (which differ in their chemical modifications and intended use in vivo) are modified single stranded antisense oligonucleotides harboring the full or partial complementary sequence to the mature miRNA, to reduce endogenous levels of the miRNA and increase expression of its mRNA targets. miRNA inhibitors/antagomirs can inhibit endogenous

Table 17.2 Microarray profiling of microRNAs after stroke

MicroRNAs	Types of stroke	Expression in stroke	Models	References
miR-7, -9, -27a, -29 (b and c), -30e, -92, -98, -101a, -137, -148b, -152a, -204, -218, -301, -338, -335, -369-5p, -376 (b and b), and -424	Ischemia/reperfusion	Down-regulation	Rat-brain	[78]
miR-134, -138, -145, 206, -210, -214, -215, -223, -290, -292-5p, -298, -324-3p, -327, -422b, -451, -494, and -497	Ischemia/reperfusion	Up-regulation	Rat-brain	[78]
miR-155, -362-3p, -223, -210	Ischemia	Down-regulation	Rat-brain	[79]
miR-10a, -182, -200b, -298	Ischemia	Up-regulation	Rat-brain	[79]
hsa-let-7f, miR-126, -1259, -142-3p, -15b, -186, -519e, -768-5p	Ischemia	Down-regulation	Human -blood	[80]
hsa-let-7e, miR-1184, -1246, -1261, -1275, -1285, -1290, -181a, -25, -513a-5p, -550, -602, -665, -891a, -933, -939, -923	Ischemia	Up-regulation	Human-blood	[80]
miR-376b-5p, -153, -29c, -98, -204, -26b, -29b, -338, -301, -341, -377, -664	Transient MCAO	Down-regulation	Rat-brain	[81]
miR-140, -145, -331, -290, -214, -324-5p, -324-3p, -344-3p	Transient MCAO	Up-regulation	Rat-brain	[81]
let-7e, miR-98, -125a-5p, -139-5p, -150, -204, -323, -329, -352, -384-5p, -539, -7a, -7b, -92a, -338 and -92b	Global cerebral ischemia/reperfusion	Down-regulation	Rat-brain	[82]
miR-143, -16, -181a and -495	Global cerebral ischemia/reperfusion	Up-regulation	Rat-brain	[82]
miR-155, -20b-3p, -200a	Hemorrhage	Down-regulation	Rat-brain	[79]
miR-298,a -200b,a - 205, a-345, -423-5p, -298,a-423-5p,a -10a, -345-5p, -674	Hemorrhage	Up-regulation	Rat-brain	[79]

miRNAs and could be applied to reduce miRNAs with pathogenic function in stressed cells or diseased tissues. Endogenous circulating miRNAs have been found to be stable because of their packaging and secretion into the blood within exosomes [33]. Increasing evidence supports the key role of miRNAs in the regulation of exosome function [35]. Our previous studies have reported the expression of

miR-126 in EPC-released microvesicles. Using miR-126 mimics or inhibitors could up-regulate and down-regulate the level of miR-126 in both EPCs and EPC-released microvesicles [90]. Moreover, we found that over-expressing of miR-126 could enhance the function of EPCs and EPC-released microvesicles. More recently, our pilot study show the similar results on the EPC-released exosomes (data not published yet). Taken together, exosomes might play an important role in stroke by transferring their carried miRNAs.

7 Exosomes and miRNAs in Stroke Pathogenesis and Pathophysiological Processes

All brain cells release exosomes [55]. Emerging data suggest that exosome-mediated intercellular communication contributes to brain remodeling by transferring cargo from source cells to target cells. Exosomes can be isolated from biofluids such as CSF and from the supernatant of cells cultured in exosome-free medium by centrifugation and other methods [55]. Exosomes are generally enriched with tetraspanin proteins (CD63, CD81), the regulator of endosomal trafficking Alix, and the chaperone protein HSP70, although the content of exosomes varies with cell origin and physiological and pathological conditions [55, 91]. Tetraspanins, Alix, and HSP70 have been used as exosomal markers [91]. Proteomic and RNA analyses have demonstrated that exosomes carry cargoes of lipids, proteins, and RNAs, including mRNAs and miRNAs [55, 91]; however, it is unclear how biological materials are loaded into individual exosomes.

More recently, miRNAs have been reported to mediate the function of exosomes and play an important role in strokes [35, 92]. For example, downregulation of miR-15a in cerebral vessels in a mouse model of focal cerebral ischemia promotes stroke-induced angiogenesis in the peri-infarct region by increasing fibroblast growth factor 2 (FGF2) and vascular endothelial growth factor (VEGF) levels [93]. VEGF released by angiogenic endothelial cells also interacts with its receptor VEGFR2 in neural progenitor cells to promote their proliferation and neuronal differentiation [94]. Moreover, cerebral endothelial cells in white matter are involved in regeneration of myelinating oligodendrocytes through brain-derived neurotrophic factor (BDNF) and FGF2 in injured brain [95]. Stroke-induced limited axonal sprouting and remyelination in the periinfarct region are also regulated by miRNAs. In vitro and in vivo studies showed that stroke-induced downregulation of miR-9 and miR-200b expression in white matter mediates remyelination [96]. Chondroitin sulfate proteoglycans (CSPGs) produced by reactive astrocytes inhibit axonal regrowth [97]. Overexpression of the miR-17–92 cluster or miR-27a in cultured cortical neurons activates neuronal intrinsic growth signals by suppressing phosphatase and tensin homolog (PTEN) and ras homolog family member A (RhoA) signals, thereby overcoming the CSPG inhibitory effect [98, 99]. In vivo studies of spinal cord injury in adult animals have shown that suppression of the PTEN signaling pathway within neurons enhances axonal sprouting even in the presence

of CSPGs [98, 100]. Thus, the miRNA and mRNA networks play a pivotal role in mediating brain-repair processes [101].

8 Exosomes and miRNAs in Regeneration and Repair After Stroke

Angiogenesis is a vital component of tissue repair processes after stroke. Besides angiogenesis, neurogenesis is another potential target for treatment of stroke. There is evidence showing that angiogenesis accompanies neuroprotection and neurogenesis during the recovery process of ischemic stroke [102, 103]. Blockade of angiogenesis attenuates the process of neuron generation [103]. Furthermore, neuroblasts migrate along these regenerated vessels to achieve neurogenesis in peri-infarct areas [103–105].

8.1 Exosomes and miRNAs in Cerebral Angiogenesis

In vitro and in vivo experiments have shown that exosomes from circulating EPCs transfer cargo mRNAs associated with the PI3K/Akt signaling pathway and proangiogenic miRNAs, such as miR-126 and miR-296, into recipient ECs [106, 107]. Within the recipient ECs, these miRNAs activate the PI3K/Akt signaling pathway, leading to angiogenesis [106, 107]. In the brain, exosomes from cultured glioblastoma cells induce angiogenesis by delivering their contents of proangiogenic proteins, mRNAs, and miRNAs into cerebral ECs [108]. Additionally, immortalized human brain microvascular ECs secrete exosomes [109]. Proteomic analysis has demonstrated that exosomes released by human cerebral ECs contain 1179 proteins, including several receptors that carry macromolecules across the BBB, such as transferrin receptor and insulin receptor [109]. The role of these exosomal proteins has not been investigated, but interactions between cerebral endothelial exosomes and pericytes have been studied [110]. Exosomes secreted by immortalized mouse cerebral ECs stimulated by lipopolysaccharide and cytokines transferred cargo miRNAs and increased VEGF-B mRNA and protein levels in recipient cerebral vascular pericytes [110].

In addition, activation of the Notch signaling pathway between cerebral ECs and pericytes is required for cerebral angiogenesis and BBB integrity [111]. For example, Delta-like 4 (Dll4), a membrane-bound Notch ligand expressed by cerebral ECs, stimulates Notch3 receptors on pericytes to keep the cerebral vascular structure quiescent [112]. Exosomes released from human microvascular ECs and human umbilical vein ECs contain Dll4 proteins and have been shown to regulate development of angiogenesis [113, 114]. The Notch signaling pathway interacts with the VEGF signaling pathway [115, 116]. Together, these data suggest that cerebral endothelial exosomes could communicate with pericytes to mediate angiogenesis and to maintain BBB integrity through the VEGF and Notch signaling pathways.

8.2 Exosomes and miRNAs in Neurogenesis

Neural stem cells in the SVZ exist in a unique niche where they contact blood vessels, neighboring cells, and CSF, constantly exchanging molecular signals [117]. There is evidence that exosomes in CSF and neural stem cells mediate neural stem cell function and immune system function, respectively, by regulating intercellular pathways [74, 118]. Exosomes isolated from embryonic CSF of rats and humans contain protein and miRNA components of the insulin-like growth factor (IGF) signaling pathway [74]. CSF-exosome cargoes, including both proteins and miRNAs, are highly conserved between rodent and human [74]. Incubation of embryonic neural stem cells with CSF exosomes activate the IGF/mTORC1 pathway in the neural stem cells and promoted stem cell proliferation [74]. Exposure of neural stem cells derived from SVZ of adult mouse to proinflammatory cytokines leads to release of exosomes enriched with mRNAs encoding components of the interferon gamma (IFN-γ) signaling pathway [118]. These exosomes activate signal transducer and activator of transcription 1 (STAT1) signaling in recipient cells through exosome-associated IFN-γ and its receptor IFNGR1 [118]. Stroke activates innate and adaptive immune responses [119]; thus, exosomes released by neural stem cells may also communicate with the immune system after stroke.

Moreover, exosomes released by cultured primary cerebral ECs and neural progenitor cells (NPCs) harvested from nonischemic and ischemic animals. Proteomic and miRNA array analyses revealed that stroke substantially changed exosomal cargo proteins and miRNAs compared with exosomes from those nonischemic cells, indicating that stroke alters exosomal contents from cerebral ECs and NPCs. Exosomes derived from ischemic NPCs promoted primary EC migration and capillary tube formation, whereas exosomes from ischemic cerebral ECs enhanced NPC proliferation and neuronal differentiation. These data suggest that exosomes secreted by cerebral ECs and NPCs contribute to the observed coupling of neurogenesis and angiogenesis during brain repair processes after stroke. In addition, cerebral endothelial exosomes could also actively engage in brain remodeling by communicating with brain cells, including neurons and glia, and with remote cells in other organs during stroke recovery [120].

8.3 Exosomes and miRNAs in Neuronal Plasticity

Neurons and glia actively communicate with each other to coordinate axonal growth and myelination. Emerging data suggest that exosomes released by neurons and glia contribute to these processes [55, 121, 122]. Exosomes released by cultured cortical neurons carried the neuronal-specific protein L1 cell adhesion molecule (L1CAM) and the glutamate receptor AMPA R2/3 (GluR2/3) subunits, but not the NR1 subunits, of glutamate receptors [123, 124]. Increasing cytosolic calcium in neurons and neuronal depolarization augmented the secretion of exosomes [123–125].

Notably, exosomes released by neurons contain alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors, while exosomes secreted from neurites of depolarized neurons are enriched with microtubule associated protein 1b (MAP1b) and miRNAs that target genes involved in neurite plasticity [124, 125]. Exosomes from neurons treated with a retinoic acid receptor β2 (RARβ) agonist had a dual effect on neurons and astrocytes to inactivate PTEN signaling, leading to enhancement of neurite outgrowth [126]. RARβ agonist inactivated cortical neuron PTEN signaling by releasing exosomes enriched with PTEN. These PTEN-enriched neuronal exosomes transferred PTEN proteins into astrocytes to suppress astrocyte proliferation [126]. In addition to neurons, exosomes released by cortical neurons transferred miR-124 to astrocytes and increased the expression of the excitatory amino acid transporter GLT-1 in astrocytes, suggesting a role for neuronal exosomes in regulating astrocyte function [127]. AMPA receptors and MAP1b are key regulators of synaptic and dendritic plasticity and axonal spouting [124, 125, 128]. The astrocyte glutamate transporter GLT-1 in rodent regulates extracellular glutamate levels and modulates synaptic activation [127]. Activation of AMPA receptors contributes to motor function recovery after stroke [129]. Suppression of neuronal PTEN signals and reduction of an astrocyte scar promote axonal sprouting in adult CNS after spinal cord injury and stroke [98, 100, 130]. Together, these data suggest that neuronal exosomes mediate synaptic and axonal plasticity by synaptic transfer of their cargo between neurons and by communication with astrocytes [124, 131], which potentially mediate axonal and synaptic remodeling in the ischemic brain.

9 Exosomes and miRNAs as New Noninvasive Biomarkers or Diagnostic Tools of Stroke

Bodily fluids are also promising sources of molecular biomarkers, which can be divided into three categories: molecules, proteins, and mRNAs/miRNAs [132]. The advantage of biomarkers obtained from bodily fluids (i.e., CSF, blood, plasma, serum, saliva, and urine) is the possibility of searching for a large number of molecules at once, for example, by the use of proteomics or genomics, at earlier stages than those could be detected by imaging. Having a biomarker that can be accessed from a body fluid has the added advantage of not relying on expensive technology (such as imaging equipment) and, in some instances (e.g., saliva and urine), of avoiding invasive method. The main disadvantage of obtaining biomarkers from body fluids is the low levels of molecules and the heterogeneity of these, as such samples arise from a wide number of tissues. Thus, to circumvent this difficulty and to improve the specificity of the biomarker, exosomes have the most potential. Exosomes can be isolated from all bodily fluids, and they carry a complex cargo consisting of miRNAs, proteins, lipids, and DNA that in part depends on the tissue of origin and its “health or disease” state [133, 134]. Catalytically active enzymes like PTEN in neurons, via can be transferred by exosomes to astrocytes to prevent

glial scar formation and induce spinal cord regeneration [126]. Exosomes carry a set of common proteins considered as "exosome markers," most of them related to their biogenesis [25, 26], such as CD36, CD9, and etc. In addition, they carry molecules that derived from their parent cells, for example, membrane and intraluminal proteins. In the case of transmembrane proteins, they can be used to immunoisolate exosomes of a specific cellular origin, separating them from other exosomes and thus improving the sensitivity of exosomes as biomarkers [31, 32]. Of all the molecules carried by exosomes, miRNAs are the ones that have gathered the most interest in the last years. One of the aspects that make miRNAs as promising biomarkers is that they can be found in body fluids that are easily accessible, such as plasma, where they appear to be transported by lipoproteins and exosomes [135]. Exosomes provide an enriched source of miRNAs for biomarker profiling [136], and miRNAs present in blood-derived exosomes have been linked to strokes [31, 137].

There are increasing reports on the application of exosome-derived miRNAs as biomarkers for vascular diseases [138]. The possibility of extracting high quality miRNAs and profiling them using well-established methods has also contributed to making them a favored area of study in the search for biomarkers [139]. Researchers have attempted to profile the miRNA identity from body fluids of patients with ischemic stroke, hemorrhagic stroke, ischemic preconditioning and hypoxic injury, however, there are important difficulties that need to be considered if some of these molecules are going to be proposed as reliable biomarkers. The methodologies used for these profiles are not consistent between laboratories, and the sample sizes are usually small; thus, validating the miRNAs associated with neurological disorders has proven difficult [140]. It is still an ongoing process with a considerable degree of variability and efforts are constantly made to increase specificity and sensitivity.

It is well known that different kind of cells produces exosomes with a specific parental molecular signature [141]. For example, B cell receptor is selectively expressed on B cell derived exosomes, as CD11c, a specific marker of DC, is present on DC-derived exosomes [142]. Similarly oligodendrocyte derived exosomes contain the myelin associated proteins PLP/DM20 [143]. As we reported, the exosomes derived from ECs contain EC specific markers, such as CD144 and CD105. Similarly, exosomes-released from EPCs show positive to the EPC specific markers, VEGFR2 and CD34 [31]. Coherently, exosome cargo depends on the physiological/pathophysiological state of the cell when produces it [144]; for instance, inflammatory and hypoxic stimuli change the protein and RNA content of EC-derived exosomes [145]. In the acute ischemic stroke, exosomal miR-223 expression in stroke patients with poor outcomes was higher than those with good outcomes. Increased exosomal miR-223 was associated with acute ischemic stroke occurrence, stroke severity, and short-term outcomes [137]. Thus, increased circulating exosomal miRNA-223 could be a novel biomarker for ischemic stroke diagnosis. Similarly, another study has found that serum exosomal miR-9 and miR-124 are promising biomarkers for diagnosing acute ischemic stroke and evaluating the degree of damage caused by ischemic injury [146]. Larger sample are needed for further verification. Moreover, so far, there is no report on the biomarker role of exosomes for the hemorrhagic stroke.

Thus, exosomes have a great potential as noninvasive diagnostic tools for stroke. More important, considering that pathologies are related to alterations of many different cells, it is intriguing to know whether changes in the molecular signature of specific cell population are induced in response to strokes. A good challenge to solve this question is to identify a specific transmembrane protein that might be used as a marker to capture peripherally the exosomes derived from different cell types. For examples, we have identified EC-exosomes and EPC-exosomes by using the microbeads and Qdot methods following by the NTA analysis. We found that the circulating EC-exosomes and EPC-exosomes are increased after stroke on day 1, 3 and 5 in a time-dependent manner [32].

10 A Novel Approach of Stem Cell-Released Exosomes in Treating Stroke

For the treatment of ischemic stroke, thrombolytic therapy is limited by a 4 h therapeutic time window, and interventional therapies, such as angioplasty and stenting, having a high rate (20%) of re-stroke within the first year [147]. At this time, there are no effective neuroprotective drugs. Unlike the ischemic infarct, which often has an acute onset, hemorrhagic stroke usually has a progressive onset. Current treatment for hemorrhagic stroke is based on the prevention of secondary brain injury, including rebleeding and secondary brain ischemia.

The aim of stroke therapy is to restore the lost neural tissue or stimulate brain plasticity to improve the functional outcome, which subsequently improves the quality of life of patients with permanent disabilities. Stem cell based therapies have been shown to be a promising approach in achieving such results [148]. Different types of stem cells also have the potential to induce or accelerate functional recovery in animal models of intracerebral hemorrhagic stroke and subarachnoid hemorrhage [149–151]. For examples, bone marrow-derived MSCs have been shown to improve neurological outcome after stroke [105, 152], and MSC therapy is in clinical trials for stroke [153, 154]. Preclinical studies have demonstrated that MSCs promote angiogenesis, neurogenesis, and white matter remodeling in the injured brain by secreting factors to trigger the signaling pathways that are involved in brain repair [105]. Intravenous infusion of MSCs inhibits intracranial hemorrhage after recombinant tissue plasminogen activator therapy for transient MCAO in rats [155]. We have reported that bone marrow-derived EPCs have also been shown to protect the brain from acute ischemic injury via cerebrovascular protection and promote neurological recovery via increasing angiogenesis and neurogenesis [64, 156]. NPCs have also been shown to offer neuroprotection by modulating the BBB and microglial functions [157], and stimulating post-ischemic angiogenesis [158].

However, one of the key limits for a noninvasive systemic therapy of strokes is the fact that several substances are not able to cross the BBB, which is a multicellular interface composed of pericytes, astrocytes, and epithelium that becomes paracel-

lularly impermeable to certain molecules (e.g., drugs) and to most of the cells of the blood stream [159]. As a novel cell–cell communicator, exosomes released from stem cell have several advantages over stem cells: (1) exosomes could cross the BBB; (2) exosomes would not cause tumorigenesis because of lacking self-proliferation; (3) ex vivo preparation of stem cell-released exosomes can avoid these limitations that the function of stem cells could be impaired by the risk factors; (4) In addition, exosomes are relatively easy to be modified, stored and administrated. Therefore, stem cell-released exosomes hold the most potential for stroke therapy.

10.1 Exosomes as Drug Delivery Vehicles to Cross the BBB

Extensive research has been done using exosomes as vehicles for therapeutic drug delivery. One study involved the use of exosomes to deliver curcumin and treat an inflammatory disease [160]. Exosomes are employed to form a complex with curcumin for the purpose of enhancing curcumin's effectiveness. Clinical trials have also shown its efficacy and safety for cancer patients [161]. Besides enhancing the properties of drugs, exosomes are also employed to carry small molecular drugs across the BBB. Indeed, 98% of potent central nervous system drugs cannot cross the BBB and their conceptual efficacy shown in labs have not been successful in clinical trials [162]. Many Nano-formulations have been employed to solve the problems associated with the permeability of drugs across the BBB. However, other problems, such as nano-toxicity and rapid drug clearance by the mononuclear phagocyte system, have also been observed [163]. To compensate for these complications, polyethyleneglycol (PEG) has been introduced to decrease mononuclear phagocyte system drug uptake. However, this resulted in reduced interaction between target cells, consequently decreasing drug distribution in the brain [164, 165]. In this case, exosomes, an product of the body's own cells, can cross the BBB, thus improving drug transport to the brain by decreasing mononuclear phagocyte system drug clearance. Meanwhile, research has been carried out encapsulating anticancer drugs such as paclitaxel and doxorubicinin to exosomes, showing the potential of exosomes for brain delivery across the BBB and explaining their transport mechanisms, using zebrafish as an animal model [166]. In that study, exosomes were isolated from various cell lines, including gliobastoma astrocytoma U-87 MG, endothelial bEND.3, neuroecto dermal tumor PFSK-1, and glioblastoma A-172, using Invitrogen® total exosome RNA and a protein isolation kit. Exosomes were loaded with rhodamine123 and paclitaxelor doxorubicin through mixing and incubation. Experiments were then performed to characterize the isolated exosomes, the cellular uptake of the exosomes containing rhodamine123, and the cytotoxicity of the delivered anticancer drugs within the exosomes of U-87 MG and bEND.3 cells. In vivo, the ability of exosomes to deliver drugs across the BBB was examined by injecting bEND.3-derived exosomes loaded with rhodamine123 and doxorubicin, orpaclitaxel, in zebrafish embryos. At the end of the experiments, brain tissue was examined for the presence of rhodamine123 fluorescence. The results undeniably

showed the drug's distribution in the brain region of the zebrafish embryos, suggesting the ability of exosomes to deliver drugs across the BBB. In subsequent experiments, a primary brain cancer model was developed using zebrafish and anticancer drugs loaded within and without exosomes, which were compared. The data showed significant therapeutic efficacy in the zebrafish brain model treated with exosomes loaded doxorubicin compared to doxorubicin alone. Overall, the results obtained from the study showed the potential of exosomes to deliver small molecule drugs across the BBB to treat both brain cancers and neurological disorders. Our pilot study showed that intravenously infused exosomes could across BBB in the stroke brain. By tagging exosomes with red fluorescence PKH26, we observed that EPC-released exosomes could merge with ECs, astrocytes and neurons dominantly in the peri-infarct area after intravenous administration.

10.2 Exosomes as Drug Delivery Vehicles for miRNAs

Exosomes are known to naturally carry miRNA and, hence, it is logical to use exosomes as a therapeutic vehicle to deliver miRNA to targeted cells. For example, let-7a was introduced into GE11 exosomes by the lipofection method. Then, GE11 exosomes containing let-7a were intravenously injected into tumor-bearing mice. Since previous studies showed let-7a inhibited tumor growth by reducing the expression level of RAS and HMGA2, the expressions of these genes were examined in injected tumor-bearing mice, using real-time reverse transcription-PCR analysis, immunoblotting, and immunostaining. The results showed that let-7a delivered by GE11-exosomes strongly inhibited the expression of HMGA2 in cancer cells.

Moreover, intranasal administration of Odyssey 800 dye-labeled exosomes derived from a glioblastoma cell line led to distribution of fluorescent particles throughout the brain, mainly in the olfactory bulb in mice [167]. Using the Cre-loxP system, studies have demonstrated that intrahippocampal injection of Cre-recombinase mRNA containing exosomes into mice with a ROSA26-lacZ reporter activated a lacZ reporter in the hippocampal neurons [168], indicating that Cre-recombinase mRNA within the exosomes activates the reporter gene in recipient neurons. Furthermore, intravenous injection of exosomes expressing a fusion protein consisting of the neuronspecific rabies virus glycoprotein (RVG) peptide with the exosomal membrane protein LAMP2B demonstrated targeting of neurons, microglia, and oligodendrocytes in the brain [169]. Intravenous administration of the RVG peptide-expressing exosomes carrying siRNA against opioid receptor mu (MOR) enhanced the movement of exosomes across the BBB and inhibited MOR expression in the brain [170]. This data suggests that exosomes not only cross the BBB, but also deliver functional cargo to trigger gene expression in specific recipient cell types in the brain.

Our study found that EPC-released exosomes (EPC-EXs) and NPCs-released exosomes (NPC-EXs) could transfer their carried miRNAs to the vascular and brain cells. For examples, EPC-EXs transfer their carried miR-126 to the ECs, neurons

and astrocytes after co-incubation. Up-regulating the exogenous miRNAs, such as miR-210, miR-126 and miR-18a, in EPCs and NPCs could increase the expression levels of miR-210, miR-126 and miR-18a in EPC-EXs and/or NPC-EXs; and the miR-126 or miR-210 enriched EPC-EXs and/or NPC-EXs could deliver those miRNAs to the ECs, neurons and astrocytes after co-incubation. This indicates that exosomes do successfully deliver their cargo to the target cells, showing promising characteristics for drug delivery [171].

10.3 MSC Derived Exosomes and Therapies

Cultured MSCs secrete a large quantity of exosomes [172]. Emerging data from independent laboratories indicate that exosomes released from MSCs provide therapeutic benefits in stroke by modulating the brain microenvironment [173–178]. Intravenous administration of MSC-derived exosomes to rats subjected to focal cerebral ischemia substantially improved neurological function by promoting neurovascular remodeling during stroke and TBI recovery [176–178]. Subsequently, the therapeutic effect of MSC-derived exosomes has also been demonstrated by independent laboratories in the mouse subjected to stroke [173, 174]. Systemic administration of MSC-derived extracellular vesicles to ischemic mice markedly reduced motor coordination deficits and enhanced angiogenesis and neurogenesis, while treatment of TBI mice with human MSC derived extracellular vesicles substantially preserved spatial leaning ability [173, 174]. Improved neurological outcomes from these MSC-derived exosome studies are comparable to the therapeutic effect observed with MSC therapy, suggesting that MSC-derived exosome–mediated cell-cell communication may contribute to the therapeutic effect of the MSC therapy.

Exosomes transfer their cargo miRNAs to recipient cells [179, 180]. The effect of engineered MSC-derived exosomes that carry elevated miRNAs on brain remodeling after stroke has been investigated in vitro and in vivo [175, 177]. Treatment of stroke models with MSCs abolished stroke-induced downregulation of miR-133b in the ischemic brain [175]. When MSCs were cultured with extracts harvested from ischemic brain tissues, they released exosomes enriched with miR-133b. Tailored MSC-derived exosomes with elevated or reduced miR-133b were harvested from the supernatant of MSCs transfected with lentiviral vectors carrying pre–miR-133b or anti-miR-133b, respectively [175, 177]. Intravenous administration of tailored MSC-derived exosomes with increased or decreased miR-133b to rats with stroke led to enhancement or exacerbation, respectively, of axonal remodeling and neurological function compared with naturally occurring MSC-derived exosomes [177]. Connective tissue growth factor (CTGF) and RhoA are putative targets of miR-133b and are known to suppress neurite growth [175]. In vitro, incubation of cortical neurons with miR-133b-elevated exosomes downregulated RhoA and enhanced neurite outgrowth, whereas treatment of astrocytes with miR-133b-elevated exosomes suppressed CTGF, which is mainly expressed by astrocytes [177, 181].

Collectively, this data indicates that MSC derived exosomes may be used as vehicles to transport miRNAs that modulate genes in the recipient neurons and astrocytes.

10.4 Hematopoietic Stem Cells (HSCs)-Derived Exosomes and Therapies

Studies from myocardial ischemia have shown that engineered exosomes with elevated sonic hedgehog (Shh) derived from CD34+ HSCs transferred functional Shh and activated the Shh signaling pathway in recipient cells, enhancing angiogenesis in the border zone of infarction and preserving cardiac function [182]. Shh [183–185] plays an important role in the regulation of adult neurogenesis under physiological and pathological conditions [186–189]. These experiments suggest that exosomes can deliver functional proteins to modulate cellular function of recipient cells and that treatment of stroke with tailored Shh-exosomes could facilitate brain remodeling.

10.5 EPC-Derived Exosomes and Therapies

EPCs, defined as bone marrow-derived immature cells with the ability to differentiate into mature ECs, are known to participate in vascular homeostasis and angiogenesis [190–192]. EPCs have been shown to have therapeutic effects on ischemic stroke by promoting angiogenesis, neuroprotection and neurogenesis during the recovery process of ischemic stroke [102, 103]. The mechanism has been partially ascribed to EPC released growth factors and EVs, including microvesicles and exosomes. Recent studies suggest that EPC-released microvesicles could promote EC survival and proliferation, and enhance EC function [107, 193]. Our in vitro studies have shown that EPC-released microvesicles protect ECs from hypoxia/reoxygenenation (H/R)-induced injury [46] and protect cardiomyocytes from angiotensin II-induced injury [194]. Additionally, another study showed that EPC-released microvesicles enhance angiogenesis of human pancreatic islets [106]. Moreover, an in vivo study demonstrated that intravenous administration of EPC-released microvesicles protects kidneys from ischemia/reperfusion injury [195] and improve neovascularization in a murine model of hindlimb ischemia [196].

Functions of EVs in physiological and pathological processes depend on the ability of EVs to interact with recipient cells to deliver their contents (proteins, RNAs and miRs) [35]. EPC-released microvesicles have been shown to enhance angiogenesis of human pancreatic islets via carried miR-126 and miR-296 [106] and protect kidneys from ischemia/reperfusion injury through transferring pro-angiogenic miR-126 and miR-296 to target cells [195]. Our recent work demonstrated the

expression of miR-126 in EPC-released microvesicles and containing high amounts of miR-126 is associated with the protective effects of EPC-released microvesicles on ECs [46]. As we know, here are two types of EVs: microvesicles and exosomes [197]. Recent studies found that most of the miRs in the circulation are carried by exosomes [35, 198]. Of not, our recent study found that EPC-exosomes have protective effects on H/R-induced EC and neuron injury.

On top of this, gene-modified exosomes that carry specific miRs have been shown to exert better efficacy than null exosomes for treating stroke. Our previous work has shown that miR-126 primed EPC-released microvesicles have better protective effects than EPC-released microvesicles on protecting EPCs from high glucose-induced injury [90]. More important, we recently found that exosomes released from miR-126 primed EPCs (miR-126-EPC-EXs) exhibited better efficacy than EPC-EXs in persevering EC and neuron functions under H/R condition (data not published). These evidence suggests that EPC-EXs or gene-modified EPC-EXs would offer a novel approach for treating ischemic stroke by protecting brain from acute injury and promoting angiogenesis and neurogenesis for the long-term recovery.

10.6 NPC-Derived Exosomes and Therapies

As one type of stem cells in the brain, NPCs residing in the subventricular zone contact the blood vessels and directly juxtapose to ECs [199]. The two types of cells could interact with each other through direct physical contact or through paracrine mechanisms with potentially different biological effects. As showed in our previous study [200], NPCs can decrease H/R-induced ROS overproduction on ECs. NPCs could also offer neuroprotection by modulating the BBB and microglial functions [157]. Besides neuronal protection, transplanted NPCs can stimulate post-ischemic angiogenesis.

Brain functions rely on intercellular communication between neural cells, astrocytes and vascular cells. The potential role of stem cell exosomes in brain "microenvironment" manifests in several aspects such as protection of brain cells (neurons, glial cells, ECs and NPCs), maintenance of BBB hemostasis, and tissue repair mechanisms (angiogenesis and neurogenesis). Our recent data revealed that NPC-EXs have protective effects on H/R-induced neuron and astrocyte injury.

10.6.1 Perspectives

Although many studies dealing with the role of exosomes in stem cell differentiation and the use of exosomes isolated from stem cells for treatment of several diseases have been published, the involved mechanisms remain largely unknown. Further understanding of these mechanisms, which include the involved cellular pathways, may improve the use of exosomes in diagnostic and treatment methods, especially for those involving stem cells.

One of the promising avenues of exosomes-based therapy for strokes might be the combination of different stem cell-released exosomes, which could provide synergistic effects for treating strokes. We have recently discovered that EPCs and NPCs act synergistically to protect cerebral ECs from H/R-induced injury through VEGF and BDNF pathways [200]. Moreover, we found that co-transplantation of EPCs and NPCs provides synergistic effects of treating ischemic stroke by alleviating infarct volume and promoting angiogenesis and neurogenesis (data not published). Altogether, these demonstrate a new conception of angioneurogenesis coupling [157, 201]. Of note, we found that EPC-EXs and NPC-EXs synergistically protect ECs and neurons from H/R-induced apoptosis. Theoretically, combination of EPC-EXs and NPC-EXs could provide a therapeutic strategy protecting neurovascular unit by targeting both the acute and later neurological recovery phases.

Overall, an exosome-based delivery system has particular benefits such as (1) specificity, as the exosomes deliver their cargo to a specific target; (2) safety, as self-derived exosomes promote no undesired immunogenicity; and (3) stability, not only the exosomes itself as nanostructures circulating in the blood were reported stable but also the content of exosomes are protected from RNases and proteases and, therefore, can be delivered in an intact form to the target cell [29]. Despite these benefits, there are some, until now, unsolved problems such as identifying and purifying a single subpopulation of endogenous cell-specific exosomes.

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Part III
Neuronal Environment, Plasticity and Repair Mechanisms

Chapter 18
Integrating Molecular, Cellular, and Systems Approaches to Repairing the Brain After Stroke

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Abstract A stroke implies a sudden and spontaneous onset of neurological symptoms due to a vascular insult. Despite the brain's inherent capacity for plasticity and spontaneous improvement, strokes still leave many patients with devastating deficits that can permanently affect independence and quality of life. This chapter focuses on ways to help restore the functionality of the central nervous system (CNS) after this type of injury. Understanding how neurons interact on both individual (i.e. cellular and molecular) and population (i.e. synapses and circuits) levels is crucial to developing successful restorative strategies, as is appreciating how these interactions change over the injury-recovery timeline. The CNS has several characteristics that make its restitution exceptionally difficult; beyond even its incredible intricacy, its parenchymal cells, or neurons, do not regenerate well after injury, and this damaged neuronal substrate embodies a consciousness system that must be engaged in its own recovery. In fact, there is now data suggesting that conscious intention, often invoked through goal-oriented rehabilitation, plays a crucial role in facilitating functional plasticity and long-range axonal sprouting. To capitalize on this principle, neural interfaces and electrical stimulation strategies are being integrated into rehabilitation paradigms to provide critically-timed feedback that

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P.A. Lapchak, J.H. Zhang (eds.), *Cellular and Molecular Approaches to Regeneration and Repair*, Springer Series in Translational Stroke Research,
https://doi.org/10.1007/978-3-319-66679-2_18

can reinvigorate injured circuits. Combining these approaches with interventions at the cellular and molecular level (e.g. immunological or genetic modulations aimed at promoting neuronal outgrowth, or stem cells that can replace damaged parenchyma) has the chance to improve neurological recovery to back toward baseline levels. Ultimately, because cells of the CNS do not regrow on their own, and because regrowth and synapse formation does not necessarily ensure restoration of function, harmonious application of synergistic approaches at both the micro- and macroscopic levels will be needed to establish long-lasting functional plasticity and meaningful recovery.

Keywords Neural repair • Neural regeneration • Stroke • Neurorehabilitation • Brain-machine interface • Brain-computer interface • Neural interface • Axonal regeneration • Neural restoration

Abbreviations

AMPA	α-Amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
AP	Action potential
BCI	Brain-computer interface
BCM	Bienenstock–Cooper–Munro
BMI	Brain-machine interface
BSDS	Brain state dependent stimulation
cAMP	Cyclic adenosine monophosphate
CIMT	Constraint-induced movement therapy
CNS	Central nervous system
CNTF	Ciliary neurotrophic factor
CPP	Cerebral perfusion pressure
CSPG	Chondroitin sulfate proteoglycans
DBS	Deep brain stimulation
DOC	Disorder of consciousness
DRG	Dorsal root ganglion
FDA	Federal Drug Administration
FES	Functional electrical stimulation
GABA	Gamma-aminobutyric acid
GAP43	Growth associated protein 43
GDF10	Growth and differentiation factor 10
ICA	Internal carotid artery
ICP	Intracranial pressure
IFG-1	Insulin-like growth factor 1
LTD	Long-term depression
LTP	Long-term potentiation

M1	Primary motor cortex
MAG	Myelin-associated
MAI	Myelin-associated inhibitory molecule
MCA	Middle cerebral artery
mTOR	Mechanistic target of rapamycin
NgR	Nogo receptor
NMDA	*N*-Methyl-D-aspartate
NSAID	Non-steroidal anti-inflammatory drug
OMgp	Oligodendrocyte-myelin glycoprotein
OPN	Osteopontin
PAS	Paired associative stimulation
PMC	Premotor cortices
PTEN	Phosphatase and tensin homolog
RGC	Retinal ganglion cell
rTMS	Repetitive transcranial magnetic stimulation
SCI	Spinal cord injury
SGZ	Subgranular zone
STDP	Spike-timing dependent plasticity
SVZ	Subventricular zone
TGF-β	Transforming growth factor beta
TGFβR	Transforming growth factor beta receptor
TMS	Transcranial magnetic stimulation

1 Introduction

The term "stroke" implies a sudden and spontaneous onset of neurological symptoms due to a vascular insult. Strokes can be ischemic from vascular occlusion or hemorrhagic from vessel rupture. Hemorrhagic strokes can be further divided by the location of extravasated blood into the subarachnoid, intraventricular, or intraparenchymal space. The mechanism and characteristics of neurological injury and resultant neurological deficits are specific to each type of stroke and its location in the brain. For example, large vessel occlusions (e.g. internal carotid artery [ICA] or middle cerebral artery [MCA]) result in cerebral infarcts with surrounding penumbras that can be devastating. On the other hand, small vessel occlusions tend to result in "lacunes," and their effects can range from asymptomatic to significant deficits (contingent on location). Hemorrhages also range from devastating to inconsequential depending on the location and size of the lesion. Because strokes occur suddenly, an inflammatory response results in edema and secondary injury over the next 2–10 days. After this process subsides, recovery begins, almost always resulting in some level of spontaneous functional improvement over the next 6–12 months [1].

Many strategies have evolved over time to either prevent (e.g. anti-hypertension therapy, diabetes control, lipid lowering agents, antiplatelet therapy) or rapidly treat (e.g. thrombolysis, thrombectomy, surgical intervention) strokes, as well as to minimize secondary injury through treatment of intracranial pressure (ICP), maintenance of cerebral perfusion pressure (CPP), and prevention of spreading depression [2]. While interventions undertaken before and during stroke have greatly improved outcomes, it has been more difficult to show clear benefit from pharmacological or other interventions undertaken to mitigate secondary injury [3]. After this phase has subsided and up until about 6–12 months, some spontaneous recovery typically occurs. Beyond this time period, the prospect for further improvement is greatly diminished [4], and the resultant neurological injury is generally considered chronic. Therefore, treatment efforts have tended to focus on the critical period of recovery (i.e. the first few months after injury). In this chapter, we focus on new discoveries in molecular, cellular, and systems neuroscience, and we discuss how their synergistic application may help further augment brain repair after stroke to improve functional outcomes in both the subacute and chronic phases of injury.

2 Molecular and Cellular Neural Repair

Although the brain demonstrates some spontaneous functional recovery after an injury via intrinsic plasticity mechanisms [1, 5–7], individual neurons of the mature central nervous system (CNS) do not self-repair. Rather, proximal axonal segments retract to form an end bulb [8–12], and distal segments initially remain electrically active before undergoing anterograde, or Wallerian, degeneration over the next 36–48 h [13]. The sparse attempts some CNS neurons make at growth cone formation and elongation after injury tend to fail because of a lack of guidance mechanisms [14–17] (although there is evidence that denervation can restore some developmental growth cues in the rat hippocampus [18]).

Though not restorative, neurogenesis does occur from niches of progenitor cells in the subgranular (SGZ) and subventricular (SVZ) zones of the healthy adult brain [19–23]. These processes support learning, memory, and olfaction. The SGZ supplies the dentate gyrus of the hippocampus, while the SVZ gives cells to the olfactory bulb and to CA1/CA3 regions of the hippocampus [24]. After a stroke, proliferation is stimulated in these areas and immature neurons are recruited to damaged sites in the striatum and cortex [25, 26]. Initially, tens of thousands of immature neurons can migrate; however, few of these cells mature and survive long-term [27, 28]. While this process is associated with some level of functional recovery, it is likely that most behavioral recovery is achieved through mechanisms other than neuronal replacement (e.g. growth factor production in local tissue and rewiring of existing connections) [22].

There are many known biological factors, both intra- and extracellular, that can alter the ability of CNS neurons to grow. Intrinsic mechanisms include transcription factors (e.g. c-Jun, Atf3, Klf family, Stat3, Sox11, and Smad1) and

regeneration-associated genes (e.g. Gap43, Cap23, Arg1, Sprr1a, Hspb1, MARCKS, stathmin family, SCG10 L1, P21/waf1, and tubulins), both of which can enhance a neuron's ability to regenerate following an injury [29–32]. Phosphatase and tensin homolog (PTEN), a tumor suppressor, plays an important role in neural regeneration, as eliminating its gene both prevents apoptosis and induces axon extension in injured retinal ganglion cells (RGC) [33, 34]. Such regeneration is dependent on the mechanistic target of rapamycin (mTOR) pathways, as inhibiting mTOR eradicates the regenerative effect of PTEN deficiency [33]. Deletion of Socs3, a suppressor of signaling through the Jak-STAT pathway, also promotes regeneration by enhancing the efficacy of ciliary neurotrophic factor (CNTF), a protein known to prevent axonal degeneration and promote neuron survival and outgrowth following injury [35, 36]. The proto-oncogene bcl-2 (and expression of its anti-apoptotic protein) also plays a key role in preventing cell death after injury, enabling axonal regrowth in RGCs with the presence of trophic factors and physiologic electrical activity [37, 38].

Extrinsic factors preventing axonal regeneration include inhibitory proteins associated with myelin (e.g. NogoA, myelin-associated glycoprotein [MAG], and oligodendrocyte-myelin glycoprotein [OMgp]), proteoglycans in the perineuronal net and glial scar (e.g. chondroitin sulfate proteoglycans [CSPGs] like aggrecan, versican, brevican, neurocan, NG2, and phosphacan), and molecules that repel axon growth during development which continue to be expressed in the mature CNS (e.g. semaphorins, ephrins, slits, netrins, robos, and Wnts) [14, 39–41]. A summary of intrinsic and extrinsic factors now to affect neural growth and inhibition is provided in Fig. 18.1 [42].

Regrettably, removal or blockage of extracellular inhibitory factors alone so far has failed to achieve extensive axonal regeneration with a few exceptions [14, 39, 43, 44]. Interestingly, a strain of dorsal root ganglion (DRG) neurons grown from CAST/Ei knockout mice are less inhibited by the same extrinsic cues listed above [40]. Cells from these mice display increased axonal growth and enhanced regenerative responses following injury when compared to C57BL/6 mice, which are abilities largely dependent on the protein activin (a member of the transforming growth factor beta [TGF-β] family) [40]. Also, deletion of receptors that bind to myelin-associated inhibitory (MAI) molecules, or Nogo receptors (NgR), has been shown to increase regeneration potential in neurons [45]. As such, anti-Nogo immunotherapies are currently of great interest [46–49]. For example, in 2014, Wahl et al. demonstrated near full recovery of skilled forelimb function in rats with large strokes after intrathecal injection of an anti-NogoA antibody followed by intensive task-specific training [49]. Injection of the NogoA neutralizing agent was shown to promote growth of corticospinal fibers from the intact forebrain motor cortex across the midline of the cervical spinal cord to the hemicord that had lost its input from the motor cortex. This new fiber sprouting was then stabilized by a goal-directed, forced-use physical therapy regimen. Importantly, sequential application of drug then training was necessary to show benefit. When immunotherapy and training were combined simultaneously, functional outcome was *poorer* compared to no treatment at all or each treatment individually, likely due to aberrant fiber branching

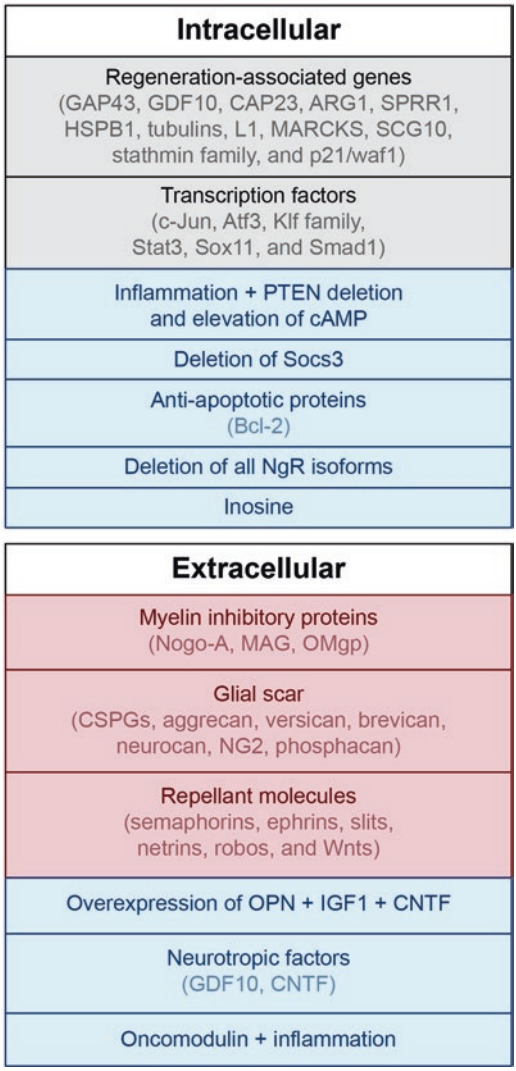

Fig. 18.1 Intra- and extracellular mechanisms of neuronal growth and inhibition. *Gray*—modulates both neuronal growth and inhibition. *Blue*—associated with neuronal growth. *Red*—associated with neuronal inhibition. Adapted with permission from Krucoff et al., 2016 [42]

(which was also seen in Maier et al. [48]). This example outlines the important distinction between regrowth and restoration of function, as well as the crucial interaction of micro- and macroscopic recovery systems.

Another example of axonal growth and synapse formation without restoration of function was demonstrated by Bei et al. when they induced adult mouse retinal axons to regrow and synapse in the superior colliculus via a PTEN/SOCS3 co-deletion and overexpression of osteopontin (OPN)/insulin-like growth factor 1 (IGF1)/CNTF [50]. However, visual function was not restored. In fact, these regenerated axons failed to conduct action potentials (AP) due to lack of myelination, and administration of voltage-gated potassium channel blockers was required to improve visual acuity.

In opposition to these inhibitory mechanisms, signals known to promote axonal growth are also present in the injured brain. For example, growth and differentiation factor 10 (GDF10) is induced in stroke and works through transforming growth factor beta receptors I and II (TGF-βRI and TGF-βRII) to promote axonal outgrowth [51]. Growth associated protein 43 (GAP43), a neuronal growth cone marker also called neuromodulin, is likewise induced in peri-infarct cortex after stroke, and it may contribute to a pro-growth environment following injury that allows for axonal sprouting and growth of dendritic trees [52–54]. Furthermore, the purine nucleoside inosine works through a direct intracellular mechanism to induce expression of genes associated with axonal growth (e.g. GAP43, L1, and α-1 tubulin) and has been shown to induce axonal reorganization and improve behavioral outcomes after spinal cord injury and stroke [55–57], as well as restore levels of GAP43 in the hippocampus in rats after stroke [34, 58, 59].

The role of inflammation in axonal regeneration is somewhat controversial. Some components of inflammation cause tissue damage and neuronal death, while others promote cell survival, axonal sprouting, and regeneration [18, 41, 60–63]. Both oncomodulin, a macrophage-derived growth factor for RGCs, and injury-induced cytokine release appear to play a role in inflammation-induced axonal regeneration [62, 64, 65]. Traditional anti-inflammatory therapies (e.g. non-steroidal anti-inflammatory drugs [NSAID]) may suppress beneficial as well as deleterious aspects of the immune response, and they can stimulate axonal regeneration via direct effects on neurons [39, 61, 66]. When combined with PTEN deletion and elevation of cyclic adenosine monophosphate (cAMP), intraocular inflammation enables some RGCs to regenerate axons from the retina to the brain and restore simple visual responses [34]. Timing of inflammation is also important, as it helps to prime the extracellular milieu for subsequent axonal entry and re-innervation for the first 10–14 days following injury [18]. This is the period when transplanted embryonic neurons have shown increased survival compared to normal brain or later transplantations [18]. For the next several months, the neural environment remains conducive to recovery due to its relatively loose extracellular space, enhanced neurotrophic factors, open synaptic sites, and probing axonal growth cones [29, 67, 68]. However, this window for recovery is limited because after 6–12 months there is less opportunity for further gain (although this doctrine is beginning to change) [69, 70]. A post-injury environment timeline is shown in Fig. 18.2.

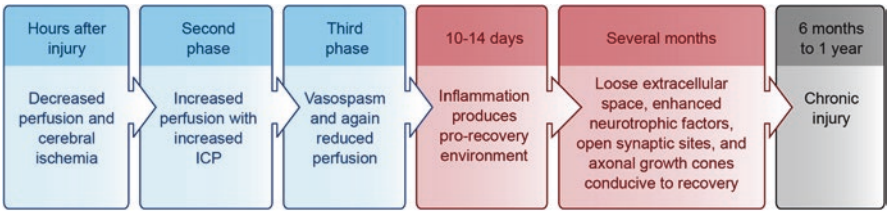

Fig. 18.2 Injury environment timeline. *Blue*—acute phase. *Red*—subacute phase. *Black*—chronic phase. Reproduced with permission from Krucoff et al., 2016 [42]

Attempts at pharmacotherapy (e.g. noradrenergic, dopaminergic, and growth factors) to limit secondary injury so far have shown limited evidence of improvement in outcomes beyond controls [71–73]. Some drugs, such as phenytoin, can even impede plasticity and recovery through suppression of growth cone-related physiological activity [3].

Cellular replacement therapy for stroke-related cell loss has the potential to significantly aid in reconstituting injured circuitry by replenishing some of the lost neuronal substrate [74]. Unfortunately, most stem cell transplants being considered in human trials are mesenchymal; in other words, they have non-neuronal origins and, thus, do not have defined mechanisms of action [75–77]. Some are even are intended to die out over time without facilitating circuit plasticity [78, 79]. Therefore, improving the stem cell derivations and defining expected mechanisms of action (e.g. facilitation of extracellular milieu, circuit reconstitution, axonal scaffolding, etc.) may lead to better results [80].

3 Neural Plasticity and Circuitry Restoration

Natural recovery after stroke occurs because the brain is inherently plastic [5, 7] and can adapt to the injury via the following mechanisms: collateral sprouting from neighboring neurons, strengthening or weakening existing synapses (i.e. long-term potentiation [LTP] or depression [LTD]), and altering concentrations of neurotransmitters, ions, gap junctions, and glial cells [81–84]. After a stroke, both hemispheres are known to assist with recovery depending on the size of the injury [85–87]. Following a small stroke within the primary motor cortex (M1), both ipsilesional dorsal and ventral premotor cortices (PMC) can reorganize themselves. However, when a lesion involves a larger portion of M1 and the dorsal PMC, the contralesional PMC appears to be critical for recovery-related reorganization [85, 87]. Initiation of post-infarct axonal sprouting from the intact cortical hemisphere to peri-infarct cortex and the contralateral dorsal striatum is signaled by synchronous neuronal activity [88]. In chronic stroke patients, activity in the ipsilesional M1 and medial-PMC is associated with good motor recovery, whereas increased cerebellar vermis activity signals poor recovery [89]. More recently, evidence has emerged for the possibility of long-range axonal sprouting in animal stroke models [49]. Context-dependent cortical activity paired with positive feedback seems to be critical for this type of axonal sprouting and lasting functional improvement. It should be noted that, while neural plasticity can contribute to functional recovery, not all re-innervations or connectivity changes are beneficial. For example, maladaptive plasticity can lead to spasticity, pathological pain, schizophrenia, and seizures [90–96].

Several avenues for functional restoration after stroke have been explored, and physical therapy plays a large role in virtually all of them [4]. For early stroke patients, constraint-induced movement therapy (CIMT) has been used to encourage use of the paretic limb by restraining the less affected one (Fig. 18.3a) [97,

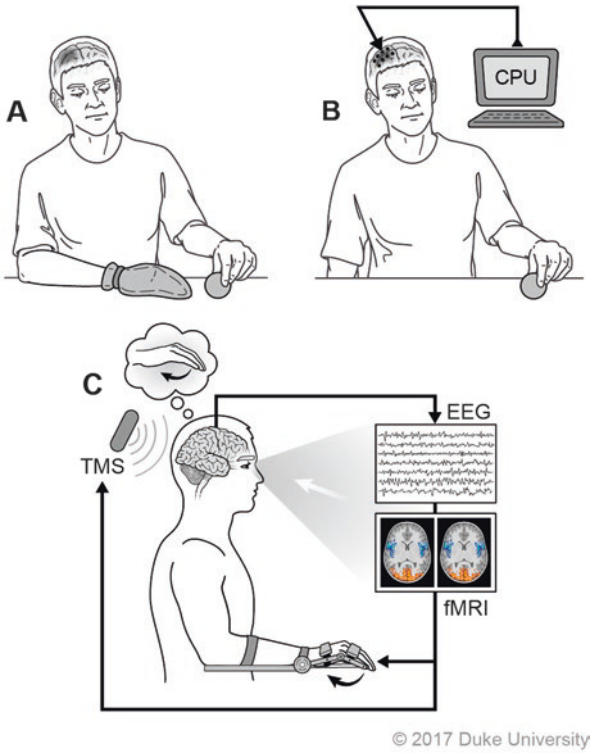

Fig. 18.3 Neurorehabilitation Modalities for Stroke. (**a**) Constraint-induced movement therapy (CIMT). (**b**) Cortical stimulation. (**c**) Closed-loop biofeedback, or brain state dependent stimulation (BSDS). Adapted with permission from Krucoff et al., 2016 [42]

98]. This is done to avoid learned non-use, as animal data has shown maladaptive changes and worse functional outcomes from allowing overcompensation with the less affected limb to dominate goal-directed tasks [99, 100]. Timing the application of CIMT appears to be crucial, however, as behavioral interventions employed too early after injury may be deleterious due to glutamate-*N*-methyl-D-aspartate (NMDA) receptor excitotoxicity in vulnerable tissue [101]. Several studies have examined the efficacy of CIMT for motor recovery in human stroke patients with mixed results [4, 102–104], and the optimal timing for its application in human stroke patients is yet to be determined [105]. Interestingly, early intensive training with immunotherapy in rat models of stroke has also been shown to be harmful due to hyperinnervation, aberrant growth, and wrong circuit connectivity, thus providing further evidence that timing of therapy is crucial yet poorly defined [49]. In yet another example, the application of γ-aminobutyric acid (GABA) antagonists or α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) agents within a few days of stroke was found to increase the size of infarction; however, when the same agents were administered 3–5 days after a stroke, motor recovery improved [106].

Because most axonal plasticity depends on electrically active growth cones [5, 7], cortical electrical stimulation as an adjunct to physical therapy has generated significant interest (Fig. 18.3b) [107, 108]. This type of brain stimulation is different from Federal Drug Administration (FDA)-approved symptomatic treatments for movement disorders, for example, where the symptoms relapse when the stimulation is stopped. In stroke recovery, brain stimulation is intended to enhance plasticity for a short duration, resulting in stable improvement even after the stimulation period. Such stimulation has shown the ability to enhance plasticity and functional recovery after stroke in rats [109, 110], squirrel monkeys [111], and some humans [107, 108]. This improvement appears to coincide with both re-emergence of movement representation in peri-infarct areas as well as the emergence of new areas of representation [112, 113]. After repeated stimulation, areas of movement representation have been seen to shift several microns and increase in size with a corresponding increase in spine density in pyramidal cell layers III and V [114, 115]. While there is some human data supporting the use of subthreshold cortical stimulation for recovery after an ischemic infarct [107], recent phase III trials have been overall negative [108]. Other forms of stimulation include deep brain stimulation (DBS) and non-invasive external brain stimulation (e.g. transcranial magnetic stimulation [TMS]), both of which may be helpful particularly during the critical recovery period [116, 117].

Closed-loop TMS paradigms have also been developed for patients who cannot participate in traditional therapy (Fig. 18.3c). In 2014, Gharabaghi et al. published a brain state dependent stimulation (BSDS) protocol in which TMS of the motor cortex and haptic feedback to a paretic hand were controlled by sensorimotor desynchronization during motor-imagery [118]. Cortex-to-cortex interfaces have also been used to bridge damaged neural pathways directly. For example, Guggenmos et al. showed that a neural prosthetic could help reconnect premotor to somatosensory cortex in an injured rat brain to restore reach and grasp functions to pre-lesion levels [109]. Both TMS and direct cortical stimulation protocols remain in the early stages of development [116, 117].

In recent years, neural interfaces, also known as brain-machine (BMI) or brain-computer interfaces (BCI), have been developed to help engage cortical circuits and enhance native motor recovery by pairing motor action (real or imaginary) with real-time positive feedback [42]. These devices directly decode information from the nervous system to generate functional outputs based on the user's intent. The rehabilitative nature of newer designs comes from pairing goal-oriented tasks with expected outcomes and re-activating lesioned circuits, thus facilitating restorative functional plasticity. This is opposed to older bypass, or assistive, BMIs which simply circumvent the lesion to perform the intended action (Fig. 18.4). As discussed above, at a molecular and cellular level, restorative functional plasticity relies on activity-dependent modulation of synaptic transmission in the forms of both LTP and LTD. As their names imply, these synaptic changes have long-lasting impacts on functional connections, and thus provide a critical substrate for the rehabilitation of damaged circuitry. The temporal relationship between pre- and post-synaptic activity is critical in these cases, a concept commonly known as Hebbian,

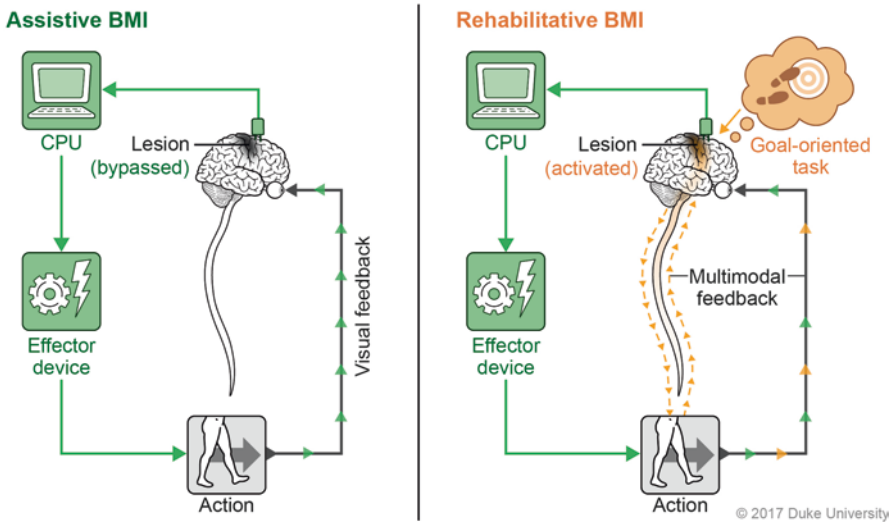

Fig. 18.4 Assistive vs. rehabilitative brain-machine interface (BMI) strategies. The assistive BMI uses nearby brain signals to bypass a neural lesion and generate an intended action. The rehabilitative BMI pairs goal-oriented tasks with positive feedback to re-activate lesioned circuits. Adapted with permission from Krucoff et al., 2016 [42]

or spike-timing dependent, plasticity (STDP) [119, 120]. Specifically, it has been shown that if pre-synaptic activity is preceded by post-synaptic activity, LTD will result. Conversely, if pre-synaptic activity is followed by post-synaptic activity, then LTP will result. This concept has been expressed in layman's terminology as, "neurons that fire together wire together," and captures the idea that synaptic strength is redistributed to favor functionally relevant pathways that are simultaneously active [121]. Mechanisms behind these observations remain largely unclear, but seem to involve the timing of NMDA receptor-mediated calcium influx into post-synaptic terminals [122]. For modeling of complex, larger scale circuits, the Bienenstock–Cooper–Munro (BCM) model can be used as a well-validated way to incorporate both pre- and postsynaptic firing rates into a circuitry stabilization metric [123, 124].

Some rehabilitative BMI approaches capitalize on STDP by using paired associative stimulation (PAS), or the act of pairing stimulation sites to promote plasticity [125, 126]. An example of a commonly used central stimulation strategy is TMS. TMS involves applying rapidly changing magnetic fields to the scalp via a magnetic stimulator. Continuous low frequency repetitive stimuli ($\leq$1 Hz rTMS) decreases excitability of targets areas (similar to LTD which is maximally evoked at 1 Hz), while bursts of intermittent high frequency stimuli ($\geq$5 Hz rTMS) enhance excitability (similar to LTP with high frequency bursts) [82, 113, 127]. These techniques have been used to induce modulation across cortico-subcortical and cortico-cortical networks through trans-synaptic spread, resulting in distant but specific changes along functional networks. Long term effects from TMS may be related to

modulation of NMDA glutamatergic receptors, similar to induction of LTP/LTD, which also modulates number, location, and properties of synaptic NMDA receptors [84]. If timed correctly, corresponding sensory inputs can be potentiated [125]. In addition to TMS, functional electrical stimulation (FES) of paralyzed muscles or electrical stimulation of the nervous system distal to the injury timed with voluntary effort has also been shown to accelerate recovery after stroke [128, 129].

4 Future Directions and Conclusions

There are many reasons to be optimistic about the potential for improving neurological recovery after stroke. Despite the brain's inherent capacity for plasticity and spontaneous improvement, strokes still leave many patients with devastating deficits that can permanently affect independence and quality of life. Advances in modern neurobiological, neural engineering, and neurorehabilitation strategies have provided hope for better outcomes, and the synergistic potential of integrated protocols is only beginning to be realized. Because most strokes occur in older individuals whose inherent capacity for neural plasticity is diminished [12], microbiological interventions are needed to prime neurons for growth cone formation, goal-directed therapy paradigms are needed to engage the consciousness system, and neural interfaces are needed to provide real-time positive feedback to help stabilize functional neural circuits. How and when to employ each of these approaches requires further study [105].

In this chapter, we have discussed evidence that goal-directed therapy is a critical to *de novo* axonal sprouting, implying that consciousness plays a direct a role in neuronal regrowth, circuitry, rewiring, and guidance [49, 130]. On a practical level, this theory has two important corollaries: (1) that patients who suffer from disorders of consciousness (DOC) (i.e. comatose or vegetative patients) may need completely different therapeutic approaches, and (2) that experiments which have failed *in vitro* may still be viable therapies when integrated into a macroscopic neurorehabilitation framework that pairs conscious intention with real-time positive feedback. For patients with DOCs, perhaps deep brain or cortical stimulation will one day be able to substitute for active participation in rehabilitation, or at least prime the system to enhance awareness. For cellular or molecular approaches, integrating these techniques into models capable of fulfilling goal-directed rehabilitation paradigms will be necessary to test their true therapeutic potential, as a potentially viable approach may otherwise fail in isolation. This applies to stem cells [131], gene therapies [132], optogenetics [133], neuronal transplantation [134], and immunotherapies [43, 48]. It should be noted that task-based rehabilitation paradigms on their own do not always demonstrate improved functional outcomes when compared to controls in certain types of strokes [135], and exactly how consciousness modulates regrowth and connectivity mechanisms remains poorly understood [136–138].

While certain molecular approaches discussed in this chapter have shown promise in the lab (e.g. multiple NgR deletions, anti-NogoA antibodies, ionosine

application, CAST/Ei knockouts), translation to bedside applications remains a challenge. This is true at least in part because replacing cells, generating plasticity, and enhancing axonal regrowth do not guarantee restoration of function by themselves [50]. The multi-year struggle to translate stem cell research into clinical benefit is an example of this challenge, as most stem cells are non-neural, do not have a defined or expected mechanism of action, and have not demonstrated the ability to alter CNS circuitry [74, 75, 77]. However, newer cells have been developed with improved neural capability that need to be tested in a strategic translational paradigm [80]. It is our hope that by combining microscopic neurobiological developments with macroscopic principles of neural circuitry and rehabilitation, success will be found where previous attempts at CNS restoration have failed [108, 135].

Conflict of Interest The authors declare they have no conflict of interest.

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Chapter 19
Neural Network Regeneration After Stroke

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Abstract Stroke remains a major cause of disability throughout the world: paralysis, cognitive impairment, aphasia, apraxia and so on. Surgical or medical intervention is curative in only a small number of cases. Stroke cases with morbidity require rehabilitation. Neurorehabilitation generally improves patient outcome, but the involved mechanisms have not been clarified. Recent advancements in technology are revealing the mechanisms of neurorehabilitation from the gene and up to neural network remodeling. Rehabilitation in clinical application, however, should be guided by convincing evidence. In this chapter we review the evidence for the regeneration of the neural network after stroke.

Keywords Cerebrovascular • Mechanism • Neural network • Rehabilitation • Reorganization • Stroke

Abbreviations

ADL	Activities of daily living
AHA	American Heart Association
AMP	Adenosine monophosphate
BDNF	Brain-derived neurotrophic factor
BOLD	Blood oxygen level dependent
CFA	Caudal forelimb area
CIMT	Constraint-induced movement therapy
DTI	Diffusion-tensor imaging
FA	Fractional anisotropy
f-MRI	Functional magnetic resonance imaging
GABA	γ-Aminobutyric acid
hBMSC	Human bone marrow stromal cells

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P.A. Lapchak, J.H. Zhang (eds.), *Cellular and Molecular Approaches to Regeneration and Repair*, Springer Series in Translational Stroke Research,
https://doi.org/10.1007/978-3-319-66679-2_19

IL	Interleukin
Muse	Multilineage differentiating stress enduring
RFA	Rostral forelimb area
TMS	Transcranial magnetic stimulation
USA	United State of America
VNS	Vagal nerve stimulation

1 Introduction

Stroke remains a major cause of disability throughout the world. It causes sudden neurological deterioration and sometimes death. From 2009 to 2012 an estimated 6.6 million Americans ≥20 years of age and 1.2 million Japanese suffered a stroke in 2014 [1, 2]. The stroke death rate has been decreasing for decades and the number of patients with a neurological deficit has thus been increasing.

The number of elderly stroke patients is also increasing, in particular in a society with progressively advancing aging [3–5]. The estimated, overall prevalence of stroke is 2.6% in the USA [2]. Previously, elderly patients were considered beyond the indication for intensive rehabilitation due to diminished treatment efficacy as compared to younger patients [6–8]. Aging is one of the key factors in stroke recovery, as older patients have less ability to fully recovered from a deficit [9]. But recent analyses suggest that inpatient neurorehabilitation provides benefits to all stroke patients, even patients over 80 years old [10]. We also found that early ambulation produces favorable outcomes and the non-demential state in elderly subarachnoid hemorrhage patients [11, 12]. Of course, not all young stroke patients will fully recover. Those who do not may still have a long life ahead. Young stroke patients also feel a greater burden of poor self-perception than do aged patients [13].

Patient neurological deficit depends on the severity and the region of stroke, not on the type of stoke. Some patients experience spontaneous recovery, but the degree of such cannot be estimated at the onset of stroke [14]. No one doubts the effectiveness of rehabilitation in counteracting a neurological deficit, but why are the outcomes of rehabilitation different in every patient? Patient-specific, stroke-type specific mechanisms of rehabilitation may well be at play, and the failure to understand the distinctions before the twenty-first century may have been the cause of therapeutic controversy.

The aim of this chapter is the clarification of the mechanisms of neural network regeneration after stroke.

1.1 Timing and Dosage of Rehabilitation in a Clinical Setting

The practice of rehabilitation started around the nineteenth century and the effectiveness of rehabilitation after stroke is common knowledge today. But the treatment strategy in rehabilitation relied on experience until a few decades ago, and the

start and dosage of rehabilitation have been controversial. In 2013 the AHA guideline for ischemic stroke recommended early mobilization of less severely affected patients, and measures to prevent subacute complications of stroke were recommended (Class I; Level of Evidence C) [15]. In 2015 the AHA guideline for spontaneous intracranial hemorrhage recommended that rehabilitation can be beneficial when started as early as possible and continued in the community as part of a well-coordinated program of accelerated hospital discharge and home-based resettlement to promote ongoing recovery (Class IIa; Level of Evidence B). The definition of 'early' was controversial. "A very early rehabilitation trial for stroke" referred to early mobilization as within 24 h, while intensive rehabilitation for non-severe ischemic and spontaneous intracerebral hemorrhage patients actually decreased favorable outcome at 3 months by four-percent as compared to typical care (46% vs. 50%) [16]. We should be concerned with the time of mobilization and, in this randomized, controlled trial (RCT), the starting time for the usual care group was from 16.5 to 29.3 h after the stroke. Neurorehabilitation should generally be carried out within 1 or 2 days after stroke.

Dromerick et al. compared three rehabilitation protocols in a single-center RCT: traditional occupational therapy involving 1 h of activities of daily living (ADL) retraining, a group with 1 h of upper extremity bilateral training activities and standard constraint-induced movement therapy (CIMT), including 2 h of shaping therapy per day and wearing a padded constraint mitten for 6 h per day, and a high-intensity CIMT group, which underwent 3 h per day of shaping and wearing the mitten for 90% of waking hours [17]. All groups continued therapy for 2 weeks and the high dosage group had significantly less improvement on day 90 [17]. Another nonrandomized, parallel-group, dosage-controlled study of aphasia therapy with distributed therapy (6 h per week; 8 weeks) showed significantly greater improvement than with intensive therapy (16 h per week; 3 weeks) [18].

In 2016 the AHA guideline for adult stroke rehabilitation and recovery did not recommend high-dosage, because very early mobilization within 24 h of stroke onset can reduce the odds of a favorable outcome at 3 months. (ClassIII, Evidence A) [19]. We need to decide on the start of and the dosage of rehabilitation for patients from the combination of the evidence and the neurological condition of the patient. Our previous clinical studies revealed that early ambulation produces physically and psychologically favorable outcomes [11, 12, 20]. Elderly stroke patients lose their physical abilities abruptly during the acute phase of stroke. At the same time, full recovery of physical and psychological functioning is difficult for elderly patients; aging diminishes tissue stem cell- and cholinergic system functioning that regenerate the central nervous system, muscle and other organs [21, 22].

1.2 Muscle Influence on Neural Networks

Muscles and bone are organs not under the direct control of the brain, but they communicate with other organs, including the brain, exerting mutual influence [23, 24]. Muscle is not only a physical organ but also a secretary organ; it secretes several

hundred peptides (e.g. interleukin (IL)-4, IL-6, IL-7, IL-15, myostatin, leukemia inhibitory factor 1, irisin, insulin-like growth factors 1, fibroblast growth factors 2, and brain-derived neurotrophic factor) [24]. Brain-derived neurotrophic factor (BDNF) is secreted by skeletal muscle, and muscle atrophy induces dementia, depression, diabetes and malignancies [21, 24–26]. BDNF protein is stored in human platelets and is released upon agonist stimulation [27]. Physical exercise training significantly increased resting concentrations of BDNF in peripheral blood, and resting concentrations of peripheral blood BDNF were significantly higher after intervention, especially in the aerobic state but not so with resistance training [28, 29]. BDNF is a contraction-inducible protein in skeletal muscle that is capable of enhancing lipid oxidation in skeletal muscle via activation of AMP-activated protein kinase [29]. Stranahan AM et al. revealed that a combination of exercise and caloric restriction increased levels of BDNF in the hippocampus, combined with an increase in dendritic spine density on the secondary and tertiary dendrites of dentate granule neurons in diabetic mice [30]. Dendrites transfected with the BDNF gene showed rapid turnover of spines, which may be a demonstration of the translation of activity patterns into specific morphological changes [31]. Rehabilitation increases circulating and intramuscular BDNF concentrations and produces neuro-reorganization in the central nervous system.

Therefore, loss of muscle strength is associated with physical disability and functional limitation [32]. In an analysis of females, reduction of both fat-free soft tissue and fat mass was associated with cognitive impairment [33]. Grip strength was also related to dementia in an aged population, especially in women [34, 35]. Careful clinical observational evaluation and treatment of muscle volume, however, is important for management. Tolea et al. showed that individuals with sarcopenia were six times more likely to experience combined cognitive impairment/physical impairment as compared to a normal control, and the effect of sarcopenia on cognition is related to low muscle strength rather than low muscle mass [21]. Sarcopenia, however, is not irreversible and we do not avoid starting rehabilitation in the chronic state. Intensive inpatient neurorehabilitation is beneficial for all stroke patients, even patients over 80 years of age [10].

1.3 Remodeling of Functional Networks Detected with MRI

Recent advanced magnetic resonance imaging (MRI) technology has revealed objective findings for rehabilitation. Rehme et al. reported functional MRI (f-MRI) scans showed robust and stronger blood-oxygen-level-dependent (BOLD) signal changes in post ischemic patients during movements of the affected or unaffected hand at various stages within 2 weeks [36]. They also revealed that the reinstatement of effective connectivity in the ipsilesional hemisphere is an important feature of motor recovery after stroke, but the supportive role of contralesional primary motor cortex into enhanced inhibitory coupling might indicate maladaptive processes that could be a target of non-invasive brain stimulation techniques [36].

In post-stroke aphasia patients, separate neuronal systems support each grammatical class—motor areas for verbs and perception areas for nouns—based on f-MRI

[37]. Semantic, feature-based rehabilitation consistently and significantly modulated the left inferior frontal gyrus in chronic post-stroke aphasia patients and in healthy volunteers [38]. Music therapy also represents a reduction in abnormal contralesional activity, an enhancement in f-MRI activity in the auditory and motor areas, and an increase in functional connectivity among several regions of the auditory-motor network in individuals suffering chronic stroke over several months [39]. Listening to music daily also significantly produced structural gray matter changes as compared to a group listening to an audiobook and a group without any listening material following middle cerebral artery stroke [40]. The gray matter reorganization in the frontal areas correlated with enhanced recovery of verbal memory, focused attention, and increased language skills, whereas gray matter reorganization in the subgenual anterior cingulate cortex correlated with reduced negative mood [40].

1.4 Transcranial Magnetic Stimulation for Stroke Patients

Transcranial magnetic stimulation (TMS) is available for the analysis of axonal integrity and to produce muscle contraction. Lefebvre et al. performed a double-blind, cross-over randomized, sham-controlled experiment with transcranial direct current stimulation [41]. Dual transcranial direct current stimulation applied during motor skill learning with a paretic upper limb resulted in prolonged shaping of brain activation, which supported behavioral enhancements in stroke patients [41]. Repetitive, continuous transcranial magnetic stimulation improved spatial neglect in stroke patients, and the effect can be evaluated by fractional anisotropy and mean diffusivity in MRI [42]. CIMT for 2 weeks yielded superior functional outcome for patients who started therapy within 9 months after stroke compared to patients who did so more than 12 months post-stroke [43]. Also, the TMS motor map shifted posteriorly in the late stroke group, and cortical reorganization did occur in those late treatment patients [43].

1.5 Neural Spine Formation in Healthy Rodents

In 2002, Trachtenberg et al. reported that new dendritic spines appeared daily in the barrel cortex of healthy mice and the elimination of some spines occurred, maintaining the density of spines [44]. Additionally, daily changes in dendritic spines in the barrel cortex *in vivo* in adult mice as well as spine sprouting and retraction are associated with synapse formation and elimination [44]. Yang et al. revealed that only 2 days of accelerated rotarod training induced new dendritic spines that were important for the reorganization of cortical circuits, and new experience led to pruning of existing synapses for the control of functional changes in cortical circuits [45]. Only 0.04% of total spines survived the first few weeks in synaptic circuits and novel experience produced pruning of a small fraction of existing spines, which promoted an integrated and stable structural basis for lifelong memory storage [45].

1.6 Animal Stroke Experiments

Animal experiments have been done to clarify among the possible mechanisms of rehabilitation. In rats with ischemic infarct in the endothelin-1 induced cortical focal ischemic model, early rehabilitation induced rapid improvement in motor function, and the forelimb motor maps were significantly enlarged compared with the no-rehab group 38 days after the stroke [46]. The authors concluded that early motor training after stroke can help shape the evolving post-stroke neural network [46]. Intensive rehabilitation significantly enhanced both the dendritic architecture and spine numbers in the adjoining rostral forelimb area in adult rats when the caudal forelimb region of the motor cortex was destroyed [22]. Those effects were diminished by cholinergic ablation [22]. Training in a forelimb reaching task leads to rapid (within an hour) formation of postsynaptic dendritic spines on the output pyramidal neurons in the contralateral motor cortex in a mouse model [47]. Forelimb skilled grasping rehabilitation did not influence the number of neurons in adult rats, but the spine density of neurons increased significantly by 22.5 ± 2.3% compared with untrained control subjects [48]. Grasping training also specifically induced spine plasticity, dendrite length and dendrite branches of C8 spine projecting corticospinal motor neurons without influencing the C4 spine situated in the same cortical layer V [48]. This synaptic plasticity contributes to neural circuits and neuro-rehabilitative outcome. Gulati et al. reported a brain-machine interface in a rat cortical focal injured model by which rats can control the firing of neurons in the perilesional cortex without motor functional recovery, and neurons form functional cell assemblies after training [49].

A time limitation for rehabilitation in various animals has been discussed. Xerri et al. reported early tactile stimulation (day 3–8) after the focal cortical ischemia reduced cortical tissue loss and digit representation loss as compared to delayed tactile stimulation (day 8–13) or without stimulation [50]. Early tactile stimulation also forced cortical re-mapping. The limitation to dendritic sprouting is 18 days after the neocortical damage [51, 52]. The immobilization of the ipsilateral forelimb (i.e. forced overuse impaired limb) after neocortical damage also prevents dendritic growth of the contralateral normal cortex and this deficiency extends beyond mobilization of the ipsilateral forelimb [53, 54]. Rehabilitation started at 5 days after the ischemic stroke achieved significant, favorable outcome as compared to starting the rehabilitation at 14 or 30 days after the stroke in the rat model [55]. An early start to rehabilitation increased the number of branches and the complexity of layer-V neurons in the undamaged motor cortex [55]. High-dosage rehabilitation treatment started at 15 days after middle cerebral artery occlusion induced improving forelimb motor function and enhanced dendritic growth of neurons at layer-V in the undamaged motor cortex [56]. Rehabilitative training for caudal forelimb area (CFA) ischemic stroke induced motor map reorganization of the rat rostral forelimb area (RFA) significantly after 2 weeks, while rehabilitative training increased the number of neurons projecting from the RFA to both the upper and lower cervical cord [57]. Four weeks of rehabilitative training after CFA ischemic stroke also

increased functional recovery and expansion of RFA due to endogenous neurogenesis in a rat model [58]. Winship et al. reported single neuron rewiring occurred after S1 cortical infarction within one month and remapping of cortical function had occurred naturally [59].

In primate focal cortical ischemic injury, 12-week rehabilitation starting 2 weeks after the stroke led to recovery to near-baseline levels, and this recovery relied on a small change in the contralesional primary motor cortex and significantly increasing motor outputs from the ipsilateral pontomedullary reticular formation [60]. Evidence from animal experiments confirms the starting time and intensity of rehabilitation in individual clinical practices.

1.7 Concept of the Cortical Network via Fasciculi

Before the twenty-first century, neurological deficit was discussed in terms of the concept of the 'eloquent' or 'non-eloquent' nucleus and specific fasciculi (i.e. the arcuate fasciculus, corpus callosum). Microsurgical tractographic anatomy and advanced magnetic resonance imaging came to represent three-dimensional white matter networks of fasciculi throughout the brain [61–67]. These findings suggested that a reconsideration of pathophysiology and the mechanism of neurological deficit was in order. Several researchers made full use of state-of-the-art diffusion-tensor imaging (DTI) technology to determine the potential neural network connectivity not only by fractional anisotropy (FA), but also using several other parameters [37, 38, 68, 69]. Steiner et al. were the first to report on the complementary nature of TMS and MRI [70]. They showed that stroke patients with a muscle evoked potentials (MEPs) reaction achieved meaningful functional recovery, but patients without MEPs combined with fractional anisotropy asymmetry that exceeded a value of 0.25 did not achieve functional recovery [70]. DTI correlated with functional recovery, and brain-computer interface training also influenced the contralesional corticospinal tract [71]. Stewart et al. compared chronic, left hemisphere stroke in ten individuals with 16 age-matched controls with respect to brain activation during right hand motor tasks by DTI [72]. The control group showed increased activation in the left inferior parietal lobule (IPL), while the stroke group showed increased activation in several right/contralesional regions, including the right IPL [72]. DTI permits an estimation of the functional recovery of patients and an evaluation of the outcome of rehabilitation [42, 71].

The semantic anatomical networks can be evaluated with MRI [73]. White matter integrity in the frontoparietal network was significantly related to haptic performance in chronic stroke, and thus the thalamus to the primary motor cortex and the precuneus interhemispheric tracts were appropriate targets for rehabilitation [74]. In an electroencephalogram experiment, weaker cortical connectivity in thalamic ischemic stroke patients suggested functional impairment of information transmission in cortical connections [75]. Both the inter-cortical network and the thalamus to the primary motor cortex may be targets for sensory rehabilitation training.

1.8 Innovative Approach to Significant Recovery

Low-dose $GABA_A$ antagonism treatment at 7 days after focal cortical ischemic injury produced 25% better functional outcome and a significant reduction in the stroke volume as compared to placebo in the rat model [76]. Left vagal nerve stimulation (VNS) paired with an isometric force task produced significant forelimb functioning in an endothelin-induced focal motor area ischemic, aged rodent model [77]. The authors speculated that the mechanism of this result is that the VNS drives neural activity in proplasticity neuromodulatory centers of the brain, including the noradrenergic locus coeruleus and the cholinergic basal forebrain [77]. VNS increases BDNF and fibroblast growth factor in the hippocampus and cerebral cortex, and activates the BDNF binding receptor (TrkB) in the hippocampus [78, 79].

Stem cell transplantation is one of the candidates for the fundamental treatment of stroke [80–86]. Human bone marrow stromal cells (hBMSC) cultured with animal protein-free medium show the same surface markers and trophic factors as BMSC cultured with animal proteins. Those cells cultured with animal protein-free hBMSC are safely available for neurotransplantation [87]. Intra carotid arterial injection of neural stem cells produced a significantly large amount of cells that migrated into brain compared to intravenous application in rat ischemic stroke [88, 89]. But the intraarterial injection of stem cells risks arterial occlusion. Animal protein-free medium cultured hBMSC survived, migrated and differentiated within infarcted brain tissue until 8 weeks after the stereotaxic implantation [90]. Uchida et al. reported early and delayed functional recovery by multilineage differentiating stress enduring (Muse) cell stereotaxic injection into an ischemic lesion in the rat model [91, 92]. We also reported Muse cell treatment in the intracerebral hemorrhage mouse model; Muse cells significantly improved neurological functioning after the early state [93]. Transplanted stem cells sprouting new axons into the cortex and spine, combined in the future with the transplantation of stem cells and neurorehabilitation, will become the fundamental treatment for stroke. Of course, we do not rush into stem cell therapy without a one-by-one evaluation of clinical trials [94].

2 Conclusion

We cannot achieve the regeneration state after stroke through one treatment alone. To rescue the brain, physicians perform thrombolysis, retrieval of the thrombus, revascularization, removal of a mass lesion or the hemostasis of a bleeding point as soon as possible after stroke (Fig. 19.1). In a next step, we medicate using several drugs to relieve edema, inflammation and free radicals. From the acute stage to the chronic stage, neurorehabilitation is done to preserve muscle volume, prevent contracture and for axonal sprouting. Consecutive control of axonal sprouting, functional compensation through the reorganization of intercortical connections,

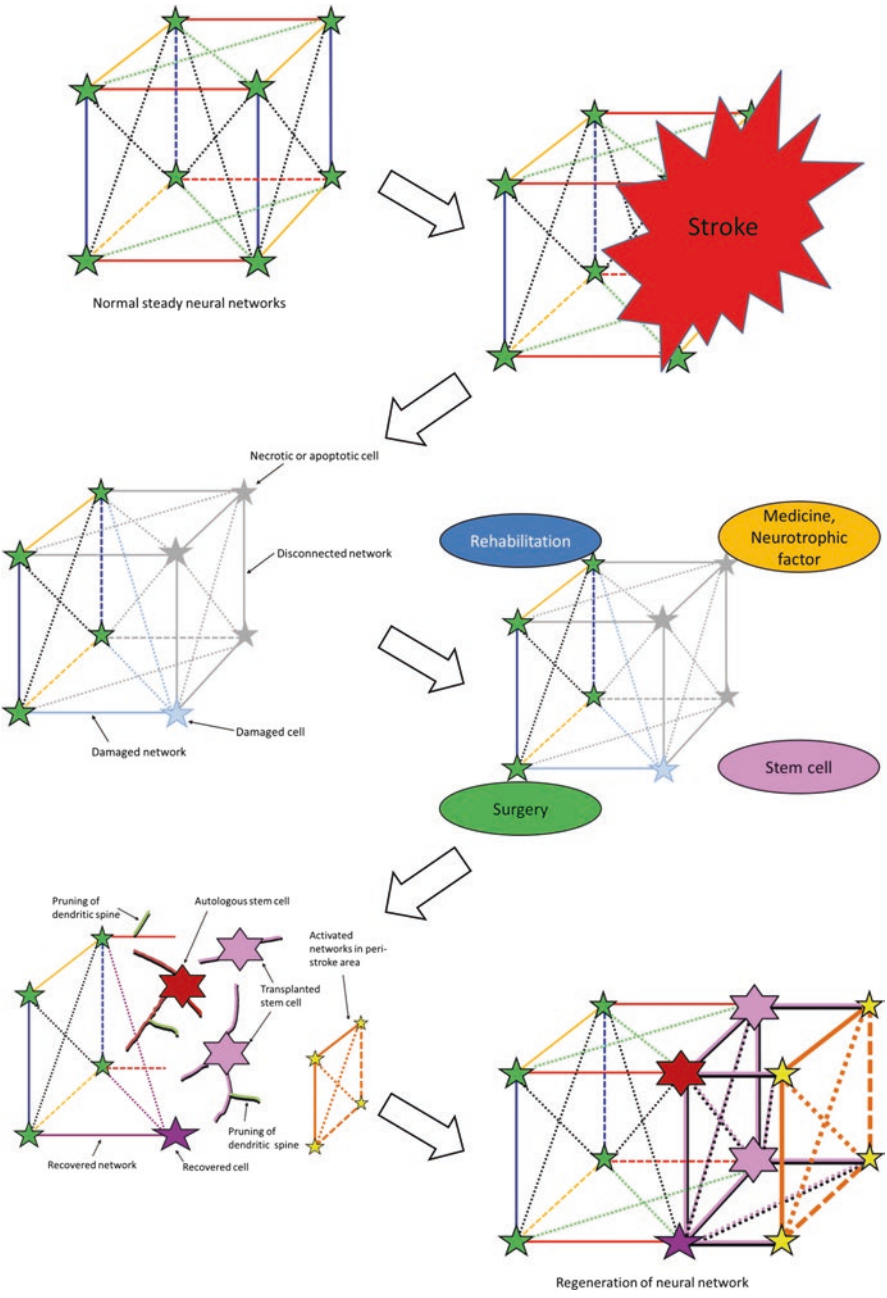

Fig. 19.1 Concept of reorganization of neural network after stroke. Several cascades are activated after the stroke and artificial influence is added. Finally, reorganization of neural networks is established. A *star* represents a neuron; *line* or *dotted line*, axonal network

reorganization through the influence of other organs, especially muscle, are indispensible processes for favorable outcome after stroke. Additionally, stem cell application to stroke provides the cellular constituents and trophic factors necessary for favorable recovery after stroke. Comprehensive and continuous treatments through surgery, medication and rehabilitation are essential for the regeneration of the neural network after stroke.

Acknowledgements We thank Mark Inglin (University of Basel) for his editorial assistance.

Competing interests We have no conflict of interest.

Funding This work was supported by Grants-in-Aid for Scientific Research of Japan, grant number 26462149 for Norihito Shimamura.

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Chapter 20
The Role of Matricellular Proteins in Experimental Subarachnoid Hemorrhage-Induced Early Brain Injury

Lei Liu and Hidenori Suzuki

Abstract Subarachnoid hemorrhage (SAH) is a serious life-threatening type of stroke caused by bleeding into the subarachnoid space surrounding the brain. It elicits a wide range of stress responses in brain tissues and results in brain injury. The term early brain injury (EBI) is a concept to explain pathophysiological changes that occur in brain within 72 h of SAH. Matricellular proteins (MCPs) are a class of nonstructural extracellular matrix proteins that exert diverse functions through binding to cell surface receptors, growth factors, cytokines and other MCPs. Until now, some of MCPs have been investigated in clinical SAH settings and laboratory studies. Here, we review the role of MCPs in post-SAH EBI by focusing on osteopontin, tenascin-C, and periostin.

Keywords Subarachnoid hemorrhage • Early brain injury • Matricellular proteins • Osteopontin • Tenascin-C • Periostin

Abbreviations

Ang	Angiopoietin
BBB	Blood-brain barrier
CCN	Cyr61/CTGF/NOV
CICES	Complement inhibiting component of *Ephedra sinica*

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P.A. Lapchak, J.H. Zhang (eds.), *Cellular and Molecular Approaches to Regeneration and Repair*, Springer Series in Translational Stroke Research,
https://doi.org/10.1007/978-3-319-66679-2_20

CSF	Cerebrospinal fluid
EBI	Early brain injury
ECM	Extracellular matrix
EGFR	Epidermal growth factor receptor
ERK	Extracellular signal-regulated kinase
FAK	Focal adhesion kinase
IL	Interleukin
ILK	Integrin-linked kinase
JNK	c-Jun N-terminal kinase
MAPK	Mitogen-activated protein kinase
MCP	Matricellular protein
MKP	Mitogen-activated protein kinase phosphatase
MMP	Matrix metalloproteinase
NF	Nuclear factor
OPN	Osteopontin
PDGFR	Platelet-derived growth factor receptor
P-gp	P-Glycoprotein
PI3K	Phosphatidylinositol 3-kinase
RGD	L-Arginyl-glycyl-L-aspartate
r-OPN	Recombinant osteopontin
SAH	Subarachnoid hemorrhage
siRNA	Short-interfering ribonucleic acid
SMA	Smooth muscle actin
SMemb	Embryonic smooth muscle myosin heavy chain
TLR	Toll-like receptor
TNC	Tenascin-C
TNKO	Tenascin-C knockout
VEGF	Vascular endothelial growth factor
VEGFR	Vascular endothelial growth factor receptor
VSMC	Vascular smooth muscle cell
ZO	Zona occludens

1 Introduction

Aneurysmal subarachnoid hemorrhage (SAH) is one of the most life-threatening diseases in the central nervous system [1]. It elicits a wide range of acute inflammatory responses in brain parenchyma and results in brain injury. The detailed mechanisms are still undefined even through there are many clinical and laboratory studies focusing on post-SAH brain injury. Recently, accumulating evidences have suggested that early brain injury (EBI), which is a concept to explain pathophysiological changes occurring in brain within 72 h of SAH, may be the primary cause of poor outcome after SAH [2].

The extracellular matrix (ECM) is a dynamic structural network and a crucial component of tissue microenvironment. Matricellular proteins (MCPs) are non-

structural ECM proteins that exert diverse functions through binding to cell surface receptors, growth factors, cytokines, and other MCPs [3]. This group of proteins includes thrombospondin, osteonectin, tenascin, osteopontin (OPN), periostin, galectin, Cyr61/CTGF/NOV (CCN) and others [3], and the members of MCPs are still increasing. During central nervous system development, the MCPs provide a microenvironment that regulates cell migration, axonal guidance, and synaptogenesis, However, upregulation of MCPs leads to blood-brain barrier (BBB) disruption and activation of inflammatory responses in pathological conditions [4].

Among MCPs, OPN, tenascin-C (TNC) and periostin have been reported to be involved in brain injury after SAH. In this chapter, we summarize the evidences regarding the role of MCPs in post-SAH brain injury.

2 Osteopontin (OPN)

OPN is a highly modified ECM glycophosphoprotein and modulates intracellular signaling pathways by binding various integrins and CD44 variants [5]. OPN plays an important role in the initiation of inflammation by affecting cell adhesion, chemotaxis, immune regulation, and apoptosis [5].

2.1 OPN Expression in SAH Brain

It has been reported that the serum level of OPN was significantly increased in intracerebral hemorrhage patients [6], but the expression level of OPN has never been investigated in aneurysmal SAH patients. However, in laboratory researches, it was reported that OPN plays a protective role in post-SAH EBI [7]. The expression level of OPN was very low in healthy adult rat brain. Conversely, OPN was obviously induced in brain after 24 h and peaked at 72 h of SAH. The trend of post-SAH OPN expression in brain was consistent with the rat's recovery of body weight losses and neurobehavior impairments as well as alleviation of BBB disruption [7]. Immunohistochemical staining showed that the expression of OPN was located at astrocytes and capillary endothelial cells after SAH in brain [7].

2.2 OPN Ameliorates EBI by Preventing BBB Disruption and Neuronal Apoptosis

The effect of endogenous OPN on EBI was investigated by suppressing and inducing OPN expression in a filament punctured SAH model. Specific OPN short-interfering ribonucleic acid (siRNA) treatment obviously aggravated neurobehavioral impairments and resulted in significantly increased BBB permeability [7]. The

mechanism study demonstrated that the blockage of endogenous OPN expression suppressed mitogen-activated protein kinase (MAPK) phosphatase (MKP)-1 induction, a kind of endogenous MAPKs inhibitor, and activated c-Jun N-terminal kinase (JNK), p38, and extracellular signal-regulated kinase (ERK) 1/2 [7]. Vascular endothelial growth factor (VEGF)-A is a pivotal regulator of angiogenesis that controls vascular growth under physiological and pathological conditions and promotes vascular permeability [8]. MAPKs not only mediated the effect of VEGF-A on BBB permeability but also induced VEGF-A expression [9]. Meanwhile, inactivating endogenous OPN decreased angiopoietin (Ang)-1 expression [7] which has a potent anti-vascular permeability property and inhibits the activation of VEGF-A [10]. These evidences indicated that endogenous OPN plays an important role in preserving BBB integrity after SAH (Fig. 20.1).

Thus, it is hopeful to prevent EBI by inducing endogenous OPN upregulation after SAH. Zuo et al. found that Complement Inhibiting Component of Ephedra Sinica (CICES), a kind of Chinese herb which has ability to inhibit the activity of the classical and alternative pathways of complement, alleviated neurological deficits, cortex cell apoptosis, BBB disruption and brain edema after 24 and 72 h of experimental SAH [11]. The neuroprotective effects of CICES on post-SAH EBI was associated with complement C3 inhibition and upregulated OPN expression as well as reduced MMP-9 expression [11]. In another post-SAH EBI study, Enkhjargal et al. demonstrated that intranasal administration of vitamin D3 improved neurological impairment and brain edema, and was associated with upregulation of endogenous OPN within 72 h after experimental SAH [12]. Advanced study found that vitamin D3 upregulates expression of endogenous brain OPN in astrocytes and that it protects BBB via CD44 splicing and P-glycoprotein in the vascular endothelial cells after SAH in rats. Blockage of vitamin D receptor and OPN exacerbated neurobehavioral impairment and BBB disruption and was associated with a decrease of mature/full-length glycosylated P-glycoprotein in rat brain vascular endothelial cells [12].

On the other hand, recombinant OPN (r-OPN) pretreatment prevented the loss of body weight, neurobehavioral impairments, BBB disruption, and brain edema formation after 24–72 h of experimental SAH [13]. The mechanism studies demonstrated that the neuroprotective effects of r-OPN were associated with inactivation of nuclear factor (NF)-κB and matrix metalloproteinase (MMP)-9, and preservation of inter-endothelial tight junction protein zona occludens (ZO)-1. Simultaneously, the expression of tissue inhibitor of MMP-1 was significantly upregulated to maintain the matrix stability [13]. MMP-9 is a key factor to cause BBB disruption by degrading the ECM of cerebral microvessel basal lamina, including collagen IV, laminin, fibronectin and ZO-1 [14–16]. Interestingly, r-OPN pretreatment did not suppress interleukin (IL)-1β induction after SAH [13] even though IL-1β has been reported to cause brain injury after SAH and IL-1β-induced MMP-9 overexpression was dependent on NF-κB transduction pathway in astrocytes [17, 18]. One possible speculation is IL-1β activation via NK-κB independent signaling pathways after SAH, such as sympathetic activation or catecholamine release [19, 20]. Thus, more researches would be needed in future.

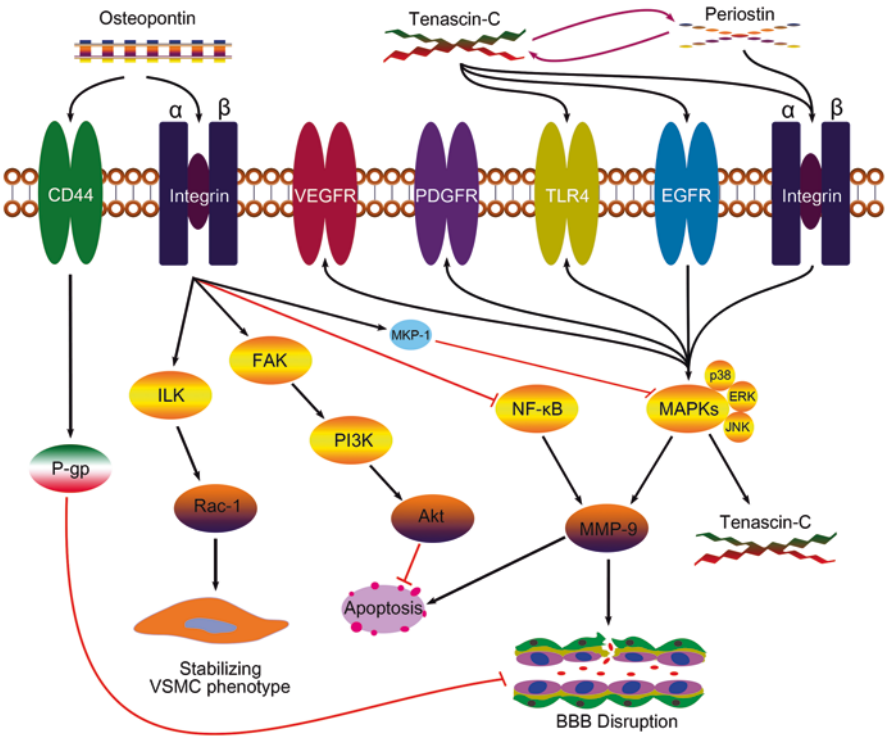

Fig. 20.1 The mechanisms for matricellular proteins to cause early brain injury (EBI) after experimental subarachnoid hemorrhage (SAH). Osteopontin prevents post-SAH EBI by (1) stabilizing vascular smooth muscle cell (VSMC) phenotype via integrin-linked kinase (ILK)/Rac-1 signaling pathways; (2) inhibiting neuronal apoptosis via focal adhesion kinase (FAK)/phosphatidylinositol 3-kinase (PI3K)/Akt signaling pathway activation; and (3) protecting blood-brain barrier (BBB) integrity via inactivating nuclear factor (NF)-κB/matrix metalloproteinase (MMP)-9 signaling pathway, via activating mitogen-activated protein kinase (MAPK) phosphatase (MKP)-1 that inhibits MAPKs/MMP-9 signaling pathway or via increasing mature/full-length glycosylated P-glycoprotein (P-gp) in brain vascular endothelial cells. Tenascin-C (TNC) induces post-SAH EBI by causing neuronal apoptosis and BBB disruption via MAPKs/MMP-9 signaling pathways. Activated MAPKs also induce TNC expression by relevant receptors, including vascular endothelial growth factor receptor (VEGFR), platelet-derived growth factor receptor (PDGFR), toll-like receptor (TLR) 4, epidermal growth factor receptor (EGFR) and integrins. Periostin induces post-SAH BBB disruption, which is mediated by MAPK signaling pathways. The interaction between periostin and TNC also plays an important role in post-SAH EBI by modulating downstream signaling pathways. *ERK* extracellular signal-regulated kinase, *JNK* c-Jun N-terminal kinase

Except for preventing BBB disruption, the noninvasive nasal application of r-OPN suggested that intranasal r-OPN treatment attenuated brain edema and neuronal apoptosis, which ultimately improved neurobehavioral impairments in rats after SAH [21]. The antiapoptotic mechanisms of r-OPN treatment possibly involved focal adhesion kinase-phosphatidylinositol 3-kinase-Akt signaling pathways activation, which inhibited caspase-3 cleavage in cortex and hippocampus (Fig. 20.1) [21].

2.3 OPN Stabilizes Vascular Smooth Muscle Cell Phenotype

The vascular neural network is a physiological unit to be considered for therapeutic development in stroke. The vascular smooth muscle is an important part of vascular neural network and may be an alternative therapeutic target for post-SAH EBI [22, 23]. The vascular smooth muscle of cerebral arteries typically switches from contractile to synthetic type after injury or hemorrhagic stroke [24]. The vascular smooth muscle cells (VSMCs) can be stained with α-smooth muscle actin (α-SMA), a marker of contractile VSMCs, and embryonic smooth muscle myosin heavy chain (SMemb)/non-muscle myosin heavy chain isoform, an accurate marker of synthetic VSMCs [25]. It is reported that the expression of α-SMA was significantly decreased and the expression of SMemb was significantly increased at 24 and 72 h after experimental SAH [26]. Unbalanced contractile/synthetic vascular smooth muscle phenotype may result in decreasing auto-regulatory capacity and aggravating brain edema [27]. r-OPN alleviated neurological impairments and protected the vascular smooth muscle phenotypic transformation, and the mechanisms involved integrin/integrin-linked kinase (ILK)/Rac-1 signaling pathways activation [26]. A L-Arginyl-glycyl-L-aspartate (RGD)-dependent integrin receptor antagonist, RGD motif-containing hexapeptide (GRGDSP), reduced r-OPN-induced ILK upregulation and Rac-1 activation, meanwhile, knockdown of ILK by siRNA or selective Rac-1 inhibition by NSC23766 abolished r-OPN-induced preservation of vascular smooth muscle phenotypic transformation [26].

In summary, OPN plays a protective role in SAH-induced EBI by protecting BBB integrity, preventing neuronal apoptosis, and stabilizing VSMC phenotype (Fig. 20.1).

3 Tenascin-C (TNC)

TNC belongs to the MCP family that is downregulated in healthy adult tissues and transiently induced during inflammatory responses [28]. It exerts various functions through binding to cell surface receptors, other MCPs, and growth factors [29].

3.1 TNC Expression in SAH Brain

In aneurysmal SAH patients, TNC expression levels were markedly increased in serum and cerebrospinal fluid (CSF). CSF TNC level peaked immediately after SAH, and the highest level in CSF occurred in the first 3 days followed by a decrease over time, whereas serum TNC level increased transiently and peaked on days 4–6 [30]. Higher TNC expression levels in CSF were observed in patients with worse admission clinical grade, more severe SAH on admission computed tomography,

acute obstructive hydrocephalus, subsequent angiographic vasospasm, delayed cerebral ischemia, chronic shunt-dependent hydrocephalus, and a worse outcome [30, 31]. In *in-vivo* studies, expression of TNC was weakly detected in normal adult animals, however, overexpressed TNC was observed in the cerebral VSMC layers and brain parenchyma after experimental SAH in rats [32, 33].

3.2 Overexpressed TNC Induces BBB Disruption After SAH

It is well known that TNC expression was obviously upregulated during acute or chronic inflammatory diseases [28]. In an experimental SAH mice model, overexpressed TNC was associated with severe neurobehavioral impairment, brain edema, and BBB disruption [34]. Endogenous TNC induction activates downstream signaling pathways, such as platelet-derived growth factor receptors (PDGFRs), MAPKs and MMP-9 [32]. It was reported that imatinib mesylate, a kind of PDGFR inhibitor, prevented cerebral vasospasm by inhibiting PDGFR activation, TNC induction, and MAPK activation [32]. Another study demonstrated that imatinib mesylate preserved BBB integrity by inhibiting JNK-mediated MMP-9 activation after experimental SAH in rats [35]. TNC knockout (TNKO) mice was used to determine the effect of TNC on post-SAH BBB disruption [34]. It was demonstrated that TNKO alleviated neurobehavioral impairments, brain edema, and BBB disruption after SAH. The following mechanism studies found that the effect of TNKO on post-SAH BBB protection was associated with MMP-9 inhibition and tight junction protein ZO-1 preservation, as well as inactivation of MAPKs in cerebral capillary endothelial cells and brain parenchyma [34]. Conversely, exogenous TNC treatment re-aggravated neurological impairments, brain edema, and BBB disruption compared with the vehicle treatment in TNKO SAH mice [34]. These findings indicated that TNC plays an important role in post-SAH BBB disruption (Fig. 20.1).

3.3 Overexpressed TNC Is Responsible for Neuronal Apoptosis After SAH

Apoptosis is one of the most significant pathological processes in EBI after SAH [36]. It is believed that there are a number of apoptotic pathways playing a role in SAH: the death receptor pathway, caspase-dependent and caspase-independent pathways, as well as the mitochondrial pathway [36]. It was reported that TNC upregulated MMPs expression in cultured VSMCs, which lead to TNC fragmentation and cell apoptosis [37]. In an experimental SAH study, it was demonstrated that TNC is a mediator of post-SAH neuronal apoptosis through the mechanism of PDGF and MAPKs, conversely, imatinib mesylate, a PDGFR inhibitor, prevented TNC induction, MAPKs activation, and neuronal apoptosis. Meanwhile, recombinant TNC administration

reactivated MAPKs pathways and induced neurons apoptosis, as well as endogenous TNC expression in SAH brain [33]. These indirect evidences suggested that the positive feedback mechanism of TNC-induced cascades reaction plays an important role in post-SAH neurons apoptosis (Fig. 20.1).

4 Periostin

Periostin is a secreted MCP that has been reported to be a critical player in the pathobiology of various diseases, including inflammatory disease [38]. Periostin plays a regulatory role rather than structural functions: binding of periostin to integrin receptors activates many intracellular signaling pathways and modulates multiple downstream proteins [39]. Previous studies suggested that periostin alternates the pathological processes by modulating MMPs expression in inflammatory diseases through different signaling pathways [40, 41].

4.1 Periostin Expression in SAH Brain

The expression level of periostin has never been investigated in aneurysmal SAH patients. In a laboratory study, the expression level of periostin was very low in sham operated mice brain, but periostin was obviously induced in brain after 24 h of SAH [42]. Immunohistochemical staining showed that periostin was weakly detected in neurons in sham mice, and was intensively expressed in brain capillary endothelial cells as well as neurons after SAH [42].

4.2 Overexpressed Periostin Causes Post-SAH EBI

To assess the role of periostin in post-SAH EBI, recombinant periostin protein and monoclonal anti-periostin antibody were administrated by an intracerebroventricular injection in a SAH mice model [42]. Recombinant periostin protein treatment obviously aggravated neurobehavioral impairments and brain edema after 24 h of SAH. However, monoclonal anti-periostin antibody treatment significantly improved neurobehavioral impairments and obviously ameliorated brain edema after 24–48 h of SAH. Immunohistochemical staining showed that periostin was effectively neutralized in brain capillary endothelial cells and neurons by anti-periostin antibody, meanwhile, BBB disruption, which was evaluated by immunoglobulin G extravasation, was significantly suppressed by periostin neutralization at 24 h of SAH [42]. The mechanism study demonstrated that overexpressed periostin activated the downstream p38/ERK/MMP-9 signaling pathways and caused ZO-1

degradation (Fig. 20.1). Thus, these evidences indicated that periostin plays an important role in post-SAH EBI.

5 Interaction Between Periostin and TNC Aggravates Post-SAH EBI

It has been reported that periostin interacts with MCPs, such as TNC and bone morphogenetic protein-1, and regulates tissue homeostasis [43, 44]. In post-SAH EBI study, administrating of recombinant periostin not only aggravated neurobehavioral impairments and brain edema but also induced TNC expression [42]. Conversely, periostin neutralization suppressed post-SAH TNC induction and EBI [42]. Furthermore, deficiency of TNC inhibited periostin induction and improved neurobehavioral impairments although periostin expression in TNKO sham mice was identical to that of wild-type sham mice [42]. These results indicated that periostin and TNC regulate each other and alter downstream signaling pathways in post-SAH EBI. Moreover, periostin and TNC may form a positive feedback mechanism to aggravate BBB disruption (Fig. 20.1).

6 Conclusions

This review summarizes the findings that MCPs, which include OPN, TNC and periostin, play important roles in SAH-induced EBI by regulating downstream signaling pathways and modulating BBB integrity and cell apoptosis. The current evidences suggest that MCPs can be therapeutic targets for preventing post-SAH EBI. However, there are many other known MCPs that have never been investigated in the context of EBI after SAH. In addition, future studies are required to determine how MCPs are involved in the pathophysiological process of SAH-induced EBI in more details.

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Chapter 21
Chemokines and Proteolysis: Implications for Stem Cell Dynamics in Ischemic Stroke

Umadevi V. Wesley and Robert J. Dempsey

Abstract Stroke still remains a significant clinical challenge, with only a small proportion of the ischemic patients benefiting from current treatments which are limited by a narrow therapeutic time window. Cerebral ischemic stroke results in severe neurological deficits due to massive loss of neurons and disruption of vasculature. Although our understanding of the stroke pathology has remarkably increased, further insight into the cellular and molecular mechanisms involved in the post-stroke brain repair is still required to identify more effective drug targets with wider time window. Cerebral ischemia and reperfusion injury alters the brain microenvironment including dysregulation of cytokines, chemokines and abnormal release of proteases leading to neuronal cell death, endothelial cell and stem/progenitor cell dysfunction, disruption of blood brain barrier and the vascular unit. Thus, delineating the timely and balanced regulation of proteases, cytokines, chemokines, and stem/progenitor cells is critical for enhancing post-stroke brain protection and repair, and neurological functional recovery. In this chapter, we will present the facts about interactions of chemokines, proteases and stem cells in the context of pathophysiology of stroke.

Keywords Stroke • Ischemic brain injury • Chemokines • Proteases • Stem cells • Matrix metalloproteases • Dipeptidyl peptidase IV • Stromal derived factor

Abbreviations

CVD	Cerebro-vascular diseases
DPPIV	Dipeptidyl peptidase 4
EPC	Endothelial progenitor cells

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P.A. Lapchak, J.H. Zhang (eds.), *Cellular and Molecular Approaches to Regeneration and Repair*, Springer Series in Translational Stroke Research,
https://doi.org/10.1007/978-3-319-66679-2_21

HIF-1a	Hypoxia-inducible factor-1α (HIF-1α)
IL	Interleukin
MCAO	Middle cerebral artery occlusion
MCP-1	Monocyte chemoattractant protein-1
MMP	Matrix metalloproteases
MSC	Mesenchymal stem cells
NPC	Neural progenitor cells
SDF1	Stromal derived factor

1 Introduction

Stroke is a leading cause of mortality and long-term disability worldwide. Stroke still remains a significant clinical challenge, with only a small proportion of the ischemic patients benefiting from current treatments which are limited by a narrow therapeutic time window [1–8]. Two major types of strokes have been identified: hemorrhagic and ischemic stroke. Ischemic stroke accounts for more than 80% of all strokes [4–9]. Cerebral ischemic stroke results in severe neurological deficits due to massive loss of neurons and disruption of vasculature. Restoration of both neuro and vascular units is thus important for functional recovery [4, 6–15]. Although our understanding of the stroke pathology has remarkably increased, further insight into the cellular and molecular mechanisms involved in the post-stroke brain repair is still required to identify more effective drug targets with wider time window.

Cerebral ischemia and reperfusion injury alters the brain microenvironment including dysregulation of cytokines, chemokines and abnormal release of proteases leading to neuronal cell death, endothelial cell dysfunction, disruption of blood brain barrier and the vascular unit [4, 6, 12–18]. Thus, delineating the timely and balanced regulation of proteases, cytokines, and chemokines is critical for enhancing endothelial and neural stem cell recruitment that contributes to post-stroke brain protection and repair. As a self-defense mechanism, ischemic-stroke upregulates chemokines that promote stem and progenitor cell migration, survival, and angiogenesis. However, this attempt to self-repair the post-stroke brain is short lived and has limited success, presumably due to disruption of chemokine activity by proteases. Indeed, through mutual balance and interactions, chemokines and proteases regulate angiogenesis and neurogenesis, and have gained much attention due to their therapeutic potential for post-stroke recovery [4, 6, 8–23]. Thus interplay between proteases, chemokines and stem cells represents important molecular-cellular cross-talk for stroke outcome. In this chapter, we will present the cross-talk between chemokines, proteases and stem cells in the context of pathophysiology of stroke.

2 Chemokines and Ischemic Stroke/Cerebral Ischemia

Chemokines are members of cytokine superfamily with distinct functional roles. Chemokines are low-molecular-weight proteins that are well established for their capability to accelerate chemotaxis or gradient directed migration for recruitment of cells including leukocytes, progenitor and stem cells. Chemokines, in addition to chemotactic activities, are also involved in autocrine and paracrine signaling and thus critically modulate cellular migration, survival, angiogenesis and neurogenesis [22–26]. Currently there are more than 50 chemokines and 20 chemokine receptors. Chemokines have been classified into four main subfamilies: CXC, CC, CX3C and XC, on the basis of the number and location of the cysteine residues at the N-terminus of the molecule. These chemokines exert their biological effects through the binding to selective G protein-coupled transmembrane receptors including CXCR, CCR, and CX3CR that are present on the surfaces of their target cells. Among these, the CC and CXC chemokines are the major mediators of cerebral injury and protection following ischemic stroke. The expression and activities of these chemokines are modulated by local and systemic inflammation. Indeed, during acute phase, ischemic-stroke induces inflammation not only in the brain, but also in the blood stream which in turn influences critical steps of the post-stroke recovery processes. The CXC and CC chemokines further exacerbate the inflammatory response in infarcted brain, as they promote recruitment and activation of inflammatory cells [25–35]. On the other hand, they also enhance stem cell homing to site of injury, neuronal and glial cell survival, and neo-angiogenesis, thus favoring brain repair [35–39]. Given these abilities chemokines represent potential therapeutic targets, and drug reagents for stroke treatment. Overall, well-orchestrated balance between the accumulation and degradation of these chemokines is integral to brain injury and repair.

Under normal physiological conditions, chemokines are expressed in brain by microglia, astrocytes and neurons, and endothelial cells at low levels, but rapidly upregulated in response to ischemic damage and re-perfusion injury. While some cytokines exacerbate cerebral injury, others are shown to provide neuroprotection [20–36]. Chemokines levels are also increased in response to signals from pro and anti-inflammatory cytokines. Higher concentration of chemokines create ligand gradient and play an important role in selectively recruiting stem cells, monocytes, neutrophils, and lymphocytes that express respective chemokine receptors. Due to their chemotactic and signaling properties, chemokines have gained much attention for investigation in many neurological disorders including ischemic stroke. Among these chemokines, Stromal cell-derived factor-1a (SDF1), Monocyte chemoattractant protein-1 (MCP-1), fractalkine (CX3CL1), and Glucagon like peptide-1 (GLP-1) macrophage inflammatory protein-1 (MIP-1), RANTES, and GRO-alpha (CXCL1) have shown to be involved in ischemic brain injury and repair [39–70]. The involvement of these chemokines in multiple biological functions that are crucial for tissue

re-modeling makes them potentially useful for improving the clinical application of stem cell therapy. Of note, the functions of many of these chemokines are tightly controlled by proteolytic events [28, 33]. Here we discuss three most widely studied chemokines, SDF1, MCP-1, and fractalkine.

2.1 Stromal Derived Factor 1 (SDF1)

The chemokine stromal-derived factor 1 (SDF1) also known as CXCL12 belongs to the CXC group of chemokines. SDF1 elicits major control over hematopoietic cells, tissue/organ-committed progenitor/stem cells, and neural stem cell migration, proliferation and survival, and angiogenesis by binding and stimulating its receptors CXCR4 and CXCR7. Indeed SDF1-CXCR4 knockout mice display defects in colonization of bone marrow stem cells, and impaired development of heart, brain, and large vessels. Thus, the SDF1-CXCR4 axis plays important roles during various biological activities including organogenesis [39]. Of particular interest, in the ischemic brain SDF1 is released at the site of injury and is shown to enhance stem/progenitor cell recruitment and survival. It has been shown that CXCR4 is expressed on neural stem cells (NSC) and neuroblasts after stroke, and SDF1 promotes directional migration of these newly formed neuroblasts to ischemic damaged areas. SDF1 expression is increased primarily in the ischemic penumbra, particularly in perivascular astrocytes. This chemokine has been suggested to provide neuroprotection also by increasing the homing of bone marrow-derived stromal stem cells to sites of injury [40–50].

Research in recent decade have demonstrated that angiogenesis is critical in improving post-stroke neurological functional recovery. Thus elucidating the underlying mechanisms of this interplay between stroke-induced neurogenesis and angiogenesis is of great importance for neuro-restorative therapy. Angiogenesis is formation of new micro-vessels from pre-existing vessels. It is a multi-step biological process, including proliferation and sprouting of endothelial cells, formation of tube-like vascular structures, and branching. SDF1 has also been demonstrated to play an important role in angiogenesis and improving local cerebral blood flow. Following ischemic stroke, the majority of SDF1 expression is shown to be associated with activated microglia in the perivascular region. In addition, in animal models of stroke, SDF1 is expressed and released, predominantly by activated astrocytes that are associated with blood vessels. SDF1 produced in brain may act as a chemoattractant for peripheral blood derived cells. SDF1 contributes to angiogenesis by recruiting endothelial progenitor cells (EPCs) from the bone marrow, and by increasing endothelial cell proliferation through a CXCR4 dependent mechanism [51–54]. These findings have linked SDF1 to the restoration of both neuro and vascular unit to areas of ischemic injury as the microvascular endothelial cells are greatly involved in this process. Thus, SDF1 is a critical player in post-stroke brain repair.

2.2 Monocyte Chemoattractant Protein-1 (MCP-1)

MCP-1 also known as CCL2 is the first identified CC chemokine of the C-C chemokine family. It signals through its cognate receptor CCR2. MCP-1 is ubiquitously expressed by many cell types including endothelial, fibroblasts, epithelial, smooth muscle, astrocytes, and microglial cells. The major source of MCP-1 are monocyte/macrophages. It regulates the migration and infiltration of monocytes, T lymphocytes, natural killer (NK) cells, and neuroblasts. Using middle cerebral artery occlusion (MCAO) model of focal ischemic stroke, we and others have demonstrated upregulation of MCP-1 expression in the rat brain. MCP-1 mRNA and protein levels increase significantly within a few hours of ischemia-reperfusion injury in the brain of the rat and it remains high for several days. Double immune-histochemical analysis in MCAO mice has revealed the increased levels of MCP-1 in neurons as early as 12 h after focal brain ischemia, but astrocytes and microglia show MCP-1 expression at a later stage following the ischemia/reperfusion. The MCP-1 levels are also shown to increase in the serum and CSF of ischemic stroke patients during the early stages of ischemic stroke. MCP-1 has been suggested to contribute to tissue damage through recruitment of inflammatory cells. These data indicate that higher levels of MCP-1 may worsen stroke outcome. In support of this idea, mice deficient in MCP-1 show significant decrease in infarct volume as a consequence of focal brain ischemia and a decrease in macrophage accumulation in the infarct area one or two weeks after the stroke. Furthermore, mice lacking the gene for the MCP-1 receptor, CCR2, exhibit reduced infarct size, edema, leukocyte infiltration and expression of inflammatory mediators following focal ischemia, indicating its role in brain injury. Detrimental effects of MCP-1 is further demonstrated in which blockade of MCP-1or its receptor CCR2 decreases the permeability of blood brain barrier (BBB) after reperfusion. Paradoxically, MCP-1 may also contribute to post-stroke brain restoration. In rats and mice, MCP-1 may promote ischemic stroke induced migration of neuroblasts derived from neural progenitors in the sub ventricular zone (SVZ-derived neuroblasts) towards the ischemic striatum and cortex. Thus, in addition to attracting leukocytes, CCL2 plays an important role in the migration of newly formed neuroblasts from neurogenic regions to the injured regions of the brain after a stroke [55–58].

2.3 Fractalkine

Fractalkine is also known as chemokine (C-X3-C motif) ligand 1. It is the only member of the CX3C chemokine family. Interestingly, CX3CL1 is widely expressed in the brains of young, but decreased in those of aged mice and rat with reduced hippocampal neurogenesis. The genetic deletion or pharmacological inhibition of CX3CR1 leads to reduced neurogenesis in the dentate gyrus of

mouse hippocampus. Fractalkine is implicated in ischemic pathophysiology and its expression is increased in neurons and in endothelial cells after a focal ischemic insult. Interestingly, its receptor CX3CR1, is expressed only in microglia/macrophages, indicating that fractalkine is involved in neuron–microglia interaction and signaling. These results suggest a possible role for fractalkine in protection against neurotoxicity produced by activated microglia. Fractalkine is shown to display neuroprotective and anti-inflammatory activities in several animal models of disease, and its expression correlates with positive outcomes in human neuro-pathologies. Fractalkine is upregulated by hypoxia and/or inflammation-induced inflammatory cytokines and it is shown to promote angiogenesis and microvasculature remodeling. On the other hand, this chemokine is shown to be involved in the activation and chemo-attraction of leukocytes and microglia into the infracted tissue. Indeed, fractalkine-deficient mice exhibit a smaller infarct size and lower mortality after transient focal cerebral ischemia. It is evident that CX3CL1 may have either beneficial or destructive potential in the CNS, depending on the activation state of the microglia cell population [59–68]. These studies indicate fractalkine (CX3CL1) and its receptor CX3CR1 as potential targets for future therapeutics for post-stroke repair.

3 Proteolytic Enzymes in Focal Ischemic Brain Damage and Repair

Proteases are generally known to be involved in the degradative processes. However, now it has become evident that proteolytic enzymes regulate key molecular and cellular signaling pathways, thus contributing to homeostasis and pathological conditions. By initiating intracellular signals through both catalytically and non-catalytically, proteases play important roles in many biological processes including injury and tissue remodeling [69–71].

Posttranslational modifications of chemokine functions have gained major attention due to their impact on physio-pathological status. Clearly, the best known type of posttranslational modification of chemokines is proteolysis by specific enzymes. Both the NH_2-terminal and the COOH-terminal end of a chemokine can be subjected to proteolysis, and internal cleavage is also carried out by endopeptidases. All forms of proteases including intracellular, membrane bound, and secreted forms of proteases are involved in truncation or degradation of proteins that modulate several critical physiological processes such as cell proliferation, cell death, injury repair, tissue remodeling, homeostasis, and immune responses [71–73].

Stroke induces significant alterations in the proteolytic events in the ischemic brain micro-environment. In particular, serine proteases and matrix metallic-proteinases (MMP) are aberrantly expressed in response to ischemic injury in brain causing time dependent effects on post-stroke brain repair and neurological func-

tional outcome. Proteolytic enzymes have gained much attention as they are involved in the catalytic processing of peptides and small proteins including chemokines in normal brain and during pathogenesis of neurodegenerative disorders. Particularly, dysregulation of these proteolytic enzymes play an important role in post-stroke brain injury and repair, and are thus potential target for therapeutic intervention in stroke [72–77]. In this chapter, we will review two key proteases involved in truncation and regulation of chemokines in ischemic stroke. The role of the matrix metalloproteases (MMPs), and dipeptidyl peptidase (DPPIV) will be discussed.

3.1 Matrix Metalloproteases (MMP)

MMPs are very well studied protease enzymes whose catalytic mechanism involves a metal. MMPs are established to have an important role in extracellular matrix (ECM) degradation and cell migration/invasion. MMPs cleave components of the ECM, such as collagen, proteoglycan and laminin, but also process a number of cell-surface and soluble proteins, including receptors, inflammatory cytokines and chemokines. Thus, in addition to their physiological roles, such as tissue remodeling, MMPs contribute to the regulation of neuro-inflammatory responses to injury [78–84].

Numerous studies have demonstrated the involvement of MMPs in ischemic stroke patho-physiology. Two members of this class of proteases, the gelatinases MMP-2 and MMP-9, have been strongly implicated in ischemic brain pathology because they contribute to the disruption of the blood brain barrier (BBB) and hemorrhagic transformation following injury both in animal models and in stroke patients. Previous studies have described increased expression and activity of MMPs in the brain following transient focal ischemia. Their protease activity was shown to increase immediately after the start of reperfusion in the regions of injured brain in a rat model of transient Middle Cerebral Artery Occlusion (MCAO). MMP enzyme activity was mainly detected in neuronal nuclei during the early stages of ischemia/reperfusion, but was detected in the cytosolic compartment and in non-neuronal glial cells at later reperfusion times. MMPs are also expressed by stromal cells and leukocytes making it a potential target for neuroprotection and repair. Indeed treatment with MMP inhibitors or MMP neutralizing antibodies showed decreased infarct volume and prevented BBB disruption after permanent or transient MCAO in rodents. During the early acute phase of stroke, MMP9 contributes to inflammation and neuronal cell death**.** Paradoxically, MMP9 is demonstrated to be beneficial during late repair phase as it promotes neuro-vascular remodeling through enhancing angiogenesis and neuroblast migration to the injured brain region [78–86]. Thus, timely regulation of expression and activity of these proteases is critical for stroke outcome.

Molecular mechanisms of MMPs are also well studied. Studies have shown that MMP-3 and 9 guide sub ventricular neuroblasts migration via the activation of PI3K/Akt and ERK1/2 signaling pathways. It has been postulated that neuroblasts express and secret MMPs that support their migration to ischemic lesion. Further studies are required to conclusively determine the role of MMPs in post-stroke brain. MMPs truncate or cleave several CC and CXC chemokines, thus altering their functions. Amino terminal proteolysis of CXCL8 (interlukin-8 (IL-8) by MMP-9 generates CXCL8 that in turn stimulates MMP-9 secretion and enhances mobilization of intracellular calcium and migration of leukocytes. MMP-8-, MMP-9-, or MMP-12 also mediate proteolysis of CXCL11and CXCL12 also called as Interferon-inducible T-cell alpha (I-Tac) and stromal derived factor (SDF) leading to decrease in their chemotactic activity. Among CC chemokines, MCPs are well known substrates for MMPs cleavage and inactivation. MMPs not only cleave chemokines but also promote degradation of the extracellular matrix [79, 85–88] that may impact post-stroke brain injury and repair.

3.2 Dipeptidyl Peptidase IV (DPPIV)

Dipeptidyl peptidase (DPP) IV was identified for the first time by Hopsu-Havu and Glenner in 1966 in livers of rats [89]. This serine protease is also called as adenosine complexing protein 2 or cluster of differentiation 26 (CD26). The DPP enzymes have been associated with a wide variety of physiological and pathophysiological processes of the immune system [90–92]. Here, we will discuss the role of DPPIV in chemokine and stem cell regulation implicated in stroke pathology.

The human DPPIV gene contains 26 exons and is located on chromosome 2q.24.3. It is a 110 kDa multi-functional protein. It is a type II integral membrane protein with a six amino acids short amino terminal domain in cytoplasm, 22 amino acids membrane span domain and a long 732 amino acids extracellular domain. It functions as a receptor, as an adhesion molecule for ECM collagen and fibronectin, as a costimulatory signal for T lymphocytes, and is involved in apoptosis [91–111]. Low oxygen levels (ischemia/hypoxia) induce DPPIV through hypoxia-inducible Factor-1α [112]. In addition, several cytokines including interferons (IFNs) and interleukins (IL-1β), differentiating factor retinoic acid, and HNF-1 can also upregulate DPPIV expression in various cell types including fibroblasts, epithelial cells, endothelial cells, and leukocytes [113, 114]. DPPIV exists in both soluble and membrane-bound form. Membrane-bound DPPIV forms homodimer and active heterodimers with fibroblast activating protein (FAPα). DPPIV also binds to adenosine deaminase (ADA), an enzyme that converts 2′-deoxyadenosine into inosine and 2′-deoxyinosine which plays a role in the development and functioning of lymphoid tissues, the activation and proliferation of T and B cells, and release of inflammatory cytokines such as IFN-γ, TNF-α, and IL-6. DPPIV is shown to bind the zymogen plasminogen that promotes its conversion to active plasmin which in turn

leads to degradation of the ECM, thus regulating cell migration and invasion. The serine protease enzyme activity of DPPIV is well studied. It selectively cleaves or truncates small peptides whose penultimate position in the NH_2-terminal amino acid sequence is occupied by a proline or an alanine residue. Several in vitro and in vivo studies have demonstrated that many cytokines and chemokines as substrates for DPPIV truncation, as many of them contain a proline or an alanine residue in the penultimate position of their NH_2-terminal sequence [92–105].

Proteolytic cleavage of N-terminal amino acids disrupts the ability of chemokines to bind their receptors. Since chemokines are involved in a number of critical cellular functions that impacts physiological and pathological status, this protease-chemokine interactions have significant impact on various metabolic and regenerative diseases including diabetes and stroke [114–117]. Due to these reasons DPPIV inhibitors have been studied both in vitro and in vivo studies.

In contrast to other proteases, much less is known about DPPIV in cerebral ischemia. DPPIV is a major regulator of cytokines and neuropeptides involved in inflammation, immunity, vascular, and stem cell function. Dysregulated DPPIV expression is associated with tumor development. We and others have shown that DPPIV inhibits tumor cell migration and angiogenic potential through blocking the SDF1-CXCR4 signaling pathways. Recent studies have suggested a role for DPPIV in organ-ischemia including lung, renal, cerebral, and limb ischemic injury [105–109]. Emerging studies have shown that inhibition of DPPIV improves cardiovascular outcomes after cerebral and myocardial ischemia-reperfusion injury [117, 118]. However, little is known about its role in cerebral-ischemia and in chemokine mediated neuro-angiogenesis.

4 Stem Cell Regulation by Proteases in Ischemic Brain

The potential contribution of stem cells in cerebro-vascular diseases has gained enormous attention within neuro-regenerative research. Endogenous progenitor/stem cells recruited from angio-neurogenic niches, or recruited from the peripheral circulation, protects from the detrimental consequences of cerebrovascular events, especially ischemia. This has led to many investigations into the role of many stem cell niches for post-stroke therapy. Indeed, emerging studies in the field of brain injury support the use of endogenous and exogenous stem cells to remodel and repair brain tissue [118–121]. Three major types of progenitor/stem cells including endothelial progenitor cells (EPC), neuronal progenitor/stem cells and mesenchymal stem cells have been largely studied in the context of stroke.

EPC are bone marrow-derived progenitor cells characterized by the expression of CD34, a stem cell marker, and the endothelial marker such as vascular endothelial growth factor receptor-2 or CD309. Endothelial progenitor cells (EPCs) through maturation into endothelial cells, mainly contribute to neo-angiogenesis, formation of new blood vessels, and thus supply required nutrients and oxygen to injured

brain. The contribution of EPCs in re-establishment of vascular unit in the brain after ischemic stroke has been well demonstrated. Increased circulating EPCs levels are shown to be correlated with reduced infarct volume, and enhanced neurological functional recovery in patients following acute ischemic stroke [122–126]. A number of studies are also exploring the role of neural progenitor/stem cells (NSCs) in ischemic stroke and have reported that NSCs protect the brain against ischemic injury, promote neurovascular repair, and improve long-term neurobehavioral outcomes. Neural progenitor stem cells differentiate into mature neurons, and thus replace the injured or dead neurons. The migration of neural stem cells is not only required for brain protection in the context of neurodegeneration but plays an important role in brain repair. Our studies have clearly demonstrated to migration of neural progenitor cells/neuroblasts to the site of injured brain following focal ischemic stroke [127–129]. Mesenchymal Stem Cells (MSCs) have gained significant attention due to their potential use in regeneration and remodeling of brain tissue. Endogenous or exogenous MSCs can differentiate into neurons, glial cells and astrocytes that support recovery of lost neurons. In addition, MSCs release various growth factors and chemokines including SDF1, brain derived neurotrophic and nerve growth factors which recruit resident neural stem cells to infarcted area, and establish network between neurons, which in turn increases the repair and recovery processes. In addition, all these stem cells regulate the immunological responses that may reduce inflammation and apoptotic cell death, which significantly reduces further neurological damage [130–132].

Physiological or pathological factors stimulate the production of bone marrow-derived EPCs and MSC, and NSCs that migrate to infarct region and attempt to repair of damaged blood vessels and neurons in ischemic tissues. However, beneficial effects of endogenous or exogenous stem cells is not fully realized in the brain likely due to their poor recruitment and survival in the injured site. The proteases MMPs and DPPIV that are induced following ischemic injury, truncate or degrade the chemotactic chemokines, thus disrupting chemokine-receptor signaling including CXCL12–CXCR4 signaling [133, 134]. This phenomenon leads to poor recruitment and proliferation of stem cells. Thus, improving the chemokine gradient from the peripheral blood to the damaged tissue may play a critical role in the recruitment of stem/progenitor cells to the ischemic brain. Taken together, inhibiting or blocking protease function rescues chemokine activities and is of benefit to promote stem cell mediated brain repair and recovery of neurological functions following ischemic stroke.

5 Concluding Remarks

Proteolytic enzymes are highly pertinent to the processes of stroke severity and functional outcome. Their interaction with other signaling molecules, particularly cytokines and chemokines plays a critical role in regulation of cross-talk between

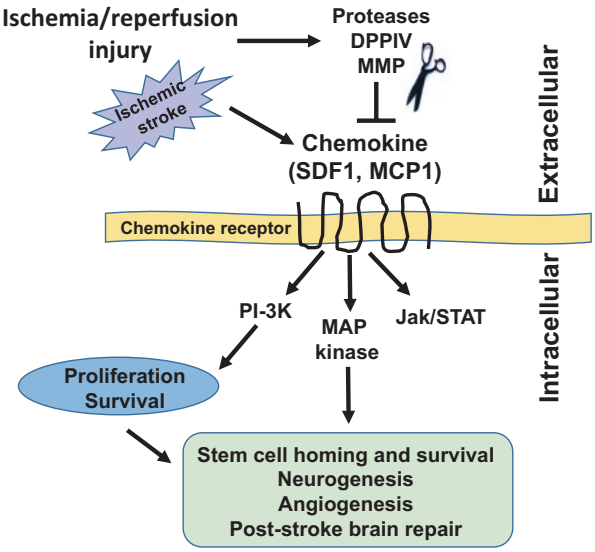

Fig. 21.1 Schematic illustration of interactions between proteases, chemokines and stem cells in response to ischemia/reperfusion injury. Ischemia/reperfusion injury is associated with upregulation of proteases and chemokines levels. Higher concentration of chemokines creates ligand gradient, and play an important role in selectively recruiting stem cells, monocytes, neutrophils, and lymphocytes that express respective chemokine receptors. This leads to activation of proliferation and survival signaling pathways. Excessive production of proteases in the ischemic microenvironment inactivates these chemokines by proteolytic events, thus disrupting chemokine-receptor signaling pathways leading to impaired stem cell homing, apoptosis, inefficient angio-neurogenesis, and poor outcome

post-stroke brain injury and ischemic microenvironment. Ischemic stroke disrupts balance between proteases and chemokines levels in the brain microenvironment. The posttranslational modifications of chemokines substantially modulate their chemotactic potential affecting various biological activities including stem cell recruitment, neurogenesis and angiogenesis. Several decades of work has established that chemokines are particularly subjected to proteolytic processing by DPPIV and MMPs. This disrupts the ability of chemokines to interact with their specific receptors, and thus impair recruitment of stem cells to injured area. In addition, truncation of chemokines disrupts survival and proliferation signaling pathways potentially leading to impaired post-stroke brain repair and recovery as illustrated in Fig. 21.1. Overall, continued research in the area of proteases, chemokines, and stem cell regulation is needed for our complete understanding of the post-stroke brain repair and recovery.

Acknowledgement Supported in part by National Institute of Health (R.J.D.) and American Heart Association (U.V.W.), and the department of Neurosurgery, UW Madison, WI 53792.

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Chapter 22
The NLRP3 Inflammasome: A Possible Therapeutic Target for Treatment of Stroke

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Abstract Ischemic stroke is a complex systemic disease causing severe long-term disability and death worldwide. Experimental and clinical data have demonstrated that inflammation is a major component of ischemic stroke pathobiology. The post-ischemic neuroinflammatory response is characterized by microglial and astro-glial activation and increased expression of inflammatory mediators. Recent findings have provided insight into a newly discovered inflammatory mechanism that contributes to neuronal and glial cell death in neurodegenerative diseases and stroke mediated by inflammasomes. Interestingly, of inflammasomes described to date, NLRP3 (nucleotide-binding domain (NOD)-like receptor protein 3) inflammasome is the best characterized multi-protein complexes and most strongly associated with sterile inflammation. In this chapter, we discuss in detail the prominent contribution and regulation of NLRP3-inflammasome activation in the pathophysiology of ischemic stroke. Furthermore, provide recent developments on the potential of NLRP3 inhibitors in the therapeutic management of stroke outcomes. The significant contribution of regulatory mechanisms of NLRP3 inflammasome with the development of stroke, may improve our understanding of NLRP3 inhibition for developing future therapies and novel drug targets for stroke.

Keywords NLRP3 • Inflammasome • Ischemic stroke • Inflammation • NOD-like receptor protein • Neuronal death • Inhibitors

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P.A. Lapchak, J.H. Zhang (eds.), *Cellular and Molecular Approaches to Regeneration and Repair*, Springer Series in Translational Stroke Research,
https://doi.org/10.1007/978-3-319-66679-2_22

Abbreviations

Aβ	Amyloid beta
ADP	Adenosine di phosphate
AIM2	Absence in melanoma
AMD	Age-related macular degeneration
AMPK	AMP-activated protein kinase
ASC	Apoptosis-associated speck like
ASK-1	Apoptosis signal-regulating kinase
ASICS	Acid-sensing ion channels
ATP	Adenosine di phosphate
BBB	Blood brain barrier
BBG	Brilliant blue G
BHB	β-hydroxybutyrate
BRCC3	BRCA1/BRCA2-containing complex, subunit 3
BTK	Bruton's tyrosine kinase
CaSR	Calcium sensing receptor
CB2R	Cannabinoid receptor 2
CLR	C-type lectin receptor
CM	Carbon monoxide
Cox2	Cyclooxygenase-2
CNS	Central nervous system
CRID	Cytokine release inhibitory drugs
CXCL1	Chemokine (C-X-C motif) ligand-1
DAMP	Damage-associated molecular patterns
DPI	Diphenylene iodonium
DUBs	Deubiquitinating enzymes
eMCAO	Embolic middle cerebral artery occlusion
EAE	Experimental autoimmune encephalomyelitis
EGCG	Epigallocatechin gallate
EP-4	Prostaglandin E2 receptor 4
ER-stress	Endoplasmic reticulum stress
FFA	Free fatty acid
FBXL2	SCF complex subunit F-box L2
GFAP	Glial fibrillary acidic protein
GLUT1	Glucose transporter-1
GPR6CA	G protein-coupled receptor family C group 6 member A
GSDMD	Gasdermin D
HCA	Hydroxy-carboxylic acid receptor 2
HIF-α	Hypoxia inducible factor-1 alpha
HRECs	Human retinal endothelial cells
HSP90	Heat shock protein 90
ICH	Intracerebral hemorrhage
IFN	Interferon

IFNAR	Interferon-alpha/beta receptor
IL-1β	Interleukin 1 beta
IP3	Inositol trisphosphate
IER-1	Inositol-requiring 1
iNOS	Inducible nitric oxide synthase
IRAK	Interleukin-1 receptor-associated kinase 1
JAC	Janus kinase
JNK	c-jun-N-terminal kinase
KO	Knock-out
LPS	Lipopolysaccharide
LRR	Leucine rich repeat domain
MAP	Mitogen activated protein
MAVS	Mitochondrial anti-viral signaling protein
MCAO	Middle cerebral artery occlusion
MFGE8	Milk fat globule-EGF 8
MI	Myocardial infarction
MAPK	Mitogen-activated protein kinase
miRNA	Micro ribonucleic acid
mtROS	Mitochondrial Reactive oxygen species
MCT	Monocarboxylate transporter 1
MMP-9	Matrix metalloproteinase 9
MNS	3,4-Methylenedioxy-β-nitrostyrene
MRI	Magnetic resonance imaging
MyD-88	Myeloid differentiation primary response 88
NADPH	Nicotinamide adenine dinucleotide phosphate-oxidase
NAD	Nicotinamide adenine dinucleotide phosphate
NADH	Nicotinamide adenine dinucleotide phosphate
NBD	Nucleotide-binding domain
NFκB	Nuclear factor kappa-B
NLR	Nucleotide-binding oligomerization domain like receptor
NLRP	NOD-like receptor proteins
NMDA	N-methyl-D-Aspartate
NOD	Nucleotide-binding oligomerization domain
NOX	Nicotinamide adenine dinucleotide phosphate oxidase
OGD	Oxygen glucose deprivation
Pal-BSA	Palmitate coupled to bovine serum albumin
PAMP	Pathogen-associated molecular patterns
PERK	dsRNA-activated protein kinase-like ER kinase
PGE2	Prostaglandin E2
PKA	Protein kinase A
PLC	Phospholipase c
PPA2	Pyrophosphatase 2
PPAR-γ	Peroxisome proliferator-activated receptor-γ
PRR	Pattern recognition receptor
PTP	Phospho-tyrosine phosphatases

P2X7R	P2X purinoceptor 7
RKIP	Raf-1 kinase inhibitory protein
ROS	Reactive oxygen species
SAR	Structure activity relationship
Ser	Serine
SGT1	Suppressor of g2 allele of skp1
SLC	Solute carriers
SCF	SKP1-cullin-F-box protein
SUR1	Sulfonylurea receptor 1
STAT	Signal transducers and activators of transcription
TAK1	Transforming growth factor beta-activated kinase 1
tMCAO	Transient middle cerebral artery occlusion
Thr	Thioredoxin
TLR	Toll-like receptor
TNF-α	Tumor necrotizing factor α
TRIF	TIR-domain-containing adapter-inducing interferon-β
Trpm4	Transient receptor potential melastatin 4
Trx	Thioredoxin
TXNIP	Thioredoxin interacting protein
WT	Wild type

1 Introduction

According to the latest statistics, stroke when considered separately from other cerebrovascular disorders is standing as the fifth cause of death, behind diseases of the heart, cancer, chronic lower respiratory disease, and unintentional injuries/accidents [1]. Around two thirds of stroke patients either die or are left disabled [2]. Of all strokes, 87% are ischemic while the rest 13% is kind of hemorrhagic. Roughly 40% of hemorrhagic cases are fatal while of those, <40% of the surviving patients may live independently in the first year after. Thrombolytic therapy is the only existing therapeutic approach, being applicable for patients with no hemorrhagic events it is advantageous only for those admitted to clinical care centers not later than 6 h after symptoms onset. Given the symptoms of either type of stroke is not quite specific, it is rational that <5% of stroke patients can get to efficient therapeutic measures [1, 3]. Therefore increasing efforts are being focused to unravel the detailed pathological process of stroke insult proving more promising therapeutic targets. Neuroinflammation has been recognized as of early implicated pathways starting from the very early beginning from stroke-associated endothelial damage and lasting for several weeks in parallel with functional shekels. For that reason it is intricately investigated in terms of its critical effectors which inherently parallel with neural loss [4, 5]. During the acute phase, referring to minutes to hours after stroke,

ischemic cell death leads to the release of a variety intracellular contents working as danger signals. Theses agent including Ca^{2+}, enzymes and cytokines (i.e., IL-1 and TNF-α) induce an initial inflammatory response which in turn, propagate the neuro-inflammatory response through activation of resident immune cells (i.e., microglia) and recruitment of inflammatory cells. Neutrophils are the first circulating cells to migrate into the brain after stroke, followed by gradual appearance of macrophages and lymphocytes in the site of injury, including core and peri-infarct regions [6, 7]. Formation of large multiprotein complexes called inflammasomes, are of the early established events efficiently amplifying the primary immune responses. Working as of main innate immune system receptors and sensors, inflammasomes are increasingly defined to act as the key mediator in detecting cellular fait and inflammatory responses after stroke [8]. Conspicuously, inflammasomes modulation could remarkably prevent neural cell death and attenuate ischemia/reperfusion (I/R) injury in *in-vivo* and *in-vitro* stroke models [9, 10], the observation that might be explained with the widespread events downstream to inflammasomes. Indeed inflammasomes activation not only instigate a variety of deteriorating and death signals, but also it is not confined to immune cells i.e. cerebral microglia. In this line, recent researches have revealed that almost all CNS territory residents including neurons, astrocytes, granulocytes and even endothelial cells also express functional inflammasomes which contribute to either CNS disorders and systemic inflammation [8]. Furthermore, upon activation, the inflammasomes induce two caspase-1-dependent inflammatory cascades: necrotic cell death (pyroptosis) and processing of IL-1β and IL-18 to active forms, both of which produce substantial consequences. Pyroptosis is the lytic cell death occurring through cleavage of gasdermin D (GSDMD) by active caspase-1. The N-terminus of GSDMD then may form pores in the plasma membrane which permits passage of fluids disturbing ion hemostasis in intracellular space besides bringing pro-inflammatory cytokines. IL-1β and IL-18 are the only cytokines produced as inactive precursors which are also processed by inflammasomes. Mature IL-1β and IL-18 cytokines activate IL-1 receptor/toll-like receptor (IL-1R/TLR) signaling. Pivotal for activation of innate immunity and inflammation, this signaling stimulate the transcription of many pro-inflammatory and antiviral genes, all contributing to amplify the background inflammatory response. IL-1β is a critical cytokine in CNS pathologies which shows true co-localization within areas of early focal neuronal injury implies that it might be the major form of IL-1 contributing to inflammation following stroke [11, 12].

In essence, this chapter would provide basic information required for understanding inflammasome function from sensing danger signals to contributing to stroke pathology. With the central role of NLRP3 inflammasome and its major implication in ischemic insult, the main effort is to demonstrate the details for its regulation during pathological processes of tissue injury in cerebral ischemia. Finally, an overview of recent advances on developing inflammasome inhibitors are provided, with a particular view on searching for promising therapeutic tools in stroke research.

2 NLRPs as Tissue Injury Sensors

NLRPs (NOD-like receptor proteins) are one of pattern recognition receptors (PRRs) in macrophages which play essential role to upregulate pro-inflammatory gene expression [13, 14]. Based on cellular location and major structural features, PRR may be classified into (a) transmembranal TLRs, (b) transmembranal C-type lectin receptors (CLRs); (c) cytosolicnucleotide-binding oligomerization domain-like receptors or NOD-like receptor proteins (NLRPs); (d) intracellular retinoic acid-inducible gene-1 (RIG-1)-like receptors (RLRs), which are primarily involved in antiviral responses; and (e) absent in melanoma 2 (AIM-2)-like receptors (or non-NLRs).

All these receptors may induce major pro-inflammatory and pro-apoptotic pathways upon activation with their cognate ligands. Depending on initial source, PPR ligands are classified to two classes named pathogen associated molecular patterns (PAMPs) or damage associated molecular patterns (DAMPs). PAMPs are motifs commonly carried by pathogens, e.g. bacterial endotoxin (or LPS) on gram-negative bacteria. Monosodium urate or cholesterol crystals and β-amyloid plaques or endogenous molecules released by necrosis like ATP are among well characterized DAMPs. The inflammation that ensues DAMPs-induced PPRs stimulation is called the sterile inflammation and either resolves the initial insult or leads to disease [15].

3 NLRP3 as the Predominant Inflammasome

Unlike many other PRRs, NLRs have not been restricted to a specific ligand or a typical cognate molecular pattern (PAMP or DAMP) [14, 16]. The NLR family is one the most extensively studied PRRs due to its major role in several pathological inflammation. NLRs are generally composed of three separate domains: 1) the N-terminal domain: which contains a pyrin domain, a caspase recruitment domain, or a baculovirus inhibitory repeat domain and has been used as a structural sub-classification for the NLR family, 2) The central NBD or nucleotide-binding domain, which is responsible for dNTPase activity and oligomerization in the presence of nucleotides, primarily ATP, 3) A Leucine rich repeat domain (LRRs) at the C terminus of NLR proteins [17]. Upon activation, the NLR protein oligomerizes with the ASC (apoptosis-associated speck like) adaptor protein which then recruits procaspase-1, allowing its autocleavage and activation. Activated caspase-1 enzyme in turn cleaves upregulated premature proinflammatory cytokines: interleukin-1 (IL-1) and interleukin-18 (IL-18) and causes their release [18, 19]. Several NLRs have the capability to activate the inflammasome in vitro, including: NLRP1, NLRP2, NLRP3, NLRP6, NLRP12, NLRC4 and NOD-2, however, only a handful of prominent NLRPs including NLRP1, NLRP3, NLRC4 and NAIP5 have been recognized as functional inflammasome activators [17]. Comparatively, in contrast to most innate immune receptors [20], NLRP3 might be activated by a variety of

pathogen- and host-derived "danger" signals including whole RNA, RNA/DNA hybrids and proteins from gram-positive and gram-negative bacteria, viruses, fungi, protozoa (as PAMPs), as well as ATP, monosodium urate crystals and calcium pyrophosphate dihydrate crystals (as DAMPs) [21, 22]. Given a diversity of stimuli may engage NLRP3, NLRP3 activation is very likely to happen downstream to many PRRs converging on a common pathway. Interestingly, of inflammasomes identified to date, NLRP3 is most strongly associated with sterile inflammation [15]. In fact the NLRP3 inflammasome is established as one of the most critical multi-protein responsible for instigating metabolic, cardiovascular, and neurodegenerative disease-associated inflammation, and is therefore named as a sensor for metabolic danger [23–25].

4 NLP3 Activation and Regulation

According to the pictorial description in Fig. 22.1, based on the most addressed models; NLRP3 activation necessitates two main signals "Priming" and "Activation" both of which initiating following sensing tissue damage through RRPs. Priming is best defined as preparing inflammasomes at transcriptional and post-transcriptional level, to get instigated by the activating signal to induce IL-β secretion from immune cells. The well-known canonical pathway for NLRP3 inflammasome activation illustrate the main dynamic interplay between tissue injury/infection, innate immune system and pathological inflammatory responses. However recent years investigations has unraveled a non-canonical pathway that influence NLRP3 inflammasome priming and activation particularly in gram negative infections. The general principles are so common though, the discriminative attributes of these two terms are detailed in the principal steps in NLRP3 priming, activation and regulation as described below.

4.1 Step 1: Priming; Canonical and Non-canonical

4.1.1 Transcriptional Priming

In physiological conditions, NLRP3 and pro-IL-1β are not expressed in sufficient amounts. That is signaling through transcriptionally active receptors (e.g.; TLRs, NOD2, TNFR1 and TNFR2) seem constitutionally necessary for NLRP3-inflammasome activation [26, 27]. Given that critical mass of NLRP3 is required for inflammasome activation, it has been shown upregulation of NLRP3 is enough for priming-independent activation [28]. Indeed, NLRP3 substantial expression lowers the threshold for NLRP3-inflammasome activation and enhances caspase-1 cleavage as well [29]. Nuclear factor kappa-B (NFκB) and mitogen-activated protein kinases (MAPKs) pathways are central for driving the gene expression and denovo

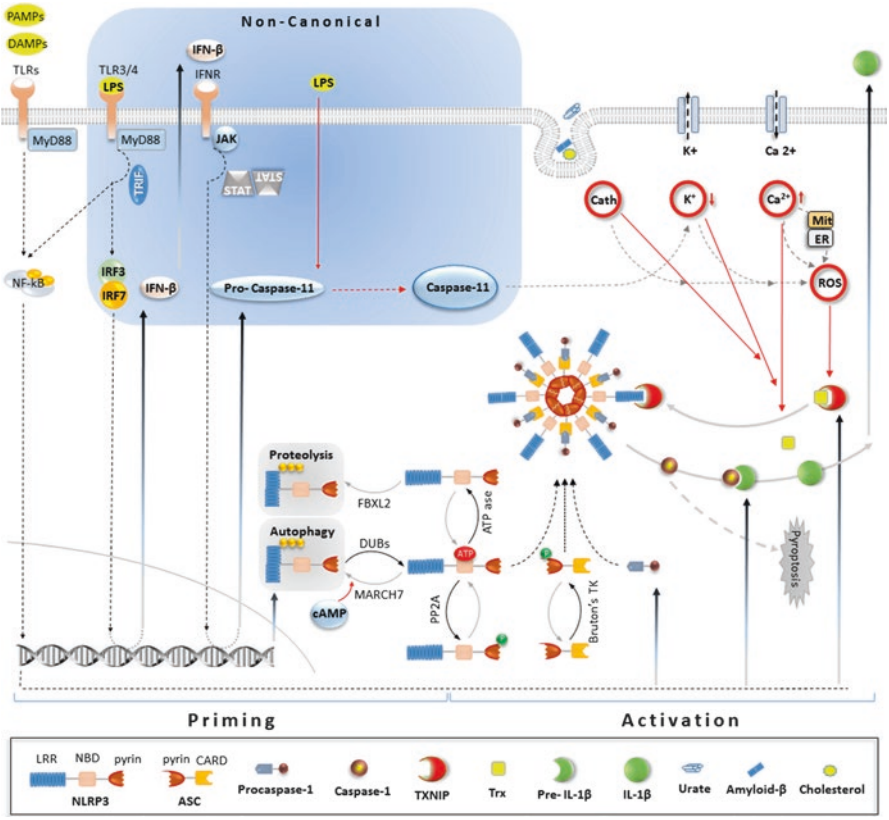

Fig. 22.1 Schematic representation of mechanisms involved in NLRP3 priming and activation. Priming step contributes to preparing NLRP3 inflammasome constituents and Pro-IL-1b (or IL-6) in transcriptional and post-translational levels. Basically TLRs stimulation by DAMPs or PAMPs (e.g. LPS) coupled with MyD 88-NFkB pathway enhances the due transcripts. Following construction in cytosolic mileu, the readily ubiquinated NLRP3 might be subject to degradation by autophagy or DUBs action. The deubiquinated NLRP3 then may recruit ASC and then pro-caspase 1 if is in a non-phosphorylated state devoid of electrostatic repulsion of pyrin domains with the ASC phosphorylated pyrin. After binding to ASC, the NLP3 ATPase activity brings ATP molecules to attach to NLRP3, the step is required for NLRP3 oligomerization. A wide range of stimuli are known to activate the NLP3 inflammasome. As shown briefly in the *up-right panel*, any factor that causes lysosomal cathepsin release following phagocytosis of non-functional particles (e.g. cholesterol crystals) and any stimuli that ends with intracellular K^+ depletion, Ca^{2+} over load and ROS generation may result in NLRP3 activation. The direct mechanism through which this effectors interface with NLRP3 to boost its caspase-1 cleavage activity is un-known. However ROS either is generated by external stimuli or downstream to other effectors has been known to explain NLRP3 inflammasome activation. Under high levels of intracellar ROS, TXNIP which is normally controlling Trx activity is translocated to NRLP3 inflammasome and instigate its activity. Following activation, NLRP3 inflammasome leads to caspase-1 activation, IL-1b release and pyroptosis. NLRP3 inflammasomes might be exported to ECF and subsequent proteolysis. Along with the basic pathways in priming and activation, there are some alternate mechanisms specific to gram negative bacteria called non-canonical as determined in light blue background. Typically LPS in gram negative

protein synthesis of NLRP3 and pro-IL-1β. Several DAMPs and PAMPs bind to pro-inflammatory receptors, including TLRs, NLRs, and tumor necrosis factor receptors 1 and 2 (TNFR1 and TNFR2), for which NF-κB or MAPKs work as transcriptional effectors. The signal is basically mediated by toll-like receptor (TLR)-adaptor molecules MyD88 (myeloid differentiation primary response 88) working along with IRAK1 and 4 (IL-1R associated kinase family members) and does not merely result in transcriptional priming but also may get involved in transcription-independent priming pathways [30, 31].

While priming step is somewhat common between canonical and non-canonical pathway, it may be differentiated based on particular transcripts might be specifically induced in the later phase. That is with the initiating stimuli to be gram negative bacteria (endotoxin), non-canonical pathway is not limited to MyD88 recruitment and IL-1β, IL-18, and NLRP3 transcription, but as well; engages toll/IL-1 receptor homology-domain-containing adapter-inducing interferon-β (TRIF) pathway. Through enhancing IRF3–IRF7 complex formation [32, 33], this elicits the expression of interferon (IFN)-α/β, and then JAK/STAT pathway activation which eventually elevates transcription of mouse caspase-11 (or human caspase 4-5). This caspase subtype acts as a prominent adjuvant activating stimulus specific to non-canonical NLRP3-inflammasome activation [34–36].

In terms of preparing licensed inflammasomes for activation, it is important to note that, the transcription of the due substrate pro-IL-1β simultaneously starts to rise taking hours to peak, but may not be temporally related to inflammasome activation. In contrast, pro-IL-18 is constitutively expressed in sufficient levels and available for inflammasome processing leading to IL-18 Release within minutes of appropriate stimulation [26, 37]. Apparently while transcriptional priming seems the essential step for inflammasome activation, transcriptional upregulation of NLRP3 and pro-IL-1β may also amplify inflammasome activity.

4.1.2 Non-transcriptional Priming

Based on numerous evidences NLRP3 inflammasome priming might take place independent of new protein synthesis, either in canonical or non-canonical pathway. That is inhibition of transcription or translation may not totally block

Fig. 22.1 (continued) bacteria activate the TLR4–MyD88 and TRIF pathways, with subsequent IRF3–IRF7 complex formation. This elicits the expression of IFN-β that binds the IFNR. The consequent JAK/STAT activation leads to elevated transcripts of caspase-11 which is then cleaved and activated by the intracellular signals of LPS11 that in turn promotes the activation of NLRP3-ASC-caspase-1 pathway. Abbreviations: *NLRP3* nucleotide-binding oligomerization domain, leucine rich repeat and pyrin domain-containing protein 3, *TLRs* toll-like receptors, *MyD88* adaptor molecules myeloid differentiation primary response 88, *TNFR* tumor necrosis factor receptor, *NF-κB* nuclear factor-κB, *ATP* adenosine triphosphate, *ROS* reactive oxygen species, *TRIF* toll/IL-1 receptor homology (TIR)-domain-containing adapter-inducing interferon-β, *IRF* interferon regulatory factor, *IFN* interferon, *IFNAR* interferon-α/β receptor, *IL* interleukin, *JAK/STAT* janus kinase/signal transducers and activators of transcription, *ER* endoplasmic reticulum, *Mit* mitochondria

inflammasome priming and activation, suggesting an important regulatory role for post translational modifications in NLRP3 inflammasome. Supporting evidence highlighting post-translational modification, it has been shown IL-18 release takes place within minutes of monocyte stimulation, which is much faster than the discernible increase in NLRP3 protein abundance [26, 29, 38]. NLRP3 receptors are constitutionally ubiquitinated following expression providing a critical regulatory mechanism for their stability and function for an immediate switch between inflammatory and silence immune responses. In fact as far as ubiquitinated, NLRP3 reside inactive in cytoplasmic milieu not capable of self-oligomerization. Upon sufficient stimuli, the main deubiquitinase enzyme BRCC3 (BRCA1/BRCA2-containing complex, subunit 3) removes the ubiquitin leading to dissociation of HSP90 and SGT1 from NLRP3 which is critical for relieving from its auto-inhibition.

Such a regulation might be more complex regarding to the fact that multiple agents may control NLRP3 ubiquitination which takes place in both NLRP3 K48- and K63 regions. (1) As mentioned above, the DUB enzyme BRCC3 works as a prominent regulatory point [39], but does not work as the only check point. (2) Instantly, SCF complex subunit F-box L2 (FBXL2) may constitutively ubiquitinate NLRP3 for degradation at the proteasome. The interaction between FBXL2 and NLRP3 might be abolished by bacterial endotoxin preserving inflammasome activity [40], (3) E3 Ubiquitin-Protein ligase, MARCH7; which also mediates anti-inflammatory function of the neurotransmitter Dopamine, ubiquitinates NLRP3 and targets it for degradation by autophagy [41].

Few other post-transcriptional alterations have been also characterized for NLRP3 activation. Intracellular kinases and phosphatases play a key role in balanced phosphorylation forms in ASC and NLRP3 for appropriate interaction. Bruton's tyrosine kinase (BTK) has been recently identified as an essential component of the NLRP3 inflammasome activation in unprimed cells, in which BTK physically needs to interacts with ASC and NLRP3 and contribute to ASC phosphorylation at pyrin domain [42]. On the contrary side, in unprimed cells, NLRP3 is phosphorylated at S5, resulting in electrostatic repulsion between pyrin domain with that of ASC. Thus PP2A induces dephosphorylation licenses NLRP3 for activation and is required before inflammasome assembly [43].

4.2 Step 2: Activation; Canonical and Non-canonical

NRP3 inflammasome activation might be defined as NRP3 oligomerization rendering it capable of pro-Caspase 1 cleavage. Following essential priming, NLRP3 may recruit the adaptor protein ASC, which has a pivotal role in the activation of pro-caspase 1 following the formation of the multiprotein complex consisting of NLRP3, ASC, and pro-caspase-1 [44–46]. Nevertheless, an efficient activating stimulus is required to initiate the assembly and promote NLRP3 inflammasome oligomerization. In specific feature of non-canonical pathway binding of lipopolysaccharide (LPS) to inactive mouse caspase-11 (or human caspase 4-5) produced through the

earlier priming, has been shown to activate the effector functions of caspase-11 to activate NLRP3 inflammasome oligomerization [47]. Nevertheless, variety of stimuli namely ATP, osmolarity or pH alterations may activate NLRP3 and ASC recruitment, might be still involved in NLRP3 activation by gram negative infections. Major classes of NLRP3 activators including several DAMPs and PAMPs end with a few alterations in intracellular territory namely potassium depletion, which in turn lead to eventual NLRP3 inflammasome-induced caspase-1 cleavage. Given most of the diverse stimuli converge on at least one of these downstream events, in the following section the discussion continues based on the type of the effectors relying downstream to one or more DAMPs or PAMPs which are addressed in this classified context.

4.2.1 Intracellular Potassium Depletion

Long before the discovery of inflammasomes, studies on LPS-primed macrophages confirmed IL-1β proteolytic processing and secretion depends on cytosolic K^+ depletion, as a common mechanism in response to different stimuli like ATP or nigericin [48, 49]. Conversely, increased extracellular K^+ concentrations was demonstrated to block NLRP3 inflammasome assembly in response to most of the identified NLRP3 triggers. Now known to be stimulated by a variety of initiating stimuli, K^+ efflux remains the best-characterized responsible stimulus for NLRP3 inflammasome activation [46]. However the exact mechanism leading to NLRP3 activation is not fully understood, recent findings has determined the Ser/Thr kinase involved in mitotic cell division, NEK7, is specifically required for NLRP3 inflammasome activation downstream to K^+ efflux, which is recruited to NLRP3 upstream of inflammasome formation [50, 51]. The following sub-sections brief the main NLRP3 activating stimuli converging on reducing the cytosolic level of K^+ ion, trough different mechanisms.

4.2.1.1 Extracellular ATP

Extracellular ATP which might increase following detrimental tissue damage and release of intracellular contents, has been established as an agonist of a ligand-gated cation channel called P2X7 receptor (P2X7R). Upon activation this channels opens and allows for an exchange of intracellular K^+ ions for extracellular Na^+ or Ca^{2+} ions. Producing a net K^+ efflux; this results in NLRP3 inflammasome activation. Activation of P2X7R may also follow opening of pannexin-1 channels, mediating caspase 11 (or human caspase 4-5) induced NLRP3 activation. That is following recognition of LPS caspase-11 cleaves intracellular domain of pannexin-1, the intramembranous receptors in monocytes, leading to opening of the channel and K^+ and ATP efflux into the extracellular space. Consequently, the leakage of K^+ ions activates the NLRP3 inflammasome and ATP acts as an agonist for the P2X7R to amplify the K^+ depletion and NLRP3 inflammasome activation. Intriguingly, it has

been demonstrated the acquired levels of ATP through this process, are much lower (nanomolar concentrations) than the amounts of ATP typically proposed to activate P2X7R receptors [52].

4.2.1.2 K^+ Ionophores

K^+ ionophores have been shown to induce NLRP3 inflammasome activation when they produce a net K^+ efflux. A variety of cytotoxic agents like nigericin, gramicidin and valinomycin are amongst the well-studied K^+ ionophores capable of NLP3 inflammasome stimulation. Nigericin, is a lipophilic ionophore existing in a free membrane-impermeant anionic form or a neutral membrane-permeant complex. In its anionic form, nigericin binds to H^+ on the outside of the cell releasing the proton on the intracellular side, leading to acidification of cytosol. Then in intracellular side, nigericin anion binds to K^+ and releases it on the outside of the cell, leading to K^+ efflux. Valinomycin, also forms equimolar complexes with K^+, however; unlike to that of the neutral nigericin-K complexes, it forms a single positive charge complex (valinomycin-K^+). Therefore, valinomycin mediated K^+ efflux is electrogenic and thus is limited to the balanced gradients between both sides of cell membrane. Gramicidin as a peptide ionophore, allows for K^+ efflux balanced by Na^+ influx in a manner electrochemically similar to the P2X7R [53, 54].

4.2.1.3 Extracellular Hypo-tonicity

Extracellular Hypo-tonicity may result from pathological edema or any event disturbing massive ion hemostasis and is shown to contribute to NLRP3 inflammasome activation mainly through intracellular K^+ depletion. Two main mechanisms may underlie the following K^+ deregulation. Firstly extracellular tonicity leads to K^+ and Cl^- channels opening, driving an efflux of K^+ and Cl^- ions to balance the intracellular and extracellular osmolarity values. Secondly Na^+/K^+-ATPase pump dysfunction may lead to increased net influx of Na^+ ions which in turn will promote an osmotic movement of water through aquaporins into the cell. Besides diluting intracellular K^+ ions concentrations and NLRP3 inflammasome activation, a strong osmosis would be deteriorating enough to end with cell death [22, 45].

4.2.2 Intracellular Ca^{2+} Overload

Some of NLRP3 inflammasome activating stimuli seem to act through increased intracellular levels of Ca^{2+}. Extracellular Ca^{2+} may influx trough different plasma membrane-resident Ca^{2+} channels namely ASICs (Acid-sensing ion channels) [55, 56]. Importantly, acidosis as well as mitochondrial and endoplasmic

reticulum (ER) stress are among the best characterized pathological features activating NLRP3 inflammasomes probably via Ca^{2+} overload. ER stress occurring following accumulation of unfolded proteins in ER compartment or disruption of the ER-Ca^{2+} homoeostasis, may lead to increased cytosolic Ca^{2+} concentration through phospho lipase C (PLC)/inositol triphosphate pathway i.e. following ATP stimulation. Mitochondria may also absorb or release Ca^{2+} under different conditions.

Pathological Ca^{2+} overload can lead to NLRP3 activation trough two different pathways: (1) it can increase mitochondrial ROS (mtROS) production, besides collapsing of the mitochondrial membrane potential [57], (2) Excessive intracellular Ca^{2+} also activate kinase TAK1 which has been shown to be involved in NLRP3 activation induced by lysosomal damage or hypotonic stimuli [45, 58, 59]. First demonstrated by Brough et al. Ca^{2+} chelator BAPTA-AM was shown to reduce IL-1β production in ATP-treated macrophages [60]. Later, it was discovered that both incubation with Ca^{2+}-free media and inhibition of intracellular ER Ca^{2+} stores by thapsigargin would prevent caspase-1 activation and IL-1β secretion by ATP, nigericin and alum [61], implying Ca^{2+} acts as a pivotal mediator for some NLRP3 inflammasome activators. However, there are still some uncertainties for significance of the hypothesis, in particular as in some studies; required concentrations of small-molecule compounds required to inhibit NLRP3 inflammasomes, significantly exceeds their IC50 values reported for other processes [62, 63].

4.2.3 Lysosomal Rupture and Pyroptosis

Insoluble particulates may manifest in extracellular milieu following exposure to environmental irritants or as a consequence of pathological tissue injury. This might include adjuvants like alum, the air pollutants such as silica, or the disease associated particles like monosodium urate and calcium pyrophosphate dehydrate are all well characterized as the NLRP3 inflammasome activators. It has been established that endocytosis of these crystalline particles triggers the disintegration of endolysosomal organelles which may lead to lysosomes rupture or pyroptosis. Based on evidences on lysosomal rupture theory, release of lysosomal contents into the cytosol, in particular for cathepsin B, could also trigger NLRP3 signaling [64–66]. In this line inhibitors of lysosomal cathepsins have been shown to block caspase-1 activation by all tested NLRP3 inflammasome inducers [67]. In the event that pyroptosis inducers rather than lysosomal deregulators, are engulfed by phagosomes, distinct cellular responses are believed to induce NLRP3 activation, involving catepsin-C instead of catepsin-1. In fact pyroptosis inducing agents are shown to induce more profound NLRP3 activation in line with caspase-B-mediated cell death, cascade of events starting with the release of proteolytic enzymes into the cytosol and leading to a loss of plasma membrane integrity and systemic immune response [68].

4.2.4 Reactive Oxygen Species

According to a pile of concrete evidences ROS is an essential signaling component required for NLRP3-inflammasome activation, besides affecting the prerequisite priming step. Xanthine oxidase, peroxisome oxidases, uncoupling of cytochrome p450 and nitric oxide synthases; in conjunction with NADPH oxidases and mitochondrial are among the multiple subcellular sources for ROS production. Of these, NADPH oxidases and mitochondrial ROS (mROS) are extensively studied and identified as sources of ROS involved in NLRP3-inflammasome activation [18, 30, 69]. Indeed empirical evidences indicating ROS is produced by a variety of NLRP3 inflammasomes activators, and works as a critical underlying mechanism in triggering NLRP3 inflammasome formation, suggest the hypothesis that ROS formation is a major mediator of a diversity of NLRP3 inflammasome activating DAMPs and PMPs [70]. Nevertheless NLRP3 is originally assumed as a cytosolic receptor with broad range of activating stimuli, it seems outlandish that NLRP3 acts as a receptor capable of directly binding to the effectors of all these remarkably different stimuli. Apparently ROS production offers a link to many different stimuli, including a variety of DAMPs and PAMPs stimulating canonical and non-canonical pathways to NLRP3 inflammasome activation [70].

In spite of the pile of evidences implicative of pivotal role of ROS in NLRP3 inflammasome's activation, little is known about the exact mechanisms by which NLRP3 senses oxidative stress. So far thioredoxin-interacting protein (TXNIP) and mitochondrial anti-viral signaling protein (MAVS) are characterized as to be associated with NLRP3 inflammasome stimulation by ROS. Demonstrated by Subramanian et al., MAVS is a mitochondrial adaptor protein to mediate the relocalization and association of NLRP3 inflammasome to mitochondria in stress conditions [71]. However, apparently MAVS mediating effect is specific to ATP and nigericin stimulation as well as few viral challenges [72].

TXNIP, the endogenous inhibitor of the antioxidant thioredoxin (Trx), may time dependently dissociate from Trx, bind to NLRP3 inflammasome to stimulate caspase-1 cleavage activity. Association between these two proteins was defined by the study of Zhou et al. as a necessary step in NLRP3 inflammasome activation in pancreatic islet cells in response to high glucose [73]. Ever since, several investigations have confirmed the necessity of TXNIP for NLRP3 inflammasome activation [74–76]. To confirm the specific role of TXNIP, shRNA transfection targeting TXNIP [77], has empirically prevented TXNIP-NLRP3 binding and subsequent homocysteine-induced glomerular injury. It might be of note, according to the existing evidences a cell-type specific manner exists for TXNIP, limiting its effects to mediate the proinflammatory effects of ROS signaling molecules, in particular cell types [78].

4.2.4.1 TXNIP and Thioredoxin System

The thioredoxin (Trx) is a thiol-reducing system consisting of NADPH (nicotinamide adenine dinucleotide phosphate-oxidase) and homodimeric seleno-protein thioredoxin reductase besides Trx [79]. There are two main critical roles for Trx

system both implicated in the pathophysiology of diseases such as diabetes, arthritis and neurodegenerative disorders. First is maintaining the reducing environment which is critical to mask proteins disulfide to protect against reactive oxygen species. Next Trx exerts antiapoptotic effects which manifests trough binding to the pro-apoptotic protein apoptosis signal-regulating kinase 1 (ASK-1) and blocking its activity. TXNIP is a critical protein for regulating Trx function. As a stress sensor, TXNIP expression can be induced by neumerous exogenous detrimental stimuli, including metabolic stress and changes in calcium and oxygen levels [75, 80, 81]. Both expression and activity of Trx is strictly controlled by TXNIP which tightly limiting Trx's diverse roles in redox-dependent processes including protein folding, regulation of apoptosis, and protection from oxidative stress [79, 82]. TXNIP provides two discrete Trx related effects: (1) Affecting Trx redox dependent signaling by limiting the availability of free sulfhydryl (thiol) group of Trx [83, 84]. (2) Translocating Trx as a member of the alpha arrestin protein family, the later highlighting a major role of TXNIP as a scaffolding protein [85]. In terms of shuttling and targeting proteins into different subcellular compartments, under increased ROS levels TXNIP may translocate Trx to the plasma membrane [86], or may shuttle from the nucleus to bind the mitochondrial Trx and mediate the inhibition of glucose uptake in response to increased ROS [87, 88].

4.2.4.2 TXNIP and NLRP3-Inflammasome Activation

As a link between increased cellular ROS levels and the proinflammatory genes expression, TXNIP has attracted many investigations for developing promising therapeutics. Through a direct interaction with NLRP3 this protein seems to convey the message from a wide variety of stimuli to NLRP3 and subsequently to caspase and Interleukines activation. The phenomena first identified in 2010 by Zhou et al. in cultured macrophages, different NLRP3-inflammasome activators including ATP, monosodium urate crystals and silica were shown to enable TXNIP to dissociate from Trx and bind to the inflammasome receptor, NLRP3; following increases in cellular ROS levels. Using pharmacological and genetic tools they provided concrete evidences implying TXNIP binding is the essential prerequisite for in vitro and in vivo NLRP3 inflammasome activation [73]. Pursued by other scientists it was further understood, more diverse type of agents could engage TXNIP translocation subsequent to ROS production. High fat diet in experimental animals [89], high glucose-exposed human retinal microvasculature [90] and thrombin-exposed BV2 cells [91] are among the most recent models have been closely inspected for involvement of TXNIP/NLRP3. Taken together with other evidences on LPS, viral and pathological stimuli the obtained findings from these investigations conclude that TXNIP works as a wide road for deteriorating insults to reach out to NLRP3 inflammasome as the governing inflammasome in inflammation and apoptosis [92–94].

4.2.4.3 Modes of NLRP3 Activation Trough TXNIP

According to several studies ROS generating stimuli may augment TXNIP/NLRP3 through enhancing TXNIP transcription and expression. Besides several data about enhanced TXNIP protein expression following exposure to NLRP3 activators [95, 96], recent findings indicate that TXNIP gene transcript are induced simultaneously with NLRP3. As part of inflammasome priming, Feng et al. showed TXNIP transcripts also raises in parallel with NLRP3 mRNA when they exposed mesangial cells to LPS [97], leading to the due proteins over expression. Thereafter in the study of potential disease modifying agents, using carbon monoxide (CM) to reduce lung inflammatory response to LPS, Jiang et al. represented in vivo evidences for elevated TXNIP mRNAs, concurrent with that of NLRP3 and IL-1β following LPS exposure which was reversed by CM [98]. A recent work also demonstrated, berberine's anti-inflammatory effects on macrophages stimulated by monosodium urate crystal is associated with reversed transcript levels of TXNIP as well as NLRP3 inflammasome [99].

Enhanced shuttering activity and TXNIP translocation to the mitochondria milieu is the other well documented behavior of TXNIP following ROS generating agents. Early after their first demonstration, Zhou et al. found ROS induced activation of the NLRP3 inflammasome is associated with increased translocation of TXNIP into the mitochondria [100]. It is well confirmed by a recent work in which intracellular shuttling of TXNIP was assessed by immunofluorescent staining using MitoTracker Red, showing enhanced translocation to mitochondria in high glucose exposure which was efficiently attenuated by antioxidants [101].

4.2.4.4 TXNIP/NLRP3 Inhibition in Inflammation and Disease

Given that TXNIP/NLRP3 pathway have been shown to be functional in several cell types and organ systems, its pharmacological inhibition trough different specific or pleiotropic agents has been shown to contribute to significant ameliorating effects. Genetic deletion of TXNIP, as a precisely specific method has established TXNIP as an incomparable effector for NLRP3 stimulation. Validated TXNIP silencing by continuous delivery of TXNIP deoxy ribozyme (DNA zyme) in a period of 12 weeks in an animal model of renal injury [102], TXNIP targeted shRNA treatment in high glucose-induced NADPH oxidase activation [94], or siRNA interference against TXNIP in rat hepatic inflammatin [103] have demonstrated successful TXNIP ablation which has resulted in substantial prevention of NLRP3 formation and disease progress.

Additionally, pharmacological inhibition of TXNIP utilizing natural or pleiotropic agents has provided putative potential therapeutic targeting TXNIP/NLRP3 pathway. Instantly quercetin (a natural antioxidant of flavonoid origin), rutin (a flavonol quercetin) or ascorbic acid all provide ameliorating effect on disease model of high glucose/fructose stress [101, 104, 105]. Xanthohumol, a principal prenyl-flavonoid is among the most recently introduced agents with substantial efficacy in TXNIP/NLRP3 blockage in acute lung injury model [92].

4.2.4.5 TXNIP as an Experimental Target in Ischemic/Reperfusion Injury

Regarding the profound generation of ROS during ischemic/reperfusion injury, there is remarkable emerging interest toward investigating novel therapeutics targeting TXNIP. Cardiac NLRP3 inflammasome activation has been shown to be associated with over-production of ROS and TXNIP in MI dogs [106]. Conspicuously, co-over expression of TXNIP and NLRP3 in animal model of cerebral stroke has been shown to be suppressed by ameliorating agent like ruscogenin, an important steroid sapogenin [107]. The ameliorating effects of umbelliferone, a natural antioxidant belonging to coumarin derivatives [108] as well as curcumin, a natural polyphenolic compound [109] have been also ascribed to attenuation of TXNIP/NLRP3 pathway, while providing neuroprotective in experimental model of cerebral ischemia and ER-stress, respectively. In a recent effort to precisely address TXNIP role in NLRP3 activation in human stroke, we utilized mice embolic stroke model, and we found TXNIP knock down or pharmacological inhibition by resveratrol leads to substabstantial protection against stroke with close implication of NLRP3 and PPARs in conjunction with inflammatory cytokines IL-1β and TNF-α [110].

4.3 Endogenous Regulators of NLRP3

4.3.1 Autophagy

The substantial intracellular degradation system autophagy, plays a key role in inflammasome inactivation. The autophagosome surrounds a portion of the cytoplasm forming the autolysosome, which leads to degradation of the contents. Engulfing several cytoplasmic molecules, autophagy may result in broad effects on inflammasomes function as a multi-compartment assembly. Autophagy have been defined to probably block inflammasomes, by its degrading activity at least at three different levels (1) activating noncanonical pathway (2) priming ASC and pro-IL-1β, (3) mature IL-1β secretion [111]. In brief, autophagy deficiency may lead to accumulation of damaged bacteria residues and enhance activation of the noncanonical inflammasome. Also it has been shown that Pro-IL-1β and IL-1β can be degraded in autophagosomes, leading to decreased inflammatory responses [112]. Interestingly, it is also proposed that autophagy preferentially controls NLRP3 activation rather than others inflammasomes. For all these reasons it is pretty rationale that autophagy impairment may contributes to caspase-1 overactivation [113] as well as NLRP3 response to various infections [114]. Some endogenous agents like cannabinoids have been proved to contribute to modulate NLRP3 inflammasome activity via autophagy induction. Cannabinoid receptor 2 (CB2R) demonstrated as a therapeutic target in inflammation-related diseases, recently has been shown to induce autophagy which may explain why activation of the anti-inflammatory CB2R attenuates NLRP3 inflammasome in mouse BV2

microglia as well as in a mouse model of EAE [115, 116]. Thus CB2R agonists (i.e., HU-308) may provide effective therapy for treating NLRP3 inflammasome-related diseases. Some naturally occurring compounds also act as autophagy inducers, and would be discussed later.

4.3.2 cAMP Enhancers

There are concrete evidences implying cAMP may inhibit NRP3 inflammasome. It is believed to bind to NLRP3, and recruits the ubiquitin ligases which polyubiquitinates NLRP3 and prepares it for autophagosomal degradation. In this line it has been shown that pharmacological activators of adenylyl cyclases [117], or agonists of GPCRs enhancing adenylyl cyclase activity [117, 118], lead to a decrease in typical NLRP3 activation. Prostaglandin E2 (PGE2) having a broad range of effects, acts as a vasodilator and facilitates tissue influx of neutrophils [119] and macrophages [120] in early phases of inflammation, however; PGE2 also has many potent immunosuppressive properties [121, 122]. NLRP3 inflammasome activation is shown to be blocked by PGE2 in human macrophages, mediated through prostaglandin E receptor 4 (EP4) and an increase in intracellular cAMP, apparently independently of protein kinase A [123]. In a minor pathway cAMP enhancers may also improve protein kinase A (PKA) which directly phosphorylate the cytoplasmic receptor NLRP3 and attenuate its ATPase function [124]. Intriguingly, dopamine may also modulate NLRP3 activation specifically, which can be ascribed to D1 receptor to produce the second messenger cAMP which in turn enhances E3 ligase induced ubiquitination of NLRP3 inflammasome and the subsequent autophagy [41, 125].

4.3.3 Micro RNAs

Micro RNAs may provide another way for regulating inflammasomes activity. MicroRNAs are 22 nt non-coding RNAs that bind to the 3′ untranslated region (3′-UTR) of protein-coding mRNAs to regulate their translation [126]. On inflammasome priming and activation, micro RNAs may interfere in several levels. Instantly, mRNA-223 binds to a conserved site in the 3′ UTR of the NLRP3 transcript, suppressing NLRP3 expression and priming and thus IL-1β production [127, 128]. By targeting TXNIP, MicroRNA-20a negatively regulates expression of NLRP3-inflammasome [129]. Reportedly, several other microRNAs are involved in the activation of the NLRP3 inflammasome, namely microRNA-133a-1 [130], microRNA-155, and microRNA-377 [131]. Repressing the levels of these factors may be advantageous in inflammasome-related disease.

4.3.4 Type 1 Interferons

Type I IFN receptor (IFNAR) is a member of the TLR family with several downstream effector proteins like Janus kinases and several kind of signal transducers and activators of transcriptions (STATs). Type 1 interferons (IFNs), including IFN-α

and IFN-β are non-specific inflammasome inhibitors, produced by specialized immune cells such as macrophages, microglia and astrocytes in response to extra cellular stimuli and irritants [132]. As a practical therapeutic tool these IFNs have been used in various auto-immune and auto-inflammatory diseases, namely multiple sclerosis and rheumatic diseases [133, 134]. However despite several investigations the underlying mechanism of inflammasome inhibition by type IFN 1 is largely unclear. A few plausible mechanisms have been suggested by Guarda et al. experiments on bone marrow-derived macrophages concluding that IFN-β may inhibit IL-1β production through at least two pathways [135]. First, NLRP1 and NLRP3 inflammasomes might be repressed following phosphorylation of STAT1 transcription. In the second alternate cascade, IL-10 levels increase in a STAT-dependent mechanism which as an immune response modulator reduces the levels of pro-IL-1α and pro-IL-1β. Conspicuously, while both IFN-α and IFN-β are non-specific inflammasome inhibitors, there are concrete evidences implicative of NLRP3 involvement in type 1 IFNs therapeutic advantages. That is IFN-β therapy might be effective in EAE mice only when the NLRP3 inflammasome contributes directly to the disease process [133].

5 Picture of Inflammasome Activation in Stroke

Based on several empirical investigations stroke induced insult has been discovered to be associated with inflammasomes activation. Based on early studies it was assumed that NLRP3 inflammasome is mainly expressed in immune cells. Nevertheless soon NLRP3, ASC, and caspase-1 expression were observed in microglia, similar to bone marrow-derived macrophages, but not in astrocytes [136]. Dietrich and co-workers in 2008 found remarkable association between expression of NLRP1, ASC, caspase-1 with IL-1β and IL-18 activation in in neurons, astrocytes, and microglia/macrophages after ischemic stroke in mice according to immunofluorescence and cellular localization experiments [137]. The co-localization of IL-1β within areas of early focal neuronal injury implies that it might be the major form of IL-1 contributing to inflammation following stroke [11]. The continuous research works by Fann et al. providing informative data in the subject of inflammasomes involvement in stroke, described an evident increase in levels of NLRP1 and NLRP3-inflammasome proteins, and IL-1β and IL-18 in stroke patient's brain in consistent with cellular and animal models of stroke [138]. This was not however in full consistency with Yang et al. findings implying that NLRP3 was expressed in microglia and endothelial cells but not in neurons in MCAO model in mice [10].

The ground breaking data from a cohort prospective 6-years long study, indicating patients carrying the NLRP3-Q705K minor allele are more likely to get affected by stroke/transient ischemic attack (TIA), suggests a link between NLRP3 inflammasome with stroke incidence [18]. This is well supported with later works implicative of a positive correlation between NLRP3 inflammasome levels with severity of coronary atherosclerosis [139]. Nevertheless NLRP3 inhibitors have been shown

efficiently ameliorating stroke induced injury, there is little consistency among findings about NLRP3 expression profiles. Instantly the NLRC4 (NLR family, CARD domain containing 4) and AIM2 inflammasomes have been demonstrated to contribute to acute ischemic brain injury, without NLRP3 inflammasome involvement [140]. Apparently the controversy mainly concerns NLRP3, where gene expression does not always collate with the protein expression at the same time point [141] and might be more confined to microglial and endothelial cells as major resources of NLRP3 inflammasomes in regions vulnerable to ischemic insult [10]. The different ischemia models and interventions, in terms of duration and severity may explain such variations. Nonetheless, regional expression of NLRP3 inflammasomes might be litteraly different in the brain. Interestingly, there are evidences implying NLRP3, ASC, and caspase-1 were abundant in the wall of human cerebral aneurysms, highlighting NLRP3 inflammasome critical involvement in the development of cerebral vascular diseases like stroke [142]. Consistently further works indicated alcohol-induced accumulation and crystallization of cholesterol activates NLRP3/caspase-1 in the cerebral vessel leading to early development of atherosclerosis [143].

The precise mechanism through which NLRP3 contributes to neurovascular damage in ischemic stroke is yet to be concluded. Fundamentally it is believed NLRP3-mediated release of IL-1β may escalate brain microvessels' endothelial cell permeability and microglia-mediated neurotoxicity. IL-1β existing at low levels in the healthy brain modulating several physiological functions; may aggravate glutamate excitotoxicity and oxidative stress in pathological levels, leading to ROS generation, ER stress and TXNIP/NLRP3 inflammasome activation [142, 144].

6 Plausible Stimulus Involved in NLRP3 Receptor Activation in Cerebral Ischemia

While there are several potential factors posited as molecular and cellular stimuli for NLRP1 and NLRP3 receptor activation during cerebral ischemia, the exact mechanism is not clear. The comprehensive view on potential mechanism of inflammasomes activation has been reviewed elsewhere [145]. As of the main interest here, the principal potential mechanisms are illustrated in Fig. 22.2 and briefly

Fig. 22.2 (continued) and ER stress which in conjunction with the preexisting excitotoxicity dramatically enhances intracellular Ca^{2+} levels (**b**). Dring the eventual blood reperfusion massive oxygen flow to the injury site is much more than the capacity of living cells to controll oxidative mitochondrial phosphorylation. For the uncoupling oxidative phosphorylation the excess O_2 would turn to superoxide mitochondria. Similarly being a substrate for NADPH oxidase the excessive O_2 would be consumed for more ROS generation (**c**). Abbreviations: *GPR6CA* G protein-coupled receptor family C group 6 member A, *ROS* reactive oxygen species, *ECF* extracellular fluids, *DAG* diacylglycerol, *IP3* inositol triphosphate, *ATP* adenosine three phosphate

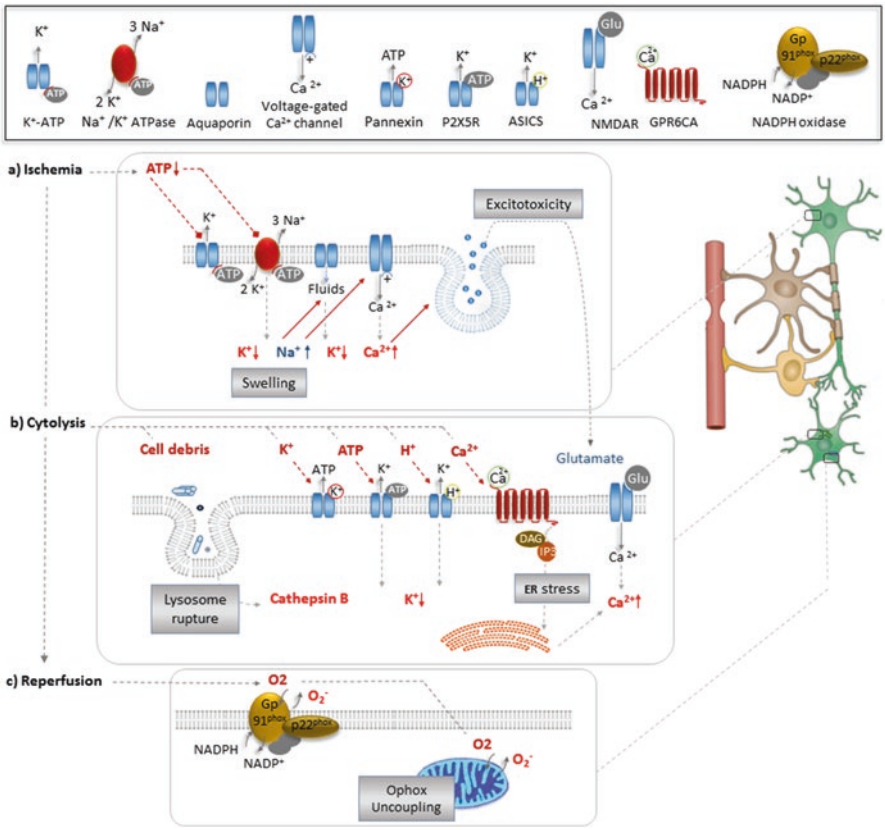

Fig. 22.2 Simplified illustration of plausible effectors involved in NLRP3 activation in acute ischemic stroke. The involved complex pathways may be described in terms of blood flow occlusion leading to ischemia (**a**) which is followed by ischemic cell death (**b**) and the spontaneous or therapeutically induced reperfusion (**c**). *Red colored letters* present the principal effectors may explain NLRP3 activation following stroke. Intracellular ATP deficiency as the major earliest manifestation results in the opening of K^+-ATP channels and leads to the channel opening and K^+ efflux. The falling ATP also leads to profound repression of Na^+/K^+ ATPase pump which in turn may trigger several NLRP3 activating pathways. First the disturbed ion exchange would result in an inward flow of ECF for the increased intracellular osmotic pressure. Besides the induced cellular edema, diluting all the cytosolic elements amplifies the intracellular K^+ depletion. Secondly, the impaired ion balance would result in a partial hyperpolarization and subsequent opening of voltage dependent Ca^{2+} channels. Ca^{2+} inward flow would instantly work to massive release of excitatory neurotransmitters affecting adjacent CNS cells (**a**). If the initial ischemic injury is deteriorating enough to end with CNS cells death the intracellular constitutional compounds and ions are released in to extracellular fluid. Non-functional particulate materials are engulfed by phagosomes primarily by glial cells leading to eventual lysosomal cathepsin B release. Cytolytic events also enriches ECF from K^+ which may activate pannexin channels exporting the intracellular ATP residues which together with the cytolytic ATP release leads to remarkable activation of P2X7 channels and massive K^+ efflux. The induced K^+ depletion is then exacerbated with ischemic induced acidosis either through lactic acidosis or cytolytic H^+ release, may activate ASICs channels permitting for more K^+ efflux. The elevated ECF Ca^{2+} levels would also activate G-protein/IP3/DAG coupled receptors

described in a classified manner in the following text. In the view of pathological start point, cerebral stroke initiates with sudden shortage of oxygen and glucose subsequent to blood flow occlusion. This result in three main specific features of stroke: ATP deficiency, cellular necrosis and reperfusion injury, each of which seemingly engage several cascades to inflammasome activation:

6.1 ATP Shortage

As the very early feature in ischemic insult, a dramatic fall in ATP generation follows the occlusive stroke. This may initiate inflammatory cascades involving inflammasome activation, at least through two interrelated pathway as follows.

6.1.1 Dysfunction of the Na^{+}/K^{+} ATPase

The NLRP1 and NLRP3 receptors can be activated by a decrease in K^{+} levels in the cytoplasm caused by dysfunction of the Na^{+}/K^{+} ATPase pump due to a decreased production of ATP. The increased influx of Na^{+} promotes the osmotic movement of water through aquaporins into the cell diluting the concentration of K^{+} in the cytoplasm; amplifying the initial K^{+} depletion subsequent to Na^{+}/K^{+} ATPase pumps dysfunction and leads to inflammasome activation. The inward movement of water through aquaporins into the cells may also end with sever cellular swelling rather than inflammasome activation.

6.1.2 Excitotoxicity

The partial hyperpolarization of cell membrane is an immediate consequent of Na^{+}/K^{+} ATPase pumps dysfunction directly affect voltage gated Ca^{2+} channels. The subsequent Ca^{2+} inward flow would instigate NLRP3 inflammasome activation. In the nervous system this intracellular Ca^{2+} overload would result in profound release of terminal glutamate vesicles leading to excitotoxicity. Trough postsynaptic receptors the excitatory neurotransmitter may augment the influx of Ca^{2+} to the cells and escalate inflammatory responses.

6.2 Necrotic Cells

Necrotic cells in the ischemic core releasing their content in surrounding space or secretions from metabolically active leukocytes may lead to enhanced extracellular concentrations of K^{+}, H^{+}, Ca^{2+}, ATP and particulate crystals all capable of stimulating NLRP3 inflammasome.

6.2.1 Acidosis

Extracellular H^+ binding onto ASIC1a on neurons and glial cells result in the influx of Ca^{2+}. Enhanced levels of Ca^{2+} ions in the intracellular environment, may activate the NLRP3 receptor through specific mechanisms. Reduction in oxygen availability also ends up with intracellular acidosis, as a result of accumulation of lactic acid following minimal anaerobic glycolysis; which may activate the NLRP3 receptor in synergy with a decreased intracellular K^+ concentration.

6.2.2 Ca^{2+} Increase

Extracellular Ca^{2+} may activate CaSRs (calcium-sensing receptor) and the orphan G-protein coupled receptor GPR6CA both leading to activate PLC/DAG/InsP3 pathway, which in turn ends with release of Ca^{2+} from endoplasmic reticulum to activate NLRP3 in different ways.

6.2.3 Extracellular ATP Increase

ATP elevation in extracellular fluid enhances the chance of binding to the plasma membrane P2X4 receptors on neurons, astrocytes or microglia, leading to K^+ efflux along with its associated K^+ decrease in the cytoplasm.

6.2.4 Extracellular K^+ Increase

P2X4R associated K^+ efflux amplifying the release of K^+ by necrotic cells, produces hyperkalemic extracellular environment and activate Pannexin 1 channels on the plasma membrane. As described before Pannexin 1 opening will lead to the release of more ATP, which can further activate more P2X4 creating a positive feedback loop for extracellular K^+ and ATP.

6.3 Lysosomal Membrane Destabilization and Rupture

As a consequence of release of damage-associated particulate materials and excessive phagocytosis, lysosomal permeation and cathepsin release is one of the expected stimuli to induce NLRP3 inflammasome in ischemic injury.

6.4 Reperfusion Injury

Reperfusion injury, the specific feature to temporary arterial occlusion, is well characterized with its associated superoxide generation. In fact blood reperfusion while rescues starving neurons works as a switch point for highly injured cells firing them by abundant ROS generation and extensive inflammatory responses. Reperfusion of the blood to the injury site brings massive oxygen flow much more than the capacity of living cells to controll oxidative mitochondrial phosphorylation and thus would be substrate for intracellular sources of ROS generation particularly mitochondria and NADPH oxidase. Accordingly oxidative stress works as one of the enormous events during ischemic injury, providing a particular pathway for ROS generation during cerebral ischemia which in turn translocates TXNIP to bind with the NLRP3 receptor leading to its activation.

7 Small Molecule NLRP3 Inhibitors and the Therapeutic Potential

Several NLRP3 inflammasome inhibitors have been designed, synthetized or discovered and evaluated in different pathological context in search for promising therapeutics. The growing number of the molecules might be classified based on different paradigms though, here we would summarize them according to their mode of action on different steps of inflammasome activation. Nevertheless given that many of these agents does not act merely trough one mechanism, it is important to note the provided classification is utterly based on the main defined mechanism concluded by recent findings which for very recent compounds are highly subject to further revisions with later investigations. Keeping in mind the main purpose is discerning on promising therapies for cerebral ischemia, in Table 22.1 a concise look has been provided to overview the reports about experimental therapeutics dealing with inflammasomes in cerebral ischemia.

7.1 Compounds Blocking NLRP3 Priming

7.1.1 Auranofin

Commonly used for the treatment of rheumatoid arthritis (RA), auranofin later was determined to antagonize NLRP3 activity at different levels. This compound suppresses pro-IL-1β mRNA expression and secretion by activated onocytes and macrophages in the synovial fluid that leads to the pathophysiological changes associated with RA [146, 147]. Auranofin has been also shown to suppress LPS-induced gene expression of NLRP3 and of IL-1 in macrophages [148], besides of other

Table 22.1 Evidences on inflammasome inhibition by experimental therapeutics against stroke

Inflammasome inhibitor	Characteristic	Disease model	Effect	Reference	Citation
In-house-made antibody	Neutralizing antibody against NLRP1	eMCAO	Reduced IL-1β/I8 levels	[137]	166
5Z-7-oxozeaenol	NF-κB inhibitor	eMCAO	Associated with a reduction in JNK and c-Jun signaling	[160]	16
		tMCAO	Improved Infarction and neurological scores	[161]	6
TAK-242	TLR4 antagonist	tMCAO	Inhibited Phosphorylation of downstream TLR4 signaling pathway, inflammatory cytokines Improved infarction and neurological scores	[259]	35
IU1	Specific USP14 inhibitor	tMCAO	Associated with reduced Protein aggregates and improved proteasome functionality Infarction and neurological scores	[164]	1
miR-124	DUB (Usp14) Silencing	tMCAO OGD (primary neurons)	Improved Resistance of neurons to in vitro ischemia, Infarction, neurological scores and neurovascular remodeling in vivo	[163]	62
ibrutinib (PCI-32765)	Bruton's tyrosine kinase inhibitors	tMCAO	Reduce IL-1β, IL-6, IL-23A And infiltrating microglia	[42]	45
Parthenolide	NF-κB inhibitor NLRP3 ATPase blocker	tMCAO	Reduced NF-κB, phosho-p38MAPK, and caspase-1 expressions improved BBB permeability and stroke outcomes	[169]	28

(continued)

Table 22.1 (continued)

Inflammasome inhibitor	Characteristic	Disease model	Effect	Reference	Citation
BAY 11-7082	NF-κB inhibitor NLRP3 ATPase inhibitor	OGD (pc12 cells)	Protects cells against ischemic injury	[172]	1
		OGD (Microglial cells)	Improved cells survival rate	[173]	
		tMCAO OGD (primary neurons)	Inhibits NF-κB and MAPK signaling, expression and activation of NLRP1 and NLRP3	[260]	2
Nicorandil	K^{+}_{ATP} Opener	OGD (BV-2 cells)	Reduced IL-1β, Caspase-1 and NLRP3	[195]	14
A438079	Selective P2X7R inhibitor	intracerebral hemorrhage	Inhibits RhoA activation	[192]	6
Brilliant Blue G	P2X7 receptor antagonist	OGD (brain section) (BV2 cells)	Protects microglial against OGD-Induced cell death	[191]	22
		tMCAO	Reduce caspase-3 dependent neuronal apoptosis	[193]	
Probenecid	Pannexin 1 inhibitor	OGD (Primary astrocytes)	Reduces NLRP3, caspase-1, and Aquporins	[261]	4
Ca-074ME	cathepsin B specific inhibitor	dMCAO	Reduced up-regulation of endosomes and apoptosis in peri-infarct neurons.	[204]	14
MCC950	ASC oligomerization inhibitor	tMCAO	Reduce caspase-3 dependent neuronal apoptosis	[193]	
		Platelet aggregation	Inhibits platelet activation/ aggregation and in vitro thrombus formation	[222]	3

BHB	ASC oligomerization inhibitor K^+ efflux inhibitor	tMCAO	Upregulates HIF-1α	[234]	110
		MCAO electrocoagulation	Depends on hydroxy-carboxylic acid receptor 2	[235]	59
Apocynin	NOX inhibitor	tMCAO	Reduced levels of NOX2, NOX4 and ROS. inhibited degradation of IκBα, NF-κBp65 nuclear localization and the expression of its target gene (COX2 and iNOS), suppressed the expression of NLRP3, ASC, caspase-1, interleukin (IL)-1β and IL-18 in	[262]	
Ac-YVAD.CMK	Caspase-1 inhibitor	tMCAO OGD (primary neuron)	Reduced inflammasome proteins, IL-1β, IL-18, and cleaved caspase-1/3 in vivo/vitro	[138]	98
Resveratrol	polyphenolic compound (Natural)	eMCAO	Inhibits TXNIP Attenuate PARP activity caspase-1/3cleavage NLRP3 activation and IL-1β release	[110]	22
Paeoniflorin	bioactive monoterpene glucoside (Natural)	OGD (hippocampal slices)	Reduce NLRP1 and NLRP3 inflammasomes, as well as IL-18, IL-1β, and caspase-3.	[263]	3

(continued)

Table 22.1 (continued)

Inflammasome inhibitor	Characteristic	Disease model	Effect	Reference	Citation
Sinomenine	Alkaloid compound (Natural)	tMCAO OGD (astrocytes/microglia)	Associates with AMPK activation Attenuates astrocytic and microglial activation in vivo Inhibits Caspase-1/3, ASC, IL-1β and NLRP3 expression in vivo/vitro	[264]	1
Ruscogenin	steroid sapogenin (Natural)	tMCAO OGD (bEnd.3 cells)	Associates with MAPK suppression Inhibits Caspase-1, IL-1β, TXNIP and NLRP3 expression in vivo/vitro Reduced ROS generation in vitro	[107]	1
Umbelliferon	coumarin derivatives (Natural)	tMCAO	Associates with PPAR-γ upregulation inhibited TXNIP/NLRP3 as well as IL-1β-18	[108]	18
Curcumin	Polyphenolic compound (Natural)	tMCAO OGD (Mice hippocampus) Glutamate Toxicity (Mice hippocampus)	Associated with IRE1α and PERK suppression Reduced ROS generation via AMPK activation attenuated glutamate neurotoxicity, TXNIP/NLRP3/Caspase-1/IL-1β in vivo/vitro	[109]	42
Chrysophanol	Anthraquinone derivative (Natural)	tMCAO	Inhibits Caspase-1/3, ASC, IL-1β and NLRP3 expression	[265]	38

17β-estradiol progesterone	Steroid hormone (Natural)	tMCAO	Inflammasomes NLRC4, AIM2 and ASC, and decreased ASC and NLRP3 proteins and transcripts of IL-1β (IL1β), IL18 and TNFα	[141]	15
Telmisartan	Angiotensin receptor blocker Insulin sensitiver	tMCAO SH Rats	Reduces NLRP3, MMP-9, GFAP positive cells	[266]	11
Ruscogenin	Steroid sapogenin	tMCAO OGD (microvascular endothelial cells)	Reduced ROS and MAPK activity, IL-1β and caspase-1, NLRP3 and TXNIP expression	[107]	1
Minocycline	Antibiotic Immunosuppressive	tMCAO OGD (BV2 microglial cells)	Attenuated NLRP3 and cerebral edema and activation of microglia	[267]	1
Cordycepin	Adenosine derivative	Intracerebral hemorrhage	Suppresses of NLRP3 inflammasome activation alleviated neurological deficits, brain edema, and peri-hematomal tissue damage	[268]	

proinflammatory cytokines, namely IL-6 and TNF-α [146]. The underlying mechanism by which auranofin blocks NLRP3 inflammasome could be explained on different targets. Firstly it has been consistently shown to block IKK phosphorylation inhibiting IκB ubiquitinylation and proteasomal degradation which prevents nuclear translocation of free NF-κB [28, 149]. At transcriptional levels, auranofin also inhibits AP-1 and IRF3, as two other major factors important for cell signaling in non-canonical pathway. Conspicuously, auranofin also has been demonstrated to prevent the homodimerization of TLR4 which is required for NF-κB and IRF3 activation [150, 151]. Besides modulating TLR4-MyD88 pathway, the drug was discovered to interact with thioredoxin reductase, a redox enzyme responsible in controlling macrophage activation, to block LPS-induced pro-IL-1β and NLRP3 gene expression.

7.1.2 TAK-242, Bromoxone, and 5Z-7-Oxozeaenol

TAK-242 the specific TLR4 antagonist [152] as well as bromoxone and 5Z-7-oxozeaenol which inhibit nuclear translocation of NF-κB p65 [153] attenuate inflammasomes activity in priming step. These are all effective NLRP3 inflammasome inhibitors that suppress LPS+ATP-induced IL-1β release upstream of IKK. For TAK-242 it takes place through binding to the intracellular domain of human TLR4 leading to its irreversible modification [154, 155] and repressed processing of pro-inflammatory cytokines like IL-1, IL-6, IL-8, and TNF-α [156, 157]. Bromoxone is a non-specific irreversible inflammasome inhibitor (NLRP1, NLRP3, AIM2). However the target of bromoxone is not well known, there are evidences showing it resides upstream of IKK. Indeed, Gong and colleagues demonstrated that bromoxone abolishes NF-κB nuclear translocation without directly affecting NF-κB or by suppressing IKKβ kinase activity. That is while 5Z-7-Oxozeaenol is known to be a TAK1 inhibitor which specifically inhibits NLRP3 inflammasome activation in response to LPS, nigericin, and alum [158].

7.1.2.1 Experimental Effects on Stroke

TAK-242 has been shown to be able to cross blood-brain barrier, block TLR4 signaling and attenuate the expression of inflammatory cytokines in animal models of stroke [159], however little is known about the importance of plausible inhibition of inflammasomes priming. 5Z-7-oxozeaenol therapeutic potential has been also evaluate in animal models of cerebral ischemia. In early studies it was shown delayed treatment, even several hours after stroke onset, was beneficiary following either intraperitoneal or intracerebroventricular administration. The TAK1 inhibitor reduced infarct size and improved behavioral, apparently independent of AMPK activation while associated with a reduction in JNK and c-Jun signaling [160]. It was later confirmed that TNF Receptor Associated Factor 3 (TRAF3) detrimental

effects through TAK1, could be blocked by 5Z-7-oxozeaenol and contributes to improved stroke outcomes [161].

7.1.3 DUB Inhibitors

While posttranslational regulation of NLRP3 is poorly characterized, inflammasome activation via deubiquitinylation discovered by Juliana and co-workers, is an established intriguing target to develop specific NLRP3 inhibitors. Among all identified deubiquitinating enzymes (DUBs), BRCC3 has been determined as the major DUB responsible for the specific deubiquitination of NLRP3 [39]. PR-619, WP1130 and eyarestatin-I are among the synthetized DUB inhibitors to be considered as promising therapeutic agents to combat diseases involving NLRP3 activation [29, 39, 162].

7.1.3.1 Experimental Effects on Stroke

Regarding the potential benefits in stroke therapy, there are few evidences about these small molecules. MicroRNAs (miRNAs) decreasing the expression of the deubiquitinating enzyme Usp14, has been shown to reduce neural injury either in cultured oxygen-glucose-deprived cortical neurons in vitro or in mice subjected to middle cerebral artery occlusion [163]. Such findings were confirmed by a recent remarkable work by Min et al. who demonstrated IU1 a specific small molecule inhibitor of USP14, attenuated ischemic stroke-caused neuronal injury, which was reflected by increased survival rate, reduced infarct volume, as well as decreased neuronal loss, while it was associated with reduced protein aggregates and enhanced proteasome functionality [164].

7.2 Compounds Blocking NBD ATPase Activity

7.2.1 Parthenolide and BAY 11-7082

The herbal sesquiterpene lactone parthenolide and BAY 11-7082 are structurally related compounds that inhibit the NF-κB pathway. However these compounds have been shown to selectively inhibit NLRP3 inflammasome activity at multiple levels independent of their inhibitory effect on NF-κB activity in macrophages [165]. Nevertheless, both compounds could also inhibit NF-κB-induced NLRP3 and pro-IL-1β gene expression. That is parthenolide directly inhibits IKK81 and NF-κB [166, 167], while BAY 11-7082 can inhibit IKK [168]. Basically, parthenolide and Bay 11-7082 inhibit ATP as well as nigericin induced NLRP3 inflammasomes with parthenolide may also directly target caspase-1.

7.2.1.1 Experimental Effects on Stroke

In the view of a potential pharmacological tool, stroke animals treated with parthenolide have been demonstrated to manifest dramatically improved neurological deficit and infarct volume, down-regulated phospho-p38 MAPK and caspase-1 expressions, and up-regulated claudin-5 expression in ischemic brain tissue. However the potential involvement of NLRP3 inhibition was not considered, NF-κB and caspase-1 modulation were identified as the potential underlying effectors for decreased stroke outcomes and BBB permeability [169]. Comparing to BAY 11-7082, the original parthenolide compound showing poor bioavailability is not a suitable compound for clinical trials and thus water soluble derivatives are being evaluated [170, 171]. This may somehow explain more recent investigations focused on BAY 11-7082 which permeate cell membranes relatively easily [165]. In a recent work by Sue et al. investigating Raf-1 kinase inhibitory protein (RKIP) neuroprotective effects against oxygen-glucose deprivation, BAY 11-7082 was shown to mimic RKIP ameliorating effects in PC12 cells, presumptively through NF-κB inhibition [172]. Further works in their lab confirmed BAY 11-7082 as a NF-κB inhibitor regulates microglial activity after oxygen-glucose deprivation (OGD) [173].

7.2.2 3,4-Methylenedioxy-β-Nitrostyrene, MNS

3,4-methylenedioxy-β-nitrostyrene, MNS was recently defined to inhibit NLRP3 ATPase activity required for ATP-dependent oligomerization of the NLRP3 inflammasome [174]. In early biological evaluation, MNS significantly inhibited NLRP3 inflammasome activation and inflammatory cytokine production in burn wounds attenuating neutrophil infiltration and accelerating wound healing [175]. As an NLRP3 inflammasome inhibitor MNS, was later shown to prevent burn sepsis by attenuating the inflammatory response in vital organs and alleviating subsequent inflammatory infiltrations [176].

7.3 Compounds Interfering with NLRP3 Activating Stimuli

7.3.1 P2X7R Antagonists

Design of P2X7R antagonists is of significant interest among medicinal chemists hence many of the due compounds have been empirically recognized as promising therapeutics. So that (AZD9056) [177], (CE-224,535) [178] and (GSK1482160) [179] are all of P2X7R blockers entering clinical trials. Nevertheless, given that there is large genetic variation in the P2X7R gene, a large variability in human responsiveness to the drugs is predictable [180, 181]. Basically by blocking activation of the P2X7R these antagonists prevent ATP-induced K^+ efflux in vitro [182] and in vivo [183, 184] attenuating NLRP3 inflammasome-mediated IL-1β maturation and release. In the absence of these

blockers, extracellular ATP causes pannexin-1 recruitment to the plasma membrane which interacts with P2X7R leading to K^+ efflux [185–187] which is sufficient for NLRP3 inflammasome activation [46].

7.3.1.1 Experimental Effects on Stroke

P2X7R has been shown to play a predominant role in anoxic depolarization after stroke and neuroinflammation [188–190]. In this line, brilliant blue G (BBG), a potent P2X7R antagonist, has been shown to protect microglial OGD-induced cell death. BBG exposure in tissue slices from P2X7R null mice also confirmed deteriorating role of P2X7R [191]. Intriguingly P2X7R suppression was also shown to protects blood-brain barrier (BBB) after intracerebral hemorrhage (ICH) through inhibiting RhoA activation [192]. Given the emerged link between P2X7R and inflammasomes, involvement of P2X7R/NLRP3 pathway has been studied and remarkable expressions of P2X7R and NLRP3 inflammasome components has been reported following stroke. Treatment of stroke animals with P2X7R antagonist BBG or NLRP3 inhibitor (MCC950); both reducing the cerebral injury neurological impairment support the hypothesis that P2X7R/NLRP3 pathway plays a vital role in caspase-3 dependent neuronal apoptosis following stroke [193].

7.3.2 K^+_{ATP} Channel Openers

ATP-sensitive potassium (K^+_{ATP}) channels are membrane associated channels that are gated by adenosine and ATP. During resting state with high ATP/ADP ratio this channels are open and contribute to inward K^+ current. In stress and ATP deficiency conditions this channel go to closing status leading to less K^+ influx. Accordingly K^+_{ATP} channel openers are investigated as efficient tools to affect inflammasome activation in various disease models. Instantly iptakalim is a K^+_{ATP} channel opener that can readily cross the blood-brain barrier. It has shown to improve neuroinflammation and neurogenesis. The beneficiary effects of iptakalim are in parallel with attenuating microglial activity as well as NLRP3-inflammasome/caspase-1/interleukin 1β axis in the hippocampus [194]. Interestingly nicorandil another K^+_{ATP} channel opener, has been demonstrated to reverse OGD-induced IL-1β production, either interfering with TLR4 or glial activation in mice [195].

7.3.3 Cathepsin B Inhibitors

The lysosomal rupture and the cytosolic release of cysteine protease cathepsin B may at least partly, mediate particle induced NLRP3 inflammasome activation. The cathepsin B inhibitor, Ca-074Me significantly suppresses NLRP3 and NLRP1 inflammasome activation [196, 197] and may represent a promising tool to prevent excessive IL-1β release. The cathepsin inhibitor Ca074Me may affect multiple

cathepsins for which it has been utilized in several biochemical and cellular examinations. That is while Ca074Me is cited as a cathepsin B-specific inhibitor in many studies and used to implicate cathepsin B in NLRP3 activation [64, 198, 199]. In fact Ca074Me, being a pro-drug methyl ester is processed in lysosomes into Ca-074, a highly cathepsin B-selective free acid. However, the very low rate of this processing provides time for Ca074Me to inhibit multiple cathepsins [200, 201]. K777 (N-methyl-piperazine-phenylalanyl-homophenylalanyl-vinylsulfone-phenyl), is a newly developed broad cathepsin inhibitor, affecting cathepsins B, L, S, C, V and K in cell-free assays [202]. Surprising recent data indicate that K777 or Ca074Me suppress IL-1β or TNF-α secretion similarly in both WT and cathepsin B-deficient PMs. Taken together with some previous evidences, these data imply the individual examined cathepsins including cathepsin B, are not sufficient for the activation of particle-induced NLRP3 inflammasome activation and IL-1β secretion [203]. However this does not rule out the potential effect of cathepsin B inhibitors, particularly regarding the recent reports on their protective effects. According to the findings of Zeng and co-workers, endosomes up-regulation following stroke is attenuated by Ca-074ME treatment in parallel with reduced apoptosis in peri-infarct neurons [204].

7.3.4 ROS Inhibitors

As emphasized in previous sections ROS play a key role in NLRP3 inflammasome activation. Almost all NLRP3 agonists induce and require ROS [205] [206]. Mitochondrial and lysosomal NADPH oxidase appears to be a good candidate for the source of ROS which can increase enormously in stress conditions [207]. The first due report in 2008 demonstrated specific knockdown of NADPH oxidase subunit p22phox as well as exposure of general ROS scavengers such as N-acetylcysteine and antioxidant ammonium pyrrolidine dithiocarbamate in THP-1 cells ends with less caspase-1 activation and IL-1b release in response to particulate stimuli like asbestos and silica [207]. The knowledge on NLRP3 involvement was later provided by Latz and co-workers in 2013, identifying NADPH oxidase (NOX) contribute to intracellular ROS generation which in turn is responsible for NLRP3 activation in response to particulate matter [208]. In line with this, several NOX inhibitors has been shown to attenuate IL-1β and/or NLRP3 inflammasome activation. Instantly NADPH oxidase inhibition either with diphenylene iodonium (DPI); a broad spectrum NADPH oxidase inhibitor, or using NOX2ds-tat blocking gp91phox subunit of NOX which is responsible for one-electron reduction of oxygen and superoxide generation; can prevent NLRP3 inflammasome activation [209–211]. Interestingly, mtROS seems to play a critical role not only in NLRP3 inflammasome activation but also in the prerequisite priming process. Studies on mouse macrophages have provided evidences indicating that TLR4 with Myd88 can prime NLRP3 through its deubiquitination. Utilizing antioxidants may efficiently block many mtROS-dependent processes [29].

7.4 Compounds Blocking ASC Oligomerization

The cytokine release inhibitory drugs (CRIDs) 1 and 2 developed by Gabel and co-workers are sort of diaryl-sufonylurea analogues showing irreversible inhibitory activity against LPS plus ATP and hypotonic stress-induced IL-1β maturation in human monocytes in vitro [212]. Continued work by Coll et al. demonstrated the related new compound MCC950. However CRIDs 1 and 2 and in particular MCC950 are all promising candidates to further develop novel therapeutics for NLRP3-dependent pathologies, no definite mechanism has been yet identified. It has been suggested that nucleophilic attack on NLRP3 cysteine residues on epoxide functional groups might explain the inhibitory effects [213]. The ketone metabolite β-hydroxybutyrate and glybenclamid the anti-diabetic sulfonylurea compound were later discovered to act as ASC oligomerization inhibitor for which yet little has been discovered as the main underlying mechanisms.

7.4.1 MCC950

MCC950 (also named as CP-456,773) was recently introduced by Coll et al. [214] as a selective NLRP3 inhibitor, blocking both canonical and non-canonical activation of the NLRP3 inflammasome. In their cutting-edge study MCC950 was shown to efficiently reduce NLRP3-induced ASC oligomerization, associated with remarkable IL-1β secretion probably through inhibiting caspase-1-dependent pathway in mouse and human macrophages. Nevertheless the exact mechanism of the compound was not ultimately specified in their experiments, several critical mechanisms were considered. Accordingly the ATP-sensitive K^+ channels as well as ATP-induced Ca^{2+} flux both of the main requirement for activation of NLRP3, was ruled out to be involved in MCC950 pharmacodynamics. NLRP3 trimerization as the requisite for efficient IL-1β production [215], was not also disturbed by MCC950. The drugs pharmacokinetic studies showed it is relatively stable following incubation with human or mouse liver microsomes, with a half-life of 3.27 h and oral bioavailability of 68% in in vivo experiments in mice. Emerging efforts to unravel the full characteristics of the drug are now on and one of the most recent works gives first insight into the SAR of MCC950 highlighting hexahydroindacene moiety as key part of pharmacophore [216].

In the earliest biological evaluations of MCC950 by Coll and colleagues, they opt for central nervous system disorders. EAE is a multiple sclerosis pathological model with typical T cell-mediated inflammation and demyelination in which IL-1 signaling and NLRP3 have been recently shown to play crucial roles. To confirm MCC950 activity in vivo, Coll and colleagues first indicated MCC950 pretreatment significantly abolished serum levels of IL-1β and IL-6 in LPS treated mice. Then in EAE mice treated with MCC950, they showed the severity of EAE was reduced and the onset was delayed [214]. The emerging interests on MCC950 as a therapeutic tool in other CNS disorders was then pursued by other studies on Alzheimers' disease, intracerebral hemorrhage and stroke. According to such investigation it was

shown MCC950 could efficiently improve cognitive function in in mice through attenuating Amyloid-β (Aβ) induced NLRP3 inflammasome activation in mice [140, 217], preventing IL-1β release from microglia and at the same time, promoting Aβ phagocytosis. In BV2 cells, the murine microglia-like cell line, MCC950 treatment was also shown to attenuate thrombin-induced cell apoptosis and expression of apoptotic proteins [91]. Aside central territory, NLRP3 inflammasome is a multi-protein complex involved in instigating inflammation in peripheral nervous system and thus MCC950 could attenuate inflammatory hyperalgesia [218].

7.4.1.1 Experimental Effects on Stroke

Sterile inflammatory responses have been long shown to be implicated in the development of myocardial ischemia, with IL-1β as an early prominent mediator [219, 220]. Recent evidence have also indicated the NLRP1 and NLRP3 inflammasome are involved in neurovascular diseases namely neurodegenerative disorders and stroke [221].

Consistently, administration of MCC950 has been shown to significantly attenuate the expression of NLRP3 components, and cleaved caspase-3 as well as neuronal apoptosis and brain infarction volume following stroke [193], with the optimal effect on 3 days after stroke. The effects of the drug have been also evaluated in thrombotic events closely associated with stroke incidence and injury. In ICH induced injury as a common event following severe stroke, MCC950 may provide protection. Interestingly, in the view of thrombus formation and incidence of occlusive strokes, it has been shown both platelet activation/aggregation and in vitro thrombus formation could be substantially inhibited by MCC950 [222]. In this line, Bruton's tyrosine kinase governing production of bioactive IL-1β and other proinflammatory cytokines; has been defined as the regulator of these NLRP3-dependent platelet effects.

7.4.2 β-Hydroxybutirate

β-Hydroxybutyrate (BHB), a ketone metabolite, has been long appeared to possess several intriguing aspects making it a molecule of interest for developing new therapies. The advantages of BHB to the body can be presumptively explained through diverse pathways. Basically, BHB could be consumed as an efficient alternative energy source by the vital organs like the heart and brain during high-intensity exercise or caloric deficiency [223, 224]. In this connection it can reduce production of mitochondrial reactive oxygen species by changing the NAD^+/NADH ratio, besides upregulation of genes involved in protection against oxidative stress via blocking histone deacetylases. Conspicuously, as a signaling molecule BHB may also influence opening of K^+ channels and regulation of Ca^{2+} channels resulting in drastic impact on many cellular functions. Indeed, the characteristic blockage of K^+ efflux from macrophages ascribes the alleviating impact of BHB on immune responses, specifically in terms of inflammasome modulation. Comparable to MCC950, BHB

also appears to block inflammasome activation by inhibiting NLRP3-induced ASC oligomerization, while BHB affects only canonical activation [225]. In fact, Youm et al. [226] revealed that the ketone metabolite BHB reduce NLRP3 inflammasome activity as well as IL-1β and IL-18 production in human monocytes. According to their in vivo experiments, BHB as well as ketogenic diet alleviate caspase-1-mediated IL-1β production and secretion without affecting the NLRC4 or AIM2 inflammasomes. To investigate about the pharmacodynamics of BHB in specific NLRP3 inhibition, they concluded BHB inhibits NLRP3 by preventing K^+ efflux and reducing ASC oligomerization and speck formation, independently of uncoupling protein-2 (UCP2) and classical starvation regulated mechanisms like AMPK and sirtuins, with no correlation with magnitude of histone acetylation in macrophages [226]. However involvement of AMPK is still under debate, as instantly in a recent study by Bae et al. in hepatoma HepG2 cells, AMPK activation was shown to be required for NLRP3 inhibition by BHB [227].

The use of BHB as a therapeutic is currently limited though, metabolism of ketone bodies including BHB has been demonstrated in full details elsewhere [224]. Low-carbohydrate, high fat ketogenic diet is a more widely used approach to alleviate disorders like seizures [228]. BHB as a monocarboxylate passes BBB through monocarboxylate transporters. Members of the SLC16 family, MCT1 (SLC16A1) and MCT2 (SLC16A7) are respectively low affinity transporters mainly in BBB and high affinity transporters mostly located in neurons [229, 230]. After entry to the CNS, all cell types use BHB in respiration which in neurons and oligodendrocytes is three times more efficient than that in astrocytes [231].

7.4.2.1 Experimental Effects on Stroke

Upon the described beneficiary effects of BHB, probably there might be a benefit to the brain being in a mildly ketotic body [232]. BHB consumption may meet baseline energy needs in the brain but may not support synaptic activity during excitotoxicity. In this connection higher on-admission BHB values have been shown to be associated with poorer stroke outcomes in stroke patients [233]. In early studies, neuroprotective properties of BHB in ischemic brain injury were primarily ascribed to the upregulation of hypoxia inducible factor (HIF)-1alpha, through a fourfold elevation of intracellular succinate a known inhibitor of HIF-1α degradation [234]. In an outstanding investigation of immune responses to ketogenic diet in stroke animals, the hydroxy-carboxylic acid receptor 2 (HCA2) on monocytes was shown to be required for the neuroprotective effect of BHB, inducing a neuroprotective phenotype of monocytes and/or macrophages [235]. These novel understanding was confirmed by another study confirming HCA2 role in neuroinflammatory diseases like multiple sclerosis [236].

7.4.3 Glyburide

Basically described as an anti-diabetes mellitus type II drug, glyburide stimulates insulin release by blocking ATP-sensitive potassium (K_{ATP}) channels in pancreatic β cells and islet chloride channels [237, 238]. Later it was shown by Gabel and colleagues that the drug may also inhibit IL-1β release in LPS-activated human monocytes [239], the findings predating the discovery of inflammasomes [240]. According to the primary SAR studies both the benzamido and sulfonyl groups of the compound contribute to inhibition of LPS+ATP-stimulated caspase-1 activation and IL-1β secretion [241]. As IL-1β secretion was later shown not significantly affected in the presence of the NLRC4 or AIM2 activators [242], glyburide was later recognized as a specific inhibitor of the NLRP3 inflammasome [241]. However the effect was confined to concentrations much higher than what can be clinically achievable. Given that K^+ efflux is required for NLRP3 inflammasome activation and K^+ channels are the main target of glyburide, [243, 244] obstruction of ATP-dependent K^+ efflux was of early assumed mechanisms for NLRP3 inhibition [245]. However, a later study showed that inhibitory activity of glyburide was maintained even when K^+_{ATP} channel subunits $Kir_{6.1}$, $Kir_{6.2}$, and SUR2 were knocked out, implying that K^+_{ATP} channels are dispensable for the effect [61]. Interestingly, not all sulfonylurea drugs inhibit inflammasome activation [241], neither sulfonylurea compounds may prevent K^+ efflux caused by NLRP3 activators [214], collectively concluding these inhibitors act in downstream of K^+ depletion and may prevent ASC oligomerization. This might occur during or upstream to NLRP3 inflammasome assembly.

7.4.3.1 Experimental Effects on Stroke

Glibenclamide (glyburide) as a NLRP3 oligomerization inhibitor, has received renewed attention in the last decade for its pleiotropic protective effects in acute CNS injury. According to several clinical studies, glyburideis is effective in preventing edema and improving outcome after focal ischemia [234–236]. Instantly glyburide has been shown to be associated with T2 fluid-attenuated inversion recovery signal intensity ratio on brain MRI, diminished tissue water and reduced blood MMP-9 level [237]. In preclinical studies, even a very low dosage of glyburide has been shown to improve functional outcomes following stroke besides improving NeuN-positive neurons in the cortex and hippocampus, and enhanced angiogenesis in the hippocampus [238]. Corroborated with evidences implicative of trivial role of blocking K^+_{ATP} channels by glyburide in the CNS, glibenclamide seems to exert its effects primarily via inhibition of the characterized Sur1-Trpm4 (sulfonylurea receptor 1-transient receptor potential melastatin 4) channel. Nevertheless, blockade of K^+_{ATP} channels have been determined to be required for neuroprotective role of microglia in the early stages of stroke [239] as well as glyburide neuroprotection against stroke induces oxidative stress and inflammatory responses [240, 241]. In fact, there are pile of concrete evidences demonstrating glibenclamide ameliorating

effects in rodent models of various CNS pathologies, importantly ischemic [242–244] and hemorrhagic stroke [245, 246], is mediated through Sur1 inhibition while there is no examination on potential implication of inflammasomes.

7.5 Compounds Blocking the Activity of NLRP3 Assembly

In 2013 Liu et al. developed Fc11a-2 a novel benzoimidazole compound, which could specifically inhibit NLRP3. Mechanistically it interferes with the proximity-induced autocleavage of procaspase-1 in NLRP3 inflammasome complex which leads to less release of activated caspase-1 [246]. Directly targeting NLRP3 complex, Fc11a-2 was shown to be highly effective in suppressing IL-1β/18 production. This promising results encouraged them to work on more benzoimidazole derivatives of which their recent publication introduced TBZ-09 and TBZ-21 as new lead compounds with comparable efficacies with Fc11a-2 [247]. All their suggested molecules provided remarkable ameliorating effects in dextran sulfate sodium (DSS)-induced experimental colitis in mice beside significant reduction in IL-1β/18 production in LPS+ATP-stimulated THP-1 cells. These encouraging effects warrant further studies to evaluate efficacy in other NLRP3 inflammasome relevant disease models. In this view caspase-1 inhibitors may be somehow regarded as NLRP3 inhibitors.

8 Naturally Occurring Inhibitors

Plant-derived natural compounds have been long considered in traditional medicine as complementary supplements in the treatment of chronic inflammatory diseases or included in preventive approaches. However according to the complexity of the effects and lack of the detailed mechanistic explanation little could be implemented as a pharmacological tool, still they provide unique promising lead compound to investigate for appropriate therapeutics. Interestingly, more than 8000 natural polyphenols have been described [248] as potential inflammasome inhibitors. Many of them existing in the form of polyphenols like resveratrol, curcumin, arglabin, EGCG, and quercetin are potent inhibitors, typically acting at more than one element in NLRP3 inflammasome regulating pathways. The comprehensive study of such naturally occurring inflammasme inhibitors have been reviewed elsewhere [249]. The exemplary well known compound, resveratrol, provides a concise instance acting at multiple levels reducing expression of NLRP3, ASC and P2X7R [250]; increasing cAMP content [251]; induce autophagy and AMPK stimulation [252]. However, as they influence a variety of pathways, it should be noted that these polyphenols have a broad biological effect all may not be explained upon inflammasome inactivation [253]. In fact, polyphenols are known to provide antioxidant effect, contribute to activate glutathione S transferases, and inhibit COX enzymes [254, 255]. To explain the complex effects of these kinds of compounds all

together in a unique feature, an interesting theory termed as “Xenohormesis” have been posited. Based on this theory, many of these polyphenols like resveratrol and curcumin are produced in stressed plants, triggering effectors of caloric restriction. Receiving such signals by consuming these compounds, might be interpreted as a potential risk of future limitation of food availability and augmentation of adapting and protecting signals [256, 257]. Nevertheless the theory needs to be validated by empirical evidences, there are some backing evidences. Interestingly, resveratrol and curcumin which are structurally unrelated, share this caloric restriction mimicking feature by activating AMPK and Sirt1 [258]. Moreover aspirin as of the most frequently used COX inhibitors, is a derivative of another stress-induced phytochemical, salicylic acid.

9 Summary and Future Directions

In summary, in light of above discussed studies, this chapter lends further credit for the central role of NLRP3-inflammasome that is a vital player in both acute and chronic diseases of the brain including stroke. These findings further suggest a potential clinical benefit of therapeutic interventions that target NLRP3 inflammasome assembly and activity. On the other hand, TXNIP-NLRP3 inflammasome activation plays a major role in mediating the pro-inflammatory response involved in the pathophysiology of the stroke. Taken together, NLPP3 represents a logical therapeutic targets for stroke, but it have never been clinically evaluated due to a lack of ideal drug candidates. Recent innovations in NLP3 inhibition and drug delivery highlight new potentials for stroke therapy. Further investigation into the mechanisms of NLRP3 components as well as pre-clinical and clinical studies of targeted inhibitors are needed for development of novel drug for stroke therapy.

Acknowledgements This chapter was supported by R01NS097800-01 to Tauheed Ishrat.
Disclosure of conflict of interest: None

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Chapter 23
Microglial Function in Intracerebral Hemorrhage Injury and Recovery

A-Hyun Cho, Neethu Michael, David H. Cribbs, and Mark J. Fisher

Abstract Intracerebral hemorrhage (ICH) accounts for 10–15% of all strokes and is a major cause of disability and mortality. Introduction of blood components (e.g., thrombin, heme, and platelets) following ICH initiates neuroinflammatory responses mainly mediated by microglia, which are the resident immune cells in the central nervous system. Microglia have been shown to have dual roles in ICH, both beneficial and detrimental. The beneficial role involves phagocytosis of cellular debris and red blood cells after the hemorrhagic incident, while the detrimental role involves the production of pro-inflammatory cytokines and chemokines resulting in neuroinflammation. These dual and contradictory roles of microglia are thought to be implemented by two distinct phenotypes: classically-activated microglia and alternatively-activated microglia. We discuss herein the role of microglia in ICH with particular emphasis on its role in brain injury and recovery after ICH.

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P.A. Lapchak, J.H. Zhang (eds.), *Cellular and Molecular Approaches to Regeneration and Repair*, Springer Series in Translational Stroke Research,
https://doi.org/10.1007/978-3-319-66679-2_23

Keywords Microglia • Intracerebral hemorrhage • Brain injury • Brain recovery • Neuroinflammation

Abbreviations

BBB	Blood-brain barrier
Bcl-2	B-cell lymphoma-2
Bcl-xl	B-cell lymphoma-extra large
CD36	Cluster of differentiation 36
CD47	Cluster of differentiation 47
CEBP α	CCAAT/enhancer-binding protein alpha
CNS	Central nervous system
CX3CR-1	CX3C chemokine receptor-1
CXCL2	Chemokine (C-X-C motif) ligand 2
HO	Heme oxygenase
ICH	Intracerebral hemorrhage
IL	Interleukin
KO	Knock-out
MHCII	Major histocompatibility complex II
mTOR	Mechanistic target of rapamycin
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
Nrf2	Nuclear factor (erythroid-derived 2)-like 2
PAR-1	Protease activated receptor-1
PI3K	Phosphoinositide 3-kinase
PPAR-γ	Peroxisome proliferator-activated receptor gamma
ROS	Reactive oxygen species
SIRPα	Signal-regulatory protein α
TGF-β1	Transforming growth factor-beta 1
TLR	Toll-like receptors
TNF-α	Tumor necrosis factor-α

1 Introduction

Intracerebral hemorrhage (ICH) accounts for ~10–15% of all strokes and is associated with high morbidity and mortality [1, 2]. Prognosis after ICH is often poor and clinical interventions are extremely limited. Mechanisms of brain injury and recovery after ICH have been described in detail [3, 4]. Primary brain injury at the time of ICH occurs within minutes-to-hours after hemorrhage development. It includes tissue disruption, mass effect due to increased intracranial pressure, and ischemia. This induces inflammation-driven breakdown of the blood-brain barrier (BBB) and,

consequently, blood components, e.g., cells (erythrocytes, leukocytes and macrophages) and plasma proteins (thrombin, complement factors, etc.), enter the brain. Thrombin contributes to early blood-brain-barrier disruption and edema formation. This in turn initiates a cascade of inflammatory reactions, comprising of both cellular and molecular components. Importantly, there is inflammatory cell migration and glial activation, followed by tissue repair responses restoring some neurologic functions [4–7].

Microglia are the critical innate immune cells in the central nervous system. They are considered to be the earliest inflammatory cells to react to ICH and the primary cell type responsible for secondary injury after ICH. Microglia in the resting state serve an immune surveillance function, and they can sense even subtle changes in the microenvironment through a variety of surface receptors and by extending and retracting their processes [8]. They can respond to these changes by producing pro- or anti-inflammatory cytokines and neurotrophic factors. In general, microglia have two different activated polarization states: a classically-activated phenotype which is pro-inflammatory, and an alternatively-activated phenotype which is interpreted to be healing or reparative. The pro-inflammatory phenotype release inflammatory factors and damage neurons by releasing oxidative metabolites and proteases. The second phenotype has anti-inflammatory and phagocytic activity and release various protective and trophic factors repairing nerve cells [4]. The involvement of microglia and its polarization in ICH has been demonstrated [4]. There is a substantial need for improvement of prognosis after ICH, and new therapeutic targets are thus of high importance. Microglia can serve as one such highly effective target in ICH treatment, owing to its critical role in the brain immune reactions. In this chapter, we will discuss the role of microglia in brain injury and repair mechanism after ICH.

2 Microglial Role in Brain Injury After ICH

2.1 Microglial Activation During Brain Injury

Microglia are the initially activated immune cells in the brain in response to ICH. There are also infiltrating macrophages entering the perihematomal tissue [9]. Microglia respond to pro-inflammatory triggers in the surrounding brain and change to an activated phenotype. Activated microglia are detected within 1 h, and peak at ~3–7 days, decrease after 7 days, and return to basal levels by 21 days [10]. They are regarded to be the primary cell type responsible for secondary injury after ICH via release of cytokines, chemokines, prostaglandins, proteases, ferrous iron, and other immunoactive molecules [11]. Microglia also create crosstalk with T cells through antigen presentation via MHCII expression [12].

Activation of TLR4, widely expressed by microglia, leads to neuroinflammation [13] which in turn causes the upregulation of pro-inflammatory genes via NF-kB

signaling [14]. Microglia are also activated by blood plasma components such as thrombin, fibrin, and heme through TLR/NF-kB pathway. TLR4 contributes to neuronal damage in ICH, and blocking of TLR4 reduces inflammatory injury and neurological deficits [14, 15]. TLR4-induced autophagy contributes to microglial activation [16]. Inhibition of the TLR4 signaling pathway may thus be a therapeutic target for ICH.

Protease activated receptor-1 (PAR-1) is involved in pro-inflammatory process, which can be detrimental to neuronal survival and are related to brain injuries including ICH. PAR-1 expression increases after ICH and PAR-1 KO mice show less brain edema and neuronal death. Thrombin-induced PAR-1 activation contributes to microglial activation/polarization toward pro-inflammatory phenotype, amplifying the release of pro-inflammatory cytokines after ICH [17].

Heme oxygenase (HO) is the rate limiting enzyme of physiological heme degradation that catalyzes heme to biliverdin, carbon monoxide, and iron. HO-1, one of the two active enzymes of HO, is found to increase after ICH, predominantly in microglia and endothelial cells. A comparison study with HO-1 KO and wild type mice showed that KO mice exhibited lesser brain injury volume and neuronal deficits, thus suggesting that HO-1 is a potent therapeutic target for ICH treatment [18]. Iron, a byproduct of the action of HO, is neurotoxic as it can produce reactive free radicals by the Fenton reaction [19]. Clearance of the iron overload in the hemorrhagic brain can thus serve as yet another target in ICH treatment.

ICH also promotes miRNA144 expression, which leads to inflammation that can affect brain function. miRNA144 is a regulator of microglial autophagy and inflammation by regulating the mTOR (mechanistic target of rapamycin) pathway, which in turn regulates the inflammatory immune response. Inhibition of miRNA144 promotes mTOR expression and decreases IL-6, IL-1β, TNF-α after ICH [20]. Chemokines such as CXCL2 produced by microglia have chemotactic activity for neutrophils and also exacerbate the inflammatory reaction [21].

2.2 Microglial Polarization

Microglia have two broad polarization states: a classically-activated pro-inflammatory phenotype and an alternatively-activated anti-inflammatory phenotype. Some have labelled these two phenotypes as M1 and M2, respectively, similar to that of macrophages. Note, however, that the use of M1 and M2 nomenclature has received criticism [22]. The classically-activated phenotype releases pro-inflammatory factors such as TNF-α, interleukin-1β, IL-6 and reactive oxygen species. It injures neurons by releasing oxidative metabolites and proteases. In contrast, the alternatively-activated microglia have anti-inflammatory and phagocytic activity, and release various protective and trophic factors repairing nerve cells. The anti-inflammatory phenotype plays an important role in brain recovery involving neurogenesis and angiogenesis [11]. However, mechanisms of phenotype shift have not yet been clearly identified. There is evidence that IL-4 is essential for

anti-inflammatory polarization after ischemia, with loss of IL-4 inducing classically-activated microglia while inhibiting alternatively-activated microglia [23, 24]. Phenotype shifts can also be triggered by extra- and intra-cellular signaling [25, 26]. There is also evidence of a novel microglial phenotype ('dark microglia') which is rarely present in the steady state condition, but present in abundance during aging, stress, and Alzheimer's disease pathology [27].

3 Microglial Role in Brain Recovery After ICH

3.1 Microglial Role in Recovery Mechanism After ICH or Other Neuronal Injury

Microglia and macrophages are among the most potent modulators of CNS repair and regeneration [28]. These cells become polarized with different phenotypes at various stages after brain injuries and have contradictory functions. The status of these functions *in vivo* is complex.

Alternatively-activated microglia absorb hematoma and cell debris by phagocytic activity. CD36, a scavenger receptor of microglia/macrophages, has been reported to mediate phagocytosis of erythrocytes. Induction of CD36 by peroxisome proliferator-activated receptor gamma (PPARγ) promotes hematoma absorption in mice [29]. Inflammatory factors that are induced by TLR signaling can regulate the expression of CD36 in macrophages [30, 31] with TLR signaling inhibiting CD36 expression and hematoma absorption [32, 33]. CD47 is an integrin-associated protein expressed on erythrocytes and other cells, which blocks phagocytosis via its interaction with signal-regulatory protein-α (SIRPα), expressed by microglia/macrophages. There is evidence that CD47 has a key role in hematoma clearance by microglia after ICH [34]. More rapid hematoma clearance after ICH is induced by intracerebral injection of blood from mice lacking CD47. Erythrocyte CD47 inhibits microglial erythrophagocytosis, and CD47 may be a potential target for its regulation [34].

Neurogenesis is induced in response to ICH and this may contribute to brain repair and functional recovery [35, 36]. Microglia have positive and negative effects on neurogenesis after brain injury. Pro-inflammatory microglia impair basal neurogenesis and secrete destructive factors that hinder neurogenesis and aggravate long-term neurological deficits after injury [37-39]. Microglia with anti-inflammatory phenotype have the potential to promote the proliferation and migration of neural progenitor cells via secretion of trophic factors [38, 40–42]. Whether both these phenotypes are pro-neurogenic or anti-neurogenic is not yet clearly demonstrated and they may have overlapping functions. The direction of their function may depend on both phenotype and the specific time after injury.

Axonal regeneration after CNS injury occurs to a limited extent. Classically-activated microglia hinder axonal regeneration [43, 44]. However, after spinal

cord injury, monocyte-derived macrophages have been shown to be critical for recovery [45].

Brain angiogenesis is yet another recovery process after ICH [46]. It is well known that tissue macrophages have proangiogenic function during wound healing [47, 48] and produce proangiogenic factors such as vascular endothelial growth factor (VEGF) and IL-8 [48, 49]. Activated microglia regulate endothelial cell proliferation [50]. However, the exact role of resident microglia in angiogenesis after ICH is still unclear.

Microglia have been shown to regulate synaptic plasticity after CNS injuries and activated microglia have an adaptive role in the regulation of synaptic homeostasis and plasticity [51]. Oligodendrocyte differentiation is promoted by the anti-inflammatory phenotype and impaired by its blockage [52]. Most of the reports describing microglia/macrophage function in repair mechanisms have been performed in ischemic brain injury or spinal cord injury models rather than in ICH models. More studies are thus needed for a more complete understanding of microglial role in ICH recovery.

3.2 Mediators of Microglia-Facilitated ICH Recovery

Identification of specific mediators of microglial activation is particularly important to determine specific therapeutic targets for attenuating microglia-mediated brain injury. Nrf2 and PPAR-γ promote transcription of antioxidant genes. Nrf2- deficient mice have severe neurological deficits with increased leukocyte infiltration, ROS production, DNA damage, and cytochrome c release [53]. Nrf2 is present in neurons, astrocytes, and microglia, and its expression is neuroprotective against early inflammatory brain injury in hemorrhagic stroke models. In addition, PPAR-γ activators reduce the expression of pro-inflammatory genes and prevent neuronal damage [29, 54]. PPAR-γ thus appears to play a protective role after hemorrhagic stroke. The chemokine receptor CX3CR-1 is required for anti-inflammatory polarization of microglia, which facilitates recovery after ICH [13]. miRNA124 is another factor which enhances alternatively-activated anti-inflammatory polarization and ameliorates ICH-induced inflammation via CEBP α pathway [55].

During the repair process, IL-4 production in the CNS controls autoimmune inflammation by inducing the anti-inflammatory phenotype [4, 56]. IL-10 is an anti-inflammatory cytokine also produced by glial cells, which inhibits pro-inflammatory cytokines of microglia. IL-10 has also been shown to prevent apoptosis by activating PI3K, enhancing apoptotic factors such as Bcl-2 and Bcl-xl, and depleting caspase-3 [57]. Intracerebral injection of IL-4 inhibits activation of the pro-inflammatory phenotype while enhancing the anti-inflammatory phenotype, along with improving neurobehavioral recovery from deficits after ICH [23]. TGF-β1 has been shown to reduce microglial inflammation, and improve functional recovery after intracerebral hemorrhage [58]. Regulatory T cells also contribute to altering microglial polarization and reducing inflammation in ICH [59]. ST2 is a

member of interleukin-1 receptor family while IL-33 acts as its ligand, and both have critical roles in inflammatory responses. Work by Yang et al. showed that ST2 receptor deficiency aggravates brain infarction and that ST2/IL-33 interaction stimulates microglial IL-10 production, which is also a marker of the anti-inflammatory microglial phenotype [60].

4 Conclusion

Microglia play a vital role in both brain injury and recovery after ICH. With its detrimental and beneficial roles (pro-inflammatory and anti-inflammatory, respectively), microglia can act as a double-edged sword. Studies of mediators of inflammatory response implicate microglia as a potential therapeutic target. Future studies emphasizing *in vivo* models and utilizing advanced imaging methods [61, 62] represent a promising approach to improve clinical outcome following ICH by targetting microglia.

Acknowledgement This work is supported by NIH R01 NS20989

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Chapter 24
The Role of T Cells in Post-stroke Regeneration

Julia V. Cramer and Arthur Liesz

Abstract The interaction of the immune system with the brain is necessary for development and surveillance of the healthy brain. The influence of the adaptive immune system on several brain diseases has been described in great detail. In ischemic stroke, a growing body of evidence has demonstrated a key role for T cells in the acute phase after stroke. Pro- and anti-inflammatory T cell subpopulations impact in this early phase the inflammatory milieu and directly affect secondary lesion progression and neuronal injury. Recently, a functional role for T cells has also become more evident also in delayed neuronal (dys-)function and late-phase recovery after stroke. Here, T cells may also affect various non-immunological pathways involved in tissue repair, neuronal plasticity and functional recovery. These pleiotropic effects of T cells on mechanisms such as neurogenesis and angiogenesis suggest T cells as potential therapeutic target to modulate post-stroke regeneration. This chapter will provide a comprehensive overview of the current knowledge about the role of T cells in stroke with a particular focus on regenerative processes in the chronic phase.

Keywords Stroke • Brain ischemia • T cell • Lymphocyte • Inflammation • Neuroimmunology • Regeneration • Cytokines

Abbreviations

APC	Antigen presenting cell
BBB	Blood brain barrier
BDNF	Brain derived neurotrophic factor
CCR	Chemokine receptor
CNS	Central nervous system
CD	Cluster of Differentiation

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P.A. Lapchak, J.H. Zhang (eds.), *Cellular and Molecular Approaches to Regeneration and Repair*, Springer Series in Translational Stroke Research,
https://doi.org/10.1007/978-3-319-66679-2_24

CTLA-4	Cytotoxic T-lymphocyte-associated Protein 4
DAMPs	Damage-Associated Molecular Patterns
GITR	Glucocorticoid-induced TNF receptor
IFNγ	Interferon gamma
IgE	Immunoglobulin E
IGF-1	Insulin like growth factor 1
IL	Interleukin
MCAO	Middle Cerebral Artery Occlusion
MHC	Major Histocompatibility Complex
MMP	Metalloproteinase
NPC	Neural Precursor Cell
RAGE	Receptor for advanced glycosylation endproducts
SGZ	Subgranular zone
SVZ	Subventricular zone
TGF-β	Transforming growth factor beta
Th cell	T helper cell
TLRs	Toll-like receptors
TNF-α	Tumor necrosis factor alpha
Treg	Regulatory T cell
VEGF	Vascular Endothelial Growth Factor

1 An Introduction to T Cells in Brain Function

1.1 T Cell Subsets and Their Physiological Role in the Body

T lymphocytes are part of the adaptive immune system and are divided in several subtypes depending on their functional properties, which are determined by transcription factors, surface markers and the cytokine secretion profile. There are two major groups, the CD8+ cytotoxic T cells and the CD4+ T helper (Th) cells. The former can act directly via cell-cell interaction dependent cytolytic pathways, involving the perforin-granzyme effector mechanism [1]. Cytotoxic T cells recognize antigens presented via MHC-I and thereby play a critical role in microbial defense [1] and primary autoimmune diseases [2, 3] (Fig. 24.1).

CD4+ Th cells have mainly indirect effector function and are able to induce proliferation of other immune cells. Despite recognizing a specific antigen, naïve CD4+ T helper cells possess still a vast plasticity. Hence, they are able to polarize into different effector and regulatory subtypes. The differentiation depends on three signals which they receive both from innate and adaptive immune cells: antigen-presentation, co-receptor stimulation and cytokine signaling. Classically it is reported, that Interferon-gamma (IFNγ) and Interleukin (IL) -12 signal can induce the pro-inflammatory T helper 1 (Th1) subtype [4, 5]. Th1 cells help resolve bacterial infection and release pro-inflammatory cytokines such as IFNγ and IL-12. In contrast, T helper 2 (Th2)

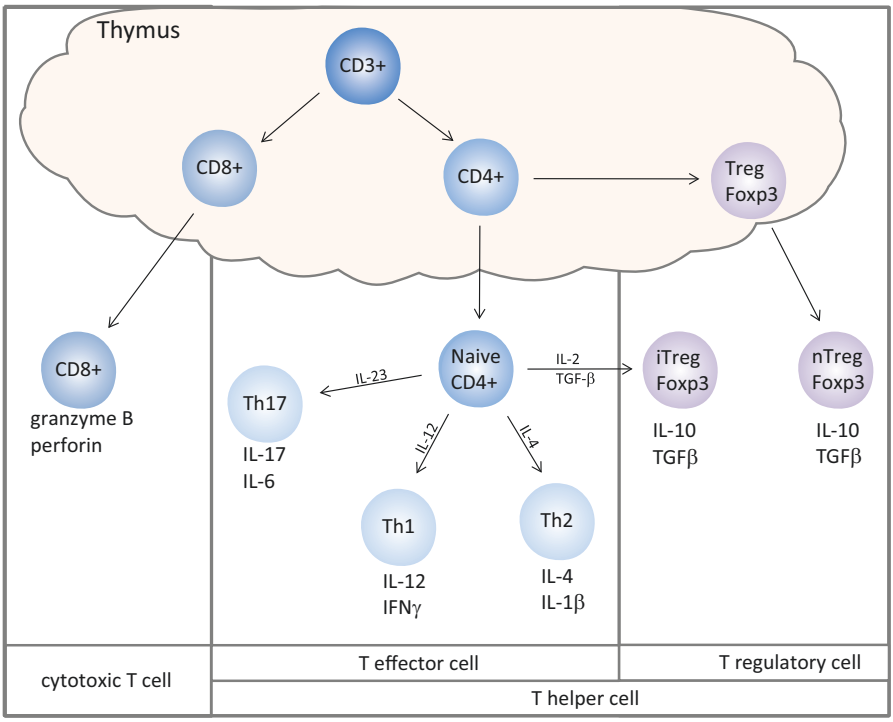

Fig. 24.1 Simplified overview of the main T cell subpopulations. Cytokines which promote polarization to the individual subsets are added to the *arrows*, cytokines released by the different cells are indicated below

polarization is mainly driven by IL-4 [6, 7]. Th2 cells are able to enhance B cell responses and initiate in synergy with IL-4 an IgE class-switch. IgE is a key mediator in mast cell activation and therefore, Th2 cells have a critical role in the pathophysiology of allergy; additionally, they provide protection against parasitosis [8]. Over the past decade a substantial variety of other CD4+ subtypes has been described, from which pro-inflammatory Th17 cells and regulatory T cells (Tregs) are the best characterized and with direct implications for neuroinflammatory diseases and modulation of the post-stroke immune response. Developing in presence of IL-23, Th17 cells produce cytokines associated with chronic inflammation such as IL-17, IL-6 and Tumor necrosis factor alpha (TNF-α) [9, 10]. In contrast, CD4+CD25+Foxp3+ Tregs play a key role in self-tolerance by suppressing autoreactive T cells under physiological conditions. Furthermore, these cells are able to dampen over-activation by attenuating T cell responses mainly by secretion of the anti-inflammatory molecules IL-10, CTLA-4 and TGF-β, in addition to other cell-cell contact dependent mechanisms [11–13].

Another subtype distinct from the classical T cells are γδ T cells. The composition of their T cell receptor by γ and δ subunits separates them from conventional

T cells in which the T cell receptor consists of α and β subunits. They are belonging to the adaptive immune cell family, but also hold some characteristics of the innate immune system in addition [14]. γδ T cells have different antigen-recognition requirements, most likely do not need antigen-processing and MHC-presentation, and can additionally recognize lipid antigens. Their adaptive immune characteristics include ability to differentially polarize. Depending on which stimuli received, they can either polarize to an IL-17 producing pro-inflammatory type or a regulatory phenotype [15, 16]. The activation of this cell population is very rapid similar to innate immune cells [15]. γδ T cells are preactivated after their development in the thymus and there is no need for clonal expansion [14].

Considering the diversity of the different T cell subsets, their complexity enables them to play a role in many physiological and pathological processes including stroke.

1.2 T Cells in the Healthy Brain and Its Homeostasis

In contrast to the previously prevailing concept of the brain as an immune privileged organ, several more recent studies have unequivocally demonstrated constant surveillance of the brain by circulating leukocytes. Patrolling leukocytes have been shown to be present in certain parts of the brain under healthy conditions and to be critical in maintaining physiological function and plasticity [17–19]. Under physiological conditions, T cells are able to migrate across the endothelium, preferentially in post-capillary venules and enter the subarachnoid and perivascular space as well as the CSF [20]. It is most likely that T cell activation is facilitating the cerebral extravasation [21]. However, lymphocytes are rarely found in the brain parenchyma itself. Highly specialized cells and membranes regulate T cell access from the perivascular space to the brain [22]. Astrocyte ensheathment of the vasculature [23] and the glia limitans, which encompass the microvessels in brain parenchyma and surface [22], restrict the perivascular space and impede immune cell infiltration [24].

Cerebral immunosurveillance by circulating lymphocytes is restricted to certain parts of the brain: In the perivascular and subarachnoid space T cells encounter resident antigen presenting cells (APCs) such as perivascular macrophages [20, 25]. Via their MHC-II molecules these APCs present antigens which are drained from the brain parenchyma [22]. Interacting with them, T cells can become reactivated, if their cognate antigen is presented [26]. Missing antigen recognition has been reported to terminate cerebral T cell patrolling and inducing T cell egress exit via the CSF to cervical lymph nodes [27] without entering the parenchyma [20]. By this mode of antigen-specific brain surveillance, the lymphocyte population presented in the brain and surrounding structures is turned over almost twice a day [20]. Some T cells, mainly CD4+ effector memory T cells, can reside in the choroid plexus with receptors specific for CNS antigens [28] and provide a cell pool for immediate immune reactions. Furthermore, the same T cell subset can be found in healthy human CSF samples [29].

Immune cells and especially T cells are known to be involved in many physiological processes in brain homeostasis. Their presence positively influences hippocampal neurogenesis and hippocampal brain-derived neurotrophic factor (BDNF) expression [17, 30]. These changes were not only observed on a biochemical or histological level. Notably, the connection between the immune system and neurogenesis has been clearly demonstrated in animal behavior studies. Behavioral deficits, especially in spatial memory tasks, are apparent in animals devoid of a functional immune system [18, 30, 31]. Interestingly, these memory deficits can at least be partially restored by T cell transfer to immunodeficient animals [31]. This finding was further verified by following studies which were able to identify that these characteristics were specifically attributable to the subpopulation of CD4+ Th cells [32]. Moreover, lymphocyte deficiency has been associated with an impact on social behavior in mice [33]. In addition to this, it was found that blocking T cell migration to the meninges was also able to evoke behavioral abnormalities in mice [30, 33, 34]. Taken together, these findings highlight the importance of T cells for physiological brain function and behavior.

Yet, the precise mechanism by which T cells cause this impact is insufficiently known. Previous reports have associated T cell cytokines such as IFNγ, IL-6 and IL-4 to the above-mentioned behavioral and structural differences between T cell-bearing and deficient animals [33–36]. Further research for the analysis of T cell mechanisms in brain homeostasis under physiological conditions will be warranted for the identification of potentially novel targets of T cell-mediated pathologies in a variety of brain disorders.

2 T Cells in Stroke

2.1 Dynamics of Leukocyte Infiltration After Cerebral Ischemia

Acute brain ischemia leads to sterile neuroinflammation attracting both local and systemic immune cells to the lesion site. In the ischemic core, destruction of the cellular integrity in necrotic cells allows intracellular content to be released into the extracellular space. These soluble components are termed damage-associated molecular patterns (DAMPs) [37]. DAMPs initiate the inflammatory cascade both locally and systemically. Pattern recognition receptors, including Toll-like receptors (TLRs) [38] and the receptor for advanced glycosylation endproducts (RAGE) [39] sense DAMPs. Interaction of cerebral DAMPs with TLRs found on microglia—the resident immune cells of the brain—induces their activation and migration to the infarct lesion [40]. Consequently, microglia generate a pro-inflammatory milieu by secretion of cytokines and chemoattractant molecules [41, 42]. Microglia-derived cytokines, such as IL-6, IL-1β and TNF-α induce two processes involved in the recruitment of circulating lymphocytes to the damaged brain [43]. Cytokines

promote the activation of peri-infarct endothelial cells and their upregulation of adhesion molecule expression. This facilitates leukocyte rolling, an initial step in extravasation of circulating leukocytes [43]. Simultaneously, microglia-derived cytokines pass on the signal to circulating peripheral immune cells which in turn become activated as well. As a consequence, activated immune cells enhance expression of proteins such as integrins, which are necessary for firm adhesion to the endothelium which is then followed by transmigration [44]. Additionally, activated T cells secrete cytokines which are also able to induce endothelial activation and thereby amplify the inflammatory response [45]. Another mechanism enabling increased leukocyte entry to the post-ischemic brain parenchyma is the degradation of the glia limitans. Under healthy conditions this membrane—which is part of the outer layer of the blood brain barrier (BBB)—is limiting the access for leukocytes to the parenchyma. After its degradation by metalloproteinases (MMPs), especially MMP-2 and MMP-9 [46], T cell infiltration to the lesion site is eased.

The dynamics of leukocyte recruitment to the brain differs substantially for different cell types of circulating leukocytes (Fig. 24.2): After stroke, invading macrophages appear within minutes to hours after ischemia at the lesion site [47, 48]. Thereafter, the number of invading neutrophils peaks at 3 days post infarct [48]. Their presence persists at least until day 7 and declines afterwards [48]. However, one report by Enzmann et al. has suggested that the majority of granulocytes attracted to the ischemic brain might not enter the brain parenchyma but are found in one murine stroke model only in the perivascular space [49]. T lymphocytes in general migrate preferentially to the lesion borders, increase in cell number of several days after ischemia [50] and can be detected throughout at least 30 days post infarct in the brain parenchyma [51]. Looking more detailed at the individual

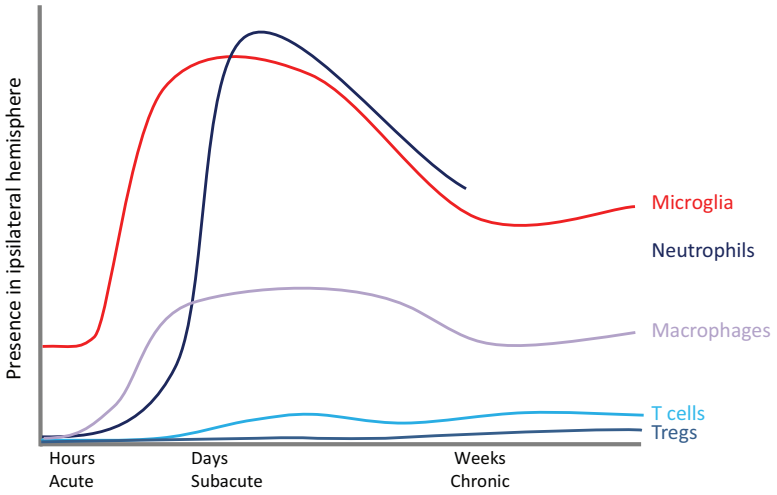

Fig. 24.2 Dynamics of immune cell infiltration into the ipsilateral hemisphere. Modified from [48, 51]

subsets of the T cells, initially, CD8+ cytotoxic T cells are recruited as early as 3 h after onset of ischemia [52]. CD4+ and Natural Killer T cells are following at around 24 h after ischemia [52]. However, Treg aggregation culminates later and this subtype is still significantly present at day 30 post lesion [51].

2.2 The Impact of T Cell Subsets in the Acute Phase

T cells play a crucial role in secondary stroke progression [41, 53, 54] albeit representing only a very small subpopulation of brain-invading leukocytes—about 3000 T cells compared to 65,000 neutrophils per hemisphere [48]. Transgenic mice deficient of lymphocytes have been consistently demonstrated to have smaller lesions after transient and permanent middle cerebral artery occlusion (MCAO) compared to immunocompetent control animals [55–60]. Further, restoring the lymphocyte population in such immunodeficient animals by adoptive cell transfer reversed the protective effect, resulting in infarct volumes comparable to WT animals [58]. Finally, antibody mediated depletion of single T cell subsets, namely CD8+, CD4+ and γδ T cells, was also able to attenuate ischemic injury [56, 61–63]. But not only depletion of leukocytes or different subsets but also changing the ratio of pro-inflammatory and anti-inflammatory subsets alleviates post-stroke neuroinflammation. Pro-inflammatory Th1 and Th17 cells constitute the majority of T cells entering the brain after stroke. Shifting the pro-inflammatory T cell polarization towards Th2 cell activation was shown to reduce secondary neuronal degeneration after stroke [64]. The processes leading to this protection are incompletely understood, but in focus of current research.

Amelioration of stroke outcome due to T cell-targeted immunomodulation was detectable as early as some hours after ischemia. However, antigen-specific clonal expansion of T cells requires approximately a week for efficient generation of antigen-specific immune response. Therefore, it is rather unlikely that antigen-specific mechanisms play a major role in post-stroke T cell immunity. It is more likely that cytokines secreted by T cells after invading the ischemic brain or already in the circulation are key contributors to T cell-mediated effects on stroke outcome. Consequently, since the individual subtypes of T cells play different physiological roles and produce characteristic cytokines, it is important to examine the functional role of individual T cell subpopulations separately.

Cytotoxic T cells have detrimental effects after stroke. Antigen-dependent activation of this cell population was associated with their invasion to the lesion site [61]. Secretion of perforin and granzymes by cytotoxic T cells leads to secondary stroke progression (Fig. 24.3a). Mracsko et al. reported that CD8+ T cells were interacting with neurons in the subacute phase after stroke and contributed to neuronal cell death [61]. Additionally, the release of IFNγ leads to upregulation of MHC-I molecules in neurons [65] potentially contributing to secondary immunological cell death by promoting interaction of neurons with cytotoxic T cells.

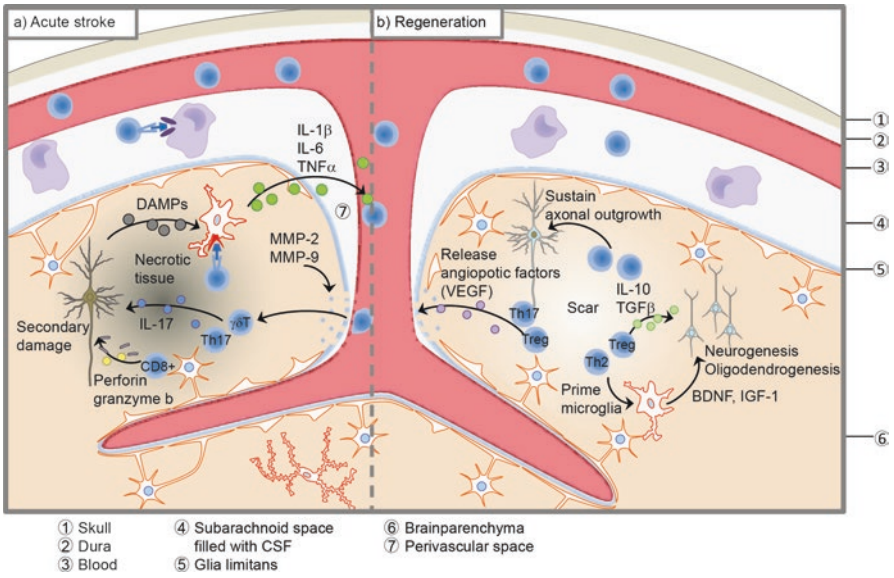

Fig. 24.3 Functional role of T cells in the acute phase and regeneration phase after stroke. (**a**) In the acute phase after stroke, necrotic cells release DAMPs which activate local microglia. Activated microglia secrete cytokines and chemoattractants facilitating T cell entry to the lesion site. T cells promote exacerbation of neuroinflammation and increase secondary cell death. (**b**) Possible roles of T cells in recovery after stroke. T cells are involved in several processes involved in tissue regeneration such as neurogenesis, angiogenesis and axonal outgrowth

γδ T cells and to a lesser extent Th17 harm the damaged tissue by secretion of IL-17 [62] which peaks at day 3 post lesion [66]. γδ T cell deficient mice—in genetic and antibody-mediated depletion models—have smaller infarct volumes compared to respective control mice [62]. Moreover, neutralization of IL-17 using specific antibodies also significantly reduced infarct size [63]. IL-17 binds to the IL-17 receptor which is upregulated in astrocytes, microglia and neurons after stroke [67]. Downstream signaling of the IL-17 receptor enhances expression of NF-κB and GSK3beta which were associated with induction of neuronal apoptosis in different neurodegenerative diseases [68–70]. NF-κB is critical in several neuroinflammatory processes after stroke including microglial activation, leukocyte trafficking and secretion of pro-inflammatory cytokines [68]. These manifold functions of IL-17 suggest a key role of the cytokine and its main producers, gamma delta and Th17 cells, in post-stroke lesion progression [71] (Fig. 24.3a). Interestingly, a second peak in IL-17 expression has been described to occur in the late phase around day 28 after stroke [66]. At this late time point mainly reactive astrocytes secreted the cytokine [66]. Lin et al. showed that at this late stage, IL-17 might improve neurogenesis in contrast to the rather detrimental function during the acute phase [66]. This example of a potentially opposing function of a specific cytokine—pro-inflammatory functions of IL-17 in the acute phase and potentially restorative

capacities in the later stages—highlights the complexity of the adaptive immune response after stroke (Fig. 24.3). While the previously discussed T cell subpopulations secrete pro-inflammatory mediators and contribute mainly to propagation of the neuroinflammatory milieu, Foxp3+ Treg have in contrary been identified to limit an overshooting immune response after stroke [55]. Although the function of Treg cells in vascular inflammation after stroke is still under debate, the vast majority of studies by now have verified a protective function of this cell type on parenchymal post-stroke neuroinflammation [72]. Several studies showed exacerbation of neuronal degeneration in models of Treg depletion including genetic or antibody-mediated depletion or selective adoptive cell transfer models [55, 73, 74]. IL-10 has been identified as the key mediator of Treg in facilitating their neuroprotective function [73, 75]. Accordingly, intraventricular IL-10 injection reversed the effect of Treg depletion [55]. In transgenic mice which overexpress IL-10, lower levels of pro-apoptotic caspase 3 were detected in the injured hemisphere on day 4 post stroke [76]. *In vitro* microglia stimulated by IL-10 enhanced neuronal survival [77]. In addition, both overexpressing IL-10 and IL-10 gene transfer lead to reduced infarct sizes and attenuated leukocyte infiltration [76, 78]. Tregs might also affect BBB integrity during acute stroke. Metalloproteinases (MMPs), in particular MMP-2 and MMP-9, are critical in degradation of extracellular matrix and contributing to vascular leakage [46]. Adoptive Treg transfer has been associated with inhibition of MMP-9 activity and as a consequence protection of the neurovascular unit integrity [79]. Taken together, Tregs play a central role in limiting the detrimental impact of neuroinflammation after stroke.

3 T Cells in Regeneration

T cells have multiple effects on other cell populations which are known to play a major role in regeneration after stroke. In the following, we will focus on the direct effect of T cells on different processes involved in post-stroke regeneration (Fig. 24.3b).

3.1 T Cells and Neurogenesis

Neurogenesis in the adult brain is limited to only few specialized localizations, namely the subventricular zone (SVZ) and subgranular zone (SGZ) of the dentate gyrus [80–82]. After stroke, neural precursor cells (NPC) have the ability to migrate to the lesion site [83, 84]. Here, NPC might not only replace dead cells [85, 86] but also—and maybe even more importantly—contribute to a pro-regenerative milieu and thereby support neuronal plasticity by secreting growth factors such as BDNF [87, 88]. Proliferation, migration and differentiation of NPC are substantially

influenced by immune cells, including T cells. Vice versa, NPC might play a role in modifying the post-ischemic immune response [89, 90]. Under physiological conditions, T cells have an impact on cerebral cell renewal [91]. Without entering the brain, lymphocytes facilitate neurogenesis [34, 92]. After brain injury, the interaction of the adaptive immune system and neuronal precursor cells (NPC) is also evident [93]. Similar to the acute phase after stroke, different T cell subsets seem to have opposing functions during recovery, in particular on NPC proliferation and differentiation. T helper cells impair regeneration after stroke and promote ineffective functional recovery. T cell deficiency, both in transgenic mice and by CD4-specific antibody-mediated cell depletion, was associated with reduced apoptosis and increased proliferation rates of NPC in a cortical lesion model [94]. A specific subset of CD4+ T helper cells expressing the glucocorticoid-induced TNF receptor (GITR) has been identified to mediate this substantial impact of T cells on post-stroke neurogenesis [95]. In contrast, Tregs have been demonstrated to support neurogenesis. Treg depletion by CD25-specific antibodies resulted in a reduced number of NPC after experimental stroke [94]. Correspondingly, the higher number of Tregs in the ventricle of the ischemic hemisphere was associated with increased proliferation of NPC and attributable to the secretion of IL-10 by Treg [96]. Taking these opposite effects of T cells on neurogenesis depending on their subtype, it reveals again the intricacy of neuroimmunological processes after cerebral ischemia.

Besides direct effects on NPC proliferation, T cells might also indirectly affect NPC via their impact on microglial cells. Cytokines released from the different T cell subsets can activate and differentially polarize microglia depending on the secreted cytokine profile [97]. In turn, activated microglia have been shown to have two main routes to influence neurogenesis: Microglia secrete IGF-1, a growth hormone which reinforces NPC proliferation in general [98]. Additionally, they are in control of balancing oligodendrogensis and neurogensis. Activated via IL-4, microglia facilitate oligodendrogenesis albeit IFNγ shifts microglia towards a neurogenesis supporting polarization type [99]. Whether these findings also affect regeneration after stroke is yet to be determined.

3.2 T Cells and Axonal Sprouting

Another essential process for neuronal repair is axonal sprouting. In spinal cord injury and transection of the perforant pathway it was shown that T cells can affect axonal outgrowth [100, 101]. *In vitro* experiments underscore their influence on sprouting, but again different subsets play distinct roles: CD4+ Th cells can enhance axonal growth, whereas CD8+ cytotoxic T cells might impair this process [102]. So far, the impact of T cells on axonal outgrowth has not been investigated in animal stroke models. Yet, better understanding of T cell-driven mechanisms in neuronal plasticity and their impact on regeneration after stroke might open new therapeutic targets for stroke patients.

3.3 T Cells and Angiogenesis

Angiogenesis is a critical mechanism in the restoration of injured tissue. After stroke, endothelial cells proliferate and immature vessels begin to outgrow to regions with stimulatory signal [103]. There is a wide variety of factors sustaining vascular growth which can be secreted by different cell types including T cells. The influence of T cell on angiogenesis was studied in different experimental conditions. Recently, it was shown in a model of hind limb ischemia that the presence of CCR7+ T cells is beneficial for effective arteriogenesis [104]. Accordingly, in transgenic mice deficient of total CD4+ cells collateral vascularization after hindlimb ischemia was reduced and associated with impaired limb function [105]. The concept of a differential impact of the individual T cell subsets described above applies also for angiogenesis. It was previously shown that pro-inflammatory CD4+ Th1 cells and their characteristic cytokine IFNγ dampen vascular growth, in models of tumor and lung ischemia [106, 107]. In contrast, anti-inflammatory Tregs promote angiogenesis not only by suppressing the impact of effector T cells [107] but also by secreting chemokines, the proangiogenic vascular endothelial growth factor (VEGF) and TGF-β [108, 109]. TGF-β signaling is known to be essential for vascular development during embryogenesis [110]. In experimental cancer research, IL-6, a classical lymphocyte derived cytokine was shown to enhance angiogenesis also via upregulation of VEGF [111]. Furthermore, T cell-derived IL-17 was shown to facilitate neovascularization of the rat cornea and promote chemotactic function in tumor vascularization by upregulation of proangiogenic factors [112]. The named mechanisms of T cells regulating angiogenesis in a variety of disease models has so far not been investigated in ischemic stroke. However, in light of the important function of angiogenesis in restoration of tissue function, further investigations on the specific role of T cells in this processes will be of high relevance.

4 Conclusion

The presence of T cells is critical in physiological brain function. A large body of evidence over the past decade has highlighted a key role for T cells in the pathophysiology of stroke in the acute phase. Within the first days after stroke, pro-inflammatory T cell subpopulations play a detrimental role and contribute to the exacerbation of neuronal damage. In contrast, immunosuppressive Treg cells are beneficial for stroke outcome by limiting the inflammatory collateral damage. While the role of T cells in the acute and subacute phase after stroke has been extensively investigated, the contribution of T cells to the chronic regenerative phase is so far barely understood. Nevertheless, studies in other research field indicated a role for T cells for several reparative processes such as neurogenesis, angiogenesis and axonal growth. Unfortunately, until now only very few studies have specifically investigated the function of T cells in post-stroke regeneration and their therapeutic

potential to improve regenerative capabilities after stroke. Yet, T cells are ideal therapeutic targets due to their excellent druggability as circulating cells and numerous T cell-targeted therapies readily available. Therefore, further studies particularly testing the function of T cells in post-stroke recovery are urgently needed for the development of novel therapeutics for stroke patients.

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Chapter 25
The Inflammatory Response and Its Effect on Rehabilitation-Induced Repair Processes After Stroke

Ali Alawieh, Farris Langley, and Stephen Tomlinson

Abstract Post-stroke inflammation is associated with a significant exacerbation of acute injury, and at the same time promotes an unfavorable environment for regeneration and recovery. Sustained inflammation after stroke is associated with poor motor and cognitive recovery and limits the ability of the brain to engage in and benefit from rehabilitation paradigms. Stroke comorbidities such as aging, diabetes, and smoking are all associated with a more robust neuroinflammatory response after stroke and poor outcomes. Preclinical and clinical studies have not yet investigated the role of post-stroke neuroinflammation in predicting the response to rehabilitation therapy. Here, we review the interaction between post-stroke neuroinflammation and determinants of response to rehabilitation therapy, we discuss the few studies that used anti-inflammatory therapy to boost the response to rehabilitative interventions, and we emphasize the need for combining anti-inflammatory therapy and rehabilitation in both therapeutic and mechanistic studies of experimental stroke.

Keywords Inflammation • Post-stroke plasticity • Stroke • Neurogenesis • Rehabilitation • Combination therapy

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P.A. Lapchak, J.H. Zhang (eds.), *Cellular and Molecular Approaches to Regeneration and Repair*, Springer Series in Translational Stroke Research,
https://doi.org/10.1007/978-3-319-66679-2_25

Abbreviations

NSAID	Nonsteroidal anti-inflammatory drug
CRP	C-reactive protein
SVZ	Sub-ventricular zone
SGZ	Sub-granular zone
CR2-fH	Complement receptor 2—Factor H
RV	Resveratrol
sTNFαR1	soluble Tumor Necrosis Factor α receptor 1
TNFα	Tumor Necrosis Factor α

1 Introduction

Despite the fact that the extent of initial deficit and the size of injury are major predictors of outcome after stroke, repair and regeneration that occurs in the brain following ischemic injury is key to limit the evolution of acute injury and promote recovery of function [1–3]. In fact, the current clinical strategy to improve recovery of stroke patients beyond the acute phase is motor and cognitive rehabilitation that targets mechanisms of repair and regeneration after stroke [4–9]. However, the detailed pathophysiological processes involved in the response to rehabilitation therapy are still largely unknown, and a standard paradigm for optimizing the response to rehabilitation in clinical settings has not yet been established.

Rehabilitation therapy has been found to promote favorable pathophysiological responses such as increased growth factor levels, increased axonal and dendritic sprouting and increased neurogenesis and neuronal migration ([10–15], reviewed in [4, 8]). However, the same processes are also expected to be under a tonic inhibitory drive by pathological inflammatory responses that persist in the infarcted brain after stroke [16–18]. Inflammatory responses sustained in the brain after the initial insult are associated with degenerative changes, and with an anti-regenerative environment that impede the response to rehabilitation therapy. Therefore, it may be hypothesized that the extent of neuroinflammation propagating beyond the acute phase of stroke may determine the response to rehabilitation therapy, and that suppression of inflammation may augment the response to rehabilitation therapy. Studies from stroke patients have shown that increased markers of inflammation, including acute phase reactants (IL6, CRP), can predict outcomes after ischemic stroke [19–21]; however, the impact of inflammation on response to rehabilitation has not yet been investigated at the clinical level. The aim of this work is to provide an overview of the interaction between inflammation and rehabilitation therapy during chronic recovery after stroke and to emphasize the need for investigating combinatory strategies of anti-inflammatory therapy and rehabilitation paradigms at preclinical levels.

1.1 Determinants of Response to Rehabilitation Therapy

In absence of treatment, stroke patients tend to show spontaneous recovery in both motor and cognitive functions beyond the acute phase. Rehabilitation therapy aims to speed up or augment the recovery trajectory and promote better outcomes. It is natural that the extent of initial injury is a key determinant of response to rehabilitation since it defines the extent of damage, the ability of patients to engage with rehabilitation paradigms, and the availability of neuronal reserve able to engage in regenerative and remodeling processes [3, 4, 22]. Reducing the extent of initial injury is a major focus in acute ischemic stroke treatment and neuroprotection therapy but beyond the acute phase when a stable infarct is defined, reducing the extent of lesion is not a major pathophysiological target of interventions such as rehabilitation therapy. Additional determinants of the response to rehabilitation include the ability to re-wire existing connections taking advantage of increased post-stroke plasticity, and the ability to form and incorporate new neurons into existing circuitry. These two components will be further discussed in the following sections to assess how inflammation contributes to the ability of the recovering brain to augment and re-organize existing resources to optimize overall outcome.

2 Pathological Inflammation After Stroke

Following cerebral ischemia and reperfusion injury leading to neuronal apoptosis and necrosis, a neuroinflammatory response is triggered that includes the expression of danger-associated molecular patterns, the release of reactive oxygen species, and the subsequent activation of cells of the innate and adaptive immune systems [23]. A prominent feature of neuroinflammatory responses is their ability to self-amplify and maintain a sustained activation, at a pathological level, that extends beyond the acute phase [24]. Post-acute inflammation is characterized by the presence of a vicious cycle that prevents the resolution of this response to allow for recovery (Fig. 25.1). Inflammatory stress to neurons kindles the growing inflammatory response, which in turn promotes more stress to recovering neurons ultimately leading to a degenerative response. Although activation of immune cells and activation products are implicated in both injury and recovery after stroke (reviewed in [1, 25]), pathologic immune activation after stroke, characterized by a delay in resolution of inflammation and a significant feed-forward mechanisms, is majorly associated with significant delay in recovery and increased degenerative responses. The fact that the same immune cells and components contribute to both reparatory and degenerative inflammation is a major hurdle for therapeutic strategies, especially those that aim at depletion of specific immune components or processes. Lessons learned from several decades of research in anti-inflammatory and neuroprotective therapies for ischemic stroke have demonstrated that efforts should be focused on strategies that promote a faster resolution of inflammation and interrupt the feed-forward amplification

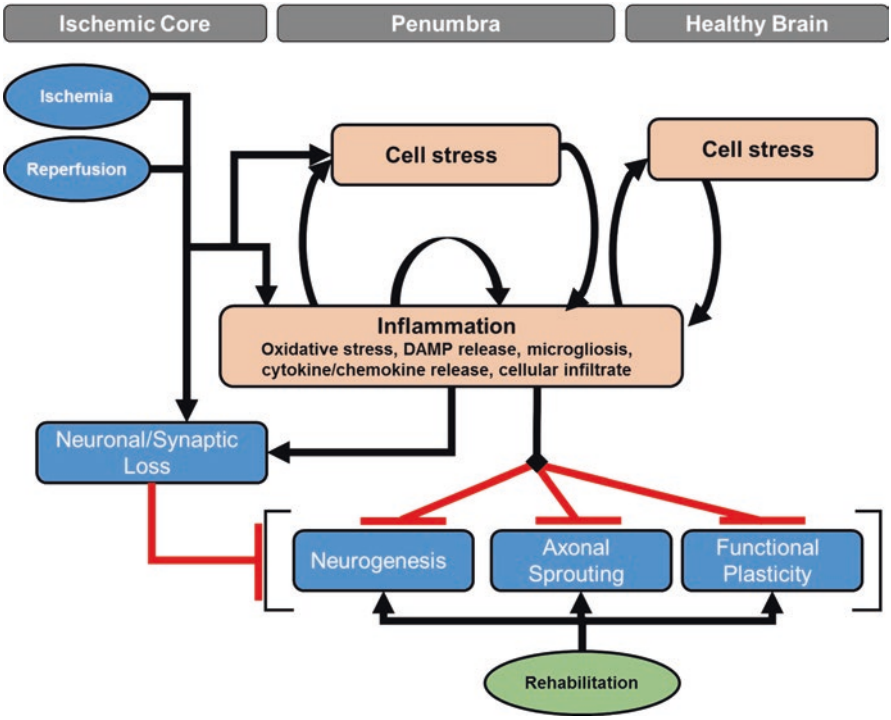

Fig. 25.1 Ischemia and reperfusion injury to the ischemic core after stroke contributes to neuronal and synaptic loss and initiates an inflammatory response that propagates to the ischemic penumbra. Inflammatory mechanisms induced after stroke have the propensity to self-amplify and elicit cellular stress on recovering neurons in the penumbra. This initiates a vicious cycle where more inflammation triggers more cellular stress and subsequent death of neurons, that in turn fosters a more pronounced inflammatory response. This cycle results in an expansion in the size of the penumbra, reducing the reserve of neurons and synapses available to engage in recovery, and promoting an anti-regenerative environment that limits the response to rehabilitative interventions

mechanisms, without eliminating pathways within the inflammatory casade that may serve important reparatory roles. The dual role of immune activation in injury and recovery after stroke has been extensively reviewed [1, 2, 25]; however, this work will review the interaction between neuroinflammation and regenerative mechanisms induced or exploited by rehabilitation interventions.

2.1 Inflammation and Post-stroke Neurogenesis

Neuronal progenitor cells in the sub-ventricular zone (SVZ) or subgranular zone (SGZ) proliferate after stroke and migrate, in the absence of inhibitory signals, to perilesional brain and populate cortical, hippocampal and striatal targets [26–29].

Newly generated neurons are capable of incorporating into existing circuitry and constitute a substrate for axonal and dendritic remodeling. Formation and incorporation of newly formed neurons after stroke leads to improvements in both cognitive and motor recovery [30–32]. However, pathologic inflammation has been consistently associated with significant reduction in basal and ischemia-induced neurogenesis, and inhibition of migration of newly formed neuroblasts [16, 33–43]. Interruption of the propagation of neuroinflammation has resulted in a significant increase in neuroblast proliferation, migration and subsequent functional outcomes [16, 33–43]. Specific inflammatory mediators that have been implicated in suppressing neurogenesis include IL-1β, IL-6, TNF-α, C3, [1, 44–46]. A few studies have implicated neuroimmune interactions such as activation of T-cells and microglia in promoting basal neurogenesis [47, 48]. This data may apply in the context of mild inflammatory responses; however, post-stroke inflammation characterized by robust activation of microglia and infiltration of immune cells leads to a detrimental effect on neurogenesis after stroke [46]. In addition, depletion of immune system components may not be an optimal strategy to investigate the role of post-stroke inflammation in neurogenesis due to potentially inhibiting beneficial homeostatic interactions. In a previous work, we demonstrated that when the ability of the complement system to self-amplify is inhibited after MCAO, there is a reduction in pathological inflammation, resulting in significantly improved neuroblasts migration from the SVZ with improved cognitive performance [43]. Complement activation is amplified by the alternative complement pathway, and we have shown that site-targeted inhibition of this pathway with CR2-fH interrupts this feed-forward mechanisms fostering uncontrolled inflammation, and is optimal to facilitate the resolution of inflammation while allowing for a regenerative environment [43]. Supporting our hypothesis, complete inhibition of complement by blocking all pathways of complement activation interrupted the migration of neuroblasts and resulted in poor recovery [43]. Although rehabilitation is implicated in promoting neurogenesis after stroke, the interaction between inflammation, rehabilitation, and neurogenesis has not been investigated. Notably, advanced age, a major determinant of clinical response to rehabilitation, is associated with a significant reduction in post-stroke neurogenesis in rodent models [49].

2.2 Inflammation and Post-stroke Plasticity

Neuronal remodeling beyond the acute phase of ischemic stroke exploits a window of neuroplasticity and allows peri-lesional neurons, or even neurons in the contralateral hemisphere, to establish new synaptic connections that can compensate for functions previously performed within the infarcted brain [50–53]. The process of remodeling has been documented in both humans using functional imaging and brain stimulation studies and in animal models [3], and has been shown to enhance recovery and restore function, at least partially. The pathophysiological mechanisms underlying neuronal re-wiring involve axonal

sprouting and subsequent synaptogenesis. In fact, extensive preclinical research has been devoted to investigate strategies to enhance axonal sprouting to promote recovery after stroke. For instance, growth inhibitory factors such as Nogo-A has been shown to suppress axonal sprouting after CNS injury, and inhibition or antagonism of Nogo-A activity has been found to enhance neuronal plasticity and improve recovery in young and adult rodents after stroke [54–59]. In parallel, rehabilitation strategies aim to exploit the window of neuronal plasticity in the brain after stroke to promote beneficial re-wiring [3], and anti-inflammatory therapies aim to lift the brakes off the growth promoting factors to extend the window and magnitude of post-stroke plasticity and potentiate the effects of rehabilitation. Chronic inflammation after stroke is considered a plasticity-impeding factor, an effect that is more prominent in aged animals [4]. Infiltration of immune cells, including CD45+, CD3+ and CD4+ cells [60, 61], increased TNF-alpha signaling [62], and increased microglial activation and release of cytokine and chemokines [60, 63], have all been implicated in suppressing plasticity and worsening recovery after stroke [4, 63]. Supporting these findings, inhibition of inflammatory pathways such as TNF-alpha, CXCL12 signaling, microglial activation, or complement activation leads to enhanced post-stroke plasticity and improved recovery [1, 61–63]. In addition, several interventions that are shown to enhance post-stroke plasticity such as erythropoietin and VEGF administration also mediate their effects, at least in part, by suppression of neuroinflammatory responses [60, 64–66]. These studies as well as others (reviewed in [67]; [4]) demonstrate that excessive inflammation after stroke is associated with depression of post-stroke plasticity, an outcome that may limit the response to rehabilitation therapy (discussed in the following section). It is also worth noting that immune activation products may also facilitate post-stroke plasticity, as seen in neurogenesis; however, this is not the hallmark of potent neuroinflammatory responses after stroke, and may be relevant for conditions with a mild inflammatory response.

2.3 Inflammation and Response to Rehabilitation in Preclinical Models

Despite the significance of assessing the interaction between inflammatory mechanisms and outcomes of rehabilitation, only a few studies have investigated the combination of anti-inflammatory therapy with rehabilitation to assess for the presence of potential cooperative, synergistic or antagonistic effects. In addition, from a therapeutic standpoint, and since most stroke patients receive some form of rehabilitation therapy after stroke, it is essential to investigate the efficacy of an anti-inflammatory or neuroprotective agent in the context of rehabilitation prior to further translation to human trials.

In one of the first studies combining anti-inflammatory therapy with rehabilitative training in animal models, Liebigt et al. [68] used a combination of minocycline

or indomethacin, and skilled reaching training during recovery from photothrombotic infarct in rats. Indomethacin is a nonsteroidal anti-Inflammatory drug (NSAID) that inhibits cyclo-oxygenases, whereas minocycline inhibits the activation of microglia after stroke. Following 4 weeks of rehabilitative training combined with minocycline or indomethacin treatment, both pharmacological treatments significantly improved motor performance on reaching and ladder tasks compared to rehabilitation alone. Notably, the effects seen with minocycline treatment were observed in the absence of a significant reduction in lesion volume at 14 or 42 days after injury. Anti-inflammatory therapy with minocycline or indomethacin was associated with a significant increase in the number of BrdU+ neurons and a significant decrease in proliferating microglia in the sensorimotor cortex compared to rehabilitation alone [68]. These findings demonstrate that anti-inflammatory therapy favorably interacts with rehabilitation training to improve outcomes after stroke, even in absence of a significant decrease in infarct size with anti-inflammatory therapy.

The interaction between NSAIDs and experience-dependent plasticity after stroke was further investigated in a rat model of photothrombotic stroke with whisker deprivation. Whisker deprivation, by clipping all but one row of vibrissae, is associated with a significant increase in cortical representation of the spared row, thus evoking a strong neuroplastic response [69]. Stroke was associated with a significant decrease in cortical plasticity after deprivation, an effect that was reversed when the NSAID, Ibuprofen, was administered following stroke. Ibuprofen showed a dose-dependent decrease in cyclo-oxygenase 2 levels in the brain, and a dose-dependent increase in the cortical representation of the spared vibrissae indicating increased cortical plasticity [69]. A similar approach was used to assess whether inhibition of TNF-alpha can also preserve cortical plasticity after stroke. Following stroke and whisker deprivation, soluble TNF-alpha receptor 1 (sTNFαR1) was osmotically infused into the murine brain without any influence on lesion volume [62]. Anti-inflammatory therapy with sTNFαR1 inhibited signaling downstream of TNFαR1, decreased TNFα levels, and restored cortical plasticity that was inhibited after stroke [62].

In a recent work, Huaifang et al. [70] used environmental enrichment and resveratrol (RV) treatment to assess the combinatory effects of both therapies on overall recovery after stroke. Enriched environment is one of the commonly used models of motor and cognitive rehabilitation that has been associated with increased neurogenesis and neuronal plasticity. Following middle cerebral artery occlusion in rats, administration of RV combined with enriched environment resulted in a more prominent functional recovery compared to either intervention alone. Combination therapy also significantly reduced oxidative stress and ERK1/2 signaling involved in exacerbating post-stroke pathology [70].

In addition to these studies investigating the combination of anti-inflammatory therapies with rehabilitation paradigms, some reports have also demonstrated a cooperative or synergistic effect of combining neuroprotective therapy and rehabilitation after stroke [71–73].

3 Conclusion

The role of neuroinflammation in modulating the response to rehabilitative interventions after stroke is still largely under-investigated. Collectively, current studies provide preliminary evidence that anti-inflammatory therapy may facilitate the response to rehabilitation paradigm; however, further and more robust behavioral and molecular characterization is still needed. Equally important is the careful distinction between pathologic inflammation, a self-amplified response with slow resolution, and mild inflammation or activation of immune cells in the brain after injury. Studies depleting specific immune cells and components showing rebound injury are not sufficient to challenge the pathologic role of excessive inflammation that occurs after stroke and constitutes a major target for intervention. Alternatively, efforts should be devoted to breaking the loop of inflammation and stimulating faster resolution of the inflammatory response by masking the triggers of sustained activation or breaking the feed-forward mechanisms along this pathway.

In addition, subsequent studies should investigate whether rehabilitation and anti-inflammatory therapies modulate different pathophysiological processes, and provide a more detailed analysis of cognitive and motor recovery measures to assess whether this interaction is consistent across the two aspects of recovery. Finally, the significance of investigating the efficacy of anti-inflammatory therapies, and neuroprotective therapies in general, in the context of rehabilitation should be further emphasized as a requirement for preclinical stroke research to help bridge the translational roadblock for many novel interventions.

Acknowledgements Authors acknowledge support from the NIH (1P20GM109040, U54 GM10494) to ST, the Department of Veterans Affairs (Merit Award 1I01RX001141 and 1BX001218) to ST, and an American Heart Association Pre-doctoral Fellowship to AA (15PRE25250009).

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Chapter 26
Complement C3a: Shaping the Plasticity of the Post-stroke Brain

Anna Stokowska and Marcela Pekna

Abstract Complement is part of the innate immune system that plays a major role in the initiation of inflammation and host defence against pathogenic bacteria. Complement activation is also a contributor to tissue damage in a range of autoimmune conditions. For those reasons, the activation of the complement system in the central nervous system (CNS) was for long considered deleterious. Based on the evidence accumulated during the past decade, this view has been dramatically changing and complement is gaining recognition for its non-immune surveillance related functions, including regulation of morphogenesis, and adult tissue regeneration. C3a is a 77 amino acid, 9 kDa peptide generated through the proteolytic activation of the central molecule of the complement system, the third complement component, C3. C3a exerts most of its functions through its canonical G-protein coupled receptor C3aR that is expressed by many cell types including neurons and glia. This chapter considers recent insights into the novel roles of the complement system, in particular C3a, in the CNS with focus on brain plasticity and recovery after ischemic brain injury.

Keywords C3a • C3a receptor • The complement system • Ischemic stroke • Neural plasticity • Recovery

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P.A. Lapchak, J.H. Zhang (eds.), *Cellular and Molecular Approaches to Regeneration and Repair*, Springer Series in Translational Stroke Research,
https://doi.org/10.1007/978-3-319-66679-2_26

Abbreviations

AMPAR	α-Amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor
C1q	Complement component 1q
C3	The third complement component
C3a	The smaller of the two fragments generated by proteolytic activation of C3
C3a-desArg	C3a after removal of C-terminal arginine residue
C3aR	C3a receptor
C3b	The larger of the two fragments generated by proteolytic activation of C3
C5a	The smaller of the two fragments generated by proteolytic activation of the fifth complement component
C5aR2	Second receptor for C5a
CD	Cluster of differentiation
CNS	Central nervous system
CR3	Complement receptor 3
ERK	Extracellular signal-regulated kinase
GAP-43	Growth associated protein 43
GFAP	Glial fibrillary acidic protein
GFAP-C3a	Glial fibrillary acidic protein promoter driven expression of C3a
IgG	Immunoglobulin G
IgM	Immunoglobulin M
IL	Interleukin
IXa	Activated coagulation factor IX
MBL	Mannose binding lectin
NGF	Nerve growth factor
XIa	Activated coagulation factor XI
Xa	Activated coagulation factor X
VGLUT1	Vesicular glutamate transporter 1

1 Introduction

The complement system is a critical constituent of the humoral innate immune response and its fundamental importance in the elimination of pathogenic bacteria, inflammation and clearance of immune complexes, as well as its role in tissue damage in various autoimmune conditions have been well recognized for decades. Research findings in the last years provide growing evidence for the involvement of immune cells and molecules, including complement proteins and their activation products, in tissue repair and regeneration. In amphibians, complement proteins or their orthologs are expressed in the regenerating limbs and lens and have been implicated in adult limb regeneration [1–3]. By controlling cell migration,

complement activation products seem to play a role in early vertebrate development and morphogenesis [4–6]. In rodents, complement is critical for hepatocyte proliferation and liver regeneration [7–9], and promotes homing [10], chemotaxis [11] and retention of hematopoietic stem and progenitor cells in the bone marrow [12]. Through its angiogenic activity, complement can support wound healing [13] but also plays a role in choroidal neovascularization [14–17], which is a typical feature of age-related macular degeneration [18]. Recent evidence points to the complement system as an important modulator of regenerative responses in the mammalian spinal cord [19–21], and implicates complement in the regulation of brain plasticity in the injured as well as healthy brain. Detailed understanding of the multiple immune as well as non-immune functions of the complement system in the CNS will be necessary for the design of complement-targeting strategies for the treatment of neurological disease conditions. The type of injury and its temporal aspects are also likely to be of critical importance, as will be the route of administration of the complement-targeting therapeutics. The potential effects on CNS of interventions targeting complement for the treatment of non-neurological disorders will also need to be carefully considered.

1.1 The Complement System

The complement system is a major effector of innate immune response that serves as a universally distributed rapid and effective first line of defense against pathogens that breach the mechanical and chemical barriers of the body as well as elimination of body's own dead cells. The complement system is a general term for a group of more than 50 soluble proteins, cell receptors and control proteins found in the blood and tissues. Their roles in innate immunity include the opsonisation and lysis of pathogens, elimination of soluble antigen–antibody complexes, stimulation of leukocyte chemotaxis and initiation of inflammation. Complement affects also adaptive immunity by regulating B and T lymphocyte function [22]. Hepatocytes are the predominant source of complement proteins is liver, however other cell types such as macrophages, lymphocytes, fibroblasts and endothelial also contribute to systemic pool of complement factors. Complement factors are also produced locally in the brain and spinal cord [22].

1.2 The Complement Cascade

Activation of the circulating complement proteins in response to injury or challenge by pathogens results in a cascade of reactions involving structural rearrangements of proteins, proteolytic cleavages, and the assembly of terminal lytic complexes on the activating/target surface. The cascade is initiated through three major pathways: the classical, lectin or alternative pathway [22]. The classical pathway of

complement activation is triggered primarily by binding of the recognition protein C1q to antigen–antibody complexes through the Fc region of IgGs and IgMs, or by antibody-independent mechanisms involving direct binding of C1q to viral envelopes, cell walls of Gram-negative bacteria, C-reactive protein, intermediate filaments and myelin. The lectin pathway is initiated by the binding of mannose-binding lectin (MBL) and ficolins to carbohydrate moieties on the surface of bacterial and yeast cells or parasite envelopes or cells. MBL can also bind to and opsonize apoptotic and necrotic cells [23]. C1q, MBL and ficolins are typical pattern-recognition molecules, which allow anchoring of C1q- or MBL-associated serine proteases that propagate the proteolytic cascade leading to the formation of C3-convertase, an enzymatic complex that cleaves and activates the central molecule of the cascade, the third complement component (C3). Alternative pathway is initiated at a very low rate in the fluid phase by a spontaneous conformational change of C3, which upon recruitment of factor B and a proteolytically-active factor D leads to the formation of an alternative C3-convertase complex on the activating surface [22]. In addition, C3 can be activated directly by MBL-associated serine protease 1 [24], non-complement proteases such as neutrophil elastase, cathepsins [25, 26], granulocyte neutral proteases [27], lysosomal enzymes, kallikrein, as well as coagulation factors XIa, Xa, IXa, thrombin, and plasmin [28, 29]. Cleavage of C3 generates a small C3a fragment, and a larger fragment, C3b, that binds to bacterial cell wall or altered mammalian cell membrane, where it participates in the amplification of the alternative pathway and triggers the terminal part of the complement cascade that results in cell lysis. As a ligand of the complement receptor 3 (CR3, CD11b/CD18)), C3b also facilitates the phagocytosis of C3b tagged target molecules or structures, including neuronal synapses [30] (Fig. 26.1).

2 Complement System Activation in the Ischemic Brain

In the CNS, complement proteins are produced by astrocytes, microglia and neurons [31–34]. Brain ischemia not only triggers systemic as well as local activation of the complement system but there is a growing body of evidence for multiple immune and non-immune functions exerted by complement proteins and their activation products in the ischemic brain parenchyma with very distinct and even opposing effects on outcome.

Ischemic stroke triggers pronounced activation of complement in the systemic circulation [35–39] and immunohistochemical evidence from human post-mortem brain tissue shows local complement activation in brain parenchyma and markedly reduced expression of membrane bound complement regulatory proteins after ischemic stroke [40, 41]. Experimental studies have been instrumental in elucidating the mechanisms involved. Mocco and co-workers demonstrated that C3 activation is the key constituent in ischemia-induced brain tissue injury and pointed to C3a as the main mediator [42]. While C1q, the complement protein initiating the classical pathway of complement activation, does not seem to be involved in complement activation in the ischemic brain parenchyma [42, 43], genetic deficiency of mannose

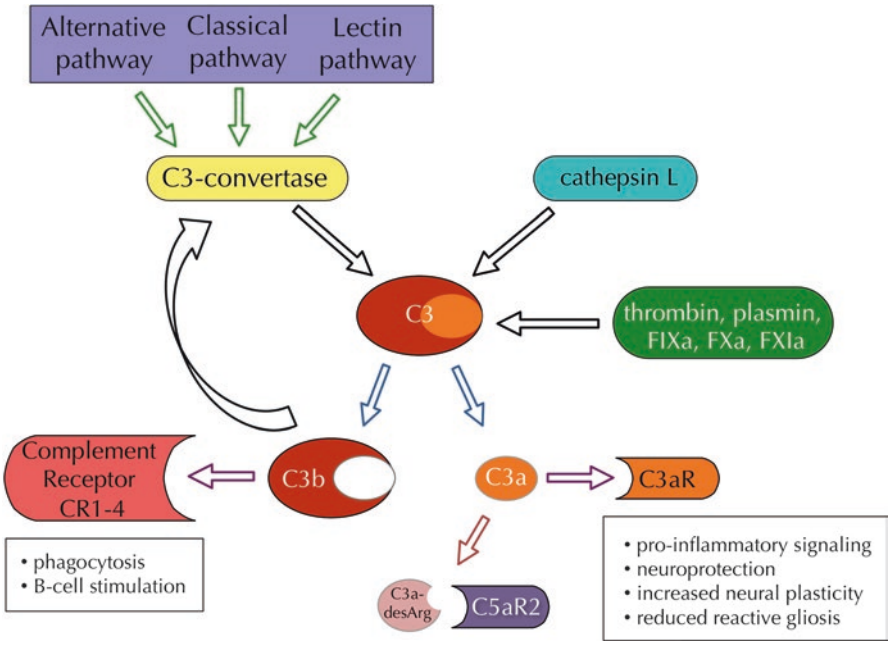

Fig. 26.1 C3a is released through the proteolytic activation of C3. C3a exerts its effects through binding to the C3a receptor (C3aR), the activation of which has been shown to induce inflammation, have neuroprotective effects and increase neural plasticity after ischemic stroke

binding lectin (MBL) abrogated C3 cleavage as well as the sub-acute accumulation of mononuclear cells in the ischemic region and improved outcome at 24 h post-stroke [44]. However, the neuroprotective effect of MBL deficiency was not sustained in the post-acute phase [44]. These findings, together with the report on lasting protection against acute brain injury conferred by acute pharmacological lectin pathway inhibition [45] imply that complement activation by the lectin pathway acutely contributes to tissue damage, but has beneficial functions in the post-acute repair phase. Although the initial steps leading to lectin pathway activation after ischemia are not fully understood, ischemia-induced appearance in brain parenchyma of neoepitopes that bind naturally occurring IgM [46] and binding of MBL to the neoepitope-bound IgM [47] seem to play an important role in this process. Indeed, IgM deposits were detected in the necrotic zones of post-mortem human brain 5–7 days after ischemic stroke [41]. How persistent is the neoepitope expression and what are the mechanisms of complement activation in the peri-infarct region and more remote brain regions in the post-acute and chronic phase are some of the outstanding questions that merit further investigation. On its own, the alternative pathway is not sufficient to initiate complement activation in the ischemic brain, but seems to propagate brain tissue injury via amplification of the cascade. Notably, deficiency of factor B as well as targeted pharmacological inhibition of the alternative pathway had sustained neuroprotective effect [48].

3 The Roles of Complement in Stroke

Given that complement activation products are powerful mediators of inflammation and neutrophil infiltration, complement activation, has been regarded as a contributor to secondary tissue damage after injury including brain ischemia [49] or trauma [50]. However, there is a growing body of evidence showing that the protective effect of complement inhibition in the acute phase after stroke is not always maintained in the subacute and chronic phases (for review see e.g. [51]). These results point to the critical role of complement in both tissue damage and repair processes after ischemic brain injury. The complement peptide C3a and its canonical receptor C3aR in particular appear to elicit multiple responses in immune and brain cells after ischemia; often with opposing effects on outcome. These consequences of C3aR activation on the long-term outcome depend on the specific cell type and the timing of the response in relation to the ischemia onset.

3.1 C3a and Its Receptor C3aR in the Healthy and Ischemic Brain

C3a is a 9 kDa, 77 amino acid peptide generated by proteolytic activation of C3 (Fig. 26.1). C3a exerts its functions through its canonical/cognate receptor, C3aR and through the considerably less studied second receptor for C5a, C5aR2 (previously known as C5L2) [52]. After its generation, C3a is rapidly cleaved by carboxypeptidases that remove the C-terminal arginine [53, 54], which ensures that the actions of C3a are tightly controlled and highly localized. While the C3a-desArg peptide does not bind to C3aR [55], it was shown to bind C5aR2 [56, 57]. C3aR is a member of the rhodopsin family of seven transmembrane G-protein-coupled receptors [58] and is widely expressed in many tissues including the brain. C3aR activation leads to increased vascular permeability, smooth muscle contraction, activation of myeloid cells such as neutrophils, monocytes/macrophages, basophils, and platelets, as well as directed migration of inflammatory cells such as eosinophilic leucocytes and mast cells [59]. C3aR expressed by endothelial cells [60, 61] regulates their expression of cytokines such as IL-8 and IL-1beta [62], and plays a critical role in endothelial activation and leukocyte recruitment into the brain by regulating the endothelial cell expression of intercellular cell adhesion molecule 1, and vascular cell adhesion molecule 1 [63]. Furthermore, stimulation of C3aR on epithelial cells of the choroid plexus has been shown to cause disruption of blood-cerebrospinal fluid barrier [64].

The high constitutive expression of C3aR on rodent as well as human neurons [60, 65–67], points to the involvement of C3a signaling in the brain beyond its established immune-related functions. Further, adult neural progenitor cells express C3aR [68], and C3a directly regulates their differentiation and migration through the extracellular signal-regulated kinase (ERK)1/2 signaling pathway [69]. C3a was

Table 26.1 Cellular expression and functions of C3aR in the CNS

Cell type	C3aR functions	References
Neural stem/progenitor cells	Neuronal differentiation Migration	[69] [69]
Neurons	Migration Neurite outgrowth Modulation of synaptic strength Modulation of dendritic morphology	[70, 71] [69] [112] [112]
Astrocytes	Intracellular signaling Cytokine expression Survival	[73] [74–76] [72]
Microglia	Intracellular calcium release NGF upregulation Regulation of phagocytosis	[77] [78] [79]
Endothelial cells	Cytokine expression Expression of cell adhesion molecules	[62] [63]
Epithelial cells of choroid plexus	Disorganization of tight junctions	[64]

also reported to accelerate the migration of granule cells of the developing cerebellum [70] and regulate neuronal migration during cortical development [71]. The expression of C3aR on astrocytes is upregulated by ischemia [60, 65, 72]. C3a affects astrocyte intracellular signaling [73] and the expression of cytokines such as interleukin (IL)-6, IL-8 and nerve growth factor (NGF) [74–76]. Through its inhibitory effect on ERK signaling-mediated apoptotic pathway and caspase-3 cleavage, C3a promotes astrocyte survival after ischemia [72]. Microglia express functional C3aR and respond to C3a stimulation with an increase in intracellular calcium concentration [77] and upregulation of NGF [78]. C3a exerts dual effect on microglial phagocytic functions such that acute C3a activation promotes, whereas chronic C3a treatment attenuates, microglial phagocytosis [79], Table 26.1.

Although unable to couple to G-proteins, C5aR2 can function as a positive modulator for both C5a- and C3a-induced responses [80]. C5aR2 was shown to be expressed on neurons and glial cells, exert anti-inflammatory functions [81], and provide neuroprotection after spinal cord injury [122].

3.2 The Dual Role of C3a in the Acute Phase After Ischemic Injury

In the acute phase after stroke, C3a can contribute to brain tissue loss as it plays a critical role in endothelial activation and leukocyte recruitment into the brain [63], and C3 deficiency as well as pre-treatment with systemically administered C3aR antagonist resulted in reduced granulocyte infiltration, reduced infarct volume and reduced neurological deficit scores in mice 24 h after transient cerebral ischemia [42]. Similarly, acute systemic administration of a low dose of a C3aR antagonist

starting prior to transient ischemia induction resulted in smaller subcortical infarcts 7 days post-ischemia [83]. These results point to the therapeutic benefit of systemic inhibition of C3aR signaling in the acute phase after stroke. Indeed, systemic C3a levels are elevated in the first days after ischemic as well as haemorhagic stroke and in some stroke subtypes show association with unfavourable outcome [36, 38, 39, 84]. Genetic studies provide further support for the involvement of C3 in the pathogenesis of ischemic stroke [85]. On the other hand, C3a was shown to be protective against excitotoxicity-induced neuronal death, an effect that appears to be mediated by astrocytes [86], to support survival of astrocytes after ischemic stress [72] and to induce neuroprotective phenotype in microglial cells as indicated by their increased production of NGF [78]. Mice expressing biologically active C3a controlled by the glial fibrillary acidic protein promoter (*GFAP-C3a*), *i.e.* expressing C3a in reactive astrocytes, were protected against shock induced by lipopolysaccharide injection [87]. In further support of the positive effects of C3a in the injured brain, *GFAP-C3a* mice showed also reduced brain tissue loss in a model of neonatal hypoxic-ischemic brain injury and single dose intraventricular treatment with C3a ameliorated neonatal hypoxia-ischemia-induced memory impairment in wild type control mice but not in mice lacking C3aR ($C3aR^{-/-}$) [88]. Notably, daily intranasal treatment with C3a for 3 days starting 1 h after hypoxia-ischemia prevented cognitive impairment in wild-type mice tested 42 days after hypoxia-ischemia and reduced injury-induced reactive gliosis in the hippocampus [89].

Jointly, these findings support the conclusion that in the acute phase after ischemic injury, C3a can contribute to tissue loss by exerting its pro-inflammatory effects on endothelial cells and recruitment of inflammatory cells from the systemic circulation. However, through its direct effects on astrocytes and microglia, C3a can increase the survival of brain cells, including neurons, thus limiting tissue damage and promoting recovery.

3.3 C3a and Post-stroke Neural Plasticity

Brain ischemia induces endogenous plasticity and repair processes that range from proliferation, differentiation and migration of neural stem and progenitor cells [90–92], to sprouting of axonal projections and establishing new synaptic contacts that form the basis for extensive rewiring of the existing neuronal connections and rearrangement of cortical maps [93, 94]. These constituents of neural plasticity play an important role in normal learning, are enhanced by the milieu created following the injury and jointly contribute to recovery of function after stroke and other CNS injuries [95]. Recent findings provide novel insights into the regulation of the various structural constituents of post-stroke neural plasticity, implicate C3a-C3aR signaling in promoting some of its important constituents and point to C3aR as a target to facilitate functional recovery after ischemic brain injury, Table 26.2.

Table 26.2 The functions of C3a in the regulation of neural plasticity and in neuroprotection

Function	References
Basal neurogenesis	[68]
Post-stroke neurogenesis	[68]
Post-stroke synaptogenesis	[113]
Post-stroke expression of GAP-43, marker of axonal and glial plasticity	[113]
Modulation of reactive gliosis	[89]
Retina regeneration	[121]
Neuroprotection, survival of astrocytes after ischemic stress	[72, 88]

3.3.1 C3a and Post-stroke Neurogenesis

There is a mounting body of evidence for the role for C3a in the regulation of adult mammalian neurogenesis. Hippocampal neural stem cells in vitro as well as migrating neuroblasts in vivo express C3aR [68] and in vitro studies show that C3a directly regulates the differentiation and migration of adult neural progenitor cells [69]. Findings that C3aR-deficient, C3aR antagonist-treated as well as C3-deficient mice had impaired basal neurogenesis, support the contention that signaling through C3aR acts as a positive regulator of adult neurogenesis [68]. In a model of permanent focal cerebral ischemia, mice deficient in C3 showed reduced ischemia-induced neurogenesis and larger infarct volume at both 7 and 21 days after ischemia [68]. These studies indicate that the products of C3 activation positively regulate post-stroke neurogenesis and protect the brain tissue after ischemia. New findings from our laboratory underscore the importance of C3aR signaling for the neurogenic response to ischemic brain injury. Using C3aR-deficient mice, *GFAP-C3a* mice expressing C3a in reactive astrocytes, and their respective wild type controls, we observed that C3a overexpression increased whereas C3aR deficiency decreased the number of newly born neurons in the peri-infarct region, implying that the stroke-induced neurogenic response is at least partially regulated by C3a-C3aR signaling (Fig. 26.2). While low levels of *GFAP* promoter activity in unchallenged adult mice likely explain unchanged basal neurogenesis in *GFAP-C3a* mice [96], pronounced and persistent reactive astrogliosis in the peri-infarct tissue [97] conceivably leads to transgene-derived C3a levels that are sufficiently high to affect post-stroke neurogenesis in this region.

It is noteworthy that daily systemic administration of a low dose of a C3aR antagonist SB 290157 [98] starting already prior to ischemia induction increased neuronal precursor cell proliferation in the ipsilesional subventricular zone of the lateral ventricle 7 days after transient middle cerebral artery occlusion in mice [83]. Given that this treatment did not affect basal neurogenesis, the positive effect of low dose C3aR antagonist treatment on post-stroke neurogenesis could be attributed to its inhibitory effect on inflammatory response and striatal infiltration by activated

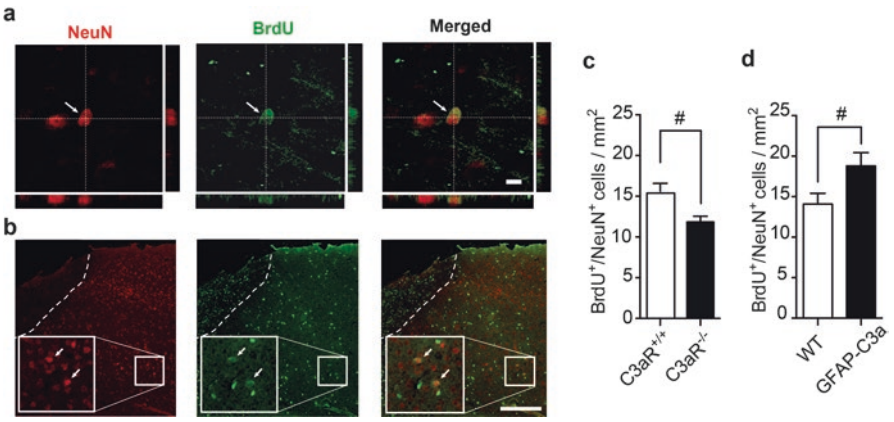

Fig. 26.2 C3a-C3aR signaling positively regulates post-stroke neurogenesis in the peri-infarct region. High (**a**) and low (**b**) magnification images of $BrdU^+/NeuN^+$ neurons. Lack of C3aR (*C3aR*$^{-/-}$) reduced (**c**), whereas over-expression of C3a in reactive astrocytes (*GFAP-C3a*) increased (**d**), the number of newly formed neurons ($BrdU^+/NeuN^+$) in the peri-infarct region. Mean ± SEM; *C3aR*$^{+/+}$ n = 10, *C3aR*$^{-/-}$ n = 14, WT n = 13, *GFAP-C3a* n = 12; #P < 0.05 determined by t test). Scale bar 10 μm (**a**) and 200 μm (**b**).

T-lymphocytes, that reduce neurogenesis, in the C3aR antagonist-treated animals rather than to the direct effect of the drug on progenitor cells [83]. In line with our previous report [68], higher dose of the C3aR antagonist, which is more likely to reach neural stem cell niche after systemic delivery, reduced the number of proliferating doublecortin-positive neural progenitor cells in the subventricular zone of unchallenged mice [83]. Further investigations need to determine the mechanism for C3a generation in an unchallenged neurogenic niche and the mechanism for C3a generation in the post-acute and chronic phase after stroke.

3.3.2 C3a and Post-stroke Synaptic Plasticity

Although the specific mechanism of complement activation in normal CNS is currently unknown, complement has a clear role in regulating the number of synapses in the developing brain. Transforming growth factor β secreted by immature astrocytes triggers the neuronal expression of complement component C1q in the developing brain [99, 100]. C1q targets an as yet unknown synapse-associated target and the synapse is tagged for elimination through the activation of the classical complement cascade, deposition of C3b and its subsequent recognition by microglial CR3 [30, 99]. Excessive classical complement pathway activity and synapse elimination have been implicated in the development of schizophrenia [101], and the C3b-CR3-mediated elimination of synapses seems to be re-activated in neurodegenerative diseases such as glaucoma [102] and Alzheimer's disease [103]. It is noteworthy that

in the hippocampus of C3-deficient mice, the excessive number of glutamatergic synapses (due to the impaired developmental synapse elimination) is compensated by their reduced release probability, which is conceivably the reason for the absence of any signs of spontaneous epileptiform activity in these mice [104]. Notably, the C3-deficient mice exhibit better hippocampus-dependent learning and memory functions [104], and are protected from age-related region-specific loss of neurons and synapses in the hippocampus, and age-related cognitive decline [105]. Similarly, adult C3-deficient mice showed faster recovery of motor functions lost after sciatic nerve transection and were protected from axotomy-induced inhibitory synapse removal [106].

Stroke leads to neuronal death, gliosis, and axonal degeneration that occur also in non-ischemic remote brain regions that have synaptic connections with the primary lesion site [107]. This secondary degeneration has been linked to neurological deficits such as depression and cognitive impairment [108, 109], and can predict motor outcome after stroke [107] but the underlying molecular mechanism are only poorly understood. Given that synaptic dysfunction and loss may be the initial step in neurodegeneration [110, 111], studies on the involvement of C3b-CR3 signaling in the elimination of synapses in the regions affected by secondary degeneration in the post-stroke brain are warranted.

While the C3b-CR3 interaction constitutes a major mechanism for synapse removal, neuronal C3aR signaling promotes α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor (AMPAR) membrane localization to increase synaptic strength, and treatment with a C3aR antagonist or C3aR deficiency in neurons reduced dendritic complexity [112]. Excessive activation of neuronal C3aR can, on the other hand, negatively alter dendritic morphology and synaptic function [112]. A recent study investigating the role of C3a-C3aR signaling in structural synaptic responses to cerebral ischemia demonstrated that C3a-C3aR signaling increases the density and size of glutamatergic pre-synaptic terminals, and presumably synapses, in the peri-infarct region as well as in the contralesional hemisphere (Fig. 26.3), but does not affect any of these parameters in an unchallenged brain, and this C3a-C3aR-mediated response to ischemia is cortical region and layer specific [113]. Daily intranasal treatment with C3a starting 7 days after ischemia induction led to robust increase in synaptic responses and was associated with faster and sustained functional recovery [113], Fig. 26.4. Intranasal administration enables rapid and non-invasive delivery of peptides to the brain. The transport of peptides occurs mainly via peri-vascular bulk flow along the olfactory and trigeminal nerves and thus permits peptides to bypass the periphery and the blood-brain barrier, reaching the brain and entering the cerebrospinal fluid within minutes [114]. The findings of the plasticity and recovery promoting effects of C3a given intranasally are particularly intriguing not only because they show that C3a can be delivered to the brain through this clinically feasible route but also that such treatment, conceivably in combination with relevant rehabilitative interventions, could provide therapeutic benefit to stroke survivors in the post-acute or even chronic phase.

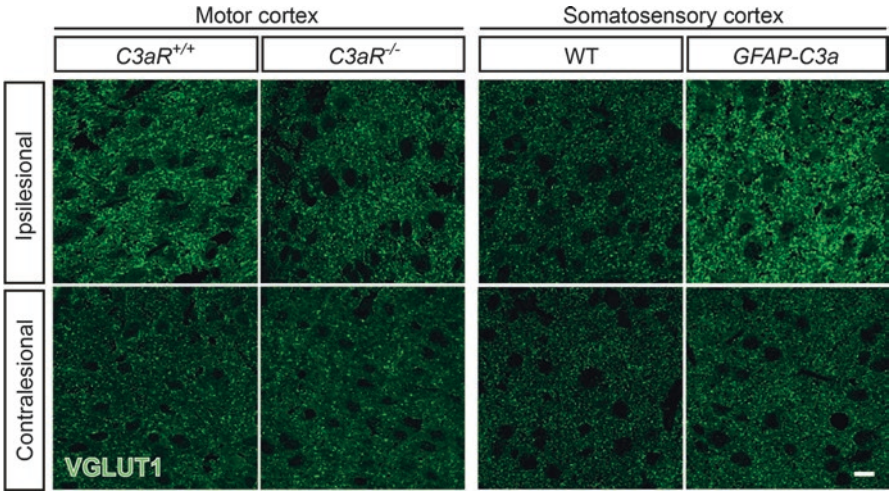

Fig. 26.3 After ischemic stroke, C3a-C3aR signaling positively regulates the density of excitatory synapses in the peri-infarct region. The expression of vesicular glutamate transporter 1 (VGLUT1), a marker of excitatory synapses, was reduced in the peri-infarct motor and somatosensory cortex in mice lacking C3aR (*C3aR*$^{-/-}$) and increased in mice over-expressing of C3a in reactive astrocytes (*GFAP-C3a*) compared with their respective controls (*C3aR*$^{+/+}$, WT). Scale bar 10 μm. Reproduced with permission from [113]

3.3.3 C3a and Post-stroke Axonal Plasticity

Axonal plasticity is a hallmark of CNS regeneration that is associated with reactivation of the intrinsic neuronal growth program and upregulation of the membrane phosphoprotein growth associated protein (GAP)-43 [115]. GAP-43 associates with axonal growth cones and is used as marker of axonal sprouting and plasticity [116, 117]. GAP-43 is also upregulated during reactive synaptogenesis [116, 118] and astrocyte-derived GAP-43 promotes neuronal survival and plasticity [119]. We have recently shown that after cerebral ischemia, the expression of GAP-43 in the peri-infarct regions is reduced in the absence of C3aR and increased when C3a is expressed in reactive astrocytes or administered intranasally [113], Fig. 26.5. As ischemia leads to upregulation of C3 in sprouting neurons [120] and C3a promotes neurite outgrowth *in vitro* [69], these findings support the conclusion that C3a signaling through C3aR plays a positive role in post-stroke axonal plasticity, possibly including axonal sprouting.

4 Concluding Remarks

Whereas complement activation and C3a in particular can contribute to tissue injury in the acute phase after cerebral ischemia, there is a mounting evidence to support the role of C3a-C3aR signaling in tissue repair and recovery by stimulating

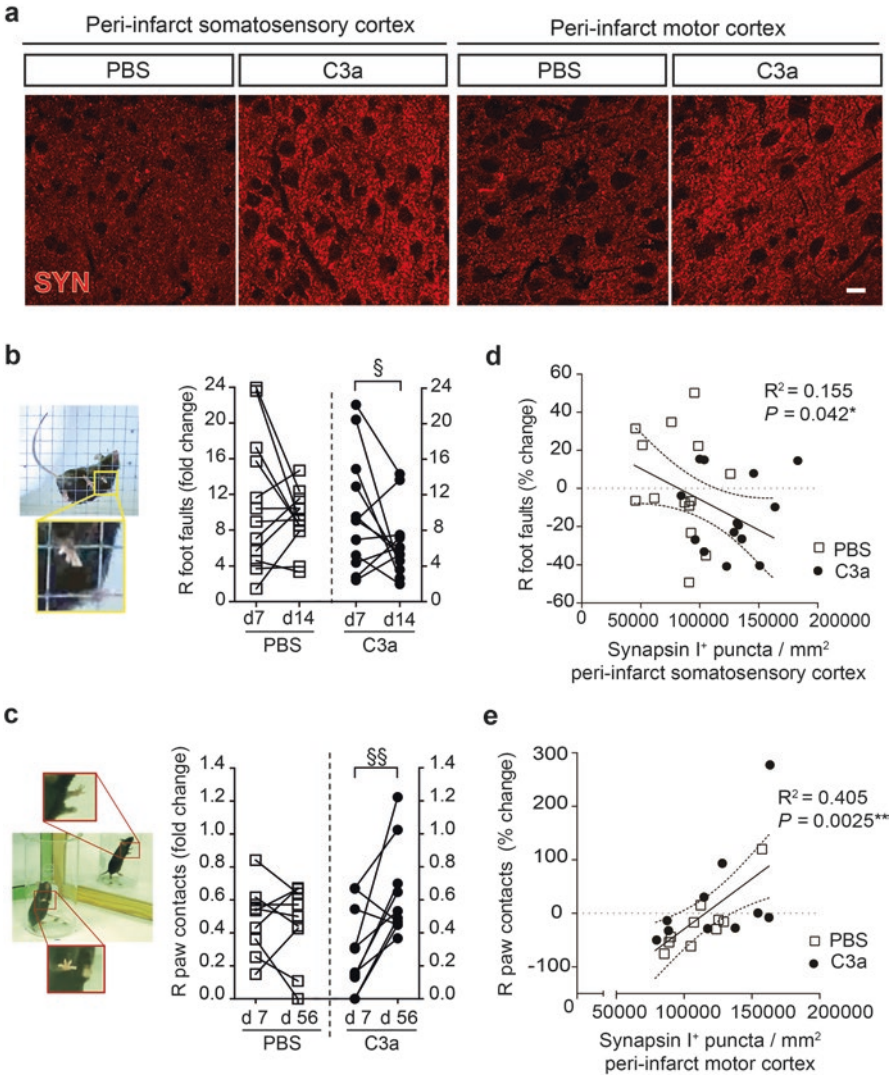

Fig. 26.4 Intranasal treatment with C3a increases pre-synaptic terminal density in the cortex and stimulates recovery of forepaw function after ischemic stroke. (**a**) Representative images of peri-infarct somatosensory and motor cortex stained with antibody against a pan-synaptic marker synapsin I on day 21 after stroke (scale bar: 10 μm). (**b**) *Left*: A typical foot fault during grid walking task; *Right*: Change in *right* (R, affected) paw foot faults in the grid walking task of individual mice between days 7 and 14 after stroke. (**c**) *Left*: An example of behavior scored in the cylinder test. *Right*: change in the performance of individual mice between days 7 and 56 post-stroke. (**d**, **e**) Scatter plots and linear regression fit of association between the density of synapsin I$^+$ puncta and change in performance between days 7 and 21 post-stroke in (**c**) grid walking test and (**e**) cylinder test. Intranasal treatment was performed between day 7 and 28 after stroke. §$P < 0.05$; §§$P < 0.01$. Reproduced with permission from [113]

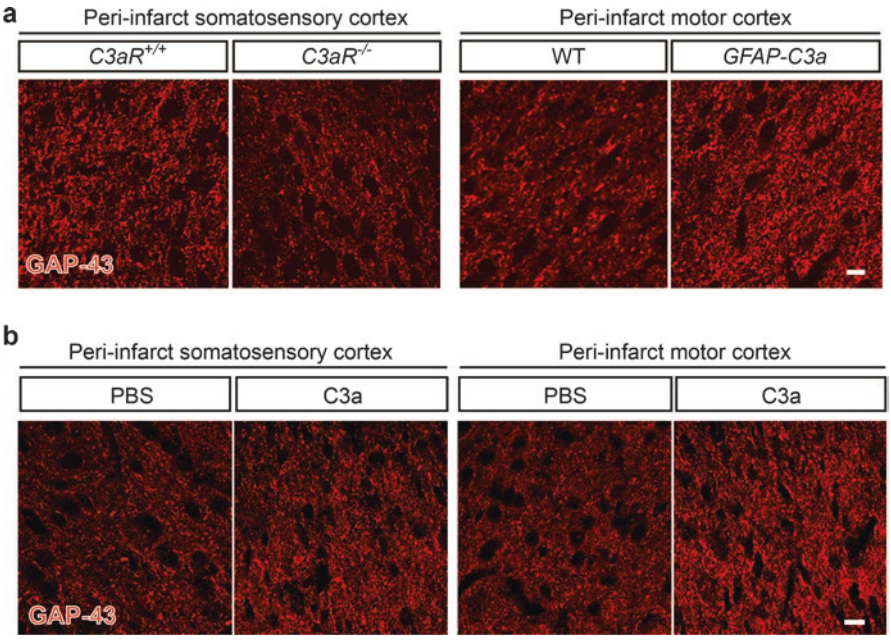

Fig. 26.5 C3a-C3aR signaling positively regulates peri-infarct axonal density after ischemic stroke. The expression of growth-associated protein 43 (GAP-43), a marker of axonal plasticity, in the peri-infarct cortex was reduced in mice lacking C3aR (*C3aR*$^{-/-}$), and increased in mice over-expressing of C3a in reactive astrocytes (*GFAP-C3a*) (**a**) as well as mice treated with intranasal C3a (**b**) compared to their respective controls (*C3aR*$^{+/+}$, WT, and PBS-treated mice). Scale bar 10 μm. Reproduced with permission from [113]

post-stroke neural plasticity including cell replacement, reorganization of axonal circuitry, and consequently, regulation of synaptic input. In light of the role of neuronal C3aR in modulation of synaptic strength and dendritic morphology [112], and the effects of C3a on neural progenitor cell differentiation and migration [69], the C3a-C3aR-mediated increase in peri-infarct neurogenesis, upregulation of expression of GAP-43 and increased number of pre-synaptic terminals, particularly glutamatergic terminals [113], are conceivably due at least in part to a direct effect of C3a on neurons. However, given the broad expression of C3aR in the brain, C3a can also exert its effects on post-stroke plasticity indirectly by modulating the functions of glial, endothelial, immune cells, stem/progenitor cells, and epithelial cells in the choroid plexus. Although the elucidation of the underlying cellular and molecular mechanism warrants additional experimental studies, the available data point to intranasal delivery of C3aR agonists in the post-acute phase as an attractive approach to improve functional recovery after ischemic stroke. While this non-invasive and directed route of administration presents clear advantages over systemic modes of delivery, further preclinical studies will need to determine the optimal therapeutic window and dose as well as the potential benefits of this type of treatment for other types of brain injuries.

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