Advances in Anatomy, Embryology and Cell Biology

Michael J. Schmeisser
Tobias M. Boeckers *Editors*

Translational Anatomy and Cell Biology of Autism Spectrum Disorder

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Preface

Autism spectrum disorder (ASD) is a complex and heterogeneous neurodevelopmental condition clinically defined by a core symptomatology including an impairment of social interaction and communication and the presence of restricted, repetitive behaviours. In addition, individuals with ASD can suffer from a variety of comorbidities ranging from intellectual disability to epilepsy and gastrointestinal dysfunction.

With a prevalence of approximately 1%, and only few therapeutic options thus far, ASD has become an increasing burden for our society. Importantly, research on ASD—ongoing since its first description in the literature in the 1940s—has made major advances throughout the last decade with the discovery of defined genetic mutations in affected individuals. Considering the fact that these mutations can be studied in model systems including cell culture and genetically modified animals, first hypotheses about the cellular and molecular underpinnings of ASD have been emerging. As many of the so-called ASD genes encode proteins essentially involved in the development of neural circuits and excitatory synapses and the observation that corresponding model systems often show neural circuit and synaptic dysfunction, it can be stated that ASD might derive from an aberrant development of brain circuitry. However, we are still at the very beginning of understanding the nature of ASD, and focused research is needed in the years to come to uncover underlying mechanisms and translate these findings from bench to bedside to further develop effective treatments.

This special volume intends to provide a state-of-the-art insight into translational ASD research with a special focus on anatomy and cell biology. International experts from the field including several members of the EU-AIMS programme launched by the Innovative Medicines Initiative of the European Union to develop new medications for ASD have contributed chapters on all aspects of translational ASD research. These include reviews on human genetics, human anatomy, stem cell-derived cellular models and a whole variety of animal models. Overall, their primary objective is to clarify how the identification of anatomical and cell

biological phenotypes of ASD in both humans and the appropriate model systems will help to translate basic mechanisms to clinical practice and efficiently treat affected individuals in the future.

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Chapter 1
Anatomy and Cell Biology of Autism Spectrum Disorder: Lessons from Human Genetics

Kristel T.E. Kleijer, Guillaume Huguet, Julie Tastet, Thomas Bourgeron, and J.P.H. Burbach

1.1 Introduction

Autism spectrum disorder (ASD) describes a group of neurodevelopmental conditions clinically characterised by impaired social interaction and communication and the presence of restricted interests and stereotyped and repetitive behaviours (Kanner 1943; Asperger 1944; Coleman and Gillberg 2012). Epidemiological studies estimate that more than 1% of the general population could receive a diagnosis of ASD (Elsabbagh et al. 2012; Developmental Disabilities Monitoring

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Network Surveillance Year Principal 2014). Individuals with ASD can also suffer from comorbidities including intellectual disability (ID), developmental delay (DD), epilepsy, motor control difficulties, attention deficit hyperactivity disorder (ADHD), tics, anxiety, sleep disorders, depression, dysmorphic features or gastrointestinal problems (Gillberg 2010; Moreno-De-Luca et al. 2013). The term ESSENCE for 'Early Symptomatic Syndromes Eliciting Neurodevelopmental Clinical Examinations' was coined by Christopher Gillberg to take into account this clinical heterogeneity and syndrome overlap (Gillberg 2010). There are 4–8 times more males diagnosed with ASD than females (Elsabbagh et al. 2012), but the sex ratio is more balanced in ASD patients with ID and/or dysmorphic features (Miles et al. 2005).

The causes of ASD remain largely unknown, but twin studies have constantly shown a high genetic contribution. Molecular genetic studies have identified more than 100 ASD risk genes carrying rare and penetrant deleterious mutations in approximately 10–25% of affected individuals (Huguet et al. 2013), and many more genes are suspected risk genes. However, the genetic landscape of ASD is shaped by a complex interplay between common and rare variants and is most likely different from one individual to another (Gardener et al. 2011). Remarkably, the susceptibility genes seem to converge on a limited number of biological pathways including protein synthesis, signal transduction, transcription/chromatin remodelling and synaptic function (Bourgeron 2009; Toro et al. 2010; Huguet et al. 2013). Here, we address advances of molecular ASD genetics and the lessons to be learned from these. We thereby focus on the convergence of expression patterns and biological pathways of specific ASD genes, thus providing a framework for the underlying pathogenesis.

1.2 From Chromosomal Rearrangements to Copy Number Variants in ASD

Studies on twins and population cohorts have provided crucial information about the high heritability of ASD in the last 40 years. They have not informed us about the identity, number and frequency of genes underlying ASD though. The first genetic studies that associated genetic variants to autism used observations from cytogenetic analyses (Gillberg and Wahlstrom 1985). However, because of the low resolution of the karyotypes (>Mb), it was almost impossible to associate a specific gene to ASD using this approach. Moreover, the prevalence of large chromosomal abnormalities is estimated to be <2% (Vorstman et al. 2006). Thanks to progress in molecular technologies such as comparative genomic hybridisation (CGH) or single-nucleotide polymorphism (SNP) arrays, the resolution in the detection of genomic imbalances has dramatically increased. Depending on the platforms, copy number variants (CNVs) of more than 50 kb are now robustly detected (Pinto et al. 2011). Several studies using the Simons Simplex Collection (SSC), a large

collection of DNA samples of individuals with ASD and their unaffected parents and siblings (https://sfari.org/resources/autism-cohorts/simons-simplex-collection) could provide an estimation of the frequency of the de novo CNVs in patients with ASD compared with their unaffected siblings (Sanders et al. 2011). All together, de novo CNVs are present in 4–7% of the patients with ASD compared with 1–2% in the unaffected siblings and controls (Glessner et al. 2009; Sanders et al. 2011; Pinto et al. 2014). Beyond ASD, large CNVs (>400 kb) affecting exons are present in 15% of patients with DD or ID (Cooper et al. 2011). Most of the de novo CNVs are private to each individual, but some are recurrently observed in independent patients. For example, three loci on chromosomal regions 7q11, 15q11.2-13.3 and 16p11.2 have been strongly associated with ASD (Ballif et al. 2007; Kumar et al. 2008; Weiss et al. 2008; Szafranski et al. 2010; Sanders et al. 2011; Leblond et al. 2014). The SSC also indicates that de novo CNVs identified in patients are most likely affecting genes, and particularly genes associated with synaptic functions and/or regulated by FMRP, the protein responsible for the Fragile X Syndrome (FXS) (Pinto et al. 2010, 2014).

In summary, 5–10% of individuals carry large chromosomal rearrangements and CNVs representing a risk of having ASD (Vorstman et al. 2006; Pinto et al. 2010, 2014). In order to progress in the identification of the ASD risk genes, (candidate) gene association studies and whole exome/genome studies have been performed.

1.3 From Candidate Genes to Whole Exome and Genome Sequencing Studies in ASD

Statistical association of a gene with ASDs has been employed in this field since 2000. Initially specific candidate genes were selected on the basis of biological, functional or genetic data or a combination of both. This approach of association studies was successful in identifying several synaptic genes (Jamain et al. 2003; Autism Genome Project et al. 2007; Durand et al. 2007). These studies moved rapidly to genome-wide association studies (GWAS) using SNPs which proved to be of limited gains in ASD genetics (Anney et al. 2010, 2012). Thanks to the advance in next-generation sequencing (NGS), we can now interrogate all genes of the genome in an unbiased manner using whole exome or genome sequencing (WES, WGS).

To date, more than 18 WES studies of sporadic cases of ASD (O'Roak et al. 2011, 2012; Chahrour et al. 2012; Iossifov et al. 2012; Neale et al. 2012; Sanders et al. 2012; He et al. 2013; Lim et al. 2013; Liu et al. 2013, 2014; Willsey et al. 2013; Yu et al. 2013; An et al. 2014; De Rubeis et al. 2014; Iossifov et al. 2014; Samocha et al. 2014; Chang et al. 2015; Krumm et al. 2015) have been performed, comprising altogether more than 4000 families (Table 1.1). In almost all these studies, the focus was on the contribution of de novo single-nucleotide variants (SNVs). All together, the average number of de novo coding SNVs per individual

Table 1.1 Summary of the main whole exome/genome sequencing (WES/WGS) studies in ASD

Studies	WES or WGS	#ASD analysed in the study	#ASD specific to this study	#ASD from other studies	#Controls	#Unaffected sibling	#Parents	Analysis of de novo variants	Analysis of inherited variants
O'Roak et al. (2011)	WES	20	20	–	–	20	38	X	–
O'Roak et al. (2012)	WES	229	209	20	–	50	418	X	–
Neale et al. (2012)	WES	175	175	–	–	–	350	X	–
Sanders et al. (2012)	WES	238	238	–	–	200	476	X	–
Iossifov et al. (2012)	WES	343	343	–	–	343	686	X	X
Chahrour et al. (2012)	WES	16	16	–	–	–	–	–	X
Yu et al. (2013)	WES	401	163	238	–	114	326	X	X
Lim et al. (2013)	WES	1496	1004	492	5474	–	–	–	X
Liu et al. (2013)	WES	1039	–	1039	869	–	–	–	X
He et al. (2013)	WES	1867	–	1867	870	593	1870	X	X
Willsey et al. (2013)	WES	1099	56	1043	–	56	112	X	X
Liu et al. (2014)	WES	1967	–	1967	870	593	2070	X	X
Samocha et al. (2014)	WES	1078	–	1078	–	343	2156	X	–

Iossifov et al. (2014)	WES	2517	1576	932	–	1911	5016	X	–
An et al. (2014)	WES	40	40	–	–	8	80	X	X
De Rubeis et al. (2014)	WES	2270	–	2270	5397	–	4540	X	X
Chang et al. (2015)	WES	932	–	932	–	593	1580	X	X
Krumm et al. (2015)	WES	2377	–	2377	–	1786	4754	X	X
Kong et al. (2012)	WGS	40	44	–	–	7	136	X	–
Michaelson et al. (2012)	WGS	20	20	–	–	–	–	X	–
Shi et al. (2013)	WGS	1	1	–	–	6	2	X	X
Jiang et al. (2013)	WGS	32	32	–	–	–	64	X	X
Yuen et al. (2015)	WGS	85	85	32	–	–	170	X	X
Nemirovsky et al. (2015)	WGS	1		–	–	–	–	–	X
Turner et al. (2016)	WGS	53	13	40	3	40	106	X	X

refers to ‘number of’ and seems significant to the authors

(including missense, splicing, frameshift and stop-gain variants) is estimated to be approximately 0.86 in female patients, 0.73 in male patients and 0.60 in unaffected male and female siblings (Krumm et al. 2014; Ronemus et al. 2014). Based on these studies (Iossifov et al. 2012; Neale et al. 2012; O'Roak et al. 2012; Sanders et al. 2012), 3.6–8.8% of the patients were shown to carry a de novo causative mutation (Iossifov et al. 2012) with a twofold increase of deleterious mutations in the patients compared with their unaffected siblings. Following these WES studies, targeted re-sequencing studies of the most compelling candidate genes were performed, and ten genes carrying de novo mutations were significantly associated with ASD (Table 1.1) (O'Roak et al. 2012).

Only few studies have analysed the contribution of inherited SNVs in ASD. In 2013, Lim et al. (2013) have analysed WES of 933 ASD cases and 869 controls for the presence of rare complete human knockouts (KO) with homozygous or compound heterozygous loss-of-function (LoF) variants ($\leq$5% frequency). They observed a significant twofold increase in complete knockouts in patients with ASD compared with controls. They have estimated that such complete KO mutations could account for 3% of the patients with ASD. For the X chromosome, there was a significant 1.5-fold increase in complete KO in affected males compared to unaffected males that could account for 2% of males with ASD (Lim et al. 2013). A couple of inherited biallelic mutations were identified by two large studies (Table 1.1) (Yu et al. 2013; Krumm et al. 2015).

To date, few whole genome sequencing (WGS) studies have been published in the field of ASD (Table 1.1). Michaelson et al. (2012) analysed 40 WGS of monozygotic twins concordant for ASD and their parents. They proposed that ASD risk genes could be hot spots of mutation in the genome and confirmed the associations between ASD and de novo mutations (Table 1.1). Shi et al. (2013) have analysed a large pedigree with two sons affected with ASD and six unaffected siblings, focusing on inherited mutations. They identified *ANK3* as the most likely candidate gene. In 2015, Yuen et al. (2015) analysed 85 families with two children affected with ASD. They identified 46 ASD-relevant mutations present in 36 of 85 (42.4%) families. Only 16 ASD-relevant mutations of 46 (35%) identified were de novo. Very interestingly, for more than half of the families (69.4%, 25 out of 36), the two affected siblings did not share the same rare penetrant ASD risk variant(s). Recently, Turner et al. (2016) performed WGS on 208 genomes of 53 families with simplex ASD and showed a significant enrichment of de novo and private disruptive mutation in putative regulatory non-coding DNA in probands. These mutations included elements of genes that had been pinpointed before by disruption of coding regions in ASD.

1.4 Rare and Common Variants in ASD

In the general population, one individual carries on average three million genetic variants in comparison to the reference human genome sequence. The vast majority of the variants (>95%) are the so-called common variants shared with more than

5% of the human population (Xue et al. 2012; Fu et al. 2013; Genome of the Netherlands Consortium 2014). Based on the results obtained from epidemiological and molecular studies, it is now accepted that the genetic susceptibility to ASD can be different from one individual to another with a combination of rare deleterious variants and a myriad of low-risk alleles (also defined as the genetic background). Most of the inherited part of ASD seems to be due to common variants observed in the general population with only a small contribution from rare variants (Fig. 1.1).

The interplay between rare or de novo variants and the background will also influence the phenotypic diversity observed in the patients carrying rare deleterious mutations. In some individuals, a genetic background will be able to buffer or compensate the impact of the rare genetic variations. In contrast, in some individuals, the buffering capacity of the background will not be sufficient to compensate the impact of the rare variants, and they will develop ASD (Rutherford 2000; Hartman et al. 2001).

The presence of multiple hits of rare CNVs, SNVs or indels (nucleotide insertions and/or deletions) in a single individual also illustrates the complexity of the genetic landscape of ASD (Vorstman et al. 2010; Girirajan et al. 2010, 2012; Leblond et al. 2012). In addition, the analysis of the WGS of multiplex families

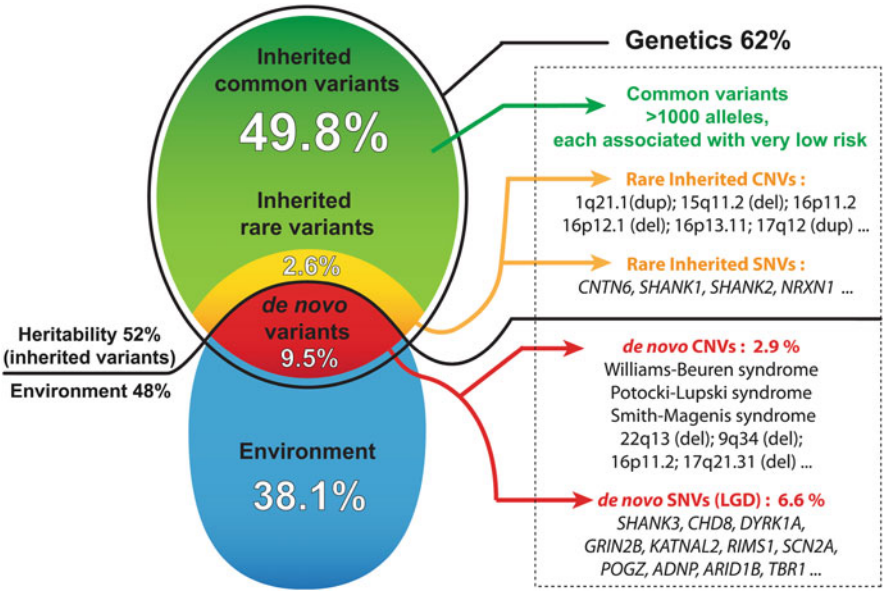

Fig. 1.1 Relative contribution of genetics and environment in ASD. Based on twin and familial studies, it is estimated that the genetic and environmental contributions to ASD are approximately 50–50%. Most of the inheritable part seems to be due to common variants observed in the general population with a small contribution to rare variants. Importantly, the de novo mutations are genetic causes of ASD, but do not contribute to the heritability since they are only present in the affected individual. These de novo events are therefore considered as 'environmental causes' of ASD, but act on the DNA molecule

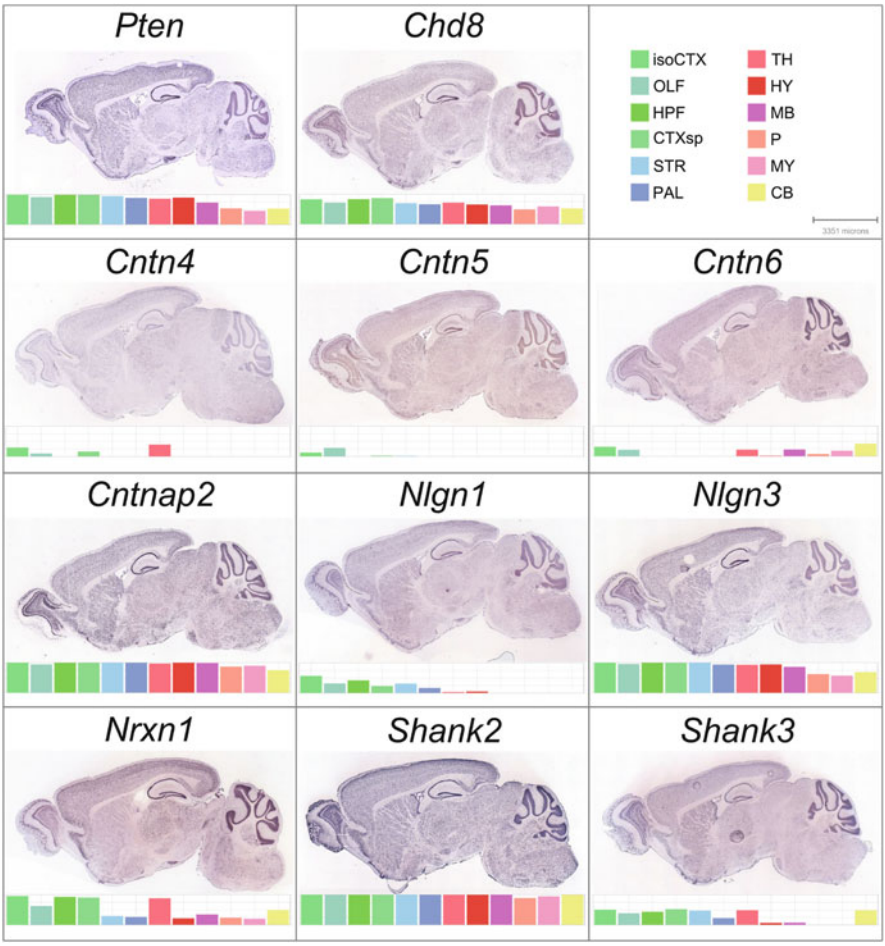

Fig. 1.2 Expression pattern of ASD genes in adult mouse brain. In situ hybridisation images of sagittal sections of the adult mouse brain (provided by the Allen Brain Atlas http://www.brain-map.org) show the expression pattern of a selection of ASD genes. Genes encoding key regulators of chromatin remodelling and gene transcription, such as *Chd8*, and genes involved in activity-driven regulation of synaptic proteins, such as *Pten*, are expressed widely throughout the adult mouse brain. Between and within functional categories of ASD-related genes, expression patterns vary in level and region specificity. For instance, cell adhesion molecules *Cntnap2* and *Nlgn3* are highly expressed throughout the adult mouse brain, whereas *Cntn4*, *Cntn5*, *Cntn6*, *Nlgn1* and *Nrxn1* show a more specified but individually different expression pattern varying in level. Even within gene families, such as in the scaffold protein Shank family, *Shank2* is expressed a high level throughout the entire brain, whereas *Shank3* expression is lower and more confined to specific brain areas in adulthood. *isoCTX* isocortex, *OLF* olfactory areas, *HPF* hippocampal formation, *CTXsp* cortex subplate, *STR* striatum, *PAL* pallidum, *TH* thalamus, *HY* hypothalamus, *MB* midbrain, *P* pons, *MY* medulla, *CB* cerebellum

also indicates that clinically relevant mutations can be different from one affected sibling to another even in a single family (Yuen et al. 2015). It is therefore still difficult to ascertain robust genotype–phenotype relationships based on our current knowledge.

1.5 Anatomy of ASD Genes

Since the brain is a highly organised organ with many distinct regions that exert specific functions, genetic mutations may have diverse effects depending on the expression pattern in the brain. Considering the anatomy of ASD and expression of ASD risk genes may provide an additional view on genotype–phenotype relationships.

Imaging studies have evaluated volume, connectivity and activation patterns of brain areas in human ASD patients. The frontal lobes, amygdala and cerebellum have most frequently been suggested as core regions. However, no consistency has been presented and a large heterogeneity dominates the field (Amaral et al. 2008). Magnetic resonance imaging (MRI) studies in mouse models found abnormalities in parieto-temporal lobe, cerebellar cortex, frontal lobe, hypothalamus and striatum. In addition, both over- and under-connectivity have been observed. However, similarly to human individuals, it has to be concluded that also in mouse models of ASD there is no converging neuroanatomical pattern (Ellegood et al. 2015). As described above, mutations in many genes have been strongly associated with ASD. Disruption of the functionality of a certain protein would mainly affect the area in which the gene is expressed and/or the areas its expressing neurons project to. Interestingly, when looking at the expression of a diverse set of ASD-related genes in the rodent (Fig. 1.2) and human brain (Fig. 1.3), it appears that the patterns vary widely between genes though. For example, the expression of *NLGN1*, *NLGN3*, *NLGN4X* and *SHANK3* in the human hippocampus is relatively high, while that of *FMR1*, *CHD8*, *CNTN4* and *CNTN5* is low (Fig. 1.3). Like the imaging studies, the overall spatial expression patterns of ASD-related genes do not point in a single direction. Interestingly, many ASD risk genes are expressed during embryonic and early postnatal development, when important processes occur, such as neuritogenesis and synaptogenesis, respectively (Table 1.2). Furthermore, expression of many ASD risk genes is found in glutamatergic neurons. However, some ASD genes such as *Chd8* and *Pten* are expressed in various cell types (Table 1.2). It should be kept in mind that the majority of cells in the brain are glutamatergic neurons and that, therefore, the chance a gene is expressed in glutamatergic neurons is larger than in other cell types.

In conclusion, human and rodent imaging data and the expression patterns of ASD genes are not sufficient to define the neuroanatomical pathology of ASD. The expression patterns of ASD genes show a large heterogeneity—similar to the genetics, symptomatology and comorbidities of ASD. Furthermore, ASD cannot

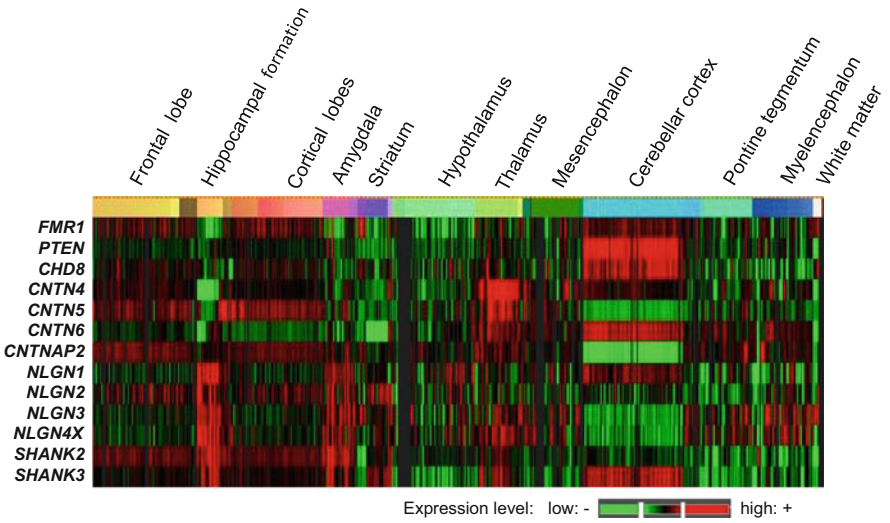

Fig. 1.3 Expression profile of ASD genes in the human brain. The plot is based on mRNA levels determined by microarrays on samples of dissected human brain regions (Hawrylycz et al. 2012) and provided by the Allen Brain Atlas (http://human.brain-map.org/microarray). The data compare expression levels and distribution of mRNAs of a number of selected ASD genes plotted as z-value scores (−, low, −1; +, high, +1)

be pinpointed to a limited number brain structures and may comprise the interplay and integration of many regions, if not the brain in a global sense.

1.6 From Genetics to Pathways Associated with ASD

While the genotype–phenotype relation remains uncertain, the main lesson is simple; when defective, a large number of genes cause or increase the risk for ASD symptoms. Therefore, this relation directly points towards pathways in the pathogenesis of ASD. Although the genes associated with ASD are numerous and show distinct spatiotemporal expression patterns, many of them function in related processes. The genetic dissection of ASD has indicated molecular pathways (function of proteins), cellular pathways (processes at the cellular level) and biological pathways (integrated functions). The latter have been indicated in various studies using unbiased pathway analysis tools. These studies have indicated that ASD risk genes are enriched in groups of proteins with specific functions (Voineagu et al. 2011; De Rubeis et al. 2014; Ronemus et al. 2014; Uddin et al. 2014; Hormozdiari et al. 2015). Below we highlight a number of seminal studies in this direction and summarise the present insights.

Pinto et al. (2014) have analysed the burden of CNVs in 2446 individuals with ASD and 2640 controls and found enrichment in genes coding postsynaptic density

Table 1.2 Spatiotemporal expression of ASD risk genes in the rodent brain

Gene name	Temporal expression			Spatial expression (at temporal peak)				Expression amongst cell types				References
	E10-19	*P0-21*	*> P56*	*CTX*	*HPF*	*STR*	*TH*	*CB*	*Glu*	*GABA*	*Glia*	
Fmr1	**Peak**	Yes	Yes	+	++	–	+/–	++	Yes	Yes	Yes	Hinds et al. (1993) Hanzlik et al. (1993) Olmos-Serrano et al. (2010)
Pten	Yes	Yes	Yes	+	+	+	+	+	Yes	Yes (p)	Yes	Kleijer et al. (2014)
Chd8	**Peak**	Yes	Yes	+	+	+	?	?	Yes	Yes	Yes	Bernier et al. (2014)
Cntn4	Yes	Yes (peak in OSN)	**Peak**	+	+	–	++	++	Yes	No	No	Felsenfeld et al. (1994) Yoshihara et al. (1995) Kaneko-Goto et al. (2008) He et al. (2009) Shimoda and Watanabe (2009a, b) Bouyain and Watkins (2010) Liu et al. (2011) Zuko et al. (2013)
Cntn5	Yes	**Peak**	Yes	+	+	++	++	+	Yes	No*	No	Ogawa et al. (2001) Li et al. (2003) Zuko et al. (2013) Kleijer et al. (2015)
Cntn6	Yes	**Peak**	Yes (peak in CB)	++	+	–	++	+	Yes	No	No	Ogawa et al. (1996) Lee et al. (2000) Cui et al. (2004) Ye et al. (2008) Sakurai et al. (2009)
Cntnap2	Yes	Yes	**Peak**	+	+	+/–	–	–	Yes	No	Yes	Poliak et al. (1999)
Nrxn1	Yes	Yes	Yes	+	+	+	+	+	Yes	No	No	Püschel and Betz (1995) Bang and Owczarek (2013) Proctor et al. (2015)

(continued)

Table 1.2 (continued)

Gene name	Temporal expression			Spatial expression (at temporal peak)				Expression amongst cell types				References
	E10-19	*P0-21*	*> P56*	*CTX*	*HPF*	*STR*	*TH*	*CB*	*Glu*	*GABA*	*Glia*	
Nlgn1	Yes	**Peak**	Yes	+	+	++	++	+	Yes	No	No	Song et al. (1999) Scheiffele et al. (2000) Varoqueaux et al. (2006) Sudhof (2008) Kwon et al. (2012) Kleijer et al. (2014)
Nlgn3	Yes	**Peak**	Yes	+	++	+	++	+/−	Yes	Yes	Yes	Gilbert et al. (2001) Budreck and Scheiffele (2007) Levinson et al. (2010) Baudouin et al. (2012) Kleijer et al. (2014) Proctor et al. (2015)
Nlgn4	No	No	**Peak**	++	++	+	+	+/−	Yes	Yes	No	Jamain et al. (2008) Hoon et al. (2011) Kleijer et al. (2014)
Shank2	No	**Peak**	Yes	++	+	−	+	++	Yes	Yes (p)	No	Böckers et al. (2004) Kleijer et al. (2014)
Shank3	Yes	Yes	**Peak**	++	+	++	++	+/−	Yes	No	No	Böckers et al. (2004) Kleijer et al. (2014)

For selected ASD risk genes, the presence of expression and highest expression (peak) is given for three developmental time windows. Further, the distribution of expression amongst brain regions is indicated by estimated levels (−, no expression detected, to ++, high expression). Data were obtained from literature sources as indicated.

E embryonic day, *P* postnatal day, *CTX* cortex, *HPF* hippocampal formation, *STR* striatum, *TH* thalamus, *CB* cerebellum, *Glu* glutamatergic, *GABA* GABAergic, *OSN* olfactory sensory neurons, *(p)* Purkinje cells

*No evidence of Cntn5 expression in rodent GABAergic cells has been shown, but expression of FAR-2 in chick Purkinje cells has been reported (Plagge et al., 2001).

(PSD) proteins and FMRP targets, amongst others. Ronemus et al. (2014) reviewed the results of four WES studies and showed an enrichment of mutated genes in chromatin modifier genes ($p = 4 \times 10^{-6}$) and FMRP targets ($p = 7 \times 10^{-6}$). Protein-protein interaction (PPI) analyses of the genes carrying loss-of-function mutations also showed enrichment in proteins involved in neuronal development and axon guidance, signalling pathways and chromatin and transcription regulation. De Rubeis et al. (2014) have also used PPI networks and showed enrichment in clusters of proteins involved in cell junctions, TGF-beta pathway, cell communication and synaptic transmission, neurodegeneration and transcriptional regulation (De Rubeis et al. 2014).

In parallel to the genetic studies, several transcriptomic analyses were performed using *post-mortem* brain tissue of individuals with ASD (Voineagu et al. 2011; Gupta et al. 2014). Several genes were differentially expressed or correlated between brain regions. Two network modules have been identified. The first module was related to interneurons and to genes involved in synaptic function. This module was downregulated in brains from ASD patients as compared to controls. The second module was upregulated in ASD patients and related to immunity and microglia activation.

Overlooking these studies, the broadness of the spectrum of proteins with different functions is remarkable. However, many of these can be aggregated in a number of cellular functions (Table 1.3). ASD-associated genes include genes encoding RNA-binding proteins, translation initiation factors and other regulators of the protein synthesis machinery pointing out that protein synthesis is one cellular

Table 1.3 Molecular functions of proteins encoded by ASD risk genes reveal defined cellular processes that can be targeted by drugs in translational treatment studies

Molecular function of proteins encoded by ASD risk genes	• Regulators of protein synthesis • RNA-binding proteins • Translation initiation factors	• GAPs and GEFs • GTPases • Kinases • Phosphatases • Receptors	• Chromatin modifiers • Transcription factors	• Cell adhesion molecules • Channels • Cytoskeletal modulators • Receptors • Scaffolding proteins • Transporters
Example genes	*FMR1*	*PTEN*	*CHD8*	*SHANK3*
Cellular process	Protein synthesis	Signal transduction	Transcription/ chromatin remodelling	Synaptic function
Principle of targeting	Inhibitors of protein synthesis	Inhibitors of Akt-mTORC1 signalling	To be investigated	Modulators of mGluR function
Example drugs	Lovastatin, 4EGI-1	Rapamycin, everolimus	To be investigated	MPEP, CDPPB

Specific genes mentioned are representative, but mere examples
GAP GTPase-activating protein, *GEF* guanine nucleotide exchange factor, *mGluR* metabotropic glutamate receptor

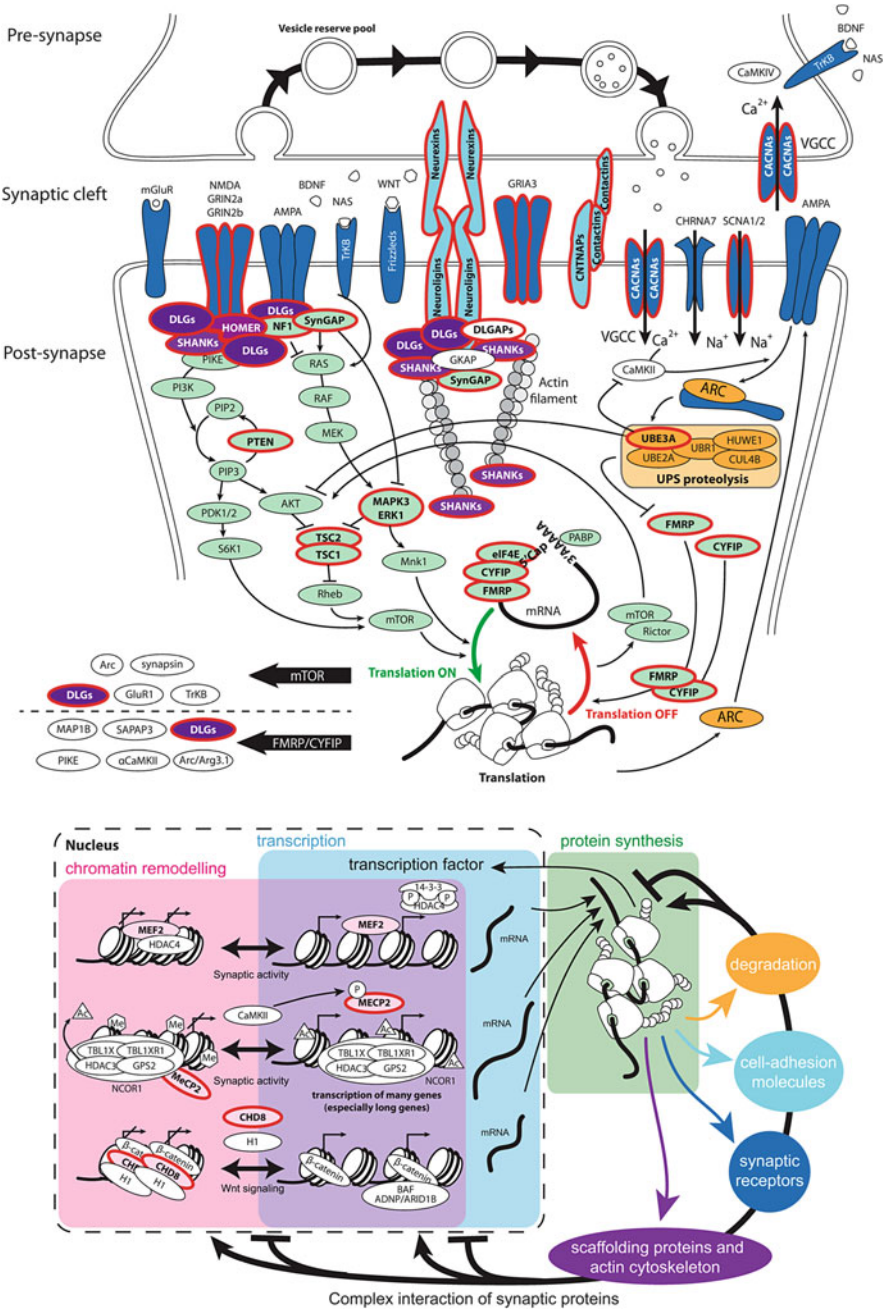

Fig. 1.4 Integration of ASD gene products in cellular pathways at the synapse. (**a**) Molecular integration. Proteins encoded by ASD risk genes (contour outlined) are positioned in molecular and cellular processes enrolling at the synapse. These proteins are found at the cell surface

process involved in ASD. Genes encoding receptors, GTPases, GTPase-activating proteins (GAPs), guanine nucleotide exchange factors (GEFs), kinases and phosphatases further indicate that signal transduction is involved. More recently, multiple genes encoding chromatin-binding and chromatin-modifying proteins or transcription factors have put transcription in focus of ASD pathogenesis. The largest group of ASD genes, however, comprises cell adhesion molecules (CAMs), scaffold proteins, cytoskeletal modulators, membrane receptors, transporters and ion channels pointing towards synaptic transmission as a central cellular process. Intriguingly, these cellular pathways are not independent from each other, but linked (Fig. 1.4). For example, transcription and translation control quantity and quality of the total pool of proteins in a cell. Signal transduction couples extracellular signals, for instance, neurotransmitter release, to intracellular responses like protein synthesis and actin dynamics. The cellular pathways affected by defective ASD genes are part of a number of biological processes that are essential for the proper functioning of the developing and mature nervous system. These include synapse, dendrite and spine dynamics, synaptic plasticity, axon guidance and neurite outgrowth. This has led to the hypothesis that abnormal synaptic plasticity and defects in neuronal/synaptic homeostasis could play a key role in the pathogenesis of ASD (Belmonte and Bourgeron 2006; Toro et al. 2010; Auerbach et al. 2011). For recent reviews, see Spooren et al. (2012), Delorme et al. (2013), Kleijer et al. (2014) and Bourgeron (2015).

Based on these results, neurobiological studies using in vitro and in vivo models have been performed in order to explore these biological processes in detail and consequently to test the therapeutic potentials of interventions by compounds.

1.7 Protein Synthesis and Synaptic Plasticity

From these data, protein synthesis, in particular the local dendritic protein synthesis (LDPS), is a common downstream process that may be core to ASD (Kleijer et al. 2014). This mechanism allows stimulus-dependent production of proteins on demand and is particularly important for an efficient and plastic synaptic function. LDPS and synaptic function are therefore tightly linked.

Fig. 1.4 (continued) (synaptic adhesion molecules, receptors, channels, transporters), at the postsynaptic protein anchoring sites (scaffolding proteins), in the signal transduction cascades triggered by synaptic activity (kinases, in the ubiquitin-proteasome pathway (ligases) and in the mRNA translation/protein synthesis machinery (initiation and elongation factors, RNA-binding proteins, kinases). These are linked functionally and can ultimately affect the rates of protein synthesis. (**b**) Cellular integration. Identity and levels of proteins are controlled by the interplay between gene expression (chromatin remodelling, transcription, translation) and degradation. This control of protein synthesis leads reversely to control of components that make up these processes

Protein synthesis in dendrites depends on extracellular signals such as neuronal activity triggered by mGluR5 activation (Greenough et al. 2001; Ma and Blenis 2009; Kao et al. 2010). Several genes involved in such activity-driven regulation of synaptic proteins have been found to be mutated in individuals with ASD (Kelleher and Bear 2008). For example, the mTOR pathway controls global mRNA translation, and its deregulation causes diseases associated with increased cell proliferation and loss of autophagy, including cancer (Ma and Blenis 2009), but also increases the risk for ASD. Remarkably, mutations in genes encoding repressors of the mTOR pathway such as NF1, PTEN, TSC1, TSC2 and SynGAP1 cause an increase of translation in neurons and at the synapse (Auerbach et al. 2011). It is known that a defective FMRP/EIF4E/CYFIP complex is the cause of FXS. Additionally, it significantly increases the risk of ASD (Budimirovic and Kaufmann 2011). This protein complex controls local translation of mRNA at the synapse and acts downstream of the Ras-ERK and mTOR signalling pathways. More than 1000 specific genes, many of which are ASD risk genes, are regulated by this complex (De Rubeis et al. 2013; Fernandez et al. 2013; Gkogkas et al. 2013; Santini et al. 2013). If this FMRP/EIF4E/CYFIP1 complex is disrupted, it creates an imbalance in the level of many synaptic proteins, which may lead to ASD. It is not surprising then that a proper regulation of LDPS is crucial for the formation, plasticity and maintenance of synaptic connections. For instance, regulation of mRNA translation by CYFIP1 is shown to be necessary for proper dendritic spine formation (De Rubeis et al. 2013). Moreover, brain-derived neurotrophic factor (BDNF) triggers transport of mRNA along dendrites and promotes their local translation. In this manner BDNF can enable both long-term potentiation (LTP) and long-term depression (LTD) (for a review, see Leal et al., 2014). Synaptic proteins, like CAMS, such as neuroligins (NLGNs) and neurexins (NRXNs), multiple cadherins (CDHs) and CAMs of the immunoglobulin superfamily (IgCAMs) as well as scaffold proteins, such as the Shanks, can impinge on signal transduction routes.

The Shank family of scaffold proteins is strongly associated with ASD. For example, deletions, duplications and coding mutations in all three *SHANK* genes (*SHANK1*, *SHANK2* and *SHANK3*) have been recurrently reported in individuals with ASD (Leblond et al. 2014). SHANK proteins assemble into large molecular platforms in interaction with glutamate receptors, CAMs and actin-associated proteins (Grabrucker et al. 2011). The consequences of mutations in the *SHANK* genes have been studied in vitro and in vivo. For example, *SHANK3* mutations identified in individuals with ASD reduce actin accumulation in spines affecting the development and morphology of dendrites and axonal growth cone motility in vitro (Durand et al. 2012). Genetic mouse models of the *Shanks* have provided further insights into their involvement in spine and synapse dynamics, and, interestingly, the mutants also show aberrant, autistic-like behaviours (Peca et al. 2011; Schmeisser et al. 2012; Jiang and Ehlers 2013; Schmeisser 2015; Wang et al. 2016).

One of the concrete functions of scaffold proteins, such as the Shanks, is the positioning of postsynaptic CAMs. NLGNs and NRXNs are an interacting set of pre- and postsynaptic CAMs, which are associated with ASD. Besides these, many

more CAMs have been related to ASD, i.e. CDHs and IgCAMs such as the Contactins (Fig. 1.4).

Most of these have not or only marginally been investigated for their engagement in ASD processes. However, new insights could be gained from further examination of their molecular and cellular functions. One example is provided by the Contactins, a subfamily of six related IgCAMs of which at least three have been associated with ASD: CNTN4, CNTN5 and CNTN6. The genetic evidence of their involvement in ASD is mostly based on CNVs found in ASD patients or a mental retardation syndrome with ASD as comorbidity (3p deletion syndrome) (Fernandez et al. 2008; Pinto et al. 2010; van Daalen et al. 2011; Guo et al. 2012). Variations in *CNTN4*, *CNTN5* and *CNTN6* have been found in multiplex and simplex families, but could not be related to ASD (Murdoch et al. 2015). These Contactins each display a distinct profile of interaction with other membrane proteins, which could engage them in signalling processes. These interacting proteins belong to protein tyrosine phosphatase receptors (PTPRs), Contactin-associated proteins (CNTNAPs), amyloid precursor proteins (APPs) and other related IgCAMs (Zuko et al. 2011, 2013; Shimoda and Watanabe 2009a, b; Mohebiany et al. 2014). Selective knockout of these Contactins results in subtle phenotypes that support roles in development and regulation of the nervous system (Oguro-Ando et al. 2017). Moreover, their brain expression is restricted and distinct. For instance, in the mouse brain, *Cntn4*, *Cntn5* and *Cntn6* are expressed in distinct but overlapping nuclei that are part of the thalamocortical system (Kleijer et al. 2015; Zuko et al. 2016), and they are expressed by various partly overlapping types of interneurons and pyramidal neurons of the mouse cerebral cortex (Oguro-Ando et al. 2017). The data on genetics and neurobiology of Contactins are suggestive for a role in the pathogenesis of ASD. However, direct links to the emerged cellular core processes in ASD, i.e. protein synthesis, signal transduction, transcription/chromatin remodelling and synapse function or a novel pathogenic mechanism, is largely lacking. This stage of knowledge is representative for many other CAMs and requires in-depth examination of molecular and cellular functions in which these proteins participate.

1.8 Conclusions

Our current thinking about the anatomy and cell biology of ASD has been guided strongly by the results of human genetic studies. In the last 10 years, we have been overwhelmed by the large number of genes associated with risk for ASD, extending the clinical notion that ASD includes very heterogeneous conditions ranging from severe intellectual disability to high-functioning forms. This heterogeneity in risk genes results in heterogeneity in affected biological processes and heterogeneity in affected brain systems underlying ASD symptoms.

Overviewing the genetics of ASD, the spectrum of functions of proteins encoded by ASD genes can be aggregated in a number of molecular and cellular functions,

which point towards essential cell biological processes (Fig. 1.4, Table 1.3). In this way protein synthesis, signal transduction, transcription/chromatin remodelling and synaptic function have emerged as major cellular processes from which ASD may originate. These cellular pathways are not independent from each other. For example, transcription, translation and degradation together control quality and quantity of the total pool of proteins of the cell. Signal transduction couples extracellular signals, for instance, neurotransmitters and growth factors, to intracellular responses like protein synthesis, degradation and transcription. These are all essential activity-dependent pathways that remain highly dynamic in adult stages (Ebert and Greenberg 2013). At an integrated level, these cellular pathways are apparent in biological functions relevant for ASD, in particular axon guidance, dendrite and spine morphology, synaptogenesis and synaptic plasticity. This has led to the hypothesis that abnormal synaptic homeostasis could play a key role in the pathogenesis of ASD.

The outstanding challenge is to exploit the current insights in genetics, anatomy and cell biology of ASD for the design of rational therapies. These insights have gained another dimension due to the notion that neurodevelopmental defects are not necessarily permanent, but reversible. There has been a long-standing view that neurodevelopmental disorders are congenital inborn errors of brain development that leave the patient with irreversible defects. This traditional view was first challenged by the group of Adrian Bird who reactivated a silenced *Mecp2* allele in mice modelling Rett syndrome (Guy et al. 2007). In adult mutants, induction of Mecp2 expression dramatically reversed behavioural and electrophysiological abnormalities. Selective reversal of abnormalities was also observed in other ASD models. For example, phenotypes in *Tsc1* knockout mice could be reversed by the mTORC1 inhibitor rapamycin, and in *Fmr1* knockout mice, a model for FXS, by treatment with mGluR antagonists (Ehninger and Silva 2010). Moreover, insulin-like growth factor I (Igf1) has been successfully used to ameliorate autistic-like phenotypes in mouse models of Rett syndrome and Phelan-McDermid syndrome (PMDS). SHANK3 is the prime culprit causing PMDS. Interestingly, selective rescue of autistic-like phenotypes was recently established by re-expression of Shank3 (Mei et al. 2016). These findings emphasise the potential of reversal of phenotypes by agents that impinge onto the aforementioned pathways and create hope that parts of ASD symptomatology might be effectively treated by pharmacotherapy in the future.

Taken together, the current efforts in genetics, anatomy and cell biology of ASD strive towards a better understanding of the pathogenesis of this group of disorders required to materialise those hopes.

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Chapter 2
Neuroanatomy and Neuropathology of Autism Spectrum Disorder in Humans

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2.1 Introduction

Autism spectrum disorder (ASD) is a lifelong, heterogeneous neurodevelopmental condition characterised by deficits in social communication and repetitive and restricted behaviours (including sensory anomalies) (Association 2013). The aetiological and neurobiological mechanisms underlying ASD are complex, and there is considerable heterogeneity in the clinical and neurobiological phenotype

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among individuals on the autism spectrum. However, research agrees that ASD is accompanied by differences in brain anatomy and connectivity.

Here, we review insights into macroscopic and microscopic neuroanatomy and structural connectivity of the brain in ASD based on human neuroimaging and neuropathological studies. In particular, we focus on the neurobiological mechanisms mediating early brain overgrowth in ASD by linking the time course of typical neurodevelopment to the neurodevelopmental trajectory observed in ASD. Moreover, we aim to relate the insights into the macroscopic pathology of the brain in ASD coming from in vivo neuroimaging studies to specific microstructural differences that have been reported by histological investigations in *post-mortem* brain tissue. In the future, such insights will be crucial for determining the validity of rodent models and/or cellular assays for ASD that provide tractable opportunities for the development of novel pharmacotherapies.

2.2 Early Brain Overgrowth in ASD During Early Childhood

Early cross-sectional structural neuroimaging studies suggest that the brain of toddlers with ASD is, on average, larger in volume than the brain of typically developing (TD) children (i.e. those without a medical or psychiatric diagnosis) between the ages of 2–4 years (Courchesne 2002; Hazlett et al. 2005), which is also accompanied by an increased head circumference (Lainhart et al. 1997; Courchesne et al. 2003). The early brain overgrowth seems to disappear around the age of 5–6 years, when growth curves of ASD individuals and TD controls converge (Courchesne et al. 2001), after which no significant enlargement of the brain in ASD is typically observed. Based on these early neuroimaging studies, it has also been suggested that the brain in ASD undergoes an atypical trajectory of maturation, which is characterised by a period of increased growth during toddlerhood, following by a period of slowed down or arrested growth during early to late childhood and possibly a period of accelerated decline across the remaining life span (Courchesne et al. 2011). The early cross-sectional neuroimaging studies, which are often confounded by large interindividual variability, have also now been confirmed by several longitudinal studies of brain development in ASD. For example, Lange et al. (2014) examined differences in brain development from early childhood into adulthood and reported (1) significantly increased brain volumes in young children with ASD and (2) significantly decreased brain volume during adolescence with (3) intersecting ASD and TD growth curves between 10 and 15 years of age (Lange et al. 2014). The particular age when the growth trajectory of the brain in ASD intersects with the trajectory of the TD brain thus varies between studies and most likely reflects variations in subject demographics (e.g. age range of examined individuals) and/or the particular model employed in the statistical analysis (e.g. linear vs. curvilinear growth trajectories).

While most traditional neuroimaging studies examining atypical brain maturation focused on toddlers with ASD (i.e. >3 years of age), more recent evidence suggests that deviations from the typical developmental trajectory of brain maturation in ASD might already occur within the first 2 years of life. For instance, Schumann et al. (2010) reported an enlargement of the brain in 2.5-year-olds with ASD, which was accompanied by a significantly increased growth rate of the cortex (Schumann et al. 2010). In a sample of 2-year-olds, a general enlargement of the cerebral cortex of about 5% was also observed by Hazlett et al. (2011). This enlargement persisted when individuals were scanned approximately 2 years later, although there was no increase in the rate of growth during this interval (Hazlett et al. 2011). It thus remains unclear whether the early brain overgrowth in toddlers with ASD reflects an ongoing neurodevelopmental process that is accompanied by an accelerated rate of growth during this period or—alternatively—reflects the end result of pathological mechanisms that happen prior to 2 years of age. Notably, a study examining infants at high familial risk for ASD (e.g. infants with an older sibling with ASD) did not find a significant increase in brain volume at 6 months of age, relative to low-risk infants (i.e. those without a sibling with ASD) (Hazlett et al. 2012). However, at the time of the study, the authors were unable to confirm which high-risk infants were diagnosed with ASD at the age of 24 months, and it thus remains unclear if these findings generalise to individuals with known diagnostic status.

While the theory of early brain overgrowth has attracted much attention in ASD research, it is important to note that macrocephaly (i.e. head circumference of more than 2 standard deviations from the average mean) is not observed in all individuals with ASD. For example, Zwaigenbaum et al. (2014) reported an average rate for macrocephaly of about 20% of individuals across studies (Zwaigenbaum et al. 2014), which implies that the brains of about 80% of individuals with ASD are not clinically enlarged. The neurobiological mechanisms that drive the early brain overgrowth may therefore not reflect a pathological mechanism that is common to all individuals on the ASD spectrum. As pointed out by Lainhart et al. (2006), it is thus not yet known if macrocephaly identifies a neurobiological subtype of ASD or if it is, in fact, just the 'tip of the iceberg' of a more general tendency towards increased head and brain size (Lainhart et al. 2006). There is also recent evidence highlighting the importance of considering wider contextual issues when interpreting the findings of traditional studies of head circumference and total brain volume. For example, a study by Raznahan et al. (2013) suggests that the increased head circumference in ASD might reflect a bias in population norm rather than a replicable pattern of dysregulated brain growth (Raznahan et al. 2013), particularly in the mental health setting (Daymont et al. 2010). Future investigations into changing population norms will thus be of importance when examining clinical populations such as ASD (Lefebvre et al. 2015). Furthermore, head circumference is significantly correlated with various other (i.e. non-ASD) measures such as gender, age, height, weight and genetic ancestry, which will also impact on population norms if not accounted for (Chaste et al. 2013). Therefore, some studies suggest that the early brain overgrowth in ASD may reflect an early pattern of

general physical (i.e. somatic) overgrowth, which has been noted in boys with ASD in particular (Surén et al. 2013; Campbell et al. 2014). Thus, while in vivo neuroimaging studies provide strong support of the early brain overgrowth hypothesis overall, various contextual factors need to be taken into account when interpreting findings so far.

2.3 Neurobiological Mechanisms Mediating Brain Growth During Early Childhood (<2 Years)

The neurobiological mechanisms that drive early brain overgrowth in ASD remain poorly understood. In the TD brain, brain maturation in the first 2 years of life is characterised by a general increase in brain volume, driven by a combination of progressive and regressive cellular events during prenatal and early postnatal brain development. These events mainly include (1) neurogenesis, neuronal migration and differentiation; (2) the formation of principal axonal and dendritic projections and subsequent axon elimination and pruning; (3) synaptogenesis and synaptic pruning; (4) apoptosis; and (5) myelination (Stiles and Jernigan 2010) (Fig. 2.1). Moreover, the specific time course of these events is highly variable across brain regions. This means that variations in total brain volume are not only driven by a combination of different cellular events but also reflect a regional mixture of progressive and regressive changes over time. While histological studies into the developing brain in ASD remain elusive, it is however possible to indirectly relate the atypical neurodevelopmental trajectory of brain in ASD to the established cellular events that drive brain maturation in the typically developing brain.

2.3.1 Cellular Mechanisms Underlying Typical Brain Maturation During Gestation and Early Childhood

In the typically developing brain, neurogenesis from neural stem cells and progenitor cells is mostly completed by midgestation (~20 weeks) (Clancy et al. 2001), when neurons start to migrate to different cortical layers and differentiate. Once neurons have reached their cortical destination, axons and dendrites start to develop in order to form synaptic connections (Stiles and Jernigan 2010; Ronan et al. 2013). Axonal growth is supported by the so-called 'growth cone' machinery, which is guided by a spatial pattern of molecular cues in the developing brain (e.g. by netrins, slit, ephrins and semaphorins) that help to establish the axonal brain circuitry, or the human 'connectome' (Lowery and Van Vactor 2009). A peak in axonal numbers is reached at birth, when axons are in abundance, shortly before a sharp postnatal decrease in growth cone markers following birth (Benowitz and Routtenberg 1997; Collin and van den Heuvel 2013), and the subsequent onset of

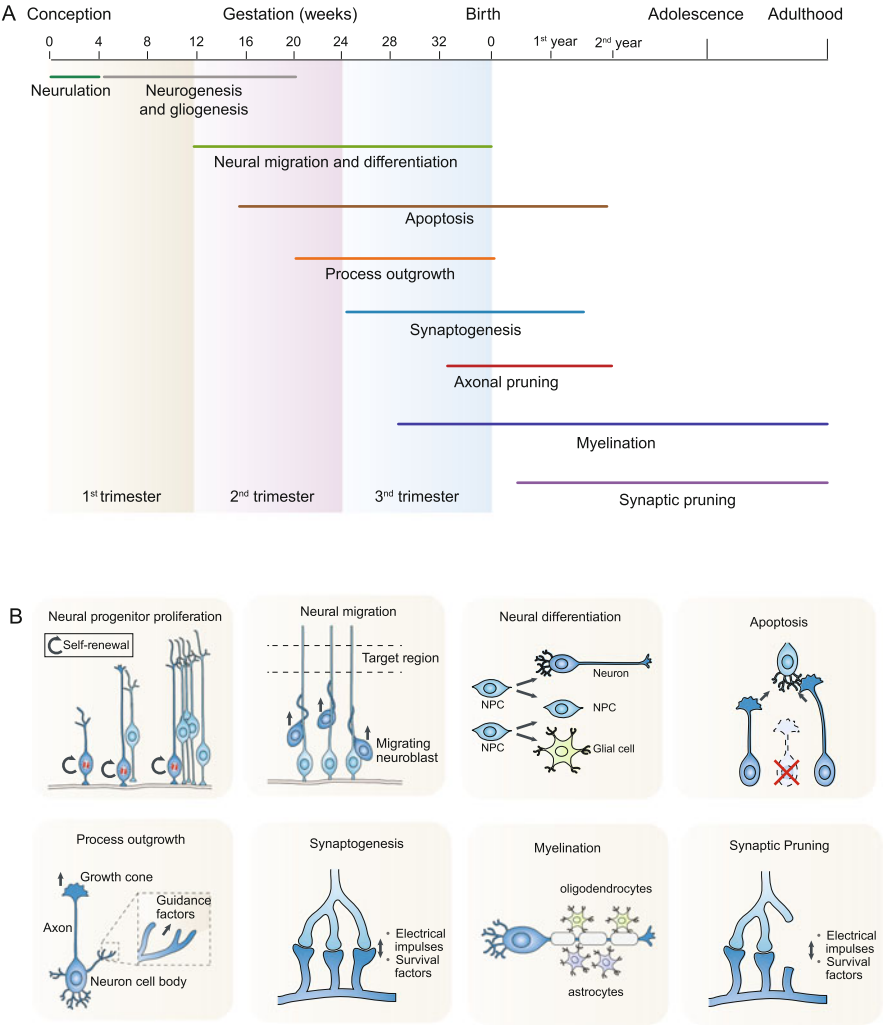

Fig. 2.1 (**a**) Timeline of human neurodevelopment across different neurodevelopmental processes. (**b**) Illustration of the different neurodevelopmental processes underlying brain maturation. Adapted from Ronan et al. (2013)

large-scale pruning of axonal branches and connections (Low and Cheng 2006). Early postnatal development is thus characterised by a generalised decrease in axonal 'connectivity' on the macroscopic scale, involving large-scale axon elimination and pruning during the first postnatal month, that continues throughout the first 2 years of life (Innocenti and Price 2005). By roughly the age of 2 years, the phase of exuberant axon removal is mainly completed, and the macroscopic layout of the human connectome is set.

Axonal projections surviving the period of elimination and pruning are subsequently strengthened by an increase in axon diameter and by formation of the

myelin sheath produced by oligodendrocytes (a type of neuroglia) (LaMantia and Rakic 1990). Myelin protein in the brain by midgestation (approximately weeks 20–28 gestation) in subcortical regions and later on in cortical regions (Iai et al. 1997). Between gestational weeks 36–40, the proportion of total brain volume that contains myelin increases from about 1 to 5% (Hüppi et al. 1998). Myelination then rapidly proceeds during the first year of life, before continuing at a slower pace in the second year of life. Notably, the time course of myelination is highly region dependent and also happens at a variable rate both across and within functional circuits (Leipsic 1901). Sensory pathways are the first to myelinate, followed by motor pathways and association areas (Leipsic 1901; Deoni et al. 2011). The most anterior parts of the frontal lobes are the last to mature in terms of white matter development, which occurs only between 7 and 11 months of age (Tau and Peterson 2010). Thus, myelination and the expansion of glial cells during the first year of life significantly contribute to the dramatic brain growth in TD newborns and toddlers and complement the maturation of the neuronal macro-circuitry of the brain during development. Notably, myelination is an ongoing process that is not completed after the first year of life, but continues across the first half of the human life span (Grydeland et al. 2013).

The changes to the brain's macroscopic circuitry during the first 2 years of life are also accompanied by changes in the local (i.e. microscopic) circuitry that mediates the communication between neurons within a restricted cortical territory via the elaboration of dendrites, spines and synapses (Innocenti and Price 2005). Synaptogenesis in the human neocortex begins during the third trimester of gestation and continues during the first 2 years of life (Huttenlocher 1990). As with most cellular events during development, there is considerable variation in the time course of synaptogenesis across the cortex. For example, the primary auditory cortex reaches peak synaptic density around 3 months postnatally, while synaptogenesis in the prefrontal cortex does not reach its peak until 15 months of age, thus continuing into the second year of life (Huttenlocher and Dabholkar 1997). It is followed by a period of synapse elimination during childhood, in which synaptic density and number decrease to about 60% of its maximum (Huttenlocher and Dabholkar 1997). The first years of life also see the arborisation of both pyramidal cells and GABAergic inhibitory interneurons (Mrzljak et al. 1990), which are the most crucial functional components of the local microcircuitry, and form so-called cortical minicolumns (Mountcastle 1997). During neurodevelopment, neurons predominantly migrate along the radial glial fibres to form ontogenetic columns arranged as radial units (Rakic 1995). As neurons mature (~3rd trimester), those within columns preferentially form functional excitatory synaptic connections with one another, rather than from adjacent cells that are not derived from the same radial founder cell (Yu et al. 2009). Within minicolumns, vertical excitatory connections predominate so that the strongest excitatory connection between pyramidal cells is vertically orientated (Buxhoeveden and Casanova 2002). The horizontal architecture of the cortex (i.e. connectivity in parallel to the cortical surface) is mostly determined by short-range excitatory connections that bind cells of neighbouring minicolumns horizontally and also by inhibitory

interneurons that contribute to the lateral separation between columns (Mountcastle 1997). The local neurocircuitry within and between columns is fine-tuned throughout perinatal and postnatal life and is hence largely experience and/or learning dependent.

Taken together, these studies examining the typically developing cortex and the formation of the cortical neurocircuitry during the first 2 years of life may provide valuable insights into the atypical development of the brain in ASD. So far, however, there is only indirect empirical evidence linking the brain in ASD to specific cellular events during neurodevelopment.

2.3.2 *Cellular Mechanisms Underlying Atypical Early Brain Maturation in ASD*

Based on the insights provided by studies examining typical brain development, it seems that early overgrowth of the brain in ASD may be related to the combined effect(s) of various cellular and/or molecular events that drive early brain maturation. For example, in vivo neuroimaging studies demonstrate that early brain overgrowth in ASD is not equally deviant across all cortical regions. Rather, there is a regional gradient of abnormal brain enlargement with frontal and temporal lobes being more affected than the parietal and occipital lobes (Carper et al. 2002). The regional neurodevelopmental trajectory of brain maturation across different areas of the cortex in ASD thus significantly differs from the typical developmental trajectory where phylogenetically older cortical areas (e.g. in occipital and parietal lobes) mature earlier than phylogenetically younger areas (e.g. higher-order association cortices in frontal and temporal lobes) (Gogtay et al. 2004). Moreover, typically, the maturation of late developing regions builds on the maturation of early developing regions (Gogtay et al. 2004). It has therefore also been suggested that deviations from the regionally and temporally ordered typical sequence of brain maturation—as is the case in ASD—may lead to a developmental 'uncoupling' of prematurely developing regions (i.e. frontotemporal regions) from the rest of the cortex (Courchesne and Pierce 2005). The early developmental perturbations to the trajectory of brain maturation in ASD may therefore not only affect the neuroanatomy of isolated brain regions but also lead to differences in brain anatomy and connectivity on a systems level (Ecker et al. 2012). ASD has therefore also been termed a 'neurodevelopmental disconnection syndrome' that is accompanied by perturbed brain connectivity (Geschwind and Levitt 2007).

Notably, the period of early brain overgrowth in ASD coincides with neurodevelopmental processes that underlie the formation of the brain's neurocircuitry, such as (1) synaptogenesis, (2) myelination and (3) axonal and synaptic pruning (Fig. 2.1). It may therefore be that ASD is accompanied by atypical synaptogenesis, pruning or differences in myelination. For example, a

recent diffusion tensor imaging (DTI) study in high-risk infants demonstrated that there are perturbations in the development of white matter circuitry from as early as 6 months of age in infants who are subsequently confirmed to have ASD at the age of 2 years relative to infants who do not have ASD (Wolff et al. 2012). Moreover, the development for most large-scale (i.e. interregional) fibre tracts was characterised by higher fractional anisotropy (FA) values—a marker of white matter integrity—at 6 months, followed by a slower change over time, so that by 24 months of age, toddlers with ASD had lower values (Wolff et al. 2012). This data suggests that there are early differences in axonal development and/or myelination that affect the development of brain connectivity prior to the age of 2 years.

Furthermore, it has been shown that ASD is also accompanied by neurodevelopmental differences within the cortical grey matter. For instance, a study by Hazlett et al. (2011) suggests that early overgrowth of the brain in terms of its grey matter volume may be driven by an accelerated expansion of the cortical surface, rather than a thickening of the cortical sheet (Hazlett et al. 2011). The distinction between cortical thickness and surface area is of importance as evidence suggests that both measures (1) are mediated by different sets of genes, (2) have differing neurodevelopmental trajectories and (3) are related to distinct aspects of neural architecture. Different cellular mechanisms for cortical expansion and laminar thickness of the cortex have been first postulated by the radial unit hypothesis (RUH) (Rakic 1995). According to the RUH, radial unit progenitor cells initially divide in the ventricular zone of the developing cortex and produce neurons that then migrate along radial glial fibres to form ontogenetic columns of neurons, or 'radial units'. Consequently, cortical thickness is determined by the neuronal output from each radial unit, produced by sequential generation of lower, middle and upper layer neurons, and is hence related to the overall number of neurons within columns. By contrast, cortical surface area is determined by the early proliferation of radial unit progenitor cells, which increases the number of ontogenetic columns, and an increase in surface area (Pontious et al. 2008). In addition, more recent studies also highlight the important role of so-called intermediate progenitor cells (IPCs), which are initially generated by radial unit progenitors that reside in the subventricular zone (SVZ) of the developing cortex (Kriegstein et al. 2006). IPCs produce neurons either directly via neurogenesis or indirectly via symmetric division and hence may amplify neuronal output exponentially. Consequently, rodent models show that increases or decreases in IPC abundance are consistently associated with parallel changes in cortical thickness, but not surface area (Pontious et al. 2008). The finding of an early enlargement of the brain in ASD, which seems to be driven by an accelerated expansion of the cortical surface area but not cortical thickness (Hazlett et al. 2011), is thus important as it points towards specific genetic and neurobiological mechanisms that might be impaired in ASD. In addition, this study also highlights the need for developing neuroimaging measures that offer a higher degree of specificity than traditional measures of cortical volume and that can hence be used to link in vivo findings to specific aetiologies and underlying neurobiological mechanisms.

2.4 Brain Development Across Late Childhood and Adolescence in ASD

While the neurodevelopmental trajectory of early brain maturation is well characterised in ASD, less is known about atypical neurodevelopment during late childhood and adolescence. In contrast to the developmental differences observed during early brain maturation in ASD, i.e. an increase in total brain volume, brain development during later childhood and adolescence in ASD seems to be dominated by an accelerated age-related decline in grey matter volume (Ecker et al. 2015). In the TD brain, overall grey matter volume continues to expand during childhood and reaches its peak during late childhood/early adolescence (i.e. around 9 years of age in girls and 11 years of age in boys (Giedd 2008). The pre-pubertal increase in grey matter volume is followed by a post-pubertal loss, resulting in an inverted U-shaped trajectory of brain development across the human life span (Giedd et al. 1999).

Histological studies suggest that the typical decline in GM volume coincides with a period of competitive and learning-dependent synaptic elimination (or pruning) during adolescence and early adulthood (Huttenlocher and Dabholkar 1997). The time course of synapse proliferation and elimination is particularly well documented in the prefrontal cortex, which continues to develop well beyond early childhood and shows a rapid attrition of grey matter volume in late adolescence (Sowell et al. 2003; Gogtay et al. 2004). In individuals with ASD, this typical attrition of grey matter volume seems accelerated during late childhood and adolescence. For example, Lange et al. (2014) reported an accelerated decrease of total grey matter in ASD individuals after the age of ~15 years, which was also accompanied by a reduced growth of the cortical white matter (Lange et al. 2014). This finding is supported by another longitudinal study by Hardan et al. (2009), who reported a significant greater decrease in total grey matter volume during late childhood and early adolescence in ASD (aged 8–12 years) over a time period of 30 months, which appeared to be driven by significant reductions in cortical thickness (Hardan et al. 2009; Ecker et al. 2014; Zielinski et al. 2014). Accelerated age-related cortical thinning has also been reported in various brain regions in ASD by other cross-sectional studies (e.g. Wallace et al. 2010). In addition, there are reports that measurements of cortical surface area decline more rapidly with age in individuals with ASD than in those without ASD at the global (i.e. total surface area) (Mak-Fan et al. 2011) and local level (i.e. vertex-wise estimates of surface area) (Ecker et al. 2014). Taken together, human neuroimaging studies suggest that the trajectory of brain maturation in ASD differs across distinct stages of development, which are dominated by an accelerated cortical expansion during early childhood, followed by accelerated decline in grey matter volume during adolescence and adulthood. Neurodevelopmentally, this period of decline in total brain volume overlaps with a phase of competitive synaptic pruning or synaptic elimination (Fig. 2.1), in which ASD individuals may differ from neurotypical controls. These different developmental stages thus need to be

accounted for when examining neuroanatomical differences in ASD across the human life span.

2.5 The Core Neural Systems Underlying ASD

By adulthood, ASD is characterised by neuroanatomical abnormalities in several large-scale neural systems. These systems include fronto-temporal and frontoparietal regions, the amygdala-hippocampal complex, cerebellum, basal ganglia and anterior and posterior cingulate regions (Amaral et al. 2008) (Fig. 2.2). Neuroanatomical differences in these regions have also been associated with particular autistic symptoms and traits. For example, many of the core regions with anatomical abnormalities in ASD overlap with the so-called 'social' and/or 'emotional' brain, which comprises a set of brain regions that mediate functions related to social cognition and emotional processing. Emotional 'core' regions (i.e. regions that are frequently observed across studies) include (1) subcortical regions including the amygdala, the nucleus accumbens and the hypothalamus and (2) cortical regions such as the orbitofrontal cortex (OFC), the anterior cingulate cortex (ACC) and the medial prefrontal cortex (mPFC) (Pessoa 2008). In addition, the neural systems in ASD include several regions of the extended limbic network, such as the hippocampus, the anterior temporal lobe (ATL), the superior temporal sulcus (STS), the somatosensory cortex and the posterior cingulate cortex (PCC) (Pessoa 2008). Some of these brain regions are also part of the so-called 'social'

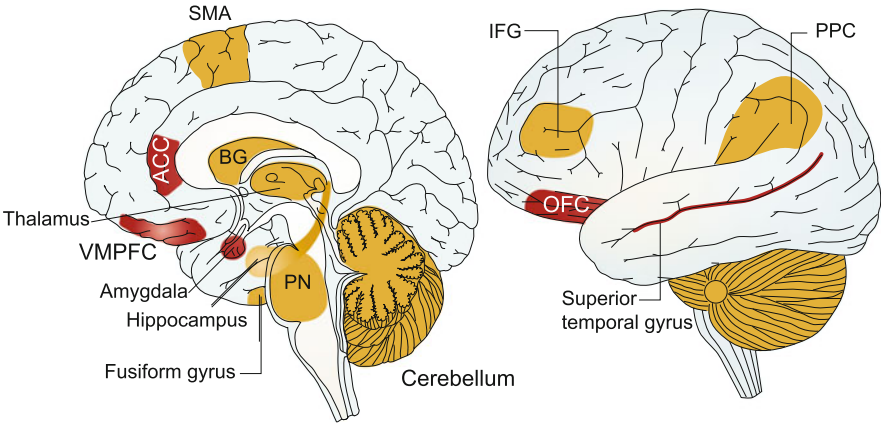

Fig. 2.2 The neurocognitive systems underlying ASD. Core ASD regions include the ventromedial prefrontal cortex (VMPFC), the orbitofrontal cortex (OFC), the superior temporal gyrus (STS), the amygdala and the anterior cingulate cortex (ACC). Extended ASD regions include the inferior frontal gyrus (IFG), the posterior parietal cortex (PPC), the cerebellum, the hippocampus, the fusiform gyrus, pons (PN), the basal ganglia (BG), the thalamus and the supplementary motor area (SMA). Adapted from Amaral et al. (2008)

brain that additionally includes the temporo-parietal junction, the inferior frontal gyrus, the intraparietal sulcus and the anterior insula (Blakemore 2008). Consequently, in ASD, anatomical abnormalities in these regions have been related to deficits in social interaction and communication (Ecker et al. 2015). These regions also subserve wider socio-cognitive functions such as theory of mind (Castelli et al. 2002), face processing (Scherf et al. 2015), biological motion (Pelphrey et al. 2003), self-referential cognition and empathy (Lombardo et al. 2010), which are typically impaired in individuals with ASD.

Moreover, there is considerable overlap between the neural systems underlying ASD and the cortical circuitry underlying obsessive-compulsive disorder (OCD). OCD is characterised by repetitive thoughts (i.e. obsessions) and repetitive behaviours (i.e. compulsions), which are also commonly observed in individuals with ASD (Russell et al. 2005). Obsessive-compulsive symptoms in OCD have been linked to the so-called cortico-striato-thalamo-cortical (CSTC) circuitry (also termed the frontostriatal model or corticostriatal model), which mainly comprises the thalamus, the OFC, the ACC and the striatum (Pauls et al. 2014). Neurodevelopmental changes in the striatum have also been reported in individuals with ASD. For example, Langen et al. (2013) found that individuals with ASD show an increase in the growth rate of the caudate nucleus between the ages of 10 years and 12 years relative to typically developing controls. Notably, the increased growth rate was significantly correlated with the severity of repetitive behaviours at the preschool age, hence suggesting that caudate nucleus growth may be a neurobiological pathway for stereotypic and repetitive behaviour in ASD (Langen et al. 2013). These findings also indicate that the anatomical abnormalities typically observed in ASD are not unique to the condition, but that there is significant overlap between the neural systems underlying ASD and the neurobiological substrates implemented in other psychiatric and/or neurodevelopmental conditions. Moreover, it seems that the phenotypic similarities in brain anatomy between ASD and other disorders might be associated with an overlap in clinical symptoms. For the development of future treatment and/or intervention strategies of comorbid symptoms, it will thus be crucial to disentangle common from distinct genetic and/or molecular pathways across disorders with similar symptom profiles.

Last, there is evidence to suggest that the neuroanatomical differences in the neural systems underlying ASD are accompanied by atypical structural connections that link the core brain regions mediating autistic symptoms and traits. By some, atypical brain connectivity has even been suggested to represent an underlying mechanism that may be common to all individuals on the autism spectrum (Abrahams and Geschwind 2008). However, the notion of atypical brain connectivity in ASD is complex, involving abnormalities within the grey and white matter (Ecker et al. 2013) and different levels of integration (Belmonte et al. 2004). There is thus considerable variation in analytical approaches employed and in the particular aspect of brain connectivity examined. Structural brain connectivity in ASD has so far mostly been examined using voxel-wise analysis of the white matter and diffusion tensor imaging (DTI). For example, voxel-based morphometry (VBM) studies show that individuals with ASD have spatially distributed reductions in

regional white matter volume during childhood (McAlonan et al. 2005), adolescence (Waiter et al. 2005) and adulthood (Ecker et al. 2012). In addition, DTI studies in ASD have reported white matter differences in the corpus callosum (Alexander et al. 2007; Freitag et al. 2009), arcuate fasciculus (Fletcher et al. 2010), inferior fronto-occipital and superior longitudinal fasciculi (Sahyoun et al. 2010), as well as in limbic pathways (Pugliese et al. 2009), connectivity of the amygdala-hippocampal complex (Conturo et al. 2008), basal ganglia (Keller et al. 2007; Langen et al. 2011), the cerebellum (Catani et al. 2008; Brito et al. 2009) and in the cortico-spinal tract (Brito et al. 2009).

Taken together, these studies suggest that the neuroanatomical differences observed in ASD are associated with atypical structural connections affecting the main white matter fibre tracks in the brain. However, it remains poorly understood whether atypical white matter connectivity is of primary or secondary aetiology in ASD, as white matter development is intrinsically linked with the development of grey matter. For example, the formation of principal axonal and dendritic projections—and subsequent myelination—builds upon the completion of neuronal proliferation, migration and differentiation (Kostovic and Rakic 1980; Tau and Peterson 2010; Molnár et al. 2014). If perturbed, as has been suggested in ASD (Pinto et al. 2010; Parikshak et al. 2013), the mechanisms that drive atypical grey matter development are thus likely to also interfere with the development of white matter (Ecker et al. 2016). Future studies are therefore needed to determine whether variations in grey matter neuroanatomy and white matter connectivity are linked or are independently modulated by ASD. Furthermore, it will be important to link the in vivo neuroimaging findings to *post-mortem* histological investigations that are able to determine the neurobiological underpinnings of ASD at a much higher level of resolution.

2.6 Microstructural Findings in the ASD Brain

So far, there are few microscopic evaluations utilising *post-mortem* brain tissue that could elucidate the specific neural mechanisms that underlie the macroscopic differences in brain anatomy and connectivity observed in ASD. The few existing studies so far are also based on a small number of tissue samples obtained from individuals at varying postnatal ages, and not all studies employed the same methodological approach. In addition, analyses were often performed on material coming from individuals with a clinical diagnosis of ASD at some stage in their lives and not on genetically defined subgroups. Consequently, the findings coming from *post-mortem* studies remain largely heterogeneous. Overall, however, there is strong agreement between the brain regions examined using microstructural techniques and brain regions that have been reported to be macroscopically different, i.e. the cerebral cortex, the limbic system and the cerebellum (Bauman and Kemper 1985; Pickett and London 2005; Amaral et al. 2008; Cheng et al. 2010; Hampson and Blatt 2015).

2.6.1 Cortical Neuropathology of ASD

The cerebral cortex contains a large number of different cell types that are arranged in distinct subregions in a complex fashion and thereby define each individual's cortical circuitry. To date, most neuropathological studies of ASD focused on subregions of the frontal and temporal lobes, which are functionally related to autistic symptoms and traits, and include the prefrontal cortex (PFC), the anterior and posterior cingulate cortex (ACC and PCC) and the fusiform gyrus (FFG) (Cheng et al. 2010). Here, cytoarchitectural differences such as (1) alterations of neuronal number and density, (2) neuronal heterotopias or (3) poorly defined and focally disrupted cortical layers have been identified in some studies (Bailey et al. 1998; Mukaetova-Ladinska et al. 2004; van Kooten et al. 2008; Simms et al. 2009; Courchesne et al. 2011; Santos et al. 2011; Oblak et al. 2011; Stoner et al. 2014).

Moreover, it is generally assumed that these neurobiological abnormalities result from an impaired cortical patterning throughout brain development and might later on contribute to subsequent impairments in cortical circuitry. For example, it has been reported that there is an increased number and altered width of so-called cortical minicolumns in ASD, which are vertically organised functional units of pyramidal projection neurons, particularly in laminae III and V of the frontal and temporal lobes (Casanova et al. 2002, 2006; Buxhoeveden et al. 2006; McKavanagh et al. 2015). These structural changes are thought to disrupt the cortical circuitry by strengthening intraregional short-range microcircuits and weakening interregional long-range macro-circuits (Casanova and Trippe 2009). The notion of perturbed long-range connections but maintained (or even enhanced) short-range connectivity is also supported by histological evidence. For example, Zikopoulos and Barbas (2010) reported fewer large myelinated axons in the deep white matter linking distant areas, but more thin myelinated axons in the superficial white matter linking nearby areas below the ACC in ASD participants (Zikopoulos and Barbas 2010). Notably, a more recent study by the same authors also found a significant reduction of parvalbumin-positive GABAergic interneurons in the dorsolateral prefrontal cortex in ASD that might contribute to an imbalance between inhibition and excitation within local microcircuits in ASD (Zikopoulos and Barbas 2013). There is also evidence of a significantly enhanced number of synaptic spines that have been detected predominantly in superficial layer II of frontal, temporal and parietal lobes in ASD, as well as in layer V of the temporal lobes (Hutsler and Zhang 2010)—and this also supports the notion of impaired cortical neurocircuitry on the synaptic level.

Last, there are histological studies reporting microglial and astroglial activation in the ASD cortex indicating neuro-inflammatory processes and/or pathologic tissue alterations (Laurence and Fatemi 2005; Vargas et al. 2005; Morgan et al. 2012; Suzuki et al. 2013). However, it remains unclear whether microglial and astroglial activation are primary of secondary to the disorder itself or may have resulted from perimortal ischaemic-reperfusion damage.

Taken together, these histological studies indicate that the macroscopic differences in neuroanatomy and connectivity observed in various cortical regions in ASD are likely underpinned by microscopic differences in neuronal cortical organisation and synaptic structure. These microstructure differences are, however, not only present in the neocortex in ASD but also in other cortical systems such as the limbic system and the cerebellum.

2.6.2 Limbic Neuropathology in ASD

Neuroanatomical differences in the limbic system—including the hippocampus and the amygdala—have repeatedly been reported by neuroimaging studies (Nordahl et al. 2012) and are known to mediate social and emotional processing in ASD. Recently, these in vivo findings have also been linked to atypical neuronal structure and composition. For example, early non-stereological studies report a reduced size, increased packing density and rarified dendritic arbours of pyramidal neurons in specific parts of the cornu ammonis (CA) region of the hippocampus in ASD (Bauman and Kemper 1985; Kemper and Bauman 1993; Raymond et al. 1996), although these early findings could not be confirmed by subsequent independent investigations (Bailey et al. 1998). Later on, it was shown that the number of defined GABAergic interneuron populations was increased in specific hippocampal subregions of ASD subjects (Lawrence et al. 2010) and that neuronal heterotopia and focal dysplasia in the ASD hippocampus may be found in some, but not all, individuals with ASD (Wegiel et al. 2010). Similarly, stereological investigations of the amygdala in ASD also showed that the neural number was reduced particularly in the lateral nucleus of this limbic structure (Schumann and Amaral 2006), while no global differences were found in the number of glial cells in the amygdala (Morgan et al. 2014).

These findings thus highlight the heterogeneity of neuropathological findings to date and the need to conduct methodologically standardised investigations in larger samples in order to detect reliable differences in the neuropathology of the limbic system in ASD.

2.6.3 Cerebellar Neuropathology in ASD

There are also histological studies examining the neuropathology of the cerebellum in ASD. Here, it has repeatedly been shown that the number of Purkinje cells (PCs)—predominantly in the cerebellar hemispheres—is significantly reduced in ASD (Williams et al. 1980; Bauman and Kemper 1985; Bailey et al. 1998; Lee et al. 2002; Whitney et al. 2008; Skefos et al. 2014). This may help explain the finding of significantly reduced cerebellar volume as reported by neuroimaging studies (McAlonan et al. 2005). There are also reports suggesting that PCs in the

cerebellum are reduced in size in ASD relative to neurotypical controls (Fatemi et al. 2002). The majority of studies to date indicate that the abnormalities in PCs in ASD result from an underlying immunological reactive mechanism. However, it has also been suggested that the selective PC loss in the cerebellum is due to developmental disturbances, especially as developmental abnormalities such as cerebellar dysplasia or neuronal heterotopia are often seen in the ASD cerebellum (Vargas et al. 2005; Whitney et al. 2008; Wegiel et al. 2010). On the functional level, a loss in PC cells might result in less inhibition of deep cerebellar nuclei, which would in turn lead to (1) increased excitation of thalamo-cortical circuits and (2) less inhibition of the inferior olivary complex (IOC) in the brainstem. Moreover, the mount of inhibition from the IOC will also impact on the remaining PCs as IOC neurons project to PC dendrites via excitatory climbing fibres.

Taken together, the cerebellar neuropathology may thus be particularly well suited to investigate the hypothesis of an imbalance between inhibition and excitation in ASD, which remains currently a topic of speculation.

2.7 Conclusions

In vivo neuroimaging studies have provided strong evidence to suggest that ASD is accompanied by an atypical development of brain anatomy and connectivity. Early perturbations to the formation of the brain's neurocircuitry are already visible within the first 2 years of life, that is, before a reliable diagnosis of ASD can be made. These findings that come from studies examining infants at high and low genetic risk of ASD are therefore of high importance, as there is now an opportunity to develop early biological markers that may predict clinical outcomes at later stages of development. However, while the neuropathology of ASD is well established on the macroscopic level, little is currently known about the molecular and cellular mechanisms or pathways that mediate the atypical development of the brain in ASD on the microscopic level. Indirect evidence links the atypical development of the brain in ASD to impaired neuronal migration, synaptogenesis and pruning, the formation of the principle axonal neurocircuitry and subsequent myelination. Yet, evidence for an involvement of these mechanisms in the neuropathology of ASD is only supported by a small number of *post-mortem* studies examining clinically heterogeneous individuals and only a subset of different brain regions. There is thus a need for combining microscopic evidence with macroscopic findings, preferably within the same set of individuals in the future, in order to identify the specific neurobiological underpinnings of ASD. Overall, however, with the advances in genetic, molecular and imaging techniques, there is no doubt that the link between micro- and macro-pathology can be established in ASD and will provide tractable opportunities for patient stratification and the development of novel pharmacotherapies in the future.

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Chapter 3
Modelling Autistic Neurons with Induced Pluripotent Stem Cells

Annie Kathuria, Carlo Sala, Chiara Verpelli, and Jack Price

3.1 Introduction

Autism spectrum disorder (ASD) is a neurodevelopmental condition primarily defined by impaired social interaction and communication, repetitive stereotyped behaviours and restricted interests and activities (Asperger 1944). As for many neurodevelopmental disorders, ASD is further characterised by a complex aetiology, a range of symptom severity and associated co-morbidities. There are no effective cures up to this date. Extensive recent epidemiological studies estimate that more than 1% of the population could receive a diagnosis of ASD (Elsabbagh et al. 2012; Developmental Disabilities Monitoring Network Surveillance Year Principal 2008). These studies also demonstrated that there are 4–8 times more males than females with ASD (Elsabbagh et al. 2012). Nonetheless, more sophisticated diagnostic tools that measure the quantitative trait using auto- or hetero-questionnaires such as the social responsiveness scale (SRS) or the autism quotient (AQ) suggest that autistic traits seem to be normally distributed in clinical cases as well as in the general population (Ronald et al. 2006; Skuse et al. 2009; Constantino 2011). Although the causes of ASD remain largely unknown, molecular genetics studies have identified more than 100 ASD risk genes carrying rare

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and penetrant deleterious mutations in approximately 10–25% of the patients (Huguet et al. 2013; Gaugler et al. 2014). Interestingly, the susceptibility genes (Huguet et al. 2013) seem to converge on a limited number of biological pathways including chromatin remodelling, protein translation, actin dynamics and synaptic functions (Bourgeron 2009; Toro et al. 2010; Huguet et al. 2013). The discovery of ASD genes has substantially contributed to the development of in vitro and in vivo models, including the use of induced pluripotent stem cell (iPSC) technology, which allows researchers to derive neurons from somatic cells obtained from patients. Considering the neurodevelopmental nature of ASD, it is essential to target treatments at specific mechanisms involved in brain development and plasticity.

In this review, we will discuss how our increased understanding of the multiplicity of molecules and effectors involved in neurodevelopment and their complex cross-interaction has highlighted several potential areas of intervention that could be exploited for this purpose with attention to the new iPSC technology that is beginning to contribute to therapeutic approaches.

3.2 The Need for Human Cellular Models

Most of our current knowledge of the molecular pathways that leads to human neurodevelopmental disorders such as ASD comes from heterologous systems and genetic animal models, in particular, mouse models. However, there are considerable differences between human and mouse physiology, thus limiting the extent to which mouse models can fully represent human disease pathophysiology. For example, it was recently found that human astrocytes are not only three times larger but also have ten times more processes than those of rodents (Zhang et al. 2016). Nonetheless, mouse models of ASD are providing insight into disease mechanisms, although these have not yet robustly resulted in translational benefits for ASD patients. The increased validity that could be brought by human cellular models would therefore be of considerable benefit.

There are multiple human cellular models available. Immortalised human cell lines have been used to overexpress mutant proteins to understand features of disease pathophysiology (Eigenmann et al. 2013). Yet, cell lines in prolonged culture acquire genetic abnormalities, and massive overexpression of human proteins does not accurately reflect human pathophysiology. Thus, this approach has significant limitations. Human embryonic stem cells (ESCs) represent another alternative. These cells are derived from embryos at the blastocyst stage, are pluripotent and can self-renew (Narsinh et al. 2011). Nonetheless, the use of human embryos for research purposes remains ethically controversial and logistically challenging because of the limited supply of human embryo donors. The last and most recent approach to generate human cellular models is to reprogram adult somatic cells to generate pluripotent stem cells (Cocks et al. 2014). The advantage

of using induced pluripotent stem cells (iPSCs) is that we can generate patient-specific iPSC lines to model complex disorders like ASD. As iPSCs retain the capacity to indefinitely renew and differentiate into cells from all three germ layers, they are most promising for disease modelling, drug discovery and personalised medicine applications (Sterneckert et al. 2014).

In 2006, Shin'ya Yamanaka (Nobel Prize in Physiology or Medicine in 2012) published a method to enforce the ectopic expression of four key transcription factors—the 'Yamanaka factors': octamer-binding protein 4 (OCT4), sex-determining region Y-box 2 (SOX2), Kruppel-like factor 4 (KLF4) and c-MYC—to reprogram somatic, terminally differentiated fibroblast cells to pluripotency (Takahashi and Yamanaka 2006; Takahashi et al. 2007). These cells were called iPSCs, in reference to their developmental potential equivalence to ESCs (Takahashi and Yamanaka 2006; Okita et al. 2007; Wernig et al. 2007). The Yamanaka approach delivered the reprogramming factors via a retroviral system. Since then, other reprogramming methods have been devised using either integrating (lentivirus) or non-integrating constructs (adenovirus, *Sendai virus*, PiggyBac, minicircle vectors, episomal plasmids). Other methods include the overexpression of reprogramming proteins (by mRNA or protein delivery), the modulation of the expression of pluripotency or reprogramming genes (with specific miRNA) and the chemical epigenetic chromatin remodelling (using chemicals like hydroxamic acid, trichostatin A and valproic acid) (González et al. 2011). The advantage of using episomal vectors over integrating vectors is the lower risk to the integrity of the cell genome, although the reprogramming efficiency that can be obtained with episomal vectors is lower than with integrating methods (Martins-Taylor et al. 2011). Because the collection of fibroblasts requires an invasive procedure, a skin biopsy, other somatic cells, such as hair keratinocytes, urinary epithelial cells and peripheral mononuclear blood cells, have been adopted successfully (Aasen and Izpisúa Belmonte 2010; Dowey et al. 2012; Zhou et al. 2012).

Quality control is important following the reprogramming procedure in order to confirm pluripotency. Typically, human iPSCs express specific surface markers like stage-specific embryonic antigens (SSEAs) 3 and 4, as well as TRA antigens (TRA-1-60 and TRA-1-81) and the endogenous expression of the transcription factors NANOG, SOX2 and OCT4. These pluripotency markers can be validated either by immunocytofluorescence (IF) staining or quantitative PCR (qPCR). Pluripotency can further be confirmed by in vitro differentiation into the three embryonic germ layers, in vivo teratoma assays or transcriptome analysis. Importantly, all generated iPSC lines should also be karyotyped to exclude the risk of acquisition of copy number variants or chromosome aberrations (Amps et al. 2011). Reliable data depends on the maintenance of genetic integrity during prolonged culture and differentiation (Zapata et al. 2012). Indeed, for ASD studies where genetic variants or CNVs are core to the pathogenesis, DNA stability is crucial.

3.3 Neural Differentiation of iPSCs

iPSCs can be induced to differentiate into different cell types including neurons for functional studies into ASD. iPSCs can be neuralised to give neural 'rosettes', two-dimensional neural tube-like structures. These neuronal precursors can subsequently be expanded for several passages and induced to differentiate into neurons or glial cells (Shi et al. 2012b; Verpelli et al. 2013). Human neuronal precursors require 4 to 12 weeks to reach their definitive phenotype in vitro, and several studies have defined the critical steps required to obtain reliable neurons for functional studies (Verpelli et al. 2013; Broccoli et al. 2014; Wang et al. 2015). Appropriate neuronal differentiation is ascertained by the detection of neuronal markers by Western blot or immunofluorescence, receptor and ion channel profiling by electrophysiology and eventually the evaluation of synaptic functions by measuring neurotransmitter metabolism, release and reuptake (Hartfield et al. 2014).

Neuronal differentiation and maturation are strongly affected by the microenvironment surrounding the cells. For example, the most frequently used artificial coating, laminin, is much less efficient than astrocyte or rodent primary neuron layers in inducing a faster and more efficient neuronal differentiation (Tang et al. 2013; Verpelli et al. 2013). These data suggest that even if the medium generally used for neuronal differentiation contains several growth factors such as BDNF, NT3, GDNF and insulin and differentiating agents such as ascorbic acid, cAMP, vitamin A and retinoic acid, direct contact of cocultured astrocytes or rodent primary neurons still promotes better differentiation and synaptic maturation.

Different differentiation protocols allow the generation of different types of neurons. Cortical pyramidal cells, GABAergic inhibitory interneurons, cerebellar Purkinje cells or dopaminergic neurons of the substantia nigra are all now possible to be generated (Zeng et al. 2010; Maroof et al. 2013; Hartfield et al. 2014; Wang et al. 2015). Despite the general success in generating human neurons in a dish, the functional reconstruction of complex organs remains a major challenge. Thus, it was a considerable achievement to demonstrate that human neurons derived from iPSCs could form 3D cultures, which self-organised into structures with the appearance of 9-week-old human foetal brain. These cerebral organoids, or 'mini brains', represent a major step from 2D cultures and are likely to improve considerably our ability to model disorders such as ASD (Lancaster et al. 2013; Lancaster and Knoblich 2014). Similarly, the generation of human neuronal microcircuits in the dish is becoming a reality (Shi et al. 2012a). Multi-electrode arrays (MEA) can record electrical impulses or stimulate the circuitry and investigate the connectivity of functional human networks in these cultures (Wainger et al. 2014). By culturing different cell types together, these circuits can replicate the dynamic interactions that take place in the brain. The use of MEA to record from neuronal assembles can be directly correlated to EEG recordings from patients. Obviously, the 'brain on a chip' does not look precisely like the natural human brain, but microcircuits have both economic and logistic advantages. A microscale version of the brain is easy to interrogate, supporting multiple variables at the same time. The system has the

potential to eliminate dependence on animal models and speed drug development, helping the exclusion of ineffective or unsafe drugs several steps earlier in the development process. In the future, doctors might no longer have to blindly estimate which of several drugs and dosages would work best for their patients—they could simply test them all out and select the one with the greatest efficacy and the fewest side effects.

3.4 Modelling ASD in a Dish Using iPSCs

Various human disorders have been successfully modelled using iPSCs, including ASD. Here, we will consider some of the principle findings.

3.4.1 Fragile X Syndrome

Fragile X syndrome (FXS) is a genetic condition caused by mutations in the *FMR1* gene, which encodes the fragile X mental retardation protein (FMRP). The protein has four RNA binding domains: a nuclear export, a nuclear localisation, a synaptic protein synthesis and a dendritic mRNA localisation sequence (Antar et al. 2005; Zalfa et al. 2005; Tabet et al. 2016). These highly suggest a role for the protein in synaptic plasticity. Most cases of FXS are caused by expansion of the trinucleotide CGG repeat in the 5′-untranslated region of *FMR1*. Expansion leads to DNA methylation of *FMR1*, which causes transcriptional silencing, and prevents the transcription of FMRP (Santoro et al. 2012). FXS symptoms include development delay, learning disabilities and cognitive impairment. Almost 30% of FXS patients report symptoms of ASD. FXS is more common in males (1 in 4000) than in females (1 in 8000), and the symptoms are also more severe in males (Pembrey et al. 2001).

Various rodent models of FXS, where the *Fmr1* gene has been knocked out (KO), have been studied. These show abnormal neuronal morphology such as thin and elongated dendritic spines in pyramidal neurons, increased spine density, fewer and shorter neurites and smaller cell body volume (Comery et al. 1997; Antar et al. 2005; Cook et al. 2011; Kazdoba et al. 2014). In addition, electrophysiological changes were also reported in *Fmr1* KO. The most common were an impaired group I mGluR-dependent long-term depression (LTD) and a decrease in the frequency of spontaneous miniature excitatory postsynaptic currents (mEPSCs) (Bassell and Warren 2008). Another study revealed a significant decrease in the AMPA to NMDA ratio during early development in *Fmr1* KO (Pilpel et al. 2009). Taken together, these data suggest that loss of *Fmr1* leads to abnormal neuronal morphology resulting in synaptic dysfunction.

Human post-mortem studies of FXS have also revealed changes in neuronal morphology including significantly longer dendritic spines and fewer shorter

dendritic spines compared to control in cortical and temporal regions of the brain (Irwin et al. 2001). In vitro models using neural progenitor cells from aborted human foetuses have also reported fewer and shorter neurites and decreased cell soma size (Castrén et al. 2005; Bhattacharyya et al. 2008). A limitation to this method is that these cells have a very short lifespan in culture.

There have been a number of iPSC studies modelling FXS (Urbach et al. 2010; Sheridan et al. 2011; Doers et al. 2014). Some have reported the disruption of structural development of neurons. For instance, Sheridan et al. (2011) studied iPSC-derived neuronal progenitors from FXS patients that had fewer, shorter neurites and more compact cell bodies. Similarly, Doers et al. (2014) also reported deficits in neurite initiation and extension in iPSC-derived forebrain neurons from FXS patients. A recent study of neurons derived from FXS human embryonic stem cells (FX-hESCs derived from FXS blastocysts) described that FXS neurons could only fire single action potentials (APs) and were unable to discharge trains of APs. The APs had longer duration and reduced amplitude than controls. This suggested reduced inward Na^+ and outward K^+ currents (Telias et al. 2015). Furthermore, using the iPSC technology, advances are being made in the development of potential treatments for FXS. For example, FXS iPSCs have been used for a high-throughput screen to find compounds that could increase the expression of FMRP1 and reverse the phenotypes reported. Out of the 5000 compounds screened, six compounds were found to modestly increase the expression of FMR1 (Kumari et al. 2015), thereby revealing new therapeutic targets.

Lately, the use of gene editing (the CRISPR/Cas9 system) on CGG repeats for both ES cells and iPSCs derived from FXS patients restores *FMR1* gene expression. This was sustained in neural precursor cells and mature neurons. After the excision of CGG repeats, extensive demethylation was reported in CpG islands of the FMR1 promoter followed by an opening of the chromatin state and transcription initiation (Park et al. 2015). This has led to a great insight into the molecular mechanism underlying FXS.

3.4.2 Rett Syndrome and MECP2 Duplication Syndrome

Rett syndrome (RTT) is neurodevelopmental disorder that affects 1 in every 10,000 births in the USA (Chahrour and Zoghbi 2007). Symptoms of RTT appear after 6–18 months and include microcephaly, ataxia, seizures, ASD-like traits and hyperventilation. RTT is known to occur mostly in females and is caused by an X-linked dominant loss-of-function mutation in the *MECP2* gene encoding the methyl-CpG-binding protein 2. This protein binds to CpG dinucleotides and modulates transcription through histone deacetylase and the corepressor SIN3A (Amir et al. 1999; Renieri et al. 2003; Feldman et al. 2016). Importantly, increased dosage of *MECP2* can also be detrimental resulting in another severe neurodevelopmental condition called *MECP2* duplication syndrome (Ramocki et al. 2009; Vandewalle et al. 2009).

Marchetto et al. (2010) used an iPSC approach to study three female patients with missense mutations and one female patient with a frameshift mutation in *MECP2*. The neurons differentiated from patient lines reported fewer synapses, altered calcium signalling and decrease in frequency and amplitude of spontaneous current. Morphological deficits were also evident, with neurons having a reduced cell soma size and reduced spine density. Three studies—Ananiev et al. (2011), Cheung et al. (2011) and Kim et al. (2011)—all characterised iPSC lines from RTT syndrome patients and showed that neurons generated from these lines had a decrease in cell soma area and neuronal maturation deficits. Additionally, Djuric et al. (2015) derived the first isoform-specific RTT iPSC model. The neurons differentiated from this line had an inactive X-chromosome and only expressed the mutant allele. The patient neurons demonstrated a reduced dendritic complexity, cell capacitance and smaller soma size. Comparable phenotypes were also reported in RTT iPSC-derived neurons in a layered 3D hydrogel system (Zhang et al. 2016). The RTT patient neurons not only had morphological deficits as previously reported but also showed neuronal migration and maturation (reduced neurite outgrowth and fewer synapses) deficits (Zhang et al. 2016).

Recently, Williams et al. (2014) generated astrocytes from RTT iPSC patient lines. The study revealed that the mutant cells affected the development of neurons and caused functional abnormalities. These were rescued by the addition of IGF-1 and GPE (an IGF-1 peptide). Similarly, Delépine et al. (2016) reported that microtubule (MT)-dependent vesicle transport was altered in both murine MECP2-deficient astrocytes and human MECP2 p.Arg294* iPSC-derived astrocytes. These defects were restored by the administration of epothilone D, a brain-penetrant MT-stabilising natural product.

Finally, a recent study used a library of compounds to reverse the cellular and molecular phenotypes observed in *MECP2* duplication syndrome. Patient iPSC-derived neurons were used as a screening system to find potential therapeutic compounds such as the histone deacetylase inhibitor NCH-51, which rescued both morphological and neuronal network synchronisation phenotypes (Nageshappa et al. 2016).

3.4.3 Timothy Syndrome

Timothy syndrome (TS) results from a mutation in the *CACNA1C* gene, which encodes the voltage-dependent calcium channel $Ca_V1.2$ that mediates cytosolic Ca^{2+} increase in the brain and heart. Clinical manifestation of the syndrome includes congenital heart defects, tachyarrhythmias, syndactyly and neuropsychiatric features including global developmental delay, seizures and ASD (Splawski et al. 2004).

CACNA1C is a large gene of 50 exons producing multiple mRNA splice variants. Alternative splicing is tissue specific (Napolitano and Antzelevitch 2011) and probably the cause of the heterogeneous phenotype found in patients

with TS. Notably, mouse models of TS do not fully recapitulate the human disorder, in particular the early postnatal death typical of TS in human patients. This is probably because of the different electrical properties of mouse versus human cardiomyocytes (Bader et al. 2011). Therefore, human iPSCs represent an important opportunity to appropriately model the disorder. Since TS affects both heart and brain functions, iPSCs offer the additional advantage of studying cellular phenotypes in different cell types derived from the same patient.

TS-derived cortical neurons show wider action potentials and increased intracellular calcium levels suggesting defects in action potential firing and calcium signalling. Furthermore, alterations in activity-dependent gene expression, increased expression of upper cortical layer markers, decreased expression of callosal projection markers and increased expression of tyrosine hydroxylase with increased production of catecholamines were observed (Paşca et al. 2011). Krey et al. (2013) further found that mutations of $Ca_V1.2$ channels caused activity-dependent dendrite retraction in both rat and human neurons. However, this phenomenon was not due to a calcium-dependent mechanism but to insufficient levels of the small GTP-binding protein Gem, causing a massive activation of RhoA, a small GTPase. These findings strongly suggest that $Ca_V1.2$ is essential for the maturation of cortical neurons in humans.

In the search for an effective rescue, Paşca et al. treated TS-derived cells with roscovitine (Ros), a cycline-dependent kinase inhibitor and atypical L-type channel blocker. Treatment with Ros resulted in restoration of electrical properties of TS cardiomyocytes and reduction of tyrosine hydroxylase-positive neurons in TS-derived neuronal cultures without affecting the total fraction of cells expressing the neuronal maker MAP2 (Paşca et al. 2011).

3.4.4 Phelan-McDermid Syndrome and SHANK3-Associated ASD

Durand et al. (2007) described three autistic patients each carrying a *SHANK3* mutation. Two were inherited missense mutations, R12C and R300C, that both disrupted the gene's N-terminal ankyrin repeats, and the third was a de novo truncating stop mutation resulting in the loss of the gene's C-terminus. Likewise, Moessner et al. (2007) screened a cohort of 400 patients with ASD and found two deletions and one de novo mutation in *SHANK3* gene. These patients exhibited severe hyperactivity and absence of speech. Additionally, a Japanese population study looked at 128 ASD patients with *SHANK3* mutations, all displaying language development delay, hypotonia and mental retardation (Waga et al. 2011). Similarly, Boccuto et al. (2013) and Gauthier et al. (2009) both reported in their ASD cohorts that more than 2% of the patients exhibited a mutation in *SHANK3* and showed signs of extreme speech delay and repetitive behaviour. Although *SHANK3* mutations are reported to be one of the leading causes of monogenic autism (Kolevzon

et al. 2011; Nemirovsky et al. 2015), there are only a few studies that have specifically studied *SHANK3* mutations leading to ASD. Most studies have characterised the role of *SHANK3* in Phelan-McDermid syndrome (PMS), also known as 22q13.3 deletion syndrome, a neurodevelopmental disorder characterised by global developmental delay, speech delay, intellectual disability, poor motor coordination and ASD (Costales and Kolevzon 2015). Among the various genes deleted in this syndrome, *SHANK3* is considered the most likely candidate for causing the neurological abnormalities observed in patients (Phelan and McDermid 2012).

The cellular and molecular phenotypes associated with PMS have been studied for the first time in iPSC lines generated from two patients. The authors of this study found that iPSC-derived PMS cortical neurons have a reduced expression of SHANK3 and impaired excitatory synaptic transmission. Both amplitude and frequency of mEPSCs were significantly reduced. Furthermore, staining of synaptic proteins revealed a decrease in both pre- and postsynaptic puncta, suggesting that these neurons had fewer synapses. Interestingly, phenotypes could be rescued by either *SHANK3* overexpression or application of insulin-like growth factor 1 (IGF-1) (Shcheglovitov et al. 2013). Two other studies generated iPSCs from PMS patients and looked at both molecular and cellular phenotypes associated with these neurons. Vicidomini et al. (2016) described a decrease in Homer1b/c puncta in PMS patient-derived cortical neurons. Moreover, Bidinosti et al. (2016) used proteomics to identify changes in the phosphoproteome of SHANK3-deficient neurons. They found an increase in the activity of Cdc2-like kinase 2 (CLK2) due to downregulation of protein kinase B (PKB/Akt)-mammalian target of rapamycin complex 1 (mTORC1). Synaptic deficits were rescued using a CLK2 inhibitor.

Yi et al. (2016) used human ESCs to engineer both homozygous and heterozygous mutations of *SHANK3*. Cortical neurons differentiated from these lines showed severely impaired hyperpolarisation-activated cation (Ih) channels. In addition, these mutations caused alterations in synaptic connectivity and neuronal morphology.

3.4.5 Non-syndromic ASD

Investigating syndromic ASD caused by defined genes is extremely important in clarifying the underlying molecular and cellular mechanisms, but they account for less than 1–2% of patients. Most ASD cases have unknown genetic causes, creating a challenge in generating in vivo and in vitro models. Several studies in mice have shown that syndromic and non-syndromic ASD share synaptic phenotypes (e.g. see Baudouin 2013). Moreover, alterations in brain anatomy and neuronal connectivity found in ASD patients suggest that ASD has a common developmental origin. By capturing the genetic heterogeneity of ASD, iPSCs represent an important tool to investigate whether different genetic variants share common cellular and molecular neuronal phenotypes.

Griesi-Oliveira et al. (2015) generated iPSCs from a non-syndromic ASD patient carrying a de novo balanced translocation disrupting the *TRPC6* gene that encodes the transient receptor potential 6 channel, a voltage-independent, Ca^{2+}-permeable cation channel involved in dendritic spine formation (Tai et al. 2008; Zhou et al. 2008). They investigated the role of TRPC6 in neuronal development and synaptic function and further compared iPSC-derived neurons from the TRPC6-mutant (TRPC6-mut) individual with those of patients with RTT. Neurons derived from TRPC6-mut iPSCs showed diminished calcium influx, decreased dendritic arborisation and fewer spines and synapses compared to control neurons. Notably reduction of spine density and glutamatergic synapse loss are similar to those previously described for loss of MECP2 function in human neurons (Marchetto et al. 2010). Moreover, RTT-derived neurons have lower expression of TRPC6 (Griesi-Oliveira et al. 2015). The defective neuronal phenotypes were rescued by treatment with either hyperforin, a specific activator of TRPC6 channels, or IGF-1, a drug, which is able to rescue the neuronal defects found in RTT iPSC models (Marchetto et al. 2010). All these data suggest that the same pathways are affected in syndromic and non-syndromic ASD.

Mariani et al. (2015) compared cortical development of iPSCs derived from patients with idiopathic ASD with iPSCs derived from their unaffected family members using 3D neural culture organoids. The iPSC lines derived from ASD patients showed decreased cell-cycle length in earlier stages of cortical neural development, suggesting a generalised increase in proliferative potential. Moreover, cortical organoids derived from ASD patients showed a higher number of GABAergic neurons, suggesting an imbalance in the glutamate/GABA neuron ratio. The authors showed that overproduction of GABAergic neurons was due to FOXG1 overexpression and reversed by interfering with FOXG1 expression, suggesting that FOXG1 could represent a potential biomarker for ASD (Mariani et al. 2015).

Taken together, all of the aforementioned studies demonstrate that iPSCs are a good model to study ASD-related neuronal phenotypes of both syndromic and non-syndromic patients and are useful for potential drug screening. Rodent models do not fully recapitulate human neuronal functions and often fail to reveal toxicity and side effects seen in human cells. Since primary human neurons remain difficult to acquire, human iPSC-derived neurons fill an important gap. Potential therapeutics can be investigated for their ability to rescue disease-associated phenotypes, thereby aiding the discovery of new pharmacological targets for these disorders. Ultimately, individual iPSCs lines may allow drugs to be tailored for specific ASD cohorts and clinical phenotypes.

3.5 Limitations of iPSCs

While we are positive regarding the potential of iPSCs in the study of neurodevelopmental disorders, we are aware of their limitation. Line-to-line variability is an issue. Since each line is derived from a different individual, patient or control, lines differ genetically (and probably epigenetically) beyond the differences associated with the trait. Even lines from the same individual differ to a degree, and like all cultured cells, iPSCs will accumulate variation with time in culture, as well as during the reprogramming process itself. One means to overcome some of this variation is through the use of isogenic controls: genome-edited lines are genetically identical to their parent lines apart from the introduced genetic variation. Thus, they provide a good control. All these considerations mean that devising an appropriately powered and controlled iPSC experiment is an evolving challenge.

A more fundamental limitation is that cell lines do not provide behaviour. ASD is defined by altered behaviour, and this obviously cannot be observed in cells in culture. So rodent studies, despite their limitations, will always have this advantage in comparison with cellular models. Not only do iPSCs not provide behaviour, they don't actually provide any true brain structure. We can study cerebral cortical cells, but not cerebral cortex. We have discussed the emergence of truly histogenic 3D cultures and the advantages they might bring, but of course, there is a trade-off here. More complex structures increase veracity, but at the same time increase cellular diversity, thereby dissipating some of the advantages of a simple cellular system. These variables are at least under our control—increasingly as more differentiation protocols become optimised—and add power to experimental design. The limitations of the iPSC approach will be balanced by their construct validity: they are human cells carrying precisely the genetic variation associated with a clinical phenotype.

3.6 Conclusions

The studies cited in this review indicate that robust data is beginning to emerge suggesting there are cellular and molecular phenotypes associated with ASD that can be observed in dissociated patient-derived cells. Though all findings are not perfectly correlated with each other, there is consensus emerging, and significantly, this consensus includes findings arising from the mouse studies. Certainly, there is some ascertainment bias in this dataset: we are finding synaptic deficits because we are looking for them. There is tautology also: we take cells with a deletion in a synaptic gene, and unsurprisingly we find a synaptic phenotype. We are some way from understanding what this tells us about ASD. Stepping from these cellular findings (in conjunction with our animal data) to an explanation of human behaviour remains a substantial leap.

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Chapter 4
Modelling Autistic Features in Mice Using Quantitative Genetic Approaches

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4.1 Introduction

4.1.1 Clinical Definition of ASD

Autism spectrum disorder (ASD) is the name for a group of neurodevelopmental conditions that are clinically defined by impairments in social interaction and communication and by restricted, repetitive and stereotyped behaviour. Most autistic behaviours become manifest in the first years of life when the development of brain circuits becomes influenced by sensory experience. The autistic spectrum is highly heterogeneous with respect to severity, timing and variability of symptom manifestation (Fountain et al. 2012). Some patients show predominantly stereotyped behaviours, whereas others may mainly suffer from deficits in social behavioural development. In addition, patients can have co-morbidities, such as epilepsy, motor abnormalities and sleep problems.

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4.1.2 Prevalence

The prevalence of autism has been increasing since first studied, when about 4.1 of every 10,000 individuals were diagnosed with a form of ASD (Lotter 1966). This increase is partly a result of changes in diagnostic routines as well as improved awareness and recognition, but an increase in unidentified risk factors cannot be ruled out (Weintraub 2011). The median worldwide ASD prevalence is currently estimated at 1–2% based on recent surveys (Baron-Cohen et al. 2009; Mattila et al. 2011; Kim et al. 2011; Blumberg et al. 2013).

4.1.3 Treatment

At this moment, therapeutic options are mostly restricted to behavioural interventions, which have proven successful in a subset of patients, especially when applied in early stages (Rogers et al. 2014; Kasari et al. 2014). Clinical biomarkers to guide treatment and patient selection are lacking, although tracking eye movements and electroencephalogram (EEG) have shown promise (Jones and Klin 2013; Jeste et al. 2015). Further understanding of neurobiological abnormalities in individual patients is essential to guide patient selection for rational treatments.

4.1.4 Risk Factors

Epidemiological studies have identified various risk factors that contribute to ASD. Advanced paternal or maternal reproductive age is probably the most accepted and consistent risk factor (Hultman et al. 2011; Sandin et al. 2012). Additionally, gestational factors that could affect neurodevelopment, such as complications during pregnancy and exposure to teratogenic agents, have been suggested to increase risk of ASD (Gardener et al. 2009, 2011; Hultman et al. 2011; Sandin et al. 2012; Volk et al. 2013; Christensen et al. 2013; Canetta et al. 2014). Many diverse conditions reflecting general compromises to perinatal and neonatal health are also associated with increased risk (Gardener et al. 2011). Recently, prevalence of ASD has also been related to urbanisation and to parents who work in the sector of information technology (Roelfsema et al. 2012). Most cases are probably the result of interactions between genetic and non-genetic risk factors.

4.1.5 Genetics

Twin studies have suggested that ASD has high heritability (more than 80%) (Ronald and Hoekstra 2011). This heritability occurs in the context of environmental risks and gene–environment interplay, since monozygotic twin rates never reach 100% concordance. The genetic architecture of ASD is beginning to be elucidated, and the amount of ASD risk genes is now estimated to range between 400 and 1000 (Geschwind and State 2015; de la Torre-Ubieta et al. 2016). A key insight into the genetic basis of ASD came from the recognition of rare medical genetic syndromes due to single genetic disorders with high penetrance. Each of these clinical syndromes is found in less than 1% of patients with ASD; however, collectively they are estimated to be found in ~5% of the total ASD population (de la Torre-Ubieta et al. 2016).

Many ASD risk genes have been identified based on rare inherited and de novo variants (de la Torre-Ubieta et al. 2016). While contributing de novo variants are found in 10–20% of cases, about 50% of ASD liability can be attributed to common genetic variation (Klei et al. 2012; De Rubeis et al. 2014; Gaugler et al. 2014). Most genetic risk factors for ASD are also found in the general population, influencing a continuum of behavioural and developmental traits (Robinson et al. 2016). The rapid progress of genetics has fuelled the development of animal model systems to enable the identification of common molecular and cellular pathways in ASD. Homologs exist for most genes and a reasonable degree of functional conservation is generally assumed (Langen et al. 2011b).

Here, we will address the level of translatability of ASD behavioural characteristics in rodent species. Furthermore, we will describe novel possibilities to study genetic background variability in a controlled manner, as opposed to the single gene/single genetic background mouse models. We will further highlight how the combination of novel more sophisticated behavioural tests in combination with recently developed mouse genetic reference populations offers powerful strategies to disentangle the genetic origin of behavioural trait variation relevant to ASD.

4.2 Animal Models Based on Environmental and Genetic Findings

Studies into ASD have been complicated by a lack of known causal factors, poor access to brain tissue and the broad clinical definition of the disorder. A logical scientific progression was to develop animal models in order to overcome some of these limitations.

4.2.1 Lesion Studies

Initial modelling approaches in nonhuman primates emphasised social and communicative deficits following neuroanatomical lesions. Rhesus monkeys with neonatal lesions to the medial temporal lobe developed severe cognitive and socio-emotional deficits, including abnormal social interaction, absence of facial and body expressions and stereotypic behaviours (Bachevalier 1994, 1996). These monkeys were characterised by memory impairments in certain types of learning tasks, which may be similar to low-functioning patients with ASD. Further investigation indicated that monkeys with neonatal damage only to the amygdaloid complex were less impaired in memory function, while developing socio-emotional deficits similar to combined amygdalo-hippocampal lesions (Bachevalier 1994).

4.2.2 Foetal Valproate Syndrome

Another line of research emerged when increased frequency of ASD symptoms was observed in children exposed to valproic acid (VPA) during pregnancy (Christianson et al. 1994; Roullet et al. 2013). Prenatal treatment of Wistar outbred rats with VPA was found to result in behavioural abnormalities such as reduced frequency of social interactions, increased latency to social contact and reduced exploratory activity, as well as repetitive behaviour and stereotypic-like movements (Schneider and Przewlocki 2005). Prenatal VPA-treated animals are still being considered as an approach to model ASD pathology in rodents (see Roullet et al. 2013 for a review).

4.2.3 Genetic Disorders

Emphasis shifted to genetic models when genetic knowledge increased and molecular technologies for engineering rodent models expanded. In the 1990s, genetic causes of neurological syndromes strongly associated with ASD were being discovered, including causes of fragile X syndrome, tuberous sclerosis and Rett syndrome (Verkerk et al. 1991; European Chromosome 16 Tuberous Sclerosis Consortium 1993; van Slegtenhorst et al. 1997; Amir et al. 1999). Subsequently, mouse models of these human genetic disorders were developed, and brought new possibilities to study ASD patho-mechanisms in animal models (Moy et al. 2006). For instance, fragile X syndrome was found to be caused by a CGG-repeat expansion in the *FMR1* gene in 1991, and associated with ASD in 35% of cases (Verkerk et al. 1991; Hagerman 2006). Subsequently, *Fmr1* knockout mice

were found to develop patho-mechanistic hallmarks such as increased spine density, immaturity of dendritic spines and abnormalities in protein synthesis-dependent synaptic plasticity (Huber et al. 2002; Kazdoba et al. 2014). Further studies revealed that *Fmr1* knockout mice also develop abnormalities in behavioural and sensorimotor traits, including deficits related to the core symptoms of ASD such as abnormal social interaction and increased perseverance during reversal learning (Bernardet and Crusio 2006; Kazdoba et al. 2014).

These genetic and non-genetic examples of animal models (neonatal medial temporal lobe lesions in monkeys, prenatal exposure to VPA in rats, and modelling genetic disorders associated with ASD in mice) greatly improved our understanding of pervasive developmental disorders, but they also have their limitations.

4.3 Strengths and Limitations of Animal Models

Historically, three main validity criteria have been developed to estimate the strength of animal models, namely, construct, predictive and face validity (Nestler and Hyman 2010). The use of animal models for ASD is complicated by the large number of implicated genetic and environmental risk factors, and neurobiological and ethological differences between species.

4.3.1 Construct Validity

Construct validity is used to estimate whether the model is based on the same pathogenic process that causes the human disease. For instance, lesions to the neonatal medial temporal lobe in monkeys were considered useful, because this brain region was also associated with autism in the human population (Bachevalier 1994, 1996).

Construct validity of the VPA animal model for ASD is based on the observation that in humans, exposure during the first trimester of pregnancy leads to a sevenfold increase in the incidence of autistic symptoms (Roullet et al. 2013). To model this aetiology, rodents can be exposed to VPA during corresponding time windows. The relevance of this construct is supported by the observation that VPA use during pregnancy is common across countries (Roullet et al. 2013), but may be limited for people with ASD that were not exposed to VPA.

Construct validity of genetic animal models for ASD is largely based on evidence from human genetic studies (‘genetic validity’). In addition, *Fmr1* gene knockout mice also display molecular and neuropathological hallmarks that have been observed in human *post-mortem* brain tissue of fragile X patients (Kazdoba et al. 2014). The existence of Mendelian disorders associated with ASD enables the

analysis of large-effect size mutations associated with the disorder, in contrast to mental illnesses such as schizophrenia. However, large-effect size mutations are rare (each seen in less than 1% of cases), and their patho-mechanistic relevance to other forms of ASD remains to be estimated based on further evidence (Baudouin et al. 2012). At this moment, many different genetic animal models for ASD have been studied (Abrahams et al. 2013), each claiming construct validity for a subset of the total ASD population.

4.3.2 Predictive Validity

The predictive validity of animal models is estimated based on the similarities in response to treatments that are known to prevent or reverse symptoms in the human disease. Predictive validity is difficult to determine for ASD animal models, given the current lack of rational treatments for ASD core symptoms. Risperidone was the first drug approved by the US Food and Drug Administration (FDA) to treat irritability associated with ASD (McDougle et al. 2008). In the *Cntnap2* gene knockout mouse model for ASD, risperidone was shown to rescue hyperactivity, repetitive grooming and perseveration in a T-maze, while having no effect on social interaction deficits (Penagarikano et al. 2011). Further studies in *Cntnap2* knockout mice have been performed, suggesting that social outcomes could be improved by acute administration of oxytocin (Brunner et al. 2015; Penagarikano et al. 2015). Rational treatments for ASD core symptoms are yet to be established, rendering the use of predictive validity infeasible to estimate the strength of an ASD animal model. The chloride reducing agent bumetanide is an interesting candidate in this respect and has shown efficacy for ASD in a number of different studies (Lemonnier et al. 2012; Bruining et al. 2015).

4.3.3 Face Validity

Face validity is used to estimate similarities in symptoms between the animal model and the human disease. For example, behavioural deficits in *Fmr1* knockout mice have similarities to the behavioural abnormalities seen in fragile X syndrome patients. Face validity in animal models of ASD is a topic of debate, given the behavioural and evolutionary differences between humans and animal models (Hyman 2014; Liu et al. 2016). ASD is a neurodevelopmental condition characterised by multifactorial aetiology and behavioural functioning as the ultimate clinical endpoint. Perhaps somewhat underestimated is the difficulty of comparing mouse to human behaviour.

Mice and humans have a shared evolutionary path, except for the last 60 million years. The sharing of biological processes between mice and humans enables the use of mouse models to study human diseases (Schughart et al. 2013). On the other

hand, mice and humans differ in their behavioural manifestation, raising the question which aspects of neurobiology and behaviour are comparable across species. Some behavioural phenotypes are frequently observed in genetic mouse models for ASD (Kazdoba et al. 2015) and therefore deemed relevant. Detailed behavioural assays to probe core features of ASD have been developed (for review, see Kas et al. 2014); however, the feasibility to achieve such direct levels of face validity remains challenging.

4.4 Evolutionary Perspective of Inherited Behaviours

An alternative strategy to overcome evolutionary distances is to focus on naturally occurring behaviours that are important to the survival of an organism and its species. The hypothesis would be that the neurobiological mechanisms regulating these behavioural strategies are conserved across species.

Environmental pressure can lead to evolution of distinct inherited behavioural traits, similar to traits related to bodily structure and form (Lorenz 1958). A recent study into behavioural evolution concerned differences in burrowing behaviour in two sister species of *Peromyscus* mice (Weber et al. 2013). Old field mice (*Peromyscus polionotus*) mostly inhabit open fields and construct unique burrows characterised by a long entrance tunnel and secondary tunnel that may serve as an escape to predators such as snakes. These mice have recently diverged from deer mice (*Peromyscus maniculatus*), which inhabit prairie and forest habitats and build *small and single-tunnel* burrows. Burrow length and the presence of an escape tunnel were studied under controlled laboratory conditions, and genetic analyses in offspring hybrids indicated four genetic loci that independently affected either burrow length or the presence of an escape tunnel. These results indicate that 'extended' and complex behaviours can evolve through evolutionary mechanisms of behavioural adaptation (Weber et al. 2013). Indeed, survival of a species in its environment depends on the proper behavioural responses to external cues.

Social interaction and communication are essential for an animal to find the best available reproduction partners and to deal with threats such as natural predators and attacks from conspecifics (Kas et al. 2007). Social cues in rodents differ from those in humans and have a differential effect on how they socially engage. For instance, mice guide their behavioural responses on the basis of olfactory signals from the environment (Keverne 2002; Silverman et al. 2010), where humans largely depend on verbal language and visual cues. Mice display vocal communications, although it is unknown to what extent these signals in mice exert a communicative function as does language in humans.

4.4.1 Communication and Social Behaviour

Different assays have been proposed to study aspects of social behaviours in mice (Silverman et al. 2010). For example, social approach is most studied in the three-chambered apparatus, through comparison of the amount of exploration of a novel conspecific placed in a wired cage versus a wired cage without conspecific (Nadler et al. 2004). Perhaps more naturalistic, reciprocal social interactions can be assessed in freely moving animals by scoring the amount of social behaviours such as nose-to-nose sniffing, anogenital sniffing and allogrooming (Silverman et al. 2010). These readouts can be used to evaluate short-term and long-term social recognition, by comparing the preference for a novel mouse over a familiar conspecific (Pearson et al. 2010; Bruining et al. 2015). Abnormalities in social approach and recognition have been described in a variety of genetic mouse models for ASD, including *Cntnap2*, *Nlgn1*, *Nlgn4*, *Pten*, *Shank2* and *Shank3* mutants (Kazdoba et al. 2015).

New approaches to study social group dynamics over time are also being implemented. For example, in a recent study, automated longitudinal tracking of animal colonies has been used to study social dominance in BTBR T+tf/J mice in a seminatural environment (Weissbrod et al. 2013). In this setup, integrated radio-frequency identification (RFID) tagging and video data enabled the analysis of locomotor behaviours in multiple socially interacting animals. Commonly observed social interactions, such as avoidance, being avoided, chasing and being chased, could be detected, and quantification of social dominance indicated reduced social hierarchy in groups of BTBR T+tf/J mice compared to control animals (Weissbrod et al. 2013).

4.4.2 Stereotyped and Restricted Behaviours

Another behavioural domain that is effected in ASD relates to restricted, stereotyped and repetitive behaviours and resistance to change. Fixed and repeatedly performed action patterns form a vital part of typical development and normal functioning across species (Langen et al. 2011b). Repetition is essential to the survival of lower species such as invertebrates, birds and rodents, and occurs in higher animals as part of normal behaviour to acquire skilled acts through practice (Turner 1999; Langen et al. 2011a). In typically developing young children, repetitive behaviours decrease over time (Langen et al. 2011b). In individuals with ASD, daily life is severely impaired by high levels of atypical stereotyped and repetitive behaviours and inappropriate adherence to interests and routines (Langen et al. 2011a; Fountain et al. 2012).

Abnormal repetitive and stereotypic behaviours are also seen in other species, for example, following environmental distress. This may be expressed as pacing in birds, rocking and self-injurious behaviour in monkeys and repetitive jumping, somersaulting and excessive grooming in mice. Some species of mice also display

repetitive behaviours under typical laboratory conditions. For instance, deer mice (*Peromyscus maniculatus*) display high levels of spontaneous stereotypic vertical jumping and backward somersaulting when reared in standard rodent cages (Tanimura et al. 2008, 2011). Interestingly, deer mice display natural variation in the expression of stereotypic behaviours, perhaps similar to continuous variation in autistic traits in the human population. In addition, high rates of stereotypies were correlated with poor performance in a T-maze reversal learning task and associated with decreased activity of the indirect basal ganglia pathway (Tanimura et al. 2008; Tanimura et al. 2011). Indeed, cognitive inflexibility and adherence to routines have also been associated with repetitive behaviours in ASD (Langen et al. 2011b)

Repetitive behaviours in genetic mouse models for ASD are predominantly assessed by the analysis of self-grooming (Silverman et al. 2010). For example, increased levels of self-grooming have been described in many genetic models, including in *Shank1*, *Shank2 and Shank3* gene knockout mice (Kalueff et al. 2016). In addition, repetitive and perseverative behaviour is assessed in the marble-burying assay based on the number of marbles buried underneath the bedding material within a defined period of time (Thomas et al. 2009). Cognitive flexibility has been assessed in genetic mouse models of ASD using a variety of reversal learning paradigms (Kas et al. 2014). For instance, choice learning and reversal may be based on olfactory, textural, visual or spatial differences between cues associated with a reward (Bissonette and Powell 2012; Brigman et al. 2013; Molenhuis et al. 2014). Additional assays are being developed to assess other relevant aspects related to inflexibility and repetitive behaviours, such as repetitive locomotor patterns, restricted interests and resistance to change (Bonasera et al. 2008; Pearson et al. 2011; Karvat and Kimchi 2012). These assays may be used to further understand the impact of ASD-related genetic variation on the development of repetitive behaviours in ASD. However, it is currently unknown how the expression of stereotyped and repetitive behaviours in rodents relates to those observed in ASD.

4.4.3 Behavioural Development

Regardless of *what* to measure, another issue is *when* to measure. A striking aspect of ASD studies is that the majority of studies characterises genetic models at adult ages, despite the fact that ASD is regarded as a neurodevelopmental condition. Mouse models offer unique opportunities to study behavioural development, because mice reach their adult stage at around 8 weeks after birth. In such a developmental analysis, BTBR T+tf/J mice and C57BL/6J control were found to display similar levels of grooming behaviour during preadolescence (4 weeks of age), but only BTBR T+tf/J mice showed increased amounts of repetitive behaviours from adolescence into adulthood (6, 8 and 10 weeks of age) (Molenhuis et al. 2014). These findings indicate that mice can show distinct developmental

trajectories of repetitive behaviours that may be relevant to those observed in the ASD patient population (Fountain et al. 2012).

4.4.4 Genetic Background and Genotype–Phenotype Relationships

Genotype–phenotype relationships are typically investigated in a single mouse inbred strain genetic background. However, a disadvantage of this approach is that the behavioural expression of a genetic disorder may very well depend on the genetic background composition of the individual (Sittig et al. 2016).

For example, expression of ASD-related behaviours in *Fmr1* knockout mice depends on the genetic background strain in which the mutation was tested (Pietropaolo et al. 2011). Deficits in social interaction and sensory hyperresponsiveness were observed on both C57BL/6 and FVB backgrounds, while aggressive tendencies and expression of repetitive behaviours were only observed in *Fmr1* knockout mice with the C57BL/6 background.

In another study, female mice with heterozygous *Fmr1* knockout in the C57BL/6J background were bred with males from six different inbred strains (A/J, DBA/2J, FVB/NJ, 129S1/SvImJ, C57BL/6J and CD-1) (Spencer et al. 2011). Across hybrids, motor stereotypies and increased marble burying were only observed in *Fmr1* knockouts with the DBA/2J background. Moreover, social interaction was only effected by deletion of *Fmr1* in the DBA/2J or C57BL/6J hybrid background, and passive social behaviours were only decreased in hybrids derived from the 129S1/SvImJ inbred strain. Genetic background effects on genotype–phenotype relationships were also suggested in other genetic models for ASD, including in *Nlgn3* (Tabuchi et al. 2007; Chadman et al. 2008) and *Shank3* (Peca et al. 2011; Mei et al. 2016) mutants.

Some phenotypes may be more resistant to genetic background effects than others. For example, sensory hyperresponsivity in *Fmr1* knockout mice is commonly observed across genetic backgrounds, which may indicate that this phenotype is more closely related to the genetic dysfunction. These examples show that genetic background and the choice of phenotypes are critical to the study of genotype–phenotype relationships in the context of ASD.

4.5 Forward Genetic Approaches in Mice

Current approaches to study ASD-related behaviours in mouse models are practically limited by the number of genes that can be studied and the amount of behavioural outcome measures that are assessed. Would it be possible to screen

all ASD-implicated human genetic mutations through an extensive behavioural testing battery, and across a wide variety of genetic backgrounds?

Indeed, human genetic studies in ASD has indicated an overwhelming diversity in the genetic landscape, with between 400 and 1000 genes estimated to be involved (Geschwind and State 2015). Rare variants have a large contribution to disease risk on an individual level, but their relevance to individuals with a different mutation, or common genetic susceptibility, may be limited. Common genetic variation may play a role in many cases but likely has a low contribution on an individual level. Understanding how all these genetic variants contribute to the expression of autistic behaviours and co-morbidities is a great challenge.

Given practical limits to the number of genes and genetic backgrounds to be studied via reverse genetic approaches in mice, systematic analysis of genotype–phenotype relationships is challenging. Another strategy to study the relation between genotypes and the expression of ASD-related behavioural phenotypes in mice would be to start with behavioural expression in mice and systematically investigate the impact of natural genetic variation, including in genes related to ASD.

4.6 Continuous Variation in ASD-Related Behavioural Traits

Autistic traits are continuously distributed in the general human population, and influenced by common and rare genetic variation implicated in ASD (Gaugler et al. 2014; de la Torre-Ubieta et al. 2016; Robinson et al. 2016). The distribution of autistic traits and associated genetic variation in the general human population suggests the possibility to study ASD-related traits in animal populations, by making use of natural genetic variation (Kas et al. 2009).

Behavioural variation is commonly observed across different inbred and outbred strains of mice. For example, the FVB/NJ strain expresses very high levels of social interaction, while A/J and BTBR T+tf/J strains are notorious for low sociability (Bolivar et al. 2007). Strain differences have also been observed in mouse exploratory behaviours and reversal learning paradigms (Moy et al. 2008). Sociability and reversal learning were found to dissociate across strains (Moy et al. 2007), which may parallel similar ‘fractionation’ of ASD-related traits across humans (Ronald and Hoekstra 2011).

These innate behavioural differences can be used to study behavioural phenotypes in mice as a quantitative trait to identify natural genetic variants contributing to continuous trait variation. For example, multiple quantitative trait loci (QTLs) for sociability and juvenile social interaction were identified using an F2 intercross of the BTBR T+tf/J and C57BL/6 inbred strains (Jones-Davis et al. 2013). The strongest detected QTL explained 10% of behavioural phenotypic variation, suggesting that social behaviour abnormalities in BTBR T+tf/J mice are caused

by a variety of genetic loci, each explaining a fraction of continuous behavioural trait variation between BTBR T+tf/J and C57BL/6 inbred strains.

In another study, genetic mapping of social recognition memory using chromosome substitution strains (CSS) revealed specific involvement of the *Pcdh9* gene in social recognition memory and sensory cortex development. This gene had previously been associated with ASD, indicating that social recognition in mice is influenced by natural genetic variation with relevance to ASD in humans (Bruining et al. 2015).

4.6.1 Modelling Genetic Diversity

A powerful tool to further dissect the genetic and neurobiological underpinning of behavioural trait variation is through the use of genetic reference populations. For example, the BXD panel consists of a large collection of mouse recombinant inbred lines with a 'mosaic' genetic structure derived from C57BL/6J and DBA/2J founder strains and captures about 10% of the known natural genetic variations in mice (Peirce et al. 2004). Forward genetic analysis in 51 BXD inbred lines led to the discovery of a reversal learning QTL on chromosome 10, and subsequent analysis of available brain mRNA levels identified that expression of *Syn3*, *Nt5dc3* and *Hcfc2* correlated with the reversal learning phenotype (Laughlin et al. 2011). Reversal learning in these lines was also correlated with expression of ventral midbrain dopamine D2 receptors, which had previously been reported in the same BXD inbred lines (Jones et al. 1999; Laughlin et al. 2011; den Ouden et al. 2013; Klanker et al. 2013).

The Collaborative Cross (CC) is a promising novel genetic reference population that consists of mouse recombinant inbred lines derived from eight founder strains. The CC population captures 90% of the known genetic variation in mice, which is equal to about two times the number of common genetic variants in the human population (Churchill et al. 2004; Keane et al. 2011; Hall et al. 2012; Schughart et al. 2013; Gralinski et al. 2015). In incipient CC lines, investigation of general behavioural traits indicated wide-ranging variation (Aylor et al. 2011). Moreover, the CC population was found to have strong power to identify quantitative trait loci underlying complex traits (Durrant et al. 2011). Three of the eight founder strains of the CC population were wild-derived. Indeed, the presence of wild-derived genetic variation gives unprecedented opportunities to study the genetic basis of fitness-driven behavioural responses, as classical laboratory strains were obtained after decades of human-driven artificial selection, inbreeding and adaptation to captivity (Chalfin et al. 2014; Chesler 2014).

Hundreds of genes contribute to ASD liability, and the additive effect of common genetic variation plays a major role. In mouse populations, natural genetic variants may contribute to variation in the expression of ASD-related behaviours. Given the homology between human and mouse genes, and the myriad of genes involved in ASD, it is likely that genes involved in ASD in humans overlap with genes

influencing ASD-related behaviours in mice. However, the extent of this overlap remains to be resolved.

Forward genetic approaches in genetic reference populations such as the BXD or the CC population can also be used to investigate molecular processes underlying behavioural trait variation relevant to ASD. For instance, it would be possible to investigate the behavioural correlates of natural variation in mTOR signalling levels, fatty acid metabolism, or oxytocin signalling. Integration of reverse and forward genetic approaches (Williams and Auwerx 2015) may aid to define the genetic relevance of phenotypic readouts for ASD-related features in animal studies.

4.7 Conclusions

Behavioural genetic studies in rodents can highly contribute to the understanding of behavioural trait variation relevant to autism spectrum disorder (ASD). In this review, the value of both forward and reverse genetic behavioural approaches has been addressed. Generally speaking, reverse genetic strategies investigate the impact of candidate human risk genes on behavioural phenotypes using genetically modified rodents with a fixed genetic background. In contrast, forward genetic strategies make use of genetic background variation to identify natural genetic variants contributing to behavioural trait variation in the mouse population. These approaches allow for systematic studies under controlled genetic and environmental conditions. However, the challenge is the selection of behavioural phenotypes and testing paradigms in view of translatability to the human ASD core features of social interaction deficits and stereotyped and restricted behavioural expression.

4.7.1 Behavioural Responses and Evolutionary Conserved Processes

Thus far, a wide variety of behavioural testing paradigms have been proposed to capture face validity for ASD-related behavioural characteristics (Silverman et al. 2010; Kas et al. 2014). Interestingly, the application of these testing paradigms in reverse genetic approaches has revealed behavioural deficits in a wide variety of mouse line mutants for human ASD risk genes. For example, *Fmr1* gene knockout mice showed aberrant social and repetitive behaviours, such as social recognition deficits and stereotyped behaviours in a marble-burying task. The outcome of these studies was variable and depended on the genetic background on which the gene knockout was generated. To address controlled genetic background variability in a systematic manner, newly generated mouse genetic reference populations, such as

chromosome substitution strains, recombinant inbred lines and the Collaborative Cross, have been generated. Applying these forward genetic strategies to a select set of phenotypes will provide insights in behavioural trait variation relevant to ASD.

Here, we propose that the selection of phenotypes that contribute to survival of a species may be a way forward to identify behavioural trait variation relevant to ASD. For example, by focusing on essential behavioural strategies, such as social recognition, candidate genes that contribute these phenotypes (e.g. the *Pcdh9* gene) have recently been identified. Subsequent studies using a reverse genetic approach with a *Pcdh9* mutant mouse line indicated a possible link between essential social behavioural phenotypes, sensory cortex development and sensory information processes (Bruining et al. 2015). In this way, translational biological processes that are relevant to ASD may be identified for further functional studies.

4.7.2 Quantitative Biological Parameters

Behavioural genetic studies provide novel insights into the relationship between behavioural and genetic variations relevant to our understanding of neurobiological and evolutionary mechanisms. However, the value of face validity with respect to translational behavioural phenotypes in rodents and human psychiatric disorders has been challenged (Kas et al. 2009) and may be limited by, for example, the lack of our understanding of species-specific behavioural responses to environment cues. To optimise the drug discovery process for neuropsychiatric disorders using integrated clinical and preclinical studies and to improve treatment efficacy, newly developed quantitative biological parameters that functionally relate to behavioural expression, such as translational measures of (EEG) neural activity, are being identified. These steps may be important to eventually parse current heterogeneous syndromes into homogeneous clusters (Insel and Cuthbert 2015).

4.7.3 Behavioural Trajectories and Therapeutic Intervention

Finally, understanding the relationship between behavioural trait variation and the large amount of ASD risk genes has to take into account the level of spatial and temporal expression of these ASD risk genes in the brain (Delorme et al. 2013). Certain ASD risk genes may be expressed at particular developmental stages that are critical for neural circuit formation. Consequently, abnormal ASD risk gene expression at these developmental stages may contribute to aberrant neural circuit formation and subsequent behavioural output. The question remains whether these developmental stages are critical in the treatment of ASD. Future rodent models

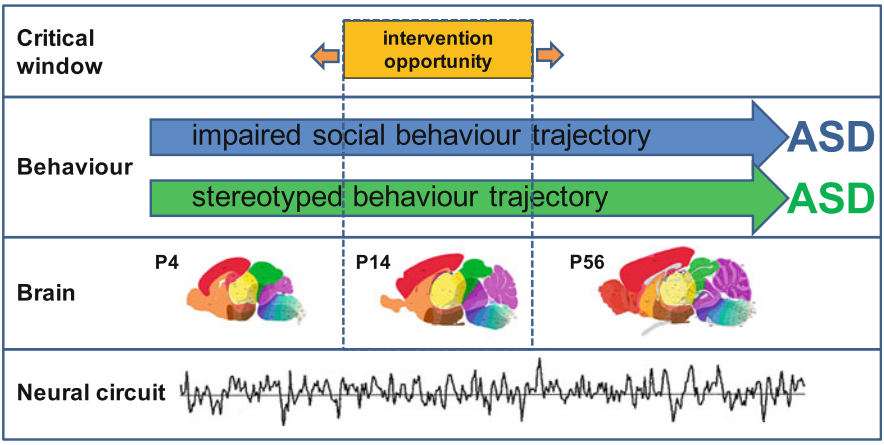

Fig. 4.1 Behavioural trajectories and therapeutic intervention. Behavioural heterogeneity in ASD has been highlighted by the identification of distinct developmental trajectories for social behaviour and for stereotyped and restricted behaviours. Understanding the pathogenic processes underlying these trajectories will be important to improve treatment efficacy through the development of aetiology-directed interventions. For example, are there specific developmental stages during which these trajectories can be reversed? Conditional (temporal and spatial) gene activation methods (Guy et al. 2007) and pharmacological interventions applied in mice at distinct developmental stages will be important to determine critical windows of treatment opportunity for these developmental trajectories of aberrant behavioural phenotypes. In addition, studying treatment efficacy will highly benefit from neural biomarkers that are proximal to the biological cause of aberrant behavioural phenotypes and that show drug target engagement. EEG neuronal activity assessments may fulfil these requirements in a biologically relevant and translational manner. The figure panel cartoon contains three brain structure diagrams from the Allen Brain Atlas (http://www.brain-map.org/)

may offer the possibility to address this question by means of conditional (temporal and spatial) risk gene expression or deletion and by pharmacological intervention studies during and outside these potentially critical windows of development (Fig. 4.1).

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Chapter 5
Behavioural Phenotypes and Neural Circuit Dysfunctions in Mouse Models of Autism Spectrum Disorder

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5.1 Introduction

Autism spectrum disorder (ASD) represents a heterogeneous group of neurodevelopmental disorders characterised by alterations in social behaviours including social interaction and social communication and stereotyped behavioural patterns and restricted interests (American Psychiatric Association 2013). These

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disorders affect more than one person in a hundred (Elsabbagh et al. 2012; US Department of Health and Human Services 2014). The most frequent comorbidities are epilepsy, attention deficit and hyperactivity disorder (ADHD) and sleep and anxiety disorders. Several hundred genes have been associated with ASD, and the majority of them encode chromatin remodelling and synaptic proteins (Toro et al. 2010; Huguet et al. 2013; Bourgeron 2015). These proteins are involved in neuronal activity-dependent gene regulation and synaptic plasticity (Bourgeron 2015). At the synapse, mutations affect cell adhesion molecules (*NLGN1-4*, *NRXN1-2*, *CNTN4-6*, *CNTNAP2-4*), scaffolding proteins (*SHANK1-3*) or channels and receptors involved in synaptic transmission (*CACNA1*, *GRIN2B*, *GABRB3*, *GABRA3*).

Mouse models carrying mutations in the murine orthologous genes were generated to better understand the mechanisms leading to ASD-like traits (Crawley 2012). These models should also facilitate the understanding of the different neuronal circuits mediating ASD-related phenotypes. At the behavioural level, two core symptoms of ASD can be evaluated in mice, namely, impaired social interactions and stereotyped behaviours (Silverman et al. 2010). Adding to a previous review on mouse models of ASD (Ey et al. 2011), we will focus on social behaviours and stereotyped behaviours and will further aim at linking them with functional and structural impairments.

5.2 Social Behaviours

In social interactions, two aspects can be distinguished. First, social motivation reflects the interest to interact with a conspecific, as shown by the active seeking of social contacts. This is usually measured by the time spent in contact with another mouse. Second, social recognition reflects the ability to distinguish between conspecifics as shown by different behavioural reactions to different individuals. This is usually measured by the decrease of sniffing duration over successive presentations of a conspecific. Social interactions are regulated by social signals, forming social communication. In mice, these signals include ultrasonic vocalisations, odours, pheromones, tactile stimuli involving whisking and body contacts and visual signals using body postures (Latham and Mason 2004; Brennan and Kendrick 2006; Portfors 2007).

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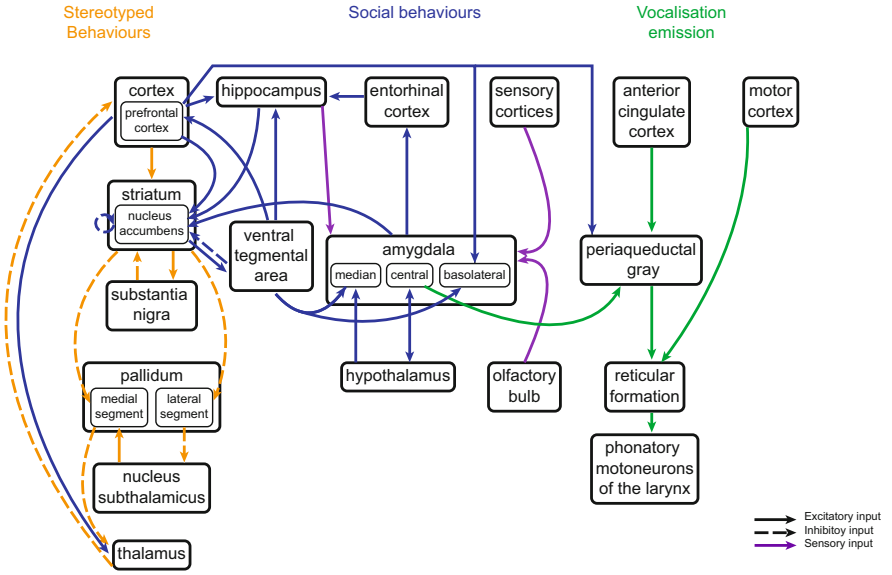

Fig. 5.1 Cortical and subcortical neuronal circuits involved in stereotyped behaviour (*left panel*), social behaviours (*central panel*) and vocal behaviour (*right panel*)

Social behaviours involve complex structural connections within the brain (Fig. 5.1; summary based on Kas et al. 2014; Wang et al. 2014a, b; Soden et al. 2016 for social interactions, and Jürgens 2002, 2009; Arriaga et al. 2012 for ultrasonic vocalisation emission).

Social interactions can have a positive or negative valence. The amygdala plays a crucial role in processing this valence in emotion-based social interactions, such as arousal, freezing and aggression. The medial amygdala is activated by positive or negative socially relevant stimuli, used in social recognition (Bosch and Neumann 2012). Oxytocin, one of the major neurotransmitters in the amygdala, favours social recognition and social bonding (Ferguson et al. 2000). The medial amygdala receives sensory information from the olfactory bulb (Keverne 1999), the preoptic area or the septum (Popik and van Ree 1991). The basolateral amygdala is involved in the processing of emotional stimuli like fear (Maren 2003). This structure projects to the mesolimbic system via the nucleus accumbens to reinforce social stimuli. It also projects to the brainstem, the entorhinal cortex (involved in the assessment of familiarity in stimuli), the prefrontal cortex (social hierarchy), the midbrain reticular formation and the periaqueductal grey (Price 2006). The activity of the amygdala is modulated by reciprocal connections with the hypothalamus.

The hypothalamus integrates cues about the external social environment and the internal evaluation of the affective state (Kruk et al. 1979). It is involved in the choice of the appropriate type of interaction that should be initiated. The medial amygdala projects to the hypothalamus, sending information about the valence of the stimuli, while the hypothalamus projects back on the medial amygdala and the

central amygdala to switch between aggressive and affiliative responses (Veening et al. 2005). The hypothalamus also projects to the nucleus accumbens.

The nucleus accumbens is part of the mesolimbic dopamine system, which reinforces functional social behaviours (Chevallier et al. 2012). It has reciprocal connections with the ventral tegmental area and the ventromedial prefrontal cortex, two other parts of the mesolimbic dopamine system (Russo and Nestler 2013). This system, also called the reward system, is involved in the mediation of social investigation, mating, maternal behaviour and formation of pair bonds (Robinson et al. 2011; Kohls et al. 2013).

The prefrontal cortex organises the temporal sequences of actions, taking into account the rapid changes in the social environment and in the emotional state of the animal (Fuster 2008). The dorsomedial prefrontal cortex, one of the major structures implicated in the establishment and maintenance of social hierarchy and dominance among littermates, projects to the basolateral amygdala (Wang et al. 2014a).

In mice, ultrasonic vocalisations are considered to be innate with very few if any evidence of learning (Hammerschmidt et al. 2012; Portfors and Perkel 2014). Mice voluntarily control the emission or absence of emission of a vocalisation through the anterior cingulate cortex, projecting to the periaqueductal grey, which itself projects to the reticular formation towards phonatory motoneurons. It is still unclear whether mouse ultrasonic vocalisations can be voluntarily modulated by the motor cortex (acting on phonatory motoneurons) (Hammerschmidt et al. 2015).

5.3 Stereotyped Behaviours

Stereotyped behaviours occur in mice under different forms: excessive self-grooming, repetitive climbing, rearing or jumping in specific locations of the cage and atypical digging in the bedding (Kelley 2001; Lewis et al. 2007). Stereotyped behaviours vary in kind but also in degree. For instance, some mouse strains mutated in ASD-risk genes perform self-grooming behaviours to such an extent that they get self-injured (e.g. *Shank3* mutant mice, Peça et al. 2011).

The basal ganglia play a crucial role in the development of stereotyped behaviours (Fig. 5.1). These structures include the striatum (caudate nucleus and putamen; e.g. Langen et al. 2011), the pallidum (globus pallidus), the nucleus subthalamus and the substantia nigra and are functionally interconnected. The basal ganglia are involved in fine-tuning of the motor impulse. Cortical and subcortical structures such as the hippocampus and the amygdala are also involved in the emergence of stereotyped behaviours (Bachevalier 1996). Cortico-striatal circuits receive multiple inputs, and each loop consists of two distinct pathways: the striatonigral “direct pathway” and the striatopallidal “indirect pathway”. Activation of the direct pathway results in an increase in thalamic activity, whereas activation of the indirect pathway results in thalamic inhibition. This means that under normal circumstances the direct pathway enhances specific movement behaviour, while the

indirect pathway inhibits specific movement. The cortico-striatal circuitry can be manipulated by targeting agents, which bind to inhibitory GABA receptors or to excitatory glutamate receptors (e.g. Presti et al. 2004). Dopaminergic drugs can also modulate stereotypic behaviours by stimulating the direct pathway or inhibiting the indirect pathway (Presti et al. 2003; Mason and Rushen 2006). Taken together, the basal ganglia are involved in fine-tuning of the required movement via the direct (activation) and indirect (inhibition) pathways. Any imbalance in those circuits can result in the emergence of stereotyped behaviours.

5.4 Meta-Analysis of ASD Mouse Models

For our meta-analysis, we scanned and collected literature from the PubMed (NCBI) database and from Web of Science. We used combinations of the following keywords: "*Nrxn1*", "*Nrxn2*", "*Nlgn1*", "*Nlgn2*", "*Nlgn3*", "*Nlgn4*", "*Shank1*", "*Shank2*", "*Shank3*", "*Cntnap2*", "*Cntnap4*", "*Cntn4*", "*Big2*", "*Cntn5*", "*NB-2*", "*Cntn6*", "*NB-3*", "*Cacna1*", "*Grin2b*", "*Gabrb3*", "*Gabra3*", "*Glra2*", "autism", "ASD", "behaviour", "mouse model" and "social". We only gathered and analysed the main studies for mouse models carrying mutations in synaptic channels and receptors, given the high number of articles for these models. For the present study, we gathered data from all age classes (pups, juveniles, adults), even if data were unbalanced for each age class.

We synthesised data on behavioural, structural and functional aspects into a table (available upon request to the corresponding author), according to the construction characteristics of the model (e.g. knockout, conditional knockout, knock-in, duplication, deletion and overexpression), the genetic background (using the MGI database) and the phenotypical traits studied. For the structural and functional aspects, we examined brain anatomy, protein expression in the different brain regions and synaptic physiology. For the behavioural aspects, we took into account social interactions (motivation and recognition) and communication as well as stereotypes.

To conduct a clustering analysis, we scored the data with the following criteria. When the trait was not examined in the model, the score was NA. These missing data were not considered. When the mutant mice did not differ significantly from wild-type animals, the score was 1. When the mutant mice differed significantly from wild-type animals (either significantly higher and/or lower performances/scores in mutant than in wild-type animals), the score was 2. When differences between mutant and wild-type mice diverged between studies (no significant difference and significantly higher and/or lower performances/scores in mutant than in wild-type animals), the score was 1.5.

Clustering was performed with the statistical software R (R Core Team (2016)) by using the *heatmap.2* function. This function connects the most similar mouse models according to the scoring of each trait. In the complete data set, the amount of missing data was large. To reduce the bias of clustering according to missing data,

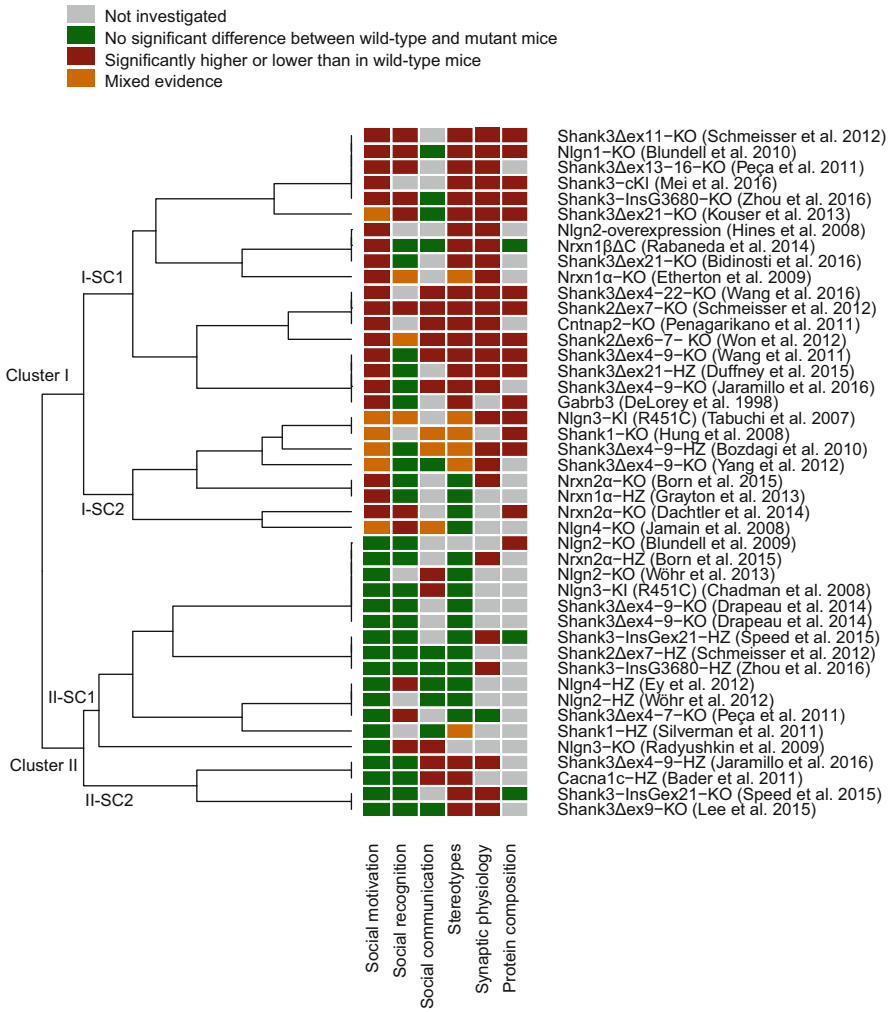

Fig. 5.2 Heatmap representation of the clustering of mouse models for ASD carrying mutations in synaptic proteins and their phenotypic traits as indicated (social interest/motivation, social recognition, social communication, stereotypes, synaptic physiology, protein composition)

we conducted the clustering analysis with the most extensively studied models (three or more behavioural and/or structural and functional traits tested). Most models with mutations in genes encoding synaptic channels and receptors failed to reach the threshold. The most extensively studied mouse models carried mutations in cell adhesion and scaffolding proteins. The heatmap (Fig. 5.2) distinguished two main clusters. The first one encompassed two subclusters (I-SC1 and I-SC2) and the second one also two subclusters (II-SC1 and II-SC2).

5.4.1 Cluster I

Cluster I includes mouse models affected in almost all traits examined. Compared to wild-type littermates, the majority of these mutant mice display reduced social interest, increased stereotyped behaviours and impaired synaptic physiology and atypical synaptic protein composition in the brain regions examined.

The *subcluster I-SC1* includes 18 animal models [*Shank3-cKI* (Mei et al. 2016), *Nlgn1-KO* (Blundell et al. 2010), *Shank3Δex11-KO* (Schmeisser et al. 2012; Vicidomini et al. 2016), *Shank3-InsG3680* (Zhou et al. 2016), *Shank3Δex13-16-KO* (Peça *et al*. 2011), *Shank3Δex21-KO* (Kouser et al. 2013; Duffney et al. 2015), *Nlgn2* overexpression (Hines et al. 2008), *Nrxn1βΔC* (Rabaneda et al. 2014), *Shank3Δex21-KO* (Bidinosti et al. 2016), *Nrxn1α-KO* (Etherton et al. 2009; Grayton et al. 2013), *Shank3Δex4-22-KO* (Wang et al. 2016), *Shank2Δex7-KO* (Schmeisser et al. 2012; Ey et al. 2013; Ferhat et al. 2016), *Cntnap2-KO* (Peñagarikano et al. 2011, 2015), *Shank2Δex6-7-KO* (Won et al. 2012; Lee et al. 2015a), *Shank3Δex4-9*-KO (Wang et al. 2011, 2014b), *Shank3Δex21-HZ*, *Shank3Δex4-9-KO* (Jaramillo et al. 2016), *Gabrb3-KO* (DeLorey et al. 1998, 2008)]. All models of the subcluster I-SC1 displayed a decrease in social interest; only one of the *Shank3Δex21-KO* mutant lines (Kouser et al. 2013; Duffney et al. 2015) displayed mixed evidence for impairment in social motivation in comparison with wild-type mice. Furthermore, only *Shank3Δex11-KO* (Schmeisser et al. 2012; Vicidomini et al. 2016), *Shank3-InsG3680* (Zhou et al. 2016), *Shank3Δex13-16-KO* (Peça et al. 2011) and both *Shank2Δex7-KO* (Schmeisser et al. 2012; Won et al. 2012; Ey et al. 2013; Lee et al. 2015a; Ferhat et al. 2016), *Nlgn1-KO* (Blundell et al. 2010), *Shank3Δex21-KO* (Kouser et al. 2013; Duffney et al. 2015) and *Nrxn1α-KO* (Etherton et al. 2009; Grayton et al. 2013) models displayed significant (or limited) decrease in social recognition in comparison with wild-type littermates.

All models also displayed high level of stereotypy such as self-grooming or jumping behaviour. Synaptic physiology was predominantly analysed in defined areas of the cortex or in the hippocampus. Most models displayed a decrease in miniature excitatory postsynaptic current (mEPSC) or field excitatory postsynaptic potential (fEPSP), except for *Nlgn2*-overexpression (Hines et al. 2008) that showed an increase in miniature inhibitory postsynaptic currents (mIPSC), in comparison with wild-type littermates. Six out of ten models investigated for social communication displayed a significantly lower call rate in comparison with wild-type littermates [*Shank3Δex4-9-KO* (Wang et al. 2011; Wang et al. 2014b), *Shank3Δex4-9-KO* (Jaramillo et al. 2016), *Shank3Δex4-22-KO* (Wang et al. 2016), *Cntnap2-KO* (Peñagarikano et al. 2011, 2015)]. Interestingly, the characterisation of the *Nrxn1α-KO* model (Etherton et al. 2009; Grayton et al. 2013) resulted in divergent results between laboratories for social recognition and stereotyped behaviours.

The *subcluster I-SC2* includes eight well-characterised mouse models [*Nlgn3-R451-KI* (Tabuchi et al. 2007; Ellegood et al. 2011; Etherton et al. 2011; Karvat and Kimchi 2012; Kumar et al. 2014; Steadman et al. 2014; Burrows et al. 2015),

Shank1-KO (Hung et al. 2008; Roullet et al. 2009; Silverman et al. 2011; Sungur et al. 2014; Wöhr 2014), *Shank3Δex4-9-HZ* (Bozdagi et al. 2010; Yang et al. 2012; Drapeau et al. 2014), *Shank3Δex4-9-KO* (Yang et al. 2012), *Nrxn2α-KO* (Born et al. 2015), *Nrxn1α-HZ* (Grayton et al. 2013), *Nrxn2α-KO* (Dachtler et al. 2014) and *Nlgn4-KO* (Jamain et al. 2008; Ey et al. 2012; Ju et al. 2014)]. These models are the ones with either divergent behavioural characterisation between studies or impaired social interactions, but non-significant or divergent differences in stereotyped behaviours. Interestingly, the divergences between studies mostly concern behavioural phenotypes (social interactions, communication, stereotyped behaviours), while results for synaptic physiology and protein composition were frequently convergent.

5.4.2 Cluster II

Cluster II includes mouse models with limited social impairments and stereotyped behaviours. Protein expression was rarely investigated in these models, and the majority of them was not characterised for synaptic physiology.

The *subcluster II-SC1* includes 14 mouse models with no significant impairment in social motivation and in stereotyped behaviours and a very limited characterisation of synaptic physiology [*Nlgn2-KO* (Blundell et al. 2009), *Nrxn2α-HZ* (Born et al. 2015), *Nlgn2-HZ and Nlgn2-KO* (Wöhr et al. 2013), *Nlgn3-R451-KI* (Chadman 2011), two models of *Shank3Δex4-9-KO* (Drapeau et al. 2014), *Shank3-InsGex21-HZ* (Speed et al. 2015), *Shank2Δex7-HZ* (Schmeisser et al. 2012; Ey et al. 2013), *Shank3-InsG3680-HZ (Zhou et al. 2016), Nlgn4-HZ* (Ey et al. 2012), *Shank3Δex4-7-KO* (Peça et al. 2011), *Shank1-HZ* (Silverman et al. 2011; Sungur et al. 2014) and *Nlgn3-KO* (Radyushkin et al. 2009)]. Surprisingly, only three models [*Nlgn3-KO* (Radyushkin et al. 2009), *Shank3Δex4-7-KO* (Peça et al. 2011), *Nlgn4-HZ* (Ey et al. 2012)] showed impaired social recognition, and three of them displayed a decreased rate of emission of ultrasonic vocalisations in comparison with wild-type mice [*Nlgn2-KO* (Wöhr et al. 2013), *Nlgn3-R451-KI* (Chadman 2011), *Nlgn3-KO* (Radyushkin et al. 2009)]. With respect to synaptic physiology, only two models displayed decreased basal transmission [*Nrxn2-HZ* mice (Born et al. 2015), *Shank3Δex4-7-KO* (Peça et al. 2011)].

The *subcluster II-SC2* includes *Shank3Δex4-9-HZ* (Jaramillo et al. 2016), *Cacna1c-HZ* (Bader et al. 2011), *Shank3Δex9-KO* (Lee et al. 2015b) and *Shank3-InsGex21-KO* (Speed et al. 2015). Two of these studies were performed on heterozygous mice. Impairments of synaptic physiology were detected in *Shank3Δex4-9-HZ* (Jaramillo et al. 2016), *Shank3Δex9-KO* (Lee et al. 2015b) and *Shank3-InsGex21-KO* (Speed et al. 2015). Stereotyped behaviours were also increased in comparison with wild-type mice in all of these models in three different tests, respectively, rearing, marbles burying and self-grooming.

Our meta-analysis provides an overview of the most extensively characterised mouse models carrying mutations in ASD-risk genes. Overall, atypical synaptic

physiology was coupled with increased stereotyped behaviours and to a lesser extent with atypical social motivation in most cases. This relationship did not hold for social recognition. Interestingly, the clustering using phenotypes did not cluster the mouse models according to their mutated genes.

5.5 Relating Behavioural Phenotypes with Structural and Functional Characteristics

One of our aims was to relate the different behavioural and structural aspects measured in mouse models of ASD to extract relevant relationships. The comparison of *Cluster I* and *Cluster II* leads to discuss the connection between the different traits measured. *Cluster I* includes models highly impaired in social interest, stereotyped behaviours, synaptic physiology and protein composition, while Cluster II includes much less impaired models. Only three models display impaired social interest without increased stereotyped behaviours [*Nrxn2α-KO* (Born et al. 2015), *Nrxn1α-HZ* (Grayton et al. 2013), *Nrxn2α-KO* (Dachtler et al. 2014)], and the four models from *II-SC2* display stereotyped behaviour without social interest impairment [*Shank3Δex4-9-HZ* (Jaramillo et al. 2016), *Cacna1c-HZ* (Bader et al. 2011), *Shank3Δex9-KO* (Lee et al. 2015b), *Shank3InsGex21-KO* (Speed et al. 2015)]. Social recognition impairments seem more variable, i.e., impaired social motivation does not mean impaired social recognition. Reversely, mouse models displaying abnormalities in synaptic physiology also display increased stereotyped behaviours. This relationship is weaker between impaired synaptic physiology and impaired social motivation. This observation can suggest that when basal circuits are deeply affected, the social motivation, but not social recognition domain, becomes affected.

We also aimed at identifying specific brain regions associated with either atypical social interactions or increased stereotyped behaviours. This was hindered by the fact that most studies analysed both structural and functional aspects only in the hippocampus (CA1, CA3 and dentate gyrus) and the cortex (sensorimotor area and prefrontal cortex). This is unfortunate especially because these brain structures are not the major ones implicated in social interactions and stereotyped behaviours. We therefore could not extract defined information about the links between brain regions and behavioural phenotypes. To link anatomical and physiological impairments with behaviours, other brain structures should be studied in the future, more specifically the striatum including the nucleus accumbens, the mesencephalon including the ventral tegmental area, the amygdala and the diencephalon. These brain regions represent relevant nodes forming a network involved in social and stereotyped behaviours. In most models, only one brain structure was studied; only few studies presented results obtained in several brain regions at the time. Standardised analyses from different brain regions and from different mouse models should allow a better understanding of the circuits affected.

5.6 Mouse Models Carrying Mutations in the *Shank* Genes

Over the last years, several mouse models with mutations in the family of *Shank* genes were published since approximately one per cent of patients with ASD carry a mutation in the *SHANK* gene family (Leblond et al. 2014). In this meta-analysis, 25 well-characterised and independent mouse models exhibited genetic disruption of *Shank1* (one knockout model and one heterozygous model), *Shank2* (two knockout models and one heterozygous model) or *Shank3* (14 knockout models, 1 knock-in and 5 heterozygous models). We tested whether phenotypic traits displayed some similarities between models carrying different mutations in the same gene family.

To date, only one *Shank1* mutant strain was reported (Hung et al. 2008). The heterozygous animals do not display any significant impairment in social interaction and communication but show a slight increase in stereotyped behaviours in old adults in comparison with wild-type littermates. In contrast, the homozygous mutant mice for *Shank1* display reduced scent-marking behaviour and atypical ultrasonic vocalisations but typical social interactions in comparison with wild-type littermates.

Two independent *Shank2* mutant strains display increased stereotyped behaviours, reduced social motivation and recognition as well as atypical social communication (Schmeisser et al. 2012; Won et al. 2012; Ey et al. 2013; Lee et al. 2015a; Ferhat et al. 2016). Although increased GluN1 expression was biochemically detected in both models at the juvenile stage (3–4 weeks), the lines display opposite results in synaptic physiology at CA1 synapses. While Won et al. (2012) and Lee et al. (2015a) identified a reduction in long-term potentiation (LTP), Schmeisser et al. (2012) highlighted an increase of LTP in comparison with wild-type littermates. These divergences remain to be elucidated.

The large panel of *Shank3* mutant models is highly interesting given that only the recent *Shank3Δex4-22-KO* is a complete knockout (Jiang and Ehlers 2013; Wang et al. 2016). The *Shank3* gene is transcribed from six promoters (Jiang and Ehlers 2013; Wang et al. 2014b), and therefore in most models some *Shank3* isoforms remain. The complete knockout model displays impairments in behavioural and synaptic traits examined (Wang et al. 2016), but the phenotypic difference with other *Shank3* mutant mice was not dramatic. Overall, the two traits observed in almost all *Shank3* mutant models are an increase in stereotyped behaviours, especially increased self-grooming, and reduced glutamatergic synaptic transmission tested mostly in the CA1 hippocampus. The presence of stereotyped behaviours seems to be more prominent in models expressing less isoforms (Peça et al. 2011; Schmeisser et al. 2012; Kouser et al. 2013; Bidinosti et al. 2016; Mei et al. 2016; Wang et al. 2016). Abnormalities in social interest are subtle, while reduced performances in social recognition do not appear to be a robust trait in these models. Protein composition at the synapse is disorganised, but it is not clearly related to the number of remaining isoforms.

Overall, *Shank3* mutant mice are best characterised by increased stereotyped behaviours and reduced glutamatergic synaptic transmission, while *Shank2* mutants are best characterised by increased stereotyped behaviours and reduced social motivation, but less obvious phenotypes in synaptic physiology. The stereotyped behaviours appear to be the trait most impacted by the modulation of the number of remaining isoforms in the *Shank3* mutant mice.

5.7 Refining the Analysis to Describe Behavioural Impairments

In mouse behavioural testing, different protocols are supposed to measure similar traits. For example, interest for social interactions or social motivation can be quantified in the three-chambered test or in free same-sex interaction. However, some studies conducting these different experiments on identical mouse models did not lead to the same conclusions. For example, *Shank2* mutant mice (Schmeisser et al. 2012) display a reduction of the interest for a conspecific during free interaction, but no significant reduction of this social interest during the first phase of three-chambered test in comparison with wild-type mice. The use of different protocols can explain the divergence between results obtained in the same models (Etherton et al. 2011; Schmeisser et al. 2012; Jaramillo et al. 2014). These divergences suggest that the interpretation of the tests should be refined to identify what each protocol actually measures. More specifically, the paradigms should be classified according to the Panksepp's system of emotional systems (Panksepp 2006) taking into account their emotional valence, positive emotions (seeking, care and playfulness) or negative emotions (fear, anger, sadness). This Panksepp's model was applied to patients with ASD, who displayed decreased playfulness and increased fear (Carré et al. 2015). Such an approach can be applied to mouse models in order to refine the characterisation of the phenotypic impairments caused by genetic mutations.

Innovative methods should also be developed to provide more ethological contexts allowing the expression of a more complete behavioural repertoire to characterise social interactions, communication and stereotyped behaviours over different age classes (pups, juveniles and adults). In such a context, both the active form (initiating social communication) and the passive form (receiving social communication) of social behaviour could be examined, the latter usually being neglected in mouse models of ASD. These innovative methods should take into account not only all the information gathered in previous experiments but also potential biases such as the different human experimenters (Sorge et al. 2014), the test cage conditions and habituation time (Ferhat et al. 2015). These biases could be reduced in complex environments where the behaviour of the individual mice is recorded continuously with few or any human intervention (Howerton et al. 2012; Ohayon et al. 2013; Weissbrod et al. 2013). In such settings, the different sensory

modalities can also be studied in more ethological conditions, with limited manipulation of the motivations of the tested animals.

As an example, focusing on social recognition in such testing conditions will be of high interest to evaluate how social cues are perceived in mouse models of ASD. New protocols should be developed to disentangle perception (whisking, sniffing or body postures during social contacts) and social cues processing deficits from common memory deficits. If mutant mice do not investigate a conspecific with all sensory modalities, they might be impaired in their ability to differentiate two individuals. Indeed, some models display social recognition deficits, but display normal spatial learning or object working memory (Peça et al. 2011; Schmeisser et al. 2012; Won et al. 2012). In this context, Engelmann and colleagues proposed to evaluate the free interactions between a tested mouse and two more or less familiar conspecifics (Engelmann et al. 2011).

5.8 Conclusions

Overall, the diversity of the mouse models carrying mutations in ASD-risk genes plays a crucial role in unravelling the links between genes, neuronal circuits and behaviour. New multi-scale approaches that include genetics, biochemistry, electrophysiology and a refinement of behavioural characterisation methods will help to dissect out biological pathways associated with ASD such as those involved in social communication and stereotyped behaviours. Altogether, it will favour a better understanding of the mechanisms behind ASD that should orient the identification of knowledge-based treatments.

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Chapter 6
Cerebellar and Striatal Pathologies in Mouse Models of Autism Spectrum Disorder

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6.1 Introduction

The definition of autism spectrum disorder (ASD) as we know today originates from behavioural observations done by Kanner (1943) and Asperger (1944). Abnormal motor behaviour and social functioning characterised these earliest observations. Modern evaluations based on the *Diagnostic and Statistical Manual of Mental Disorders (DSM-V)* include persistent difficulties in the social use of verbal and non-verbal communication, impaired social interaction as well as restricted and/or repetitive behaviours (American Psychiatric Association 2013). Even though hundreds of abnormal genes have been identified that are correlated with the presence of ASD, understanding of the aetiology has not advanced much further since these earliest behavioural characterisations. Still, enough experimental insights have been gathered to establish the following: ASD is a heterogeneous

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neurodevelopmental condition most probably caused by dysfunctional neural circuits involving multiple brain areas. Within these circuits, the timing and location of insult, being of genetic nature or otherwise, are of great importance for the progress of the pathology. This temporal aspect has recently been proposed to be critical during postnatal development in the cerebellum (Wang et al. 2014). Abnormalities in striatal circuits, being an integral brain region for sensory input and reward-related behaviour, have also been put forward as a critical area in the aetiology of ASD phenotypes (Fuccillo 2016). In this review, we discuss several major findings of both cerebellar and striatal abnormalities found in ASD mouse models.

6.2 Human Cerebellum in ASD

Cerebellar functioning has traditionally been linked to the timing, coordination and learning of motor tasks. Contributions of cerebellum-related motor impairments to ASD have been identified in patients through impaired eye-blink conditioning (Sears et al. 1994; Oristaglio et al. 2013), eye movement abnormalities (Takarae et al. 2004; Schmitt et al. 2014), motor learning deficits (Marko et al. 2015; Mostofsky et al. 2000) and balance and posture difficulties (Memari et al. 2014; Stins et al. 2015; Molloy et al. 2003). Still, an accumulating amount of evidence points towards dysfunctions that reach beyond the readily observable symptoms of motor problems. For instance, damage in the adult cerebellum has also been shown to lead to deficits in language, executive function and impaired emotions, a condition termed cerebellar cognitive affective syndrome (Schmahmann 2010). ASD patients commonly show cerebellar pathologies like widely distributed reduction of Purkinje cells (PCs) from early on (Allen 2005; Bailey et al. 1998; Silvestri et al. 2015). Apart from the cell loss during development, there is also evidence for reduced size and density of PCs (Palmen et al. 2004; Fatemi et al. 2002). In addition to the PC abnormalities, there is proof for a developmental reduction of granule cells (GCs) and abnormal cerebellar nuclei (Allen 2005; Bauman et al. 1995). Beyond the body of work on the contribution of cerebellum-related behaviour in ASD, there is support for a crucial role during development that ranges from ASD risk gene co-expression in the cerebellum (Menashe et al. 2013) and early-stage isolated cerebellar damage in infants, leading to motor, language, cognitive as well as social behaviour deficits (Limperopoulos et al. 2007). Finally, emerging evidence also points towards a role of the cerebellum in the maturation of cerebral cortical networks that could play a crucial role in the development of ASD (Wang et al. 2014).

6.3 Cerebellum in ASD Mouse Models

Given the extensive behavioural and pathological changes in ASD that can be associated with the cerebellum, we still have relatively little understanding as to how these contribute to the aetiology of ASD. To tackle this issue, different cerebellar mouse models have been generated and tested in recent years; these include models of both syndromic (i.e. part of a set of symptoms that characterise the disease) and non-syndromic forms of ASD.

6.3.1 Mouse Models for Syndromic ASD

One of the well-known syndromes associated with ASD is fragile X syndrome (FXS). Approximately 5% of FXS patients fit within the ASD classification (Budimirovic and Kaufmann 2011; Garber et al. 2008). FXS is associated with abnormalities in the cerebellum, including decreased cerebellar volume (Mostofsky et al. 1998) and PC loss (Sabaratnam 2000; Greco et al. 2011). Additionally, cerebellum-dependent eye-blink conditioning is impaired in FXS patients (Koekkoek et al. 2005; Tobia and Woodruff-Pak 2009). Morphological changes in the *Fmr1* KO cerebellum have been described as a reduction of volume, cell loss in the cerebellar nuclei (CN) (Ellegood et al. 2010) and longer dendritic spines have been identified among PC dendrites (Koekkoek et al. 2005). Further evidence for cerebellum-related pathophysiology includes cerebellar-prefrontal deficient dopamine release (Rogers et al. 2013), enhanced LTD induction at the parallel fibre to PC synapse (Koekkoek et al. 2005) and abnormal presynaptic vesicle dynamics (Broek et al. 2016). In addition, pathology of non-neuronal cerebellar cells such as increased expression of myelination proteins in astrocytes may also contribute to the phenotype of ASD (Pacey et al. 2015). Such phenomena may partially reflect compensation for decreased myelination in the cerebellum as occurs in *Fmr1* KO mice.

Another syndromic disorder regularly presenting with ASD is Rett syndrome (RTT), which is primarily caused by a mutation in the *MECP2* gene (Amir et al. 1999). Assessment of these individuals has shown motor impairments during the developmental phase (Segawa 2005). A significant body of evidence points towards cerebellar pathology in RTT including loss and abnormal size of PCs (Oldfors et al. 1990; Murakami et al. 1992; Bauman et al. 1995). In *Mecp2* KO mice, motor coordination and learning, as assessed by rotarod, are impaired (Goffin et al. 2012). Additionally, a reduced cerebellar volume (Belichenko et al. 2008) and smaller and more dense GCs have been reported in these mutants (Chen et al. 2001). *Mecp2* exhibits a defined cerebellar expression pattern with early expression in PCs and relatively late expression in GCs (Mullaney et al. 2004). In line with the finding that *Mecp2* has been shown to regulate hundreds of genes in the cerebellum (Ben-Shachar et al. 2009), this temporal expression pattern in PCs and GCs could

reveal sensitive periods that are crucial for key developmental processes of the cerebellar network.

Mutations in the maternal *UBE3A* gene lead to Angelman syndrome (AS), a developmental disease with a high prevalence of ASD (Peters et al. 2004). In addition to the observations that AS and RTT have similar behavioural abnormalities, there is evidence for functional pathway overlap between *Ube3a* and *Mecp2* at the molecular level (Kim et al. 2013). Cerebellar involvement is probably also implicated in AS, as revealed by the abnormal motor behaviour of AS patients (Dan et al. 2004) and abnormal GABA receptor functioning in their cerebellum (Holopainen et al. 2001). In mice, *Ube3a* maternal deficiency leads to phenotypes that include impaired motor coordination (Miura et al. 2002; Heck et al. 2008) and orofacial activity (Heck et al. 2008). These behavioural impairments are in line with deficits found in the morphology (Dindot et al. 2008) and firing rates of their PCs (Cheron et al. 2005), possibly further causing abnormal functioning in the cerebellar cortical network. However, recent evidence indicates that the motor-related impairments in maternal *Ube3a* might not be due to impairments in PCs alone, as PC-specific *Ube3a* ablation did not result in significant motor phenotypes (Bruinsma et al. 2015). Other physiological impairments may be explained by decreased tonic inhibition due to a deregulation of GABA transporter 1 (GAT1) in the GCs (Egawa et al. 2012). The results of this study show that due to excessive GAT1 expression, there is more GABA reuptake from the extra synaptic space resulting in less inhibition of the GCs. The motor deficits resulting from these physiological impairments could be reversed by applying the THIP $GABA_A$ receptor agonist, suggesting a possible therapeutic strategy (Egawa et al. 2012).

The dup15q syndrome, another ASD-related disease that has been modelled in mice, involves chromosome 15q11–13 duplication. This duplication is of particular interest as it has a high prevalence in the ASD population (Cook and Scherer 2008). A mouse line modelling the 15q11–13 duplication, the *patDp*/+ mutant, shows impaired social interaction, behavioural inflexibility and abnormal communication (Nakatani et al. 2009). In a recent study, *patDp*/+ mutants were used to evaluate possible cerebellar phenotypes. Intriguingly, these mice indeed show impairments in motor coordination, learning and eye-blink conditioning (Piochon et al. 2014). Disruption of cerebellar physiology was supported by impaired LTD at the parallel fibre to PC synapse and abnormal climbing fibre elimination of the PCs.

Tuberous sclerosis complex (TSC) is caused by mutations in *TSC1* or *TSC2* and results in benign tumour growth across the body, including the brain. A large portion of TSC patients is diagnosed with ASD (Harrison and Bolton 1997). The extent of TSC-related ASD has been shown to correlate with the severity of cerebellar pathology (Eluvathingal et al. 2006). The *TSC* gene products hamartin and tuberin both inhibit mTOR activity and can thereby regulate protein biosynthesis. Because of their relatively high expression in the murine cerebellum (Gutmann et al. 2000), it was hypothesised that mutations in *TSC1* or *TSC2* could have a major impact on cerebellar function. Tsai et al. created a PC-specific *Tsc1* mutant mouse model to assess the importance of cerebellar dysfunction in TSC (Tsai et al. 2012). This was the first study to show that a PC-specific mutation of a

syndromic ASD gene results in social, repetitive and communicative impairments in mice, mimicking the human condition. The behavioural deficits are furthermore supported by reduced excitation, abnormal spine density and apoptosis of PCs. Similarly to the *Tsc1* mouse model, a PC-specific mutation of *Tsc2* shows comparable results (Reith et al. 2013, 2011). Interestingly, in both the *Tsc1* and *Tsc2* mutants, the behavioural and PC-specific abnormalities can be rescued using the mTOR inhibitor rapamycin (Tsai et al. 2012; Reith et al. 2013, 2011). Another ASD-related gene involved in mTOR signalling is *PTEN* (McBride et al. 2010). In support of the central role of PC dysfunction in ASD, a recent study investigated PC-specific deletion of *Pten* and found that mutants show abnormalities of PC structure, disrupted climbing and parallel fibre synapses and death of PCs (Cupolillo et al. 2016). These mutant mice exhibited impaired sociability, repetitive behaviour and deficits in motor learning.

6.3.2 Mouse Models for Non-syndromic ASD

The *SHANK* family of postsynaptic scaffolding proteins comprises three main genes with homologous functional domains that have alternative splicing variants. They are crucial for the morphology, transmission and plasticity of glutamatergic synapses (Sheng and Kim 2000; Grabrucker et al. 2011). Genetic disruption of the three different *SHANK* genes has been related to ASD, whereas cerebellum-related motor problems were predominantly occurring in individuals with mutations in the *SHANK2* gene (Leblond et al. 2014). A mouse model based on global and PC-specific *Shank2* deletion was recently investigated for physiological and behavioural phenotypes in two different studies (Ha et al., 2016; Peter et al. 2016). In one of the studies (Peter et al. 2016) the authors reported deficits in motor learning, social interaction and task-specific repetitive behaviour. Interestingly, the latter phenotype was also reflected in a lack of flexibility and adaptability on the ErasmusLadder, while the baseline motor performance during locomotion appeared intact. Physiological experiments indicated impaired LTP at the parallel fibre to PC synapse and dysfunctional intrinsic plasticity. Moreover, a notable increase in simple spike PC firing irregularity can be seen in posterior lobules. This increase in irregularity was accompanied by increased inhibition. The differentiation between anterior and posterior phenotypes in the cerebellum is of particular interest as posterior abnormalities have been reported in patients (Stoodley 2014) and could therefore indicate a biological vulnerability in the aetiology of ASD. In the second study (Ha et al. 2016) the authors report that PC-specific deletion of *Shank2* results in reduced PSD number within the molecular layer as well as motor coordination, repetitive and anxiety-like behavioural phenotypes. However, these mutants do not show any impaired social interaction. The differences between the studies, as noted by the authors, could be due to different exon deletion strategies and genetic backgrounds of the mice used.

Mutations in *NLGN3* and *NLGN4* encoding the postsynaptic cell adhesion molecules Neuroligin3 and 4 have been identified in individuals with ASD (Südhof

2008). Through their role as cell adhesion molecules (CAMs), which bind neurexins and thereby physically connect pre- and postsynaptic terminals, they are crucially involved in different aspects of synapse function. Recently, similarities were reported between syndromic and non-syndromic ASD by analysing molecular phenotypes in the Neuroligin3-deficient cerebellum (Baudouin et al. 2012). In this study, the authors show that *Nlgn3* KO mice exhibit cerebellum-related motor impairments and impaired cerebellar mGluR-dependent synaptic plasticity—a phenotype that is similar to the one found in *Fmr1* KO (Koekkoek et al. 2005).

6.3.3 *Converging Cerebellar Phenotypes Among Several Mouse Models*

ASD patients have deficient processing of multiple sensory stimuli, which might contribute to their characteristic social and cognitive abnormalities. In a recent report, Kloth et al. (2015) investigated five different ASD mouse models including *Shank3*+/Δ*C*, *Mecp2*$^{R308/Y}$, *Cntnap2*$^{-/-}$, *patDp*/+ and *L7-Tsc1* mutants in their ability to acquire conditioned eye-blink responses, a form of associative learning where proper integration of sensory information is crucial, the acquisition and expression of which depend on the cerebellum (ten Brinke et al. 2015). They provided data showing that dysfunctional sensory integration in ASD could be related to various genetic mutations that result in cerebellar dysfunction (Kloth et al. 2015). In the light of these results, and the relatively high expression of

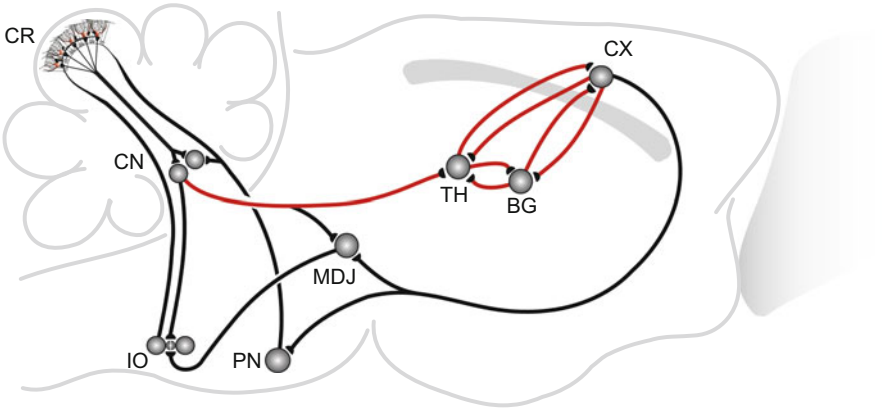

Fig. 6.1 Simplified illustration of several key connections that are implicated in ASD. Here, the cerebellar output through cerebellar nuclei and the reciprocating connections between the thalamus, basal ganglia and the cerebral cortex are central. These connections require in-depth investigation that focuses on timed circuit insults as well as cell specificity of these insults. Both of these aspects (i.e. location and timing) are believed to be at the core of ASD aetiology. *CR* cerebellar cortex, *CN* cerebellar nuclei, *MDJ* mesodiencephalic junction, *PN* pontine nuclei, *IO* inferior olive, *TH* thalamus, *BG* basal ganglia, *CX* cerebral cortex

ASD-related genes in the cerebellum (Menashe et al. 2013), it is quite plausible that the sensory integration problems in ASD may be caused by a dysfunctional cerebellar circuit, appearing early in life and preventing proper functional development of downstream structures like the thalamus and sensorimotor cerebral cortex (Fig. 6.1).

6.4 Human Striatum in ASD

Similarly to the cerebellum, the striatum, a relatively large structure that is part of the basal ganglia, has been functionally related to different aspects of motor control (Doya 1999, 2000). Just like the cerebellum, the striatum receives input from both cortical and thalamic structures and is also implicated in control of cognitive processes, reaching beyond motor control functions (Middleton and Strick 2000). Considering that ASD has such a complex aetiology involving multiple brain areas, the striatum has also been recognised as a promising target for further investigation. Differential abnormal changes in the volume of the striatum in ASD have been reported previously (Mehmet et al. 2006; Hollander et al. 2005; Sears et al. 1999). In support of these changes, a longitudinal study provided evidence for a developmental correlation of striatal volume change and repetitive behaviour in ASD patients (Langen et al. 2014). The idea that the striatum is involved in repetitive behaviours is not new; in fact, over the years different lines of evidence have been accumulated in favour of dysfunctional corticostriatal pathways in mice (Peça et al. 2011; Wan et al. 2014), which could well be related to impulsive and stereotypical behaviours in humans (Bienvenu et al. 2009; Abelson et al. 2005). To advance our understanding of the underlying mechanisms in the striatum-related dysfunctional behavioural phenotypes in ASD, several different mutant mouse models have been utilised over the years.

6.5 Striatum in ASD Mouse Models

One major group of mouse models that has provided insight into striatum-related pathology in ASD are the different *Shank* mutants. As mentioned above, genetic disruptions of *SHANK* genes are highly relevant for ASD, especially mutations in *SHANK3* (Leblond et al. 2014). Interestingly, Shank3 is highly expressed in the striatum, and several different *Shank3* mutant lines exhibit core ASD-like behaviour and various deficits at corticostriatal synapses (Peça et al. 2011; Jaramillo et al. 2016a, b; Wang et al. 2016). Importantly, reintroducing *Shank3* has recently been shown to rescue both certain behavioural phenotypes and corticostriatal deficits, indicating a certain degree of flexibility in the adult mutant brain (Mei et al. 2016). In addition, recent work also suggests promise for the use of the pharmacological enhancement of metabotropic glutamate receptor 5 in the reversal of behavioural and physiological corticostriatal abnormalities in another *Shank3* mutant

(Vicidomini et al. 2016). Two lines of *Shank2* mutants also show ASD-like behavioural deficits (Won et al. 2012; Schmeisser et al. 2012). Interestingly, Schmeisser et al. (2012) indicate in their model that absence of *Shank2* in the striatum results in striatal upregulation of Shank3, while the absence of major *Shank3* isoforms results in striatal upregulation of Shank2. In terms of circuit pathology, an important role for early cortical hyperactivity has been revealed in *Shank3B*$^{-/-}$ mutants (Peixoto et al. 2016). Here, the authors provide evidence for a surprising early maturation of excitatory inputs of striatal spiny projection neurons in *Shank3B*$^{-/-}$ mutants and propose that this corticostriatal hyperactivity during development could be central in ASD models where dysfunctional cortical activity could be part of the aetiology implicating cognitive impairment. Because *SHANK3* mutations have been associated with both ASD and schizophrenia, differential mutations of the *SHANK3* gene have recently been attributed to different disorder-associated symptoms in mice (Zhou et al. 2016). This work provides important clues as to how different alleles of the same *SHANK3* gene could in fact be responsible for ASD and schizophrenia-related symptoms, where distinct phenotypes can be found for striatal (ASD) and cortical (schizophrenia) impairments in physiology.

Apart from the above-discussed cerebellar findings in *Nlgn3* KO, these mice have also been instructive in unravelling the striatal role in ASD. Specific deletion of *Nlgn3* in medium spiny neurons (MSNs) of the direct, but not indirect, pathway of the ventral striatum (i.e. nucleus accumbens) resulted in enhanced repetitive behaviours (Rothwell et al. 2014). Deletion of *Nlgn3* in the dorsal striatum did not induce repetitive behaviours, which is surprising, as dysfunction of the dorsal striatum has been implicated in the development of repetitive behaviour in related ASD models, such as those of *Shank3* (Peça et al. 2011). The Rothwell et al. (2014) study therefore sheds light on the complexity of circuit involvement in the generation of behavioural phenotypes and adds insight into the role of a specific cell type within both a defined circuit and defined subregion of the brain in this context.

An additional feature of ASD is impaired communication. As the basal ganglia are in general strongly implicated in the process of language (Chan et al. 2013), aberrations of striatal functions have also been suggested as one of the prime causes for the communication problems in ASD. Mouse models that could prove useful involve genetic abnormalities of the *Foxp* genes, which seem to be strongly associated with language. *FOXP1* and *FOXP2* encode transcriptional factors, and their genetic disruption is involved in lower IQ, verbal dyspraxia and ASD in humans (Chien et al. 2013; Hamdan et al. 2010; Tsang et al. 2013; Lozano et al. 2015; Watkins et al. 2002; Vargha-Khadem et al. 1995). Interestingly, a recent report on the ablation of the *Foxp1* gene selectively in the mouse brain has shown a substantial reduction of striatal volume in an early postnatal phase, disrupted learning and memory, reduction of social interest and a higher occurrence of repetitive behaviours (Bacon et al. 2015). Furthermore, *Foxp2* mutants show impaired motor function and ultrasonic vocalisations (USV) abnormalities (Kurt et al. 2012; Fisher and Scharff 2009). Possibly, impairments in striatal LTD (Groszer et al. 2008) and negative modulation of firing rates in striatal neurons (French et al. 2012) contribute to the behavioural phenotype in these *Foxp2* mutants.

6.6 Conclusions

The cerebellar and striatal morphological and physiological abnormalities found in ASD-related mouse models are not uniform of character (Ellegood et al. 2015). Experimental data indicates that different gene dysfunctions can lead to differential, and sometimes opposing, physiological and morphological abnormalities in the same brain areas, while the impaired behavioural consequences appear to be similar. This indicates that the underlying nature of the insult is probably not overly informative in the unravelling of ASD aetiology, even though it might provide therapeutic intervention strategies in those specific insults. Instead, for the purpose of unravelling ASD aetiology, it seems prudent to investigate two major questions: Which neuronal circuits are core suspects in the development of ASD? And during what time are these circuits especially vulnerable? The cerebellum and striatum have traditionally been viewed as structures controlling motor function. Yet, over the years multiple lines of evidence provide support for a functionally relevant interaction of both areas in nonmotor-related tasks. Evidence from functional imaging and viral tracing studies (Bostan et al. 2013) further indicates a strong interaction between both brain regions, which could have significant implications for neuropsychiatric diseases. Importantly in this context, physiologically relevant data have been published that underline a short latency modulation of the basal ganglia through deep cerebellar nuclei stimulation (Chen et al. 2014). Given the evidence of striatal involvement in repetitive behaviours in ASD (Langen et al. 2014), it seems parsimonious to hypothesise that the striatum-related phenotypes of repetitive and cognitive behaviours may be modulated by cerebellar output and vice versa. Indeed, together they could derail coherent activity in the cerebral cortex from early on (Fig. 6.1), which is one of the hallmarks of autism (Khan et al. 2015; Robertson et al. 2014; Carson et al. 2014). The ASD mouse models discussed here (Table 6.1) give potential insight into the pathology that could result from neural circuit dysfunction. However, one of the main obstacles in elucidating the contribution of a brain region to specific behavioural phenotypes is the lack of cell specificity in the targeting strategies. Future studies need to utilise cell-specific mutants in both brain regions and investigate circuit morphology and physiology. *Shank* mutants are a promising model for that purpose, as global KO have already been used to evaluate either cerebellar or striatal abnormalities (Won et al. 2012; Schmeisser et al. 2012; Peça et al. 2011; Ha et al, 2016; Peter et al. 2016). Cell-specific *Shank* mutants should provide further insights into both behavioural and neurobiological abnormalities that might directly derive from dysfunction of specific cerebellar or striatal cell types. This type of approach will offer key insight in both motor and cognitive abnormalities in ASD, and more specifically, this might lead to the identification of the major circuits involved.

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Table 6.1 A short list of some of the more frequently used ASD mouse models and their main morphological and physiological impairments in cerebellum and striatum

Model	Morphology		Physiology		References
Cerebellar phenotypes in ASD mouse models					
Fmr1	N/R	Increased PC spine length	P15–P30	Increased PF-PC LTD	Koekkoek et al. (2005)
	P30	Reduced volume and cell loss in DCN	–	N/I	Ellegood et al. (2010)
	–	N/I	N/R	Reduced cerebellar-mediated PFC dopamine release	Rogers et al. (2013)
	–	N/I	P98	Increased synaptic vesicle unloading	Broek et al. (2016)
Mecp2	P10–P70	Smaller and more dense GCs	–	N/I	Chen et al. (2001)
	P21	Reduced cerebellar volume	–	N/I	Belichenko et al. (2008)
Ube3a	–	N/I	P300–P390	Increased oscillations in the cerebellar cortex	Cheron et al. (2005)
	P49–P56	Reduced PC spine density	–	N/I	Dindot et al. (2008)
	–	N/I	P25–P28	Decreased tonic inhibition in GCs	Egawa et al. (2012)
	–	N/I	P30–P32	Decreased tonic inhibition in GCs	Bruinsma et al. (2015)
Patdp/+	P10–P12 and P63–P70	Reduced developmental elimination of CFs	P25–P120	Absent PF-PC LTD, restored after initial LTP induction	Piochon et al. (2014)
L7-Tsc1	P30–P120	Increased PC spine density and degeneration	P42	Reduced PC excitability	Tsai et al. (2012)
L7-Tsc2	P30–P280	PC degeneration	–	N/I	Reith et al. (2011, 2013)
L7-Pten	P180–P270 P30–P270	Reduced number of PCs Increased PC size	P84–P126	Reduced PC excitability	Cupolillo et al. (2016)
L7-Shank2	P30–P90	No abnormalities	P20–P60 P90–P150	Reduced PF-PC LTP Increased irregular firing of PCs in posterior lobules	Peter et al. (2016)
Nlgn3	P60–P90	No abnormalities	P21–P28	Reduction in PC mEPSCs and absent PF-PC LTD	Baudouin et al. (2012)

(continued)

Table 6.1 (continued)

Model	Morphology		Physiology		References
Striatal phenotypes in ASD mouse models					
Shank3 ex13–16	P35	Reduced MSN spine density	P42–P49	Reduced corticostriatal transmission	Peça et al. (2011)
Shank3 ex13	–	N/I	P90–P120	Decreased NMDAR/AMPAR ratio	Jaramillo et al. (Jaramillo et al. 2016a, b)
Shank3 ex13–16	–	N/I	P10–P14	Increased corticostriatal transmission	Peixoto et al. (2016)
Shank3 ex11	–	N/I	N/R	Reduced mGluR5 transmission in MSNs	Vicidomini et al. (2016)
Shank3 ex4–22	P56	Reduced MSN spine density, PSD length and thickness	P60–P150	Reduced sEPSCs and MSN LTD	Wang et al. (2016)
Shank3 InsG3680		Reduced MSN spine density	P14 P240	Reduced field population spikes dorsolateral striatum Reduced mEPSCs in MSNs	Zhou et al. (2016)
Nlgn3	–	N/I	P120	Reduced inhibitory synaptic currents in D1 MSNs	Rothwell et al. (2014)
Foxp1	P1–P300	Reduced striatal volume	–	N/I	Bacon et al. (2015)
Foxp2	–	N/I	P90–P180	Absent LTD in MSNs	Groszer et al. (2008)
	–	N/I	P60–P180	Decreased firing rate modulation	French et al. (2012)

P postnatal age of mice in days, *PC* Purkinje cell, *PF* parallel fibre, *CF* climbing fibre, *GC* granule cell, *DCN* deep cerebellar nuclei, *PFC* prefrontal cortex, *MSN* medium spiny neuron, *LTP* long-term potentiation, *LTD* long-term depression, *PSD* postsynaptic density, *N/I* not investigated, *N/R* not reported in study

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Chapter 7
Neurotrophic Factors in Mouse Models of Autism Spectrum Disorder: Focus on BDNF and IGF-1

Dominik Reim and Michael J. Schmeisser

7.1 Introduction

Autism spectrum disorder (ASD), a complex and heterogeneous neurodevelopmental condition, affects approximately 1% of the human population. The two core features are impaired social interaction and repetitive behaviour. Frequent co-morbidities include intellectual disability (ID), epilepsy, anxiety, sleep disturbance, hyperactivity and abnormal sensory perception (Zoghbi and Bear 2012; Geschwind and State 2015). Importantly, ASD has a strong genetic component and can be either non-syndromic or syndromic (Huguet et al. 2013). The latter means that autistic traits are part of a rare monogenic disorder or syndrome. In translational ASD research, several animal models have been developed to get a broader understanding of the underlying anatomy and cell biology.

Neurotrophic factors are secreted proteins that were first discovered as factors, promoting neuronal growth and survival during development. Interestingly, they are also known to regulate the maintenance of neuronal cell biology throughout the entire lifetime and therefore are of particular importance for development, homeostasis and plasticity in the CNS. Neurotrophic factors basically include members of the neurotrophin family. However, additional proteins, such as insulin-like growth

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factors, can also be considered as neurotrophic factors, since they are also involved in essential processes of neurons like survival and growth (Kwon 2002; Reichardt 2006; Nickl-Jockschat and Michel 2011; Park and Poo 2013).

Neurotrophins were first discovered in the early 1950s as target-derived proteins, which are promoting growth and survival of neurons. Today, the neurotrophin family comprises at least five members: nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF) and the neurotrophins 3–5 (NT-3, NT-4, NT-5). As a consequence of their high similarity, NT-4 and NT-5 are often merged to NT4/5. In addition, intense research has revealed that neurotrophins are involved in nearly all aspects of neural circuit development and function. These include cell proliferation and differentiation, neurito- and synaptogenesis, synaptic function and synaptic plasticity (Lu et al. 2005; Reichardt 2006; Nickl-Jockschat and Michel 2011; Park and Poo 2013).

As most secreted proteins, neurotrophins are synthesized as pre-pro-neurotrophins with a subsequent cleavage to pro-neurotrophins that are further converted to the mature form. However, the underlying mechanisms and enzymes involved are controversially discussed (Nagappan et al. 2009; Dieni et al. 2012; Park and Poo 2013). Secretion of neurotrophins is usually induced by membrane depolarization during neuronal activity. Additionally, neurotrophin secretion can also be provoked by extracellular factors, including neurotrophins themselves, thereby representing a self-amplifying autocrine manner of neurotrophin secretion (Kruttgen et al. 1998; Lessmann et al. 2003; Cheng et al. 2011).

Here, we will review the role of neurotrophic factors in mouse models of ASD. As the majority of studies in this context have addressed murine BDNF and IGF-1, we will also focus on these two molecules and outline their potential for translational therapy.

7.2 Neurotrophic Factors in Individuals with ASD

Neurotrophic factors, especially those of the neurotrophin family, are essentially involved in the regulation of neural circuit development. As many studies point towards the fact that ASD derives from impaired neural circuit development, a better understanding of neurotrophic factors and their role in ASD is of high interest for the identification of biomarkers and for the understanding of underlying pathomechanisms alike.

An initial study compared the levels of neurotrophins in children with ASD or ID to healthy controls (Nelson et al. 2001). They reported higher levels of both BDNF and NT4/5 in the blood of children with either ASD or ID. In 99% of the ASD group and 97% of the ID group, at least one of the examined neurotrophins exceeded the levels of healthy control children. This study was further supported by various subsequent studies, mainly reporting higher levels of BDNF in the blood (Miyazaki et al. 2004; Connolly et al. 2006; Nishimura et al. 2007; Correia et al. 2010) or brain (Perry et al. 2001) of individuals with ASD. Conversely, some studies have reported

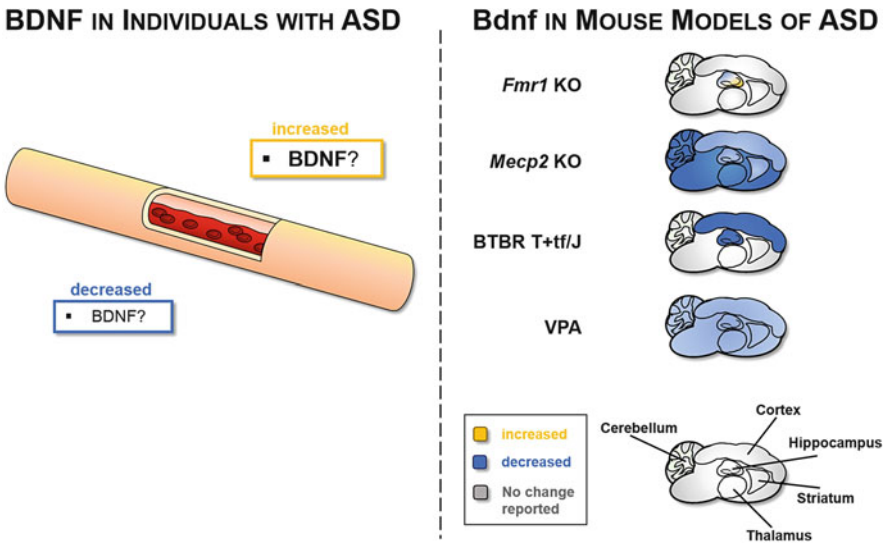

Fig. 7.1 BDNF protein levels in the blood of human individuals with ASD and the brain of ASD mouse models. Most studies have shown that BDNF levels were increased in the blood of ASD patients, but some studies also reported decreases (*left panel*). BDNF protein levels have also been analysed in the brain of ASD mouse models including *Fmr1* KO, *Mecp2* KO, the BTBR T + tf/J model and the valproic acid (VPA) model as indicated

unaffected or lower levels of BDNF (Riikonen and Vanhala 1999; Hashimoto et al. 2006; Nelson et al. 2006; Ray et al. 2011; Abdallah et al. 2013; Taurines et al. 2014) (Fig. 7.1). Interestingly, some authors provide evidence that increased BDNF levels in the blood of individuals with ASD positively correlate with milder phenotypes (Al-Ayadhi 2012; Kasarpalkar et al. 2014), female gender or hyperactivity (Spratt et al. 2015).

With respect to NT-3, NT4/5 and the insulin-like growth factors IGF-1 and IGF-2, not as many studies have been performed. However, results also diverge as some studies report higher, some unaffected or lower levels of these neurotrophic factors in blood, cerebrospinal fluid (CSF) or brain of individuals with ASD (Nelson et al. 2001; Vanhala et al. 2001; Riikonen et al. 2006; Mills et al. 2007; Sajdel-Sulkowska et al. 2011; Tostes et al. 2012; Abdallah et al. 2013; Segura et al. 2015).

There are several possible explanations for the aforementioned discrepancies such as different study design and populations or different methodological approaches. Furthermore, levels of neurotrophic factors, especially BDNF, vary among the lifetime in human subjects (Nelson et al. 2006), which might also have a significant impact on the results obtained within study groups with a high diversity in age. However, most of the studies do report alterations in neurotrophic factor levels in individuals with ASD. Such alterations—increases or decreases—might indeed have an impact on the formation and maturation of neural circuits during pre- and postnatal brain development. We can only speculate at this point if defined

alterations are beneficial or detrimental, cause or consequence or possibly only an epiphenomenon. As most of the studies in patients are merely descriptive and in general don't exceed mRNA and/or protein level determination of the neurotrophic factor(s) of interest, it is of upmost importance to study their neuronal cell biology in more detail in animal models of ASD.

7.3 BDNF in Mouse Models of ASD

7.3.1 BDNF in Mouse Models of Fragile X Syndrome

Fragile X syndrome (FXS) is one of the most frequent and best characterized monogenic causes of both ASD and ID. The first mouse model for FXS—the *Fmr1* knockout (*Fmr1* KO)—has initially been generated more than 20 years ago (The Dutch-Belgian Fragile X Consortium 1994). Phenotypical hallmarks include ASD-like behaviour, cognitive deficits, hyperactivity and a decreased seizure threshold as well as abnormal increases in the number of immature dendritic spines, mGluR5 activity and synaptic protein synthesis (Krueger and Bear 2011; Kazdoba et al. 2014; Santos et al. 2014; Richter et al. 2015).

Interestingly, BDNF/TrkB signalling has been under investigation in the *Fmr1* KO since 2002, when Castren et al. analysed *BDNF* and *TrkB* mRNA in the hippocampus of both developing and adult *Fmr1* KOs for the first time (Castren et al. 2002). Since then, studies have been performed in neural progenitors and hippocampal and cortical neurons, and both RNA and protein levels of BDNF and TrkB have been analysed (Castren et al. 2002; Lauterborn et al. 2007; Selby et al. 2007; Yuskaitis et al. 2010; Louhivuori et al. 2011; Uutela et al. 2012, 2014; Yang et al. 2012; Pietropaolo et al. 2014; Sun et al. 2014, 2016). Despite some discrepancy among studies including brain- and cell-type-specific effects, one overall finding was that BDNF expression in *Fmr1* KO is increased in younger mutants but declines with ageing. This has been most extensively analysed in the hippocampus (Uutela et al. 2012) (Fig. 7.1). Interestingly, several studies also report an increase in TrkB expression levels. It can therefore be hypothesized that in *Fmr1* KO, BDNF/TrkB signalling is hyperactive at younger ages, which could have both beneficial and detrimental effects for later life. This theory is strongly supported by the behavioural characterization of *Fmr1* KO with a modified BDNF expression of only 50% (Uutela et al. 2012). Compared to *Fmr1* KO, cognitive deficits worsen, but hyperactivity and sensorimotor deficits improve in these animals. Importantly in this context, several studies provide convincing data on both direct application of BDNF or—due to poor BDNF pharmacokinetics in vivo—TrkB agonists and indirect augmentation of BDNF levels in *Fmr1* KO by other agents to ameliorate and/or restore neurobiological and behavioural phenotypes in these mutants (Lauterborn et al. 2007; Yuskaitis et al. 2010; Pietropaolo et al. 2014; Sun et al. 2014, 2016). However, when considering the findings on the *Fmr1* KO expressing

only 50% of BDNF, there could be certain time periods during development that don't allow these treatments and certain brain regions, cell types or circuits that might rather be deteriorated than stabilized. More detailed studies are therefore needed to further understand the cell biology of BDNF and its therapeutic potential in *Fmr1* KO and FXS.

7.3.2 BDNF in Mouse Models of Rett Syndrome

Individuals with Rett syndrome (RTT), a complex neurodevelopmental disorder, often show symptoms of ASD. Importantly, in most of cases, a loss-of-function mutation in the X-linked *MECP2* gene is the underlying cause. The first mouse models for RTT—*Mecp2* knockouts (*Mecp2* KO)—have been generated 15 years ago (Chen et al. 2001; Guy et al. 2001). Phenotypes mimic RTT and include microcephaly, loss of motor function, cognitive deficits, seizures, impaired respiratory function, neuronal hypotrophy and decreased excitatory synapse number and plasticity (Lombardi et al. 2015).

The finding that Mecp2 is involved in the transcriptional regulation of *BDNF* expression (Chen et al. 2003; Martinowich et al. 2003) fostered several studies that report a progressive reduction of BDNF levels as well as impaired trafficking and secretion of BDNF in *Mecp2* KO neurons over time (Chang et al. 2006; Wang et al. 2006; Ogier et al. 2007; Chahrour et al. 2008; Kondo et al. 2008; Schaevitz et al. 2010; Li et al. 2012; Roux et al. 2012; Xu et al. 2014). Some of these deficits are both spatially and temporally restricted (Chang et al. 2006; Wang et al. 2006; Ogier et al. 2007; Kline et al. 2010; Deogracias et al. 2012) (Fig. 7.1). In support of a functional Mecp2-BDNF interplay in vivo, Chang et al. reported earlier symptom onset after deletion of *BDNF* in *Mecp2* KO and improvement of phenotypes in *Mecp2* KO with increased expression of *BDNF* (Chang et al. 2006). These results prompted BDNF-targeted translational treatment studies in *Mecp2* KO primarily aiming at augmentation of endogenous BDNF levels or at direct activation of TrkB. One study, for example, found that treatment of *Mecp2* KO with the ampakine CX546 increased BDNF levels in the vagal sensory nodose ganglion and reversed respiratory tachypnea in mutant animals (Ogier et al. 2007), while another one provided evidence for a positive effect of the sphingosine-1-phosphate receptor agonist fingolimod on both BDNF levels and RTT-like phenotypes in *Mecp2* KO (Deogracias et al. 2012). Furthermore, cysteamine, a substance increasing the secretion of BDNF vesicles, was shown to improve both lifespan and motor defects in *Mecp2* KO (Roux et al. 2012). Interestingly, environmental enrichment at early stages of postnatal development also resulted in both increased BDNF levels and amelioration of RTT-like phenotypes in *Mecp2* KO (Kondo et al. 2008, 2016; Lonetti et al. 2010). With respect to TrkB activation, it was further reported that the small molecule TrkB agonist LM22A-4 restored both synaptic dysfunction in the brainstem and breathing abnormalities in *Mecp2* KO (Schmid et al. 2012; Kron et al. 2014) and that inhibition of the protein-tyrosine phosphatase 1B (PTP1B) by

the small molecule inhibitor CPT157633 resulted in both increased TrkB phosphorylation and amelioration of RTT-like phenotypes in *Mecp2* KO (Krishnan et al. 2015).

7.3.3 BDNF in Other Mouse Models of ASD

Due to behavioural phenotypes that mimic the core features of ASD, the inbred mouse strain BTBR T + tf/J (BTBR) is also used as model system for the disorder. Several studies have reported a BDNF and TrkB decrease in the BTBR forebrain (Stephenson et al. 2011; Scattoni et al. 2013; Jasien et al. 2014; Daimon et al. 2015) (Fig. 7.1). Interestingly, this might be an age-dependent phenomenon as elevated BDNF levels were observed in the fetal BTBR brain (Hwang et al. 2015).

Similar results have been obtained for the valproic acid (VPA) model of autism generated by application of VPA to pregnant wild-type mice. Offspring analysis revealed that BDNF levels were increased in the fetal brain but decreased in the adult cortex of these animals (Roullet et al. 2010; Almeida et al. 2014) (Fig. 7.1).

Furthermore, deficits in axonal BDNF secretion are the major cellular phenotype in an ASD mouse model expressing an exon 3-skipped form of the Ca^{2+}-dependent activator protein for secretion 2 (CAPS2) as it has been found in autistic individuals (Sadakata et al. 2007, 2012, 2014).

7.4 IGF-1 in Mouse Models of ASD

7.4.1 IGF-1 in Mouse Models of Rett Syndrome

Besides BDNF, IGF-1 is another neurotrophic factor at focus in *Mecp2* KO. A selective reduction of IGF-1 protein was found in the hippocampus of *Mecp2* KO, while no differences were observed in cortical subregions and the striatum (Nag et al. 2008; Schaevitz et al. 2010). A recent study further described reduced *IGF-1* mRNA levels in the cerebellum (Mellios et al. 2014), although no changes were reported for cerebellar IGF-1 protein (Nag et al. 2008). Interestingly, two studies also found reduced IGF-1 serum levels in both young and adult *Mecp2* KO animals (Castro et al. 2014; Mellios et al. 2014).

In line with the observed reductions of IGF-1 levels, several studies have evaluated the therapeutic effects of human IGF-1 or IGF-1 derivatives on behavioural and neuronal phenotypes in *Mecp2* KO (Fig. 7.2). In an initial study, an active peptide fragment of IGF-1, (1–3)IGF-1, was chronically applied to *Mecp2* KO from 2 weeks of age onwards. Intriguingly, this treatment resulted in a significant improvement of survival, behavioural, autonomic and synaptic impairments in the mutant animals (Tropea et al. 2009). Subsequent studies further

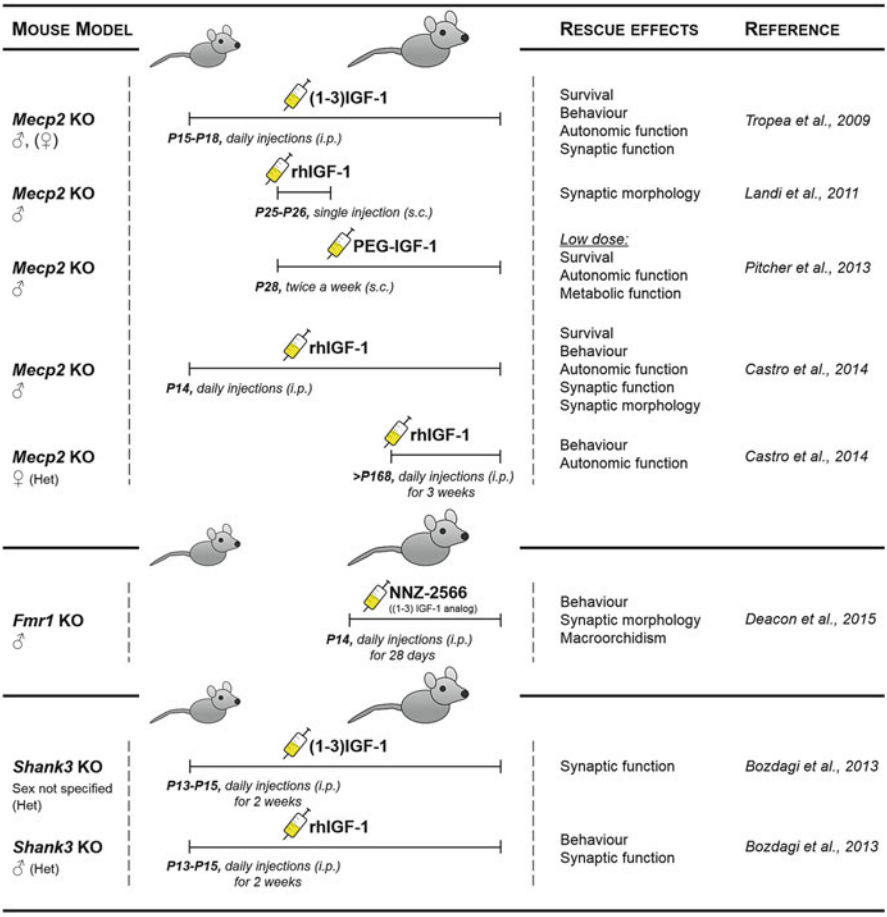

Fig. 7.2 Beneficial effects of treatment with IGF-1 or its analogues in ASD mouse models. The figure shows the respective ASD mouse models, the gender of the mice included in the studies, the treatment strategy including the substance used and the resulting rescue effects after treatment

analysed the effects of full-length recombinant human IGF-1 (rhIGF-1) in *Mecp2* KO. Impaired spine dynamics, for example, were restored in 3–4-week-old *Mecp2* KO 24 h after a single-dose application of IGF-1 (Landi et al. 2011). Another study used polyethylene glycol-coupled IGF-1 (PEG-IGF-1) to improve its pharmacokinetics and reported differential effects on varying doses after chronic treatment, starting at 4 weeks of age. Intriguingly, low doses of PEG-IGF-1 resulted in an improvement, high doses in an exacerbation of phenotypes especially with respect to a newly identified metabolic syndrome in *Mecp2* KO (Pitcher et al. 2013). Two additional studies evaluated the effects of rhIGF-1. The first one reported beneficial effects on RTT-like phenotypes in *Mecp2* KO after long-term rhIGF-1 application in young male and short-term rhIGF-1 application in adult female mutants (Castro

et al. 2014). The second one showed that the β2-adrenergic receptor agonist clenbuterol can ameliorate reduced endogenous IGF-1 levels in *Mecp2* KO, has similar beneficial effects on mutant phenotypes when compared to rhIGF1 and could be used for a combination therapy with rhIGF1 (Mellios et al. 2014).

Studies of iPSC-derived neurons or astrocytes from individuals with RTT further support these findings as the observed neurobiological phenotypes in these cultures could be rescued by application of IGF-1 (Marchetto et al. 2010; Williams et al. 2014). Importantly, rhIGF-1 is not only safe to apply and well tolerated by individuals with Rett syndrome, but has already been shown to ameliorate breathing abnormalities and neurological phenotypes in these patients (Pini et al. 2012, 2014, 2016; Khwaja et al. 2014).

7.4.2 IGF-1 in Other Mouse Models of ASD

Targeting the IGF-1 pathway as therapeutic option has intensely been investigated in Rett syndrome but might also be of use for other ASD variants. In this context, IGF-1 or its derivatives/analogues have been successfully used to ameliorate behavioural and neurobiological phenotypes in mouse models for FXS and Phelan-McDermid syndrome (PMS), respectively (Fig. 7.2).

Administration of NNZ-2566, a synthetic analogue derived from (1–3)IGF-1, for example, corrected various phenotypes including learning and memory deficits, hyperactivity, social impairment, increased spine density, overactive Erk and Akt signalling and macroorchidism in *Fmr1* KO (Deacon et al. 2015).

Moreover, both (1–3)IGF-1 and full-length rhIGF-1 were able to restore hippocampal LTP deficits, while rhIGF-1 also restored reduced rotarod performance in heterozygous *Shank3* mutants, serving as a mouse model for PMS, which is caused by *SHANK3* haploinsufficiency (Bozdagi et al. 2013). These findings are strongly supported by a following study showing that application of IGF-1 to iPSC-derived neurons from individuals with PMS restored excitatory synaptic transmission defects (Shcheglovitov et al. 2013). Intriguingly, a pilot trial on the effects of IGF-1 in individuals with PMS also revealed a significant improvement of behavioural impairments (Kolevzon et al. 2014).

7.5 Conclusions

Despite a certain degree of discrepancy among studies, alterations of neurotrophic factors are a well-established fact in both individuals with ASD and corresponding animal models. In this context, murine BDNF and IGF-1 signalling has been thoroughly analysed, and augmentation or application of these molecules has proven to evoke beneficial effects in several mutant mice. Moreover, human

IGF-1 has already been well tolerated in individuals with either Rett or Phelan-McDermid syndrome and shown to ameliorate some phenotypes. Such efforts provide an extremely important basis to develop more and broader translational treatment studies in the future. However, there is still limited knowledge about the precise cellular and molecular effects and signalling mechanisms related to both endogenous alterations and exogenous application of neurotrophic factors. A better understanding of these phenomena and the determination of defined timeframes throughout pre- or postnatal development during which the targeting of neurotrophic factor pathways is most effective will be essential to shape the appropriate translational treatment studies in the future.

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Chapter 8
The Role of the Oxytocin/Arginine Vasopressin System in Animal Models of Autism Spectrum Disorder

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8.1 Introduction

Autism spectrum disorder (ASD) is a heterogeneous neurodevelopmental condition primarily characterised by impairments in social interaction and communication as well as repetitive/stereotypic patterns of behaviour. The exact aetiology of ASD is unknown, but there are good indications that both genetic and environmental risk factors contribute to its pathogenesis (Hallmayer et al. 2011). Among all methods currently available for the treatment of ASD, rehabilitation training is the only method that has been proven effective. Although some drugs can relieve some of the co-morbid symptoms while also causing adverse reactions, they don't improve the social interactions or language abilities in most cases. Therefore, it is important to better understand the aetiology and pathophysiology of ASD in more detail to find better therapies. Several lines of evidence suggest that the central oxytocin (OXT) and arginine vasopressin (AVP) system might be involved in the development of ASD since both neuropeptides play important roles in regulating social behaviours.

8.2 OXT/AVP Systems and ASD

In mammals, OXT and AVP are nonapeptides with a six-member disulfide ring between Cys residues on positions one and six. There are high levels of sequence homology between OXT and AVP with only two amino acids difference between them (Harony and Wagner 2010). Through evolution, the two neuropeptides might have arisen from a gene duplication event, making OXT and AVP known as "twin" neuropeptides (Donaldson and Young 2008). Both are mainly synthesised in the hypothalamic supraoptic and paraventricular nuclei (SON and PVN, respectively). CD38, an ADP-ribosyl cyclase, was recently found to mediate OXT release in the brain (Jin et al. 2007), and oxytocinase (human leucyl/cystinyl aminopeptidase, LNPEP) is the enzyme that metabolises OXT and AVP (Tsujimoto and Hattori 2005). The oxytocin receptor (OXTR) is widely distributed in the brain including the hippocampus, amygdala, striatum, suprachiasmatic nucleus, bed nucleus of stria terminalis and brainstem. In the periphery, the OXTR is mainly found in the uterus, mammary gland and the heart. The receptors for AVP are classified into three subtypes named AVPR1A, AVPR1B and AVPR2 (Thibonnier et al. 2002). AVPR1A is highly expressed in the brain and plays important roles in the modulation of mammalian social behaviour and cardiovascular functions; AVPR1B is expressed in the brain and pituitary gland, and several studies revealed that AVPR1B is involved in the regulation of stress. AVPR2 is mainly expressed in the kidneys and is associated with water retention (Carter 2007; Harony and Wagner 2010; Meyer-Lindenberg et al. 2011).

After being synthesised in magnocellular neurons of hypothalamic nuclei, OXT and AVP are transported and processed along axonal projections to the posterior

lobe of the pituitary gland. Here they are stored and released into the blood stream to mediate uterine contractions and milk ejection (OXT) or fluid homeostasis and blood pressure control (AVP) (Aoyagi et al. 2009; Hew-Butler 2010). Moreover, OXT- and AVP-expressing magnocellular neurons were also found to project to the amygdala. OXT and AVP are further synthesised in parvocellular neurons of hypothalamic nuclei that project to the hippocampus, amygdala, striatum, suprachiasmatic nucleus, bed nucleus of stria terminalis and brainstem to regulate glucose metabolism (Cai and Purkayastha 2013), feeding behaviour (Chaves et al. 2013), sexual behaviour (Veening et al. 2015), learning and memory (Chini et al. 2014) and pain perception (Tracy et al. 2015). AVP also participates in the modulation of feeding, pain perception and aggression behaviour (Ray et al. 2015). In addition, there is accumulating evidence suggesting that both OXT and AVP play important roles in the regulation of complex social behaviours, such as social cognition and attachment (Insel and Young 2001), social exploration and recognition (Winslow and Insel 2004), social approach (Pagani et al. 2011; Eskandarian et al. 2013), social preference and avoidance (Lukas and Neumann 2014), maternal aggression (Eskandarian et al. 2013), affinitive behaviour [i.e. pair-bonding (Scheele et al. 2012)] and maternal behaviour (Rich et al. 2014). Importantly, some of these behaviours are also impaired in ASD.

Data have been obtained showing that plasma levels of OXT (Modahl et al. 1998; Jacobson et al. 2014) and AVP (Al Ayadhi 2005) were lower in autistic children compared to typically developing children. Moreover, plasma levels of OXT are positively correlated with the degree of core symptoms in ASD patients using the Childhood Autism Rating Scale (CARS) (Alabdali et al. 2014). Lower levels of OXT and AVP were also found in mothers of autistic children, showing a negative correlation with their children's autistic behaviour scores (Xu et al. 2013). However, in a mixed child and adolescent population, ASD was associated with higher levels of OXT (Taurines et al. 2014) and AVP (Momeni et al. 2005) indicating that there are multiple factors determining the plasma levels of these peptides including age (Miller et al. 2013), gender (Jacobson et al. 2014) and methodological issues (Szeto et al. 2011).

Genetic variants have also been described for the *OXTR* gene in ASD populations from different ethnical backgrounds (Wu et al. 2005; Jacob et al. 2007; Liu et al. 2010; Campbell et al. 2011). The current largest and most comprehensive meta-analysis included 3941 individuals with ASD and showed significant associations between ASD and the single nucleotide polymorphisms (SNPs) rs7632287, rs237887, rs2268491 and rs2254298 in the *OXTR* gene (LoParo and Waldman 2015). For the *AVPR1A gene*, a weak association with ASD was reported in some ethnical groups (Yirmiya et al. 2006; Tansey et al. 2011).

In order to gain a better understanding of the roles of OXT and AVP in the pathophysiology of ASD, various animal models have been established. In this respect, two main strategies have been followed: one is to inactivate rodent Oxt and Avp system-related genes and analyse putative ASD-like phenotypes; the other is to find out whether there are changes in the rodent Oxt and Avp systems in existing ASD animal models. Assays to test for ASD-like symptoms in animal models are,

for example, the three-chamber test to measure social interaction, the analysis of ultrasonic vocalisations (USVs) to evaluate vocal communication, the documentation of increased self-grooming, jumping or repeated circling to analyse stereotypic repetitive behaviours and the measurement of prepulse inhibition (PPI) of the startle reflex to screen for abnormal sensory perception.

8.3 Oxt and Avp System-Related Animal Models of ASD

8.3.1 Genotype-Based Models: Oxt System

There are three critical genes of the Oxt system that have been identified so far: *Oxt*, *Oxtr* and *Cd38*. Accordingly, these genes have all been manipulated in mice to study Oxt system-related phenotypes (Modi and Young 2012).

The first two *Oxt* germline knockout (KO) mouse lines were created independently in 1996 focusing on peripheral phenotypes, i.e. female KO of both lines showed normal parturition but no postpartal milk ejection (Nishimori et al. 1996; Young et al. 1996). The following studies found that *Oxt* KO mice were responding to psychogenic stress with overexpression of c-fos and CRH (Nomura et al. 2003; Amico et al. 2008). Moreover, maternal behaviour was impaired (Pedersen et al. 2006), and *Oxt* KO females were not able to discriminate parasitised male odour (Kavaliers et al. 2003). In addition, *Oxt* KO mice were more aggressive, anxiety was exaggerated and they failed to develop social memory (Ferguson et al. 2000, 2001; Winslow et al. 2000; Amico et al. 2004; Ragnauth et al. 2005). However, olfactory detection, spatial memory capabilities and sexual behaviour seemed to be unaltered (Ferguson et al. 2000; Winslow and Insel 2002; Becker et al. 2013). Interestingly, the amygdala was found to be involved in the described alterations of social behaviour (Becker et al. 2013; Mantella et al. 2004). *Oxt* KO mice further showed signs of metabolic impairments affecting glucose homeostasis (Amico et al. 2004; Camerino 2009), hydration status (Rinaman et al. 2005) and thermoregulation (Kasahara et al. 2007).

Germline *Oxtr* KO lines were more specifically characterised regarding ASD-like phenotypes and showed impaired cognitive flexibility, social deficits, increased aggression and increased seizure susceptibility (Sala et al. 2011). Importantly, other studies confirmed the social deficits (Pobbe et al. 2012a, b). *Oxtr* KO females were fertile and showed normal reproductive behaviour, but a high level of pup abandonment was seen (Rich et al. 2014). Compared with the *Oxtr* null genotype, heterozygous *Oxtr* mutants showed normal cognitive flexibility and aggression but impaired social interaction (Sala et al. 2013). A selective ablation of the Oxtr in the forebrain using conditional *CamkIIα-Cre-Oxtr* mutants resulted in a prominent reduction of the target gene in the lateral septum, hippocampus and ventral pallidum but not in the medial amygdala. Interestingly, males from this

Table 8.1 Summary of the main behavioural phenotypes relevant to ASD in genotype-based models of the OXT/AVP system

Model	Gender	Main behavioural phenotypes relevant to ASD	References
Oxt$^{-/-}$	♂ and ♀	Social memory ↓ Maternal behaviour ↓ (♀) Response to psychogenic stress ↑ Aggressive behaviour ↑	Ferguson et al. (2000), (2001) Pedersen et al. (2006) Nomura et al. (2003), Amico et al. (2008) Winslow et al. (2000), Ragnauth et al. (2005)
Oxtr$^{-/-}$	♂ and ♀	Social interaction ↓ USV number during infancy ↓ (♂) Pup abandonment ↑ (♀) Cognitive flexibility ↓ Aggressive behaviour ↑	Sala et al. (2011), Pobbe et al. (2012a, b) Sala et al. (2011) Rich et al. (2014) Sala et al. (2011) Sala et al. (2011)
Cd38$^{-/-}$	♂ and ♀	Social recognition ↓ (♂) USV number during infancy ↓ (♂) Maternal behaviour ↓ (♀) Paternal behaviour ↓ (♂) Locomotor activity ↑	Higashida et al. (2011) Liu et al. (2008) Lopatina et al. (2011) Akther et al. (2013) Liu et al. (2008)
BB rat	♂ and ♀	Social recognition ↓ Emotional reactivity ↓ PPI deficits	Engelmann and Landgraf (1994) Williams et al. (1985) Birkett and Pickering (1988)
Avpr1a$^{-/-}$	♂	Social interaction ↓ Social recognition ↓ Spatial memory ↓	Egashira et al. (2007) Bielsky et al. (2004) Egashira et al. (2007)
Avpr1b$^{-/-}$	♂ and ♀	Social recognition ↓ Social memory ↓ (♀) Social motivation ↓ USV modulation ↓ Maternal behaviour ↓ (♀) Aggressive behaviour ↑ Locomotor activity ↑ PPI deficits	Wersinger et al. (2002) Wersinger et al. (2008) Wersinger et al. (2004) Scattoni et al. (2008) Wersinger et al. (2007a, b) Wersinger et al. (2002), Caldwell and Young (2009) Daikoku et al. (2007) Egashira et al. (2005)

conditional mutant line failed to recognise individual mice implicating a specific deficit of social recognition behaviour (Lee et al. 2008).

Cd38 is a transmembrane glycoprotein with ADP-ribosyl cyclase activity, which regulates the Ca^{2+}-dependent secretion of Oxt in the hypothalamus. It was shown that *Cd38* KO mice exhibited markedly lower ADP-ribosyl cyclase activity in both the hypothalamus and pituitary gland. The plasma level of Oxt, but not Avp, was significantly decreased in *Cd38* KO mice, and depolarisation-induced Oxt secretion and Ca^{2+} elevation in oxytocinergic neurohypophysial axon terminals were disrupted (Jin et al. 2007). Further analysis of these mice revealed a significant impairment of maternal behaviour in females (Lopatina et al. 2011) and paternal

Table 8.2 Stimuli of Oxt/Avp release within defined brain regions

Stimulus	Species	Brain regions	Neuropeptides	References
Physiological stimuli				
Parturition	Sheep	SN, OB, CSF	Oxt↑	Kendrick et al. (1988) Kendrick et al. (1991)
	Rats	PVN, SON	Oxt↑, Avp-	Neumann et al. (1993b) Neumann et al. (1996)
Suckling	Rats	SON, septum, hippocampus	Oxt↑, Avp-	Neumann et al. (1994a) Landgraf et al. (1992) Neumann and Landgraf (1989) Moos et al. (1989)
	Rats	MPOA, BNST	Oxt-, Avp↑	Bosch et al. (2010)
Hyperosmotic stress	Rats	SON	Oxt↑, Avp↑	Neumann et al. (1993a) Ludwig et al. (1994) Neumann et al. (1995) Ludwig et al. (1996)
	Rats	Septum	Avp↑	Demotes-Mainard et al. (1986)
Social/emotional stimuli				
Maternal defence	Virgin rats Lactating rats	PVN	Oxt↑	Bosch et al. (2004)
Maternal aggression	Lactating rats	CeA	Avp↑	Bosch and Neumann (2010)
Mating	Rats	PVN	Oxt↑	Waldherr and Neumann (2007) Nyuyki et al. (2011)
	Voles	NAc	Oxt↑	Ross et al. (2009)
Social discrimination	Rats	LS	Avp↑	Lukas et al. (2011)
Social fear	Mice	DLS	Oxt↑	Zoicas et al. (2014)
Social defeat	Rats	LS	Oxt↑ Avp-	Ebner et al. (2000)
	Rats	SON	Oxt↑	Engelmann et al. (1999)
	Rats	PVN	Oxt- Avp↑	Wotjak et al. (1996)
Physical stimuli				
Electrical stimulation	Rats, in vitro	Isolated neurohypophyses	Oxt↑ Avp↑	Han (2003)
Restraint stress	Rats, voles	PVN	Oxt↑	Babygirija et al. (2012a, b) Smith and Wang (2014)
Shaker stress	Rats	PVN	Oxt↑ Avp-	Nishioka et al. (1998)
Forced swimming	Rats	PVN, SON	Oxt↑ Avp↑	Wotjak et al. (1998)
	Rats	SCN, septum, CeA	Avp↑	Ebner et al. (1999) Ebner et al. (2002) Engelmann et al. (1998)
Haemorrhage	Rats	PVN	Avp↑	Ota et al. (1994)

(continued)

Table 8.2 (continued)

Agents	Species and administration	Brain regions	Neuropeptides	References
Mc4r agonists	Voles, i.p.	NAc	Oxt↑	Modi et al. (2015)
alpha-MSH	Rats, in vitro	Isolated SON	Oxt↑	Sabatier et al. (2003)
5-HT	Rats, i.c.v.	PVN	Oxt↑	Jorgensen et al. (2003a) Jorgensen et al. (2003b)
CCK-8	Rats, i.v.	SON	Oxt↑ Avp↑	Neumann et al. (1994b)
Interleukin-1β	Rats, i.c.v	SON	Oxt↑ Avp↑	Landgraf et al. (1995)
Neurosteroid	Rats, in vitro	Isolated SON	Oxt↑ Avp↑	Widmer et al. (2003) Wang et al. (1995)
$GABA_A$ receptor agonist (muscimol)	Rats, in vitro	Isolated SON	Oxt↑	Widmer et al. (2003)
Angiotensin	Rats, i.c.v.	SON, PVN	Avp↑	Moriguchi et al. (1994)
OXT agonist	Rats, in vitro	Isolated SON	Oxt↑	Moos et al. (1984)
AVP analogue	Rats, local administration	SON	Avp↑	Wotjak et al. (1994)
Naloxone	Rats, s.c., i.p.	SON, hippocampus	Oxt↑ Avp-	Neumann et al. (1991) Douglas et al. (1995)
Histamine $H_{1/2}$	Rats, local administration	PVN	Oxt↑	Bealer and Crowley (1999)

SN substantia nigra, *OB* olfactory bulb, *CSF* cerebrospinal fluid, *MPOA* medial preoptic area, *BNST* bed nucleus of stria terminalis, *CeA* central amygdala nucleus, *NAc* nucleus accumbens, *LS* lateral septum, *DLS* dorsolateral septum, *SCN* suprachiasmatic nucleus, *MC4R* melanocortin receptor 4, *5-HT* serotonin, *CCK-8* cholecystokinin, *s.c.* subcutaneous, *i.c.v.* intracerebroventricular, *i.v.* intravenous, *i.p.* intraperitoneal

behaviour and social recognition in males (Higashida et al. 2011; Akther et al. 2013). The animals also showed increased levels of locomotor activity and less frequent USVs in male pups (Liu et al. 2008), while no deficits in lactation or milk ejection were found in *Cd38* female KO mice. Overall, the social deficits of *Cd38* KO were less severe than that in *Oxt* or *Oxtr* KO mice (Liu et al. 2008). For a summary of phenotypes, see Table 8.1 and Table 8.2.

8.3.2 Genotype-Based Models: Avp System

Within the Avp system, three genes have been under focus in animal model studies: *Avp*, *Avpr1a* and *Avpr1b*.

The Brattleboro (BB) rat is the most extensively studied Avp-deficient animal model, which lacks the ability to synthesise Avp because of a single-base-pair deletion in the coding region of the *Avp* gene (Birkett and Pickering 1988; Feifel and Priebe 2001). These rats exhibit a series of behavioural deficits including decreased emotional reactivity (Williams et al. 1985), altered motivation and attention (Williams et al. 1983), impaired social recognition (Engelmann and Landgraf 1994) and PPI deficits (Birkett and Pickering 1988).

The *Avpr1a* germline KO mouse exhibits profound impairments in social recognition and social interaction (Bielsky et al. 2004; Egashira et al. 2007), subtle olfactory deficits (Wersinger et al. 2007a, b) and impaired spatial memory (Egashira et al. 2004).

Avpr1b germline KO mice also exhibited reduced social motivation and impaired social recognition with no change in olfactory discrimination (Wersinger et al. 2002, 2004). It has also been suggested that female *Avpr1b* KO mice may have some social memory deficits since they failed to terminate pregnancy in the presence of an unfamiliar male (this pregnancy block is also referred to as the Bruce effect) (Bruce 1959; Wersinger et al. 2008). The ability to modulate USVs within different social contexts was also impaired in *Avpr1b* KO mice, and maternal potentiation of USVs was absent in *Avpr1b* KO pups. Adult female *Avpr1b* KO mice further emitted fewer USVs during the resident-intruder test (Scattoni et al. 2008). Additionally, *Avpr1b* KO mice displayed markedly reduced social forms of aggression, including intermale aggression and maternal aggression (Wersinger et al. 2002, 2004, 2007a, b; Caldwell and Young 2009). Besides these behavioural alterations, *Avpr1b* KO mice also exhibited deficits of PPI of the startle reflex (Egashira et al. 2005) and a higher locomotor activity (Daikoku et al. 2007). For a summary of phenotypes, see Table 8.1.

8.3.3 The Oxt/Avp System in Monogenic Mouse Models of ASD

Importantly, the Oxt/Avp system has also been under investigation in some monogenic mouse models of ASD. For example, the number of Oxt-positive cells was decreased in the PVN of *Fmr1* (Francis et al. 2014), *Cntnap2* (Penagarikano et al. 2015) and *Magel2* KO mice (Meziane et al. 2015). Oxtr expression was also altered in several ASD mutant mice including *Fmr1*, *Dhcr7*, *Ube3a*, *Oprm1* and *Mecp2* mutants (Kotulska and Jozwiak 2011; Gigliucci et al. 2014). These findings indicate that an altered Oxt/Avp system may be common feature of monogenic ASD models.

8.3.4 Phenotype-Based Models

In contrast to the comparative exploration of inbred mouse strains, there are animals with natural variations in social behaviour, which may result from changes in Oxt/Avp system.

Microtine rodents (voles), for example, show a natural diversity in social behaviour. Therefore, these animals have become an important model to study the neurobiology of social behaviour in general, but also the specific role of hormones like Oxt can be analysed. Prairie voles (*Microtus (M.) ochrogaster*) are a highly partner-oriented rodent species characterised by a socially monogamous mating strategy and high levels of alloparental care. The analysis of the Oxt system in these animals showed that Oxtr density was highest in the prelimbic cortex, bed nucleus of the stria terminalis, nucleus accumbens (NAc), midline nuclei of the thalamus and the lateral aspects of the amygdala. In contrast, low levels of Oxtr binding in the NAc have been demonstrated in nonmonogamous rodent species, including meadow voles (*M. montanus* and *M. pennsylvanicus*), mice and rats (Insel and Shapiro 1992; Insel and Young 2001). The dense distribution of Oxt-immunoreactive fibres in the NAc is conserved in voles, mice and rats, and it is speculated that the differences of social performance might be due to remarkable species differences in Oxtr binding in this specific region (Ross et al. 2009).

The application of various chemical compounds has also been used to develop animal models of ASD. For example, valproic acid (VPA) was given to pregnant rats. The offspring of these rats showed decreased social interactions and fewer social contacts with both familiar and unknown animals (Schneider and Przewlocki 2005; Dufour-Rainfray et al. 2010). We found that the levels of *Oxt* and *Avp* mRNA and Oxt and Avp peptide were significantly lower in the PVN and SON of the hypothalamus in a VPA-induced rat model of ASD (unpublished data). However, opposite observations have also been reported indicating that adult VPA rats had an increased expression of Oxt in the SON and PVN and an increased expression of Oxtr in some brain regions including the basolateral and basomedial amygdala (Stefanik et al. 2015).

The Oxt/Avp system is also involved in some other animal models that exhibit social behavioural deficits. Results from our research group suggested that sex hormone levels during pregnancy might play a role in the susceptibility of the foetus to ASD (Xu et al. 2013, 2015). Rats or mice prenatally exposed to higher levels of testosterone or bisphenol A (BPA) displayed fewer social interactions as compared to controls. The analysis of gene expression revealed that *Avp* mRNA levels were fourfold diminished in F1 embryonic brains exposed to BPA (Wolstenholme et al. 2012; Xu et al. 2015). Phencyclidine (PCP) also induces a dose-dependent disruption of social behaviour and an increase of stereotyped behaviour in rats. Interestingly, subchronic PCP administration significantly reduced the density of Avpr1a binding sites in several brain regions in rats (Sams-Dodd 1995; Tanaka et al. 2003). Mutations in the *MECP2* (methyl-CpG-binding protein 2) gene are causative for Rett syndrome (Amir et al. 1999). It has

been suggested that this gene can also regulate Avp expression within the hypothalamus (Murgatroyd et al. 2009; Forbes-Lorman et al. 2012).

8.4 Therapeutic Strategies for Targeting the OXT/AVP System

8.4.1 Acute Administration

Clear improvements in ASD-like symptoms following acute administration of OXT or AVP have been reported in multiple animal models. For example, single intraventricular injections of OXT but not AVP could rescue social memory in *Oxt* KO mice (Ferguson et al. 2000), while injection of both OXT and AVP could lower aggression and fully reverse ASD-like behaviour in *Oxtr* KO mice. A subcutaneous injection of OXT could further rescue social memory and maternal care in *Cd38* KO mice (Jin et al. 2007). From these experiments it has been suggested that OXT can also bind to the Avp receptor in the *Oxtr* KO model (Sala et al. 2011). Moreover, administration of AVP by microdialysis into the septum could significantly improve social recognition in BB rats (Engelmann and Landgraf 1994). In monogenic ASD models such as *Oprm1* KO mice, single intranasal administration of OXT could rescue social impairments (Gigliucci et al. 2014). It has further been demonstrated that AVP could increase partner-oriented behaviour in male prairie voles, an effect that was not seen in male montane voles (Young et al. 1999).

The re-expression of the *Avpr1a* gene in the lateral septum of *Avpr1a* KO mice using a viral vector system resulted in a complete rescue of social recognition (Bielsky et al. 2005). The introduction of the entire human *AVPR1A* locus (with all the surrounding regulatory elements) could also rescue the PPI impairments in *Avpr1a* KO mice that were showing increased reciprocal social interactions (Charles et al. 2014).

8.4.2 Chronic Administration

Regarding chronic treatment, it was reported that daily administration of OXT in the first postnatal week was sufficient to prevent deficits in social behaviour and led to a more lasting behavioural recovery in adult *Cntnap2* mutant mice (Penagarikano et al. 2015). Other labs reported that chronic administration of OXT has no therapeutic effects, and sometimes it can even cause impairments in social behaviour. In the BTBR mouse model of autism, for example, intranasal administration of OXT for 30 days starting on P21 did not lead to any improvements in ASD-like phenotypes including social interaction, repetitive behaviour and fear-conditioned

learning and memory except for female sniffing in the three-chamber social interaction test (Bales et al. 2014). Moreover, intranasal administration of OXT in prairie voles given from P21 (weaning) to P42 (sexual maturity) with low (0.08 IU/kg), medium (0.8 IU/kg) and high (8.0 IU/kg) dosages resulted in dosage-dependent deficits in partner preference behaviour (Bales et al. 2013). These results indicate that the effects of chronic OXT administration may vary with dosage, age, duration and course of treatment.

8.4.3 Stimuli Enhancing Synthesis or Release of Oxt and Avp in Animals

Although direct administration of OXT or AVP is potentially beneficial for ASD patients, there are several hurdles preventing both from being used as therapeutic agents: (1) short half-life of both peptides (about 20 min in the brain and 5 min in the periphery, Mens et al. 1983), (2) poor penetration of the blood-brain barrier because of the size and charge (Landgraf and Neumann 2004) and (3) the receptors are desensitised after chronic administration. Therefore, the enhancement of the physiological synthesis or release of endogenous OXT and/or AVP by various stimuli may provide an alternative and possibly even more effective therapeutic strategy.

In rodents, several push-pull perfusion and microdialysis studies have proved that there is a local release of Oxt and Avp within the hypothalamus and other limbic brain regions in response to physiological stimuli [suckling, (Moos et al. 1989; Neumann and Landgraf 1989; Landgraf et al. 1992; Neumann et al. 1994a), parturition (Neumann et al. 1993b; 1996), hyperosmotic challenge (Neumann et al. 1993a, b, 1995; Ludwig et al. 1994)], social or emotional experience (maternal defence, Bosch et al. 2004, and social defeat, Engelmann et al. 1999) and physical stimuli (such as chronic homotypic stress, Babygirija et al. 2012a, b, and forced swimming, Wotjak et al. 1998). Some pharmacological agents also stimulate release of neuropeptides by activating hypothalamic Oxt and/or Avp neurons. For example, the exogenous administration of melanocortin receptor (Mcr) agonists to mice selectively activated Oxt neurons in the hypothalamus (Kublaoui et al. 2008) and enhanced the central release of Oxt. This can further be blocked by a melanocortin-4 receptor (Mc4r) antagonist (Sabatier 2006). In addition, the stimulation of Mc4r facilitated Oxt-dependent partner preference formation in the prairie vole (Modi et al. 2015) and improved social interaction in the *Cntnap2* mutant mouse model of autism (Penagarikano et al. 2015). The serotonin system is also involved in the regulation of Oxt secretion. Serotonergic fibres and 5-HT receptors are found in PVN and SON. Both animal and human studies demonstrated that 5-HT agonists elevated peripheral OXT/Oxt (Van der Kar et al. 2001; Lee et al. 2003) and AVP/Avp levels (Jorgensen et al. 2003a, b). The central administration

of 5-HT increased the excitability of PVN magnocellular neurons (Ho et al. 2007) and promoted the synthesis and release of Oxt and Avp (Jorgensen et al. 2003a, b).

OXT and AVP are mainly synthesised and stored in the PVN and SON of the hypothalamus. Several stressors, including forced swimming, immobilisation and long-term dehydration, increased *Oxt* and/or *Avp* mRNA concentrations in the rodent hypothalamus (Wotjak et al. 2001; Babygirija et al. 2012a, b). Interestingly, both single and repeated exposure to restraint stress resulted in the upregulation of *Oxt* (Zheng et al. 2010) and *Avp* (Jezova et al. 1995) mRNA expression in the rat PVN. A recent study in our lab also reported increased number of Oxt-immunoreactive cells in the PVN in response to stress (unpublished observation), whereas Avp-immunoreactive cells in PVN or SON were not affected. During the development of the OXT/AVP system, the production of these neuropeptides might be especially vulnerable to early-life manipulations. Both environmental (such as sensory experience, Zheng et al. 2014) and pharmacological (Bales and Carter 2003; Yamamoto et al. 2004; Penagarikano et al. 2015) manipulations during early postnatal life enhanced Oxt production as well as social behaviours during adult life.

Acupuncture, an important component of traditional Chinese medicine, is also used as a therapeutic option for a wide range of clinical conditions. It has been suggested that acupuncture or electroacupuncture (EA) stimulation with unique frequencies facilitates the release of frequency-specific neurochemicals in the central nervous system (CNS) eliciting profound physiological effects (Han 2003). Rats exposed to 30 min of EA treatment showed increased Oxt levels both in cerebrospinal fluid (CSF) and in the plasma (Uvnas-Moberg et al. 1993). This increase also occurred in certain brain regions, including the hypothalamic suprachiasmatic nucleus, the hypothalamic ventromedial nucleus and periaqueductal grey (Yang et al. 2007). A study in our lab showed that single EA intervention potentiated Oxt and Avp gene expression in the SON, but not in the PVN of adult rats. Repeated sessions of EA resulted in the upregulation of *Avp* mRNA levels and increased Oxt and Avp content in the SON. Interestingly, the EA-induced elevation of neuropeptide levels was accompanied with a social behavioural improvement of rats (Zhang et al. 2015). Despite these encouraging findings on EA in rats, several critical questions still need to be clarified in future studies including the optimal parameters of EA stimulation.

8.5 Translational Medicine of OXT and AVP

Since OXT and AVP are strongly involved in the modulation of social behaviours, these neuropeptides have been considered as potential therapeutic agents (Bartz and Hollander 2008; Macdonald and Macdonald 2010; Meyer-Lindenberg et al. 2011; Anagnostou et al. 2014; Gumley et al. 2014; Guastella et al. 2015; Neumann and Slattery 2016).

8.5.1 *Effects of Single-Dose Administration of OXT on Social Cognition in Humans*

A large body of research suggested the benefit of OXT nasal spray or intranasal administration for improving social behaviours including attachment (Buchheim et al. 2009), social memory (Guastella et al. 2008; Rimmele et al. 2009), facial expressions (Evans et al. 2010; Marsh et al. 2010), emotion recognition (Di Simplicio et al. 2009), empathic accuracy (Bartz et al. 2010) and trusting (Kosfeld et al. 2005; Mikolajczak et al. 2010). Neuroimaging studies in this context focused on the activity of the amygdala and the functional coupling between the amygdala and other brainstem regions that mediate autonomic and behavioural aspects of fear (Kirsch et al. 2005; Domes et al. 2007).

8.5.2 *Effects of Acute OXT Administration in Adult Patients with ASD*

Hollander et al. (2003) were the first to report on the effects of intravenous (i.v.) administration of OXT on facilitating the retention of social cognition in adult participants with Asperger syndrome. They found a significant reduction of repetitive behaviour and an enhanced ability to accurately assign emotional significance to speech intonation on the speech comprehension task (Hollander et al. 2007). Subsequent studies from other labs demonstrated that i.v. (Andari et al. 2010; Hall et al. 2012) or intranasal administration (Guastella et al. 2010) of OXT improved the symptoms in adolescents or children with Asperger or Fragile X syndrome (Tachibana et al. 2013).

8.5.3 *Multiple-Dose Studies of Intranasal OXT in Patients with ASD*

Most OXT interventions were studied in adult patients with ASD carefully considering ethical and safety factors. Open-label case studies and uncontrolled cohort studies imply potential benefits of repeated nasal OXT to treat ASD symptoms (Kosaka et al. 2012). However, later pilot trials showed controversial results with either positive (Watanabe et al. 2015) or negative (Dadds et al. 2014; Guastella et al. 2015) outcomes. Recently, a clinical trial in children raised the hope of a successful OXT treatment in ASD. In this study, 32 children with ASD received a 5-week OXT or placebo nasal spray. This resulted in significant improvements of caregiver-rated social responsiveness in the OXT-treated group with mild adverse events (thirst, urination and constipation). In summary, the human studies are quite similar to the animal studies showing that the benefit of single dosing could not yet

been translated to repeated OXT treatment. Further studies are needed to determine the optimised regimen and route of application for OXT.

For the therapeutic potential of AVP in ASD, data on experimental studies with patients are still missing. The prominent feature of peripheral AVP is to maintain blood pressure by its antidiuretic and vasopressor activity (Thompson et al. 2004). Therefore, the safety of this neuropeptide has to be carefully considered, especially when it is applied to children.

8.5.4 Endogenous Release of OXT/AVP in Humans

Due to ethical and methodological restriction, it is difficult to obtain local peptide concentrations in peptide-producing nuclei or CSF from human brains. Up to now, there are no studies on the central release of OXT or AVP in humans in response to exogenous stimuli. The reviewed literature is therefore mainly focusing on alterations of peripheral (plasma, saliva and urine) peptide concentrations.

Birth and suckling, two classical physiological stimuli, are known to induce the release of OXT from the neurohypophysis into the peripheral circulation. Dehydration leads to an increased osmotic pressure, which triggers AVP secretion into the blood stream. Social stimulation such as social vocalisations (Seltzer et al. 2010), parent-child contact (Feldman et al. 2014), spouse/partner support (Grewen et al. 2005; Light et al. 2005), empathy towards strangers (Barraza and Zak 2009) and interpersonal touch (Scheele et al. 2014) triggers peripheral OXT release. Recent studies in our lab indicated that transcutaneous electrical acupoint stimulation (TEAS) is also potent to increase plasma AVP levels in children with ASD and alleviate their social interaction impairments (Zhang et al. 2012). However, there is no direct evidence that OXT and AVP levels in the periphery reflect the levels and functions of these neuropeptides in the CNS. Therefore, the interpretation of peripheral neuropeptide levels with respect to CNS availability of these neuropeptides needs more experimental evidence (Horvat-Gordon et al. 2005; Henricson et al. 2008).

8.6 Conclusions

The OXT/AVP system plays a critical role in social cognition in mammals. Alterations of OXT/AVP, their receptors or upstream mediators lead to severe impairments of social behaviour that are reminiscent of clinical symptoms seen in ASD. Rodent animal models for ASD oftentimes show a clear dysfunction of their Oxt/Avp system, suggesting an involvement in the formation of social behaviour. Based on the findings in several animal models, OXT or AVP have been acutely administered to experimental cohorts. These studies revealed obvious positive effects on social memory or interaction both in animals and humans. Chronic

treatment, however, thus far resulted in contradictory results that might be explained by the complex pharmacological properties and pathway modulation of OXT and AVP. Stimulating endogenous synthesis and release of OXT and AVP may therefore be a more promising therapeutic strategy for the treatment of patients with ASD.

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Chapter 9
Extracerebral Dysfunction in Animal Models of Autism Spectrum Disorder

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9.1 Introduction

ASD is a neurodevelopmental condition characterised by the following core symptomatology: deficits in social interactions and communication and repetitive, stereotypic behaviours and restricted interests. In addition, patients commonly experience a wide range of comorbidities including gastrointestinal (GI) issues, altered metabolism and immune system dysfunction. The majority of ASD cases are diagnosed by the age of 3 (Landa 2008; Barbaro and Dissanayake 2010) with developmental signs evident between 2 and 6 months (Jones and Klin 2013). Impairment in developmental processes occurring before birth or during early infancy may therefore play a significant role in the pathophysiology of ASD (Grabrucker 2012).

Large-scale molecular genetic studies have provided evidence that the pathogenesis of ASD has a strong genetic component (Delorme et al. 2013). However,

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although genetic factors might be largely responsible for the occurrence of ASD, it is likely that in addition to a combination of ASD-related genes, specific nongenetic factors act as risk factors triggering the development and/or modifying the disorder. Here we discuss potential mechanisms underlying gastrointestinal (GI) issues and immune system abnormalities, inflammation and metabolic alterations including amino acid imbalances and the role of zinc during brain development.

9.1.1 Enteric and Immune System Abnormalities in ASD

GI symptoms are a significant issue and affect a large proportion of ASD patients. Vela and colleagues recently reported that 42% of children with ASD show GI problems (Vela et al. 2015) and previous figures have indicated as many as 90% are affected (Buie et al. 2010). In addition, ASD patients are more likely to be hospitalised for GI dysfunction than non-autistic individuals (Kohane et al. 2012) and have a higher incidence of constipation than age-matched controls (Ibrahim et al. 2009). Diarrhoea and abdominal pain are also more prevalent in ASD patients than controls (Horvath and Perman 2002; Parracho et al. 2005; Valicenti-McDermott et al. 2006; Wang et al. 2011), and the severity of GI disturbance reportedly correlates with the severity of the ASD phenotype (Adams et al. 2011; Gorrindo et al. 2012). Although altered brain function (evident as elevated stress/anxiety) undoubtedly impacts on GI issues, evidence for extracerebral mechanisms involving immune and metabolic dysfunction, gut dysmotility and altered synaptic activity in the peripheral nervous system in ASD is growing. These observations highlight the need to understand underlying biological mechanisms contributing to these symptoms with the aim that identifying effective treatments will increase quality of life for patients and families.

Furthermore, there is significant evidence indicating dysregulation of the immune system in ASD patients. A recent systematic review and meta-analysis identified increased concentrations of interleukin (IL)-1beta, IL-6, IL-8 and other serum cytokines in plasma and serum of participants with ASD compared to healthy controls (Masi et al. 2015). It is also relevant in the context of ASD to consider interactions between synaptic function and immune abnormalities (Voineagu and Eapen 2013) as multiple synaptic genes are implicated in ASD. Microglia are the principal immune cells in the brain, and in addition to performing an immunoregulatory role, it is now known that they are essential for synaptic pruning during development (Schafer et al. 2012). A higher incidence of autoantibodies including those against neuronal progenitor cells (Rossi et al. 2011; Mazur-Kolecka et al. 2014) suggests a potential immune dysfunction in ASD. Indeed, maternal inflammation induced by lipopolysaccharide (LPS) treatment of pregnant mice during critical periods of embryonic development causes brain overgrowth and ASD-like behaviours in the offspring. For example, affected pups from LPS-treated mice displayed increased NADPH oxidase (NOX)-PI3K pathway signalling,

hyperproliferation of neural stem and progenitor cells and increased forebrain microglia numbers (Le Belle et al. 2014).

In addition, corticotropin-releasing factor (CRF) and neurotensin (NT) are significantly increased in serum of ASD children pointing towards a role for brain mast cells (MCs) in the pathogenesis of ASD (Theoharides et al. 2015a). MCs release inflammatory and neurotoxic mediators in response to CRF and NT that disrupt the blood-brain barrier, activate microglia and cause focal inflammation.

Similarly, in Rett syndrome (RTT), where patients frequently exhibit ASD-like behaviour, there is evidence for dysregulation of the immune system early in life. Here, reduced microglial phagocytosis and decreased levels of BDNF have been measured. Luteolin is an inhibitor of microglia and MC activation and displays BDNF-like activity. Interestingly, treatment of a maternal immune mouse model with luteolin inhibited ASD-like behaviour and further improved sociability in children with ASD (Theoharides et al. 2015b). Further studies support these findings of increased inflammatory markers and dysregulated markers of innate immunity in ASD patients. For example, large-scale transcriptomics analysis identified microglial genes potentially driven by type I interferon responses in cortical brain regions (Gupta et al. 2014).

Relevant to these observations, the GI tract is the largest immune organ of the body and houses the majority of immunoglobulin producing cells. Increased infiltration of immune cells into the GI tract has been reported in ASD patients (Furlano et al. 2001; Torrente et al. 2002, 2004; Ashwood et al. 2003). Furthermore, recent research has highlighted that microglia in the CNS are constantly regulated by the gastrointestinal microbiota (Erny et al. 2015). In light of these findings, developing a better understanding of extracerebral mechanisms, including those involving GI function and contributing to ASD, is crucial.

9.1.2 Abnormal Amino Acid Metabolism in ASD

Amino acids are not just the building blocks of proteins but can also function as neurotransmitters, signalling molecules and source of energy. Thus, the brain is dependent on a continuous supply of amino acids during development and in adulthood. The need for aa may vary at different developmental stages and requires tight regulation throughout life. Recent genetic studies have revealed abnormal amino acid metabolism as a cause of neurodevelopmental disorders, including ASD (Novarino et al. 2012; Ghaziuddin and Al-Owain 2013; Castellan Baldan et al. 2014). The exact pathophysiological mechanisms underlying these pathologies remain unclear in most cases; however, a prompt identification of these causes may lead to effective disease prevention or treatment. Although rigorous studies are still lacking, abnormal amino acid and nutrient metabolism may also be one of the consequences of GI tract dysfunction and thus deserves more attention in the field of ASD.

9.1.3 Trace Metals in ASD

Trace metals, especially Zn^{2+}, play an essential role in the brain by acting as neuromodulators of synaptic function and in various signalling pathways. However, given that all essential trace metals must be absorbed via the GI system from dietary sources, an imbalance in trace metals can be also considered as an extracerebral mechanism potentially contributing to ASD. GI system abnormalities are tightly linked to trace metal malabsorption; however, they can also be influenced by maternal Zn^{2+} deficiency. As for many essential biological pathways, Zn^{2+} deficiency has broader effects on components of the immune system and is linked to physiological and psychological stress. The interaction of these components could form a vicious cycle to facilitate the development of ASD-like phenotypes when activated in the offspring of Zn^{2+}-deprived individuals (Vela et al. 2015). Thus, GI abnormalities, immune system dysfunction, stress and Zn^{2+} deficiency may be highly interrelated processes contributing to the development of ASD.

9.2 Overview of the Enteric Nervous System in ASD

The enteric nervous system (ENS, Fig. 9.1) plays an important role in regulating digestion, including secretion and absorption of nutrients, and motility. Although alterations in brain function may affect GI function via the sympathetic and parasympathetic nervous systems (reviewed in Furness et al. 2014), the ENS can operate independently of the CNS. Of the large number of mutations in synaptic genes identified in ASD patients, many are also expressed in the ENS and therefore may contribute to gastrointestinal issues (Gershon and Ratcliffe 2004).

During development, the human ENS is likely highly sensitive to environmental influences as it forms in the first trimester (Wallace and Burns 2005) and continues to develop after birth (Burns et al. 2009; Hao et al. 2013). In particular, factors such as altered zinc homeostasis, maternal inflammation and genetic influences during this period may contribute to ASD phenotypes.

The gut is a sensory organ with direct exposure to the external environment and is part of the body's first line of defence to many potential pathogens. The ENS is in close communication with immune cells in the GI tract (Lomax et al. 2005; Muller et al. 2014; Sharkey and Savidge 2014), and these interactions may be modified in ASD. Furthermore, growing evidence for a prominent role of the microbiota in influencing behaviour is being investigated in the context of ASD. Interactions between the ENS and the microbiota of the gut are well documented (Kunze et al. 2009; Mao et al. 2013; McVey Neufeld et al. 2015), and microbes influence mood and behaviour via the CNS (Cryan and Dinan 2012, 2015). This gut-brain communication occurs via sensory pathways (e.g. the vagal nerve) (Ratcliffe et al. 2011; Forsythe et al. 2014; Furness et al. 2014) as well as via non-neural pathways (Bravo et al. 2012; D'Mello et al. 2015).

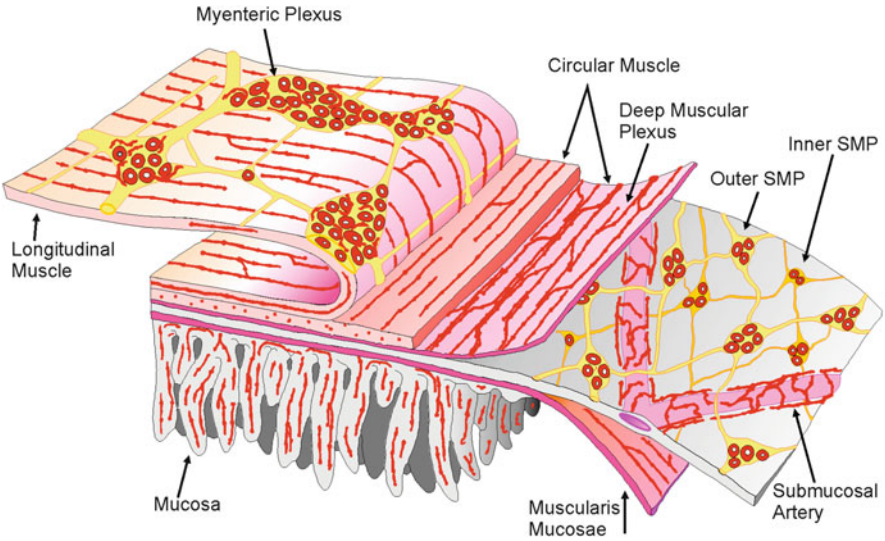

Fig. 9.1 The organisation of the ENS of human and medium-large mammals. The ENS has ganglionated plexuses, the myenteric plexus between the longitudinal and circular layers of the external musculature and the SMP that has outer and inner components. Nerve fibre bundles connect the ganglia and also form plexuses that innervate the longitudinal muscle, circular muscle, muscularis mucosae, intrinsic arteries and the mucosa. Innervation of gastroenteropancreatic endocrine cells and gut-associated lymphoid tissue is also present, which is not illustrated here. The myenteric plexus is largely involved in regulating motility, whereas the SMP predominantly regulates secretion in the GI tract. Abbreviations: *ENS*, enteric nervous system; *SMP*, submucosal plexus (with permission from Furness 2012)

Dietary factors also influence ENS function and likely contribute to GI issues in ASD patients. For example, enteric neuronal function is modified by fatty acids in the gastrointestinal lumen, which bind a range of G-protein-coupled fatty acid receptors to alter neurotransmitter release (reviewed in Furness 2012).

Although a complex network of interactions between the ENS, immune system, microbiota and the CNS ultimately affect behaviour, further research is needed to elucidate these pathways. A greater understanding of these processes may provide targets for potential therapies for ASD patients.

9.3 Animal Models of Enteric Dysfunction in ASD

Many synaptic gene families associated with ASD and characterised in the CNS (Jamain et al. 2003; Betancur et al. 2009; Betancur 2011; Grabrucker et al. 2011) are also expressed in the ENS regulating the gastrointestinal tract. Examples include the neurexins, neuroligins, L-CAMs and Shanks (Raab et al. 2010; Zhang et al. 2013; Seifi et al. 2014; Borhorquez et al. 2015).

It is hypothesised that mutations in synaptic genes contribute to GI dysfunction in patients via alterations in the regulation of secretion and motility via the ENS (Gershon and Ratcliffe 2004; Ellis et al. 2012; Argyropoulos et al. 2013). A striking example of this is the recent study of zebrafish expressing a mutation in the *CHD8* gene encoding the CHD8 neuronal cell adhesion protein mutant and its impact on GI function (Bernier et al. 2014). *CHD8* is expressed in both the CNS and ENS and is thought to play a role in synaptic adhesion and axon outgrowth. Mutant zebrafish show reduced numbers of enteric neurons and slow GI transit in addition to other relevant phenotypic traits (e.g. increased inter ocular space) (Bernier et al. 2014). Although the spatiotemporal expression profiles of the majority of these synaptic function genes have not been explored in the ENS, they are presumably present at the time of maturing synaptic function (Hao et al. 2013). Similarly, mice expressing an ASD-associated point mutation in the *Nlgn3* gene, encoding neuroligin-3 (NL3^{R451C} mice), express the mutated protein in the ENS and demonstrate altered gastrointestinal motility (Ellis et al. 2012) (Table 9.1).

9.4 Overview of Maternal Infection and Inflammatory Processes in ASD

One proposed aetiology for ASD is maternal viral infection (Grabrucker 2012). For example, in human studies, a strong association is found between rubella infection and ASD (Berger et al. 2011).

IL-6 was shown to be increased in the cerebellum of individuals with ASD and might affect synapse formation (Wei et al. 2011). Given that IL-6 is a prominent proinflammatory cytokine, events triggering increased inflammation and cytokine release may have similar consequences. Indeed, several cytokines that are released during the activation of immune cells can also induce the synthesis of cytokines in the brain. Many of them are involved in basic aspects of brain physiology. For example, in vivo and in vitro administration of exogenous IL-1, IL-2, interferon α and γ, TNFα, IL-6 and IL-18 inhibits LTP, thus affecting synaptic plasticity (Besedovsky and del Rey 2011). Increased levels of *IL-6* mRNA were also found in neonatally Borna disease virus (BDV)-infected rats (Sauder and de la Torre 1999).

Besides infection, another important factor mediating inflammatory responses is the mature GI system. Here, altered intestinal barrier function along with an increased intestinal permeability (D'Eufemia et al. 1996; de Magistris et al. 2010) may enable metabolites and bacterial waste products to enter the blood stream, which may lead to an elevated immune system response. Potential causes for such inflammatory responses in the context of ASD include prenatal zinc deficiency, altered enteric neural control of epithelial barrier function and changes in sympathetic and parasympathetic activity via the vagal nerve pathway.

Table 9.1 Overview of ASD animal models; extracerebral traits and behavioural phenotypes

Physiological trait	Animal model	Phenotype	Reference
GI abnormalities	Neuroligin-3 R451C mice	ASD-like behavioural abnormalities, altered $GABA_A$ receptor-mediated gastrointestinal motility	Tabuchi et al. (2007) Chadman et al. (2008) Ellis et al. (2012) Etherton et al. (2011) Rothwell et al. (2014) Burrows et al. (2015)
	CHD8 antisense knockdown zebrafish	Reduced enteric neuronal numbers and slow GI transit	Bernier et al. (2014)
Inflammation	Prenatal viral infection (rats and mice)	ASD-like behavioural abnormalities in the offspring	Pletnikov et al. (2002) Shi et al. (2003)
	poly(I:C)/MIA mice	Increased intestinal permeability, increased IL-6 in gut, altered microbiota, increased levels of 4-EPS (4-ethyl phenylsulphate) in serum, ASD-like behavioural abnormalities in the offspring	Smith et al. (2007) Hsiao et al. (2013)
	Prenatal LPS injection (rats and mice)	ASD-like behavioural abnormalities in the offspring, altered HPA axis	Kirsten et al. (2012) Babri et al. (2014) Gao et al. (2015)
	Maternal IL-6 injection (rats and mice)	Deficits in prepulse inhibition (PPI) and ASD-like behavioural abnormalities in the offspring	Samuelsson et al. (2006) Smith et al. (2007) Meyer (2014)
	BTBR mouse	Inflammation in gut ASD-like behavioural abnormalities	McFarlane et al. (2008) Careaga et al. (2015)
Abnormal amino acid metabolism	*Bckdk* knockout mice	Growth retardation, tremors and seizures, hind limb clasping	Joshi et al. (2006) Novarino et al. (2012)

(continued)

Table 9.1 (continued)

Physiological trait	Animal model	Phenotype	Reference
Trace metal dyshomeostasis	Prenatal zinc-deficient animals	ASD-like behavioural abnormalities in the offspring	Grabrucker et al. (2014) Grabrucker et al. (2016)
	MT3 knockout mice	Deficits in social interactions, reduced prepulse inhibition (PPI), more susceptible to kainic acid-induced seizures	Koumura et al. (2009)
	ZnT3 knockout mice	Male mice show reduced sociability and interest in social novelty and display ASD-like behavioural abnormalities (in the reciprocal social interaction test, open field test and marble-burying test)	Yoo et al. (2016)
Toxin exposure-related effects	Prenatal VPA exposure (mice)	Inflammation in gut, altered microbiota ASD-like behavioural abnormalities in male offspring	de Theije et al. (2014) Lucchina and Depino (2014)

Due to constant exposure to potential pathogens, the immune system associated with the gut is extensive, and it is therefore important to understand how inflammation (and accompanying changes in mucosal permeability) affects neural activity in central and peripheral nervous systems in ASD.

9.5 Animal Models of Maternal Infection and Inflammation

Indeed, animal models displaying certain autistic features can be generated by maternal infection. Neonatal BDV infection of the rat brain (Pletnikov et al. 2002) or influenza virus infection (Shi et al. 2003) of pregnant mice results in ASD-like behavioural abnormalities in the offspring. However, given that no virus has been detected in the brain of neonatal mice born to infected mothers, viral infection is likely to perturb fetal brain development through secondary processes. For example, viral infection may lead to alteration of the immune response of the mother and/or the offspring (Libbey et al. 2005).

In line with this, another model has been established involving activation of the immune system of pregnant mice in order to mimic the effects of maternal infection. Here, the maternal immune system is stimulated by injection of the viral mimic poly-cytidylic acid (poly (I:C)) during pregnancy (Malkova et al. 2012). This maternal immune activation (MIA) produces offspring with abnormalities in behaviour (Smith et al. 2007). For example, offspring of MIA mice show deficits in all three core features of ASD, social and communicative behaviour as well as a high level of repetitive behaviour. The mechanism by which MIA induces

behavioural deficits in the offspring might be related to elevated cytokine levels, in particular IL-6. Reduction of IL-6 by application of antibodies or genetic knockout significantly ameliorates the behavioural changes seen in the offspring of mice with MIA (Smith et al. 2007). The offspring of MIA mice also have decreased permeability of the gastrointestinal tract, altered microbiota and serum metabolome, with increased levels of IL-6 in the colon (Hsiao 2014). However, it is not known whether intestinal motility or secretion is affected, or if the ENS is altered in any way in these mice.

The role of commensal bacteria in the MIA model requires further investigation as feeding *Bacteroides fragilis* to MIA offspring improves intestinal permeability (and restores serum metabolome changes), decreases IL-6 and improves some of the behavioural phenotypes (Hsiao et al. 2013). These alterations in mucosal permeability could potentially result from a disruption in enteric neural function, an area that remains unexplored in this model. For example, it has been reported that the rate of renewal of epithelial stem cells in the base of mucosal crypts is under neuronal control (Bohórquez and Liddle 2015; Lundgren et al. 2011). Dysregulation of this process could therefore contribute to altered permeability, and further analyses in terms of enteric neural function (known to regulate GI motility and secretion) may also shed light on potential mechanisms.

Another way to induce immune system activation is the use of lipopolysaccharide (LPS). LPS is a bacterial endotoxin and induces a cytokine-associated bacterial-like acute phase response after systemic maternal administration. Indeed the adult offspring of mothers challenged with LPS displays behavioural abnormalities related to ASD such as decreased social and exploratory behaviour (Meyer 2014). Additionally, prenatal LPS-exposed pups had a significant decrease in the number and duration of ultrasonic vocalisations on postnatal day 3 and 5 (Baharnoori et al. 2012), and an increase in marble burying (a test of repetitive behaviour) was observed in male offspring (Xuan and Hampson 2014).

Given that an increase in cytokines and in particular IL-6 seems to be an important factor in various non-genetic models for ASD, the response to a direct increase of IL-6 in animal models was investigated (Samuelsson et al. 2006; Smith et al. 2007). Indeed, administration of exogenous IL-6 to pregnant animals is sufficient to induce structural and functional abnormalities in the offspring. Furthermore, the observed phenotypes were very similar to those induced by prenatal exposure to poly(I:C). For example, a decrease in social and exploratory behaviour, working memory and selective attention was observed (Meyer 2014) (Table 9.1).

9.6 Overview of Amino Acid Metabolism in ASD

The main function of the GI tract is to extract and absorb nutrients needed to support bodily functions. While poor nutrition has been shown to affect brain development (Prado and Dewey 2014), the impact of nutritional deficits in ASD remains poorly explored. In the last few years, however, genetic studies have shown that abnormal metabolism of nutrients, including vitamins, fatty acids and amino

acids, may lead to neurodevelopmental disorders, including ASD (Novarino et al. 2012; Ghaziuddin and Al-Owain 2013; Castellan Baldan et al. 2014).

A notable example of amino acid-associated neurodevelopmental disease is phenylketonuria (or PKU), a disease caused by genetic mutations leading to the inability to process the amino acid phenylalanine. When not treated, phenylalanine accumulation leads to intellectual disability and other neurological symptoms.

Recently, mutations in the gene *branched chain keto-acid dehydrogenase kinase* (*BCKDK)*, regulating the serum and brain branched-chain amino acid (BCAA) levels, have been implicated in ASD (Novarino et al. 2012). The branched chain amino acids are a group of essential amino acids (i.e. valine, leucine and isoleucine) involved in several fundamental biological processes. In eukaryotic systems, a tightly controlled catabolic pathway is in place to keep the level of the BCAA within a well-defined physiological range. The *BCKDK* gene encodes for a kinase that can shut down the BCAA catabolic pathway by phosphorylating and thus inactivating the enzyme catalysing the rate-limiting step of this pathway. Individuals carrying homozygous-inactivating mutations in the *BCKDK* gene show a significant reduction of serum BCAA levels accompanied by impairment in the three core areas implicated in ASD. In addition, BCKDK-deficient individuals may present with recurrent seizures and intellectual disability. Unfortunately, despite the fact that the BCAA are well-known molecules, the exact molecular mechanisms leading to ASD in patients lacking functional expression of BCKDK remain unclear.

BCKDK deficiency is not the first inborn error of amino acid metabolism to be associated with ASD. For example, mutations in the X-linked gene *trimethyllysine hydroxylase epsilon* (*TMLHE*), encoding for a protein implicated in the biosynthetic pathway of carnitine, have been reported as a risk factor for autism (Celestino-Soper et al. 2012). Carnitine is an amino acid derivative implicated in fatty acid transport, a function important in neurogenesis and synapse maturation. However, while based on its physiological activity it is tempting to assign carnitine an important role in the development of ASD, the very low penetrance (2–4%) of *TMLHE* mutation suggests that other unknown factors may have a major impact in determining sensitivity to carnitine deficiency.

Regardless of the gene affected, the implication of abnormal amino acid metabolism in ASD prompts to speculate that in the presence of genetic risk factors, environmental factors such as exposure to specific gut microbiota or malnutrition, neurological symptoms associated with ASD may be exacerbated.

9.7 Animal Models for Amino Acid Metabolism in ASD

The identification of a genetic mutation implicated in ASD enables the generation of accurate animal models that can be employed to study potential treatments and molecular mechanisms underlying ASD. This was certainly the case for *BCKDK*-associated ASD.

Mice lacking functional expression of *Bckdk* show a pathology resembling that observed in human patients, including decreased serum BCAA levels and neurobehavioural deficits (Novarino et al. 2012). In addition, metabolic studies in brain tissue of *Bckdk* knockout mice showed decreased levels of BCAAs and increased levels of other large neutral amino acids due to abnormal flux through the facilitative transporters present at the blood-brain barrier (BBB). BCAAs are transported across the BBB mainly by the heterodimeric amino acid transporter *SLC7A5/SLC3A2* (or LAT1). By studying *Bckdk* knockout mice, it was shown that LAT1 plays an essential role in the pathophysiology of individuals carrying *BCKDK* mutations.

Finally, *Bckdk* knockout mice were also employed to explore potential strategies to treat neurological symptoms associated with *BCKDK* deficiency. Using this approach, it was shown that branched chain amino acid supplementation can successfully rescue neurological phenotypes observed in mice and patient treatment was started accordingly (García-Cazorla et al. 2014) (Table 9.1).

9.8 Overview of Zinc Biology in the Brain and GI Tract

The imbalance of essential and putative toxic trace metals shows a strong association with ASD but also other neuropsychiatric disorders such as schizophrenia (SCZ) and depression (Pfaender and Grabrucker 2014). For ASD, genetic studies as well as the investigation of trace metal levels in patient biosamples reveal an influence of trace metal homeostasis on the ASD pathology.

In genome-wide association studies and other genetic investigations, several candidate genes for ASD have been identified that are part of pathways regulating metal homeostasis. For example, the zinc transporter 5 [ZnT5, solute carrier family 30 member 5 (SLC30A5)] has been identified by exome sequencing of ASD patients (O'Roak et al. 2011; Sanders et al. 2012). Furthermore, mutations and copy number variation of metal-regulatory transcription factor 1 (MTF1) and copper metabolism (Murr1) domain containing 1 (COMMD1) have been associated with ASD (Serajee et al. 2004; Levy et al. 2011). Additionally to these ASD candidate genes that imply trace metal imbalances as a risk factor, altered trace metal levels have been reported in numerous studies assessing blood, hair, cerebrospinal fluid (CSF) and other tissues of individuals with ASD. In particular, fluctuation of the Cu/Zn ratio seems to be an important factor given that significantly elevated Cu^{2+} levels and/or Zn^{2+} deficiency are commonly reported in individuals with ASD (Pfaender and Grabrucker 2014). Moreover, the magnitude of Cu/Zn imbalance was found to correlate with the severity of the ASD phenotype (Faber et al. 2009; Russo et al. 2012; Li et al. 2014). Besides the alterations of the essential trace metals Zn^{2+} and Cu^{2+}, and possibly facilitated by these abnormalities, elevated concentrations of nonessential and putative toxic metals have been found in patient samples. For example, an increase in Al, As, Cd, Hg and Pb was noted (Pfaender and Grabrucker 2014). A strong association of the levels of toxic

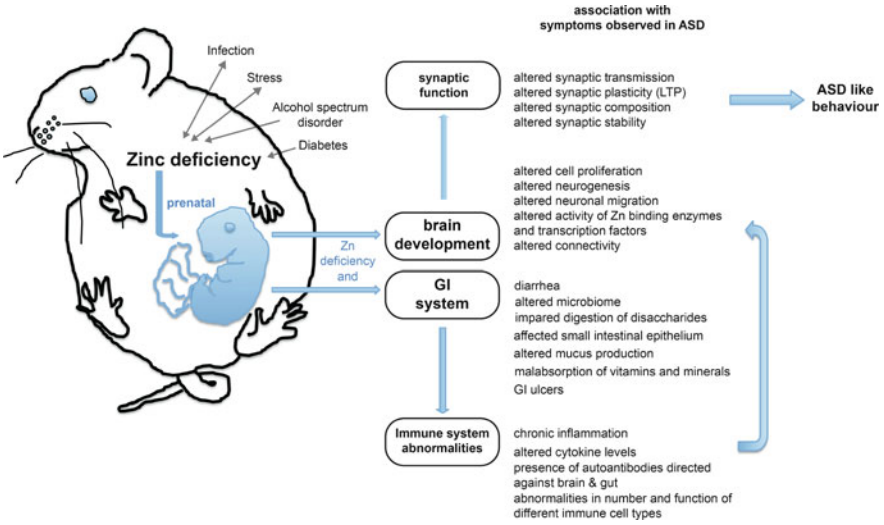

Fig. 9.2 Association of prenatal zinc deficiency with ASD. Maternal zinc deficiency has not only been associated with malnutrition but also infection, stress, alcohol consumption and diabetes. In the offspring, zinc deficiency impairs several mechanisms, given that Zn^{2+} is a cofactor of many enzymes and almost 10% of the human genome encodes for Zn^{2+}-binding proteins. However, the developing brain and GI system seem to be especially vulnerable to zinc deficiency. In the GI tract, this may lead to several problems seen in ASD patients such as frequent diarrhoea, an altered microbiome and malabsorption as well as GI ulcers. Further, due to a 'leaky gut', inflammatory processes may be triggered leading to chronic inflammation and the release of cytokines as well as the presence of autoantibodies. In the brain, zinc deficiency may impair cell proliferation, neurogenesis and neuronal migration altering overall brain connectivity and ultimately affecting synapse function in terms of transmission, composition, stability and overall plasticity

metals with variation in the degree of severity of autism was reported (Adams et al. 2013).

However, the most prominent trace metal altered in ASD is Zn^{2+} (Bjorklund 2013; Grabrucker 2012). Given that many autistic children display a Zn^{2+} deficiency especially in young age with a decline in the incidence rate until adulthood (Yasuda et al. 2011), prenatal or perinatal exposure to Zn^{2+} deficiency may contribute to the aetiology of ASD later in life by influencing the function of various ASD-associated pathways (Fig. 9.2).

9.8.1 Prenatal Zinc Deficiency and Synaptic Function

The Zn^{2+} content in the brain is relatively high compared to other tissues, with Zn^{2+} and Fe^{2+} as prevalent trace metals (Pfeiffer and Braverman 1982). Five to fifteen percent of brain Zn^{2+} is concentrated in synaptic vesicles of glutamatergic (zincergic) neurons. These neurons are highly enriched in the hippocampus and neocortical region of the mammalian brain (Frederickson and Moncrieff 1994).

There, Zn^{2+} signalling is able to influence synaptic plasticity (Xie and Smart 1994; Lu et al. 2000) and has an important role in the formation and maintenance of the postsynaptic density (PSD) (Jan et al. 2002; Baron et al. 2006; Gundelfinger et al. 2006; Grabrucker 2014). In this way, Zn^{2+} participates in the processes of neurogenesis, neuronal migration and differentiation.

Of particular interest, Zn^{2+} is released along with neurotransmitters during synaptic activity into the synaptic cleft at high μM concentrations but also increases within the postsynapse by secondary mechanisms. Zn^{2+} in the synaptic cleft is able to bind to specific Zn^{2+} receptors such as mZnR/GPR39 (Besser et al. 2009) and neurotransmitter receptors such as the NMDA subtype of glutamate receptor (NMDAR) (Peters et al. 1987; Westbrook and Meyer 1987) or enters the postsynaptic cell via various routes, including ion channels (Sensi et al. 1997). Several targets of Zn^{2+} have been associated with ASD, such as TrkB/BDNF signalling (Koh et al. 2014), ERK activity (Faridar et al. 2014; Nuttall and Oteiza 2012), NMDAR activity (Lee et al. 2015a), and Shank2 and Shank3 regulation (Grabrucker 2014). Recently, male ZnT3 knockout mice lacking presynaptic vesicular Zn^{2+} were reported to display abnormal BDNF levels and ASD-like phenotypes (Yoo et al. 2016).

Intriguingly, transsynaptic Zn^{2+} mobilisation using clioquinol is able to rescue social interaction in two independent mouse models of ASD (Lee et al. 2015b). Moreover, Zn^{2+} partially reverses functional deficits of an ASD-associated human dopamine transporter (hDAT) mutation (variant T356 M) in vitro (Hamilton et al. 2015).

Zn^{2+} might also be present at synapses of the ENS, given that the expression of the presynaptic vesicular Zn^{2+} transporter ZnT3 (SLC30A3) was detected in both myenteric and submucosal ganglia in the jejunum and duodenum of pigs (Wojtkiewicz et al. 2012a, b). However, so far, the role of synaptic vesicular Zn^{2+} in the ENS has not been well investigated.

9.8.2 Prenatal Zinc Deficiency, Gastrointestinal Function and Inflammation

Gastrointestinal development is influenced by an adequate supply of Zn^{2+} (Vela et al. 2015). In particular, Zn^{2+} deficiency affects the structure and function of the small intestinal epithelium and is accompanied with mucosal cell death and ulceration, inflammation, edema and structural alterations of villi, as well as changes in intestinal length and mucosal cell proliferation (Southon et al. 1984, 1985; Koo and Turk 1977). Additionally, several alterations in the activity of brush border enzymes have been reported, and Zn^{2+}-deficient animals showed an altered composition of intestinal mucin (Quarterman et al. 1976) that has an important role in innate host defence. Thus, it is likely that Zn^{2+} deficiency will ultimately cause structural alterations that have been associated with the so-called leaky gut syndrome and lead to inflammation based on GI problems.

Via the NF-κB (nuclear factor 'kappa-light-chain-enhancer' of activated B-cells) pathway, Zn^{2+} is able to downregulate the production of inflammatory cytokines (Prasad et al. 2011), and thus, this anti-inflammatory feature is absent in Zn^{2+} deficiency. Under normal conditions, NF-κB target genes are turned on by translocation of NF-κB dimers into the nucleus, where they bind to κB sites located within target gene promoters. The translocation is enabled by activation of the I kappa B kinase (IKK) complex, which includes IKKα, IKKβ and NF-kappa-B essential modulator (NEMO). This activation results in IκB phosphorylation and degradation and release of phosphorylated NF-κB dimers (Hayden and Ghosh 2008). Zn^{2+} inhibits IKKβ (Liu et al. 2013) and thus prevents downstream translocation of NF-κB and target gene activation. Intriguingly, IL-6 is a target gene of NF-κB (Libermann and Baltimore 1990).

LPS treatment induces maternal hypozincemia (Kirsten et al. 2015a). However, cytokine release might be balanced by adequate or increased Zn^{2+} levels. Indeed, prenatal Zn^{2+} supplementation reduces the stress response observed in adult offspring from rats exposed to LPS during gestation (Galvão et al. 2015), as well as prevents communication impairments, and social and cognitive autistic-like behaviours (Kirsten et al. 2015a, b). On a molecular level, disturbance in BDNF levels and the striatal dopaminergic and mTOR systems are prevented (Kirsten et al. 2015a, b).

9.9 Animal Models for Prenatal Zinc Deficiency in ASD

Today, an estimated 17.3% of the population worldwide is at risk of developing Zn^{2+} deficiency (Wessells and Brown 2012) with a high number also in industrialised countries. Among the major risk factors to develop Zn^{2+} deficiency in industrialised nations are ageing and pregnancy. In particular, meeting the necessary Zn^{2+} requirement during pregnancy can be challenging for the mother given that both endogenous losses and the increased demand by the embryo must be covered by absorption of Zn^{2+} from dietary sources. Similarly, during lactation, the metabolic daily requirement of Zn^{2+} is significantly increased in the mother. Meeting the requirement of Zn^{2+} is challenged by the consumption of diets low in Zn^{2+} and/or intake of dietary constituents that reduce the availability of Zn^{2+} such as competing metals, phytates or folic acid (Vela et al. 2015).

Although in humans no epidemiological studies have been performed so far that directly investigate a link between the occurrence of maternal Zn^{2+} deficiency and neuropsychiatric disorders in offspring later in life, animal models for prenatal Zn^{2+} deficiency display relevant behavioural alterations. Teratogenic effects have been shown in rodent models after severe prenatal Zn^{2+} deficiency (Hurley et al. 1971; Warkany and Petering 1972); however, these are less marked after mild Zn^{2+} deficiency. Early reports on the behaviour of prenatal Zn^{2+}-deficient mice tested later in adulthood indicate altered learning and memory, emotionality, attention and impaired social behaviour (Hagmeyer et al. 2014). Today, in light of the findings on Zn^{2+} deficiency in individuals with ASD and the increasing interest in ASD, these

data might have already been interpreted as indicating an ASD-like phenotype. In line with this, recent investigations of potential ASD-like behaviours of prenatal Zn^{2+}-deficient mice using state-of-the-art testing paradigms (designed to evaluate the core features of ASD and the most common comorbidities) indeed reveal impairments in ultrasonic vocalisation, reduced maternal behaviour, increased aggression (Grabrucker et al. 2014), increased anxiety, cognitive deficits and altered social behaviour (Grabrucker et al. 2016), reflecting most, but not all, phenotypes of ASD (Table 9.1).

However, given the wide variety of Zn^{2+}-dependent pathways in the body and the various effects of Zn^{2+} deficiency, i.e. on the immune system, GI tract and brain, the major molecular mechanisms contributing to pathological effects of prenatal Zn^{2+} deficiency are hard to decipher. Most likely, the contribution of many Zn^{2+}-driven processes in combination will shape the outcome of Zn^{2+} deficiency, dependent on the onset, duration and severity of Zn^{2+} depletion. Nevertheless, regarding ASD, two major sites of action have been proposed, namely the contribution of Zn^{2+} deficiency as a risk factor via impaired synaptic function in the brain and via gastrointestinal development (Grabrucker 2014; Vela et al. 2015).

9.10 Role of the Microbiome

The gastrointestinal microbiota plays an important role in modulating mood and behaviour; however, the precise pathways by which this occurs remain to be clarified. Diet has a major effect on the microbiota (Kashyap et al. 2013; Cox et al. 2013; Scott et al. 2013). Furthermore, it is clear that the microbiota interacts closely with both the immune and nervous systems, which are in constant bidirectional communication (Bienenstock et al. 2015).

An abnormal composition of gut microbiota has been reported in individuals with ASD as a contributing factor to GI problems. For example, lower levels of beneficial *Bifidobacter* species, an increase in *Bacteroides* and a decrease in *Firmicutes* and altered *Clostridium* species numbers and types were found compared to controls (Finegold et al. 2002, 2010; Song et al. 2004; Parracho et al. 2005; Adams et al. 2011; Mayer et al. 2014; De Angelis et al. 2015).

Recently, a role for alterations in the microbiome was also proposed in the pathology of the offspring of MIA mice. The human commensal *Bacteroides fragilis* corrected gut permeability, altered microbial composition and ameliorated several features of ASD-like behaviour such as impairments in communication, stereotypic behaviours, anxiety and sensorimotor abnormalities (Hsiao et al. 2013). Various bacterial species are known to alter gastrointestinal neuronal function (Khoshdel et al. 2013; Mao et al. 2013), and the interaction of the microbiome with the host nervous system remains to be fully explored (Kashyap et al. 2013).

Interactions of the gastrointestinal microbiota with the host highlight the importance of the gut-brain pathway in overall mood and behaviour (Cryan and Dinan 2012, 2015) (Fig. 9.3). At the level of the GI tract, more research is needed to

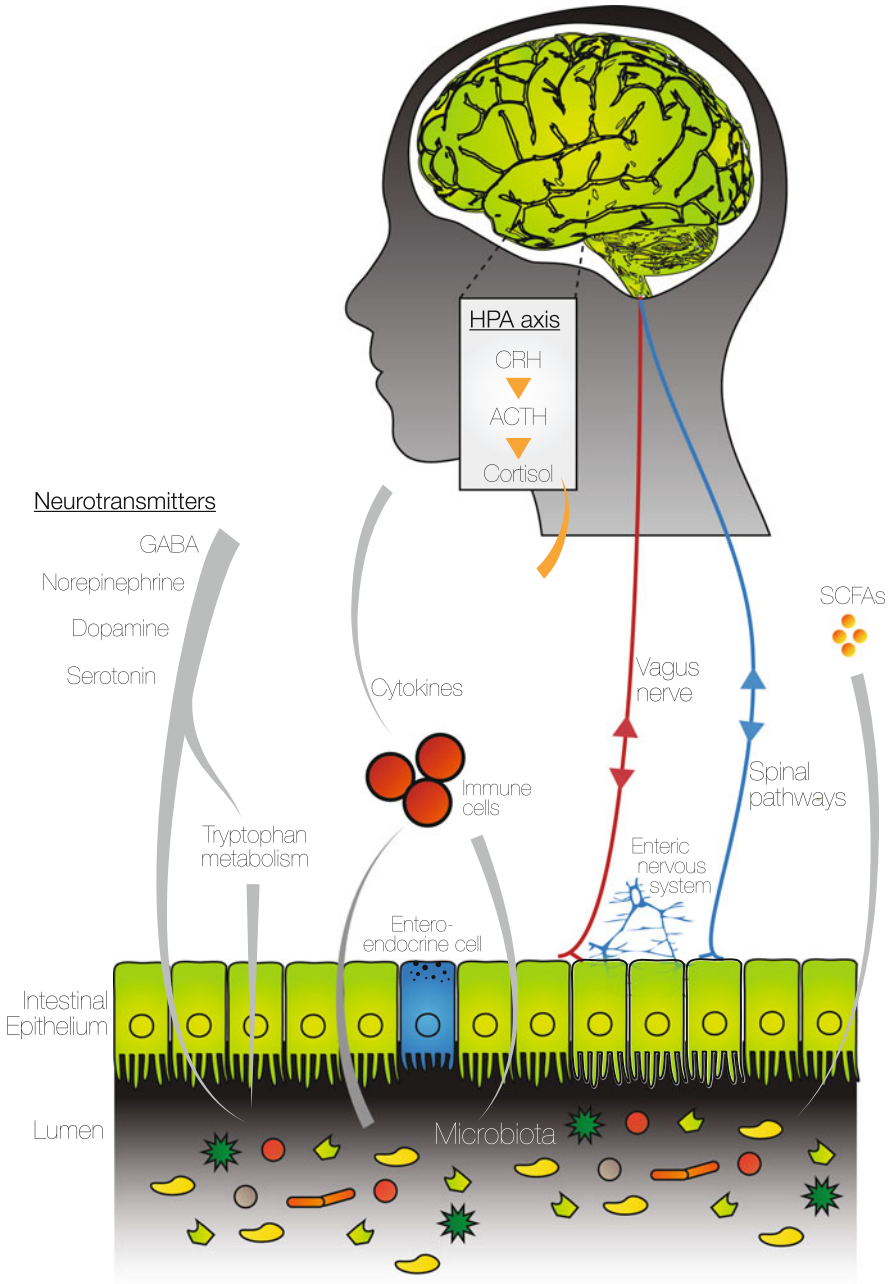

Fig. 9.3 Key pathways involved in microbiota-gut-brain signalling. Many potential direct and indirect pathways exist through which the gut microbiota are able to modulate the gut-brain axis. They include endocrine (cortisol), immune (cytokines) and neural (vagus and enteric nervous system) pathways. The gut microbiota and probiotic agents can alter the levels of circulating cytokines, and this can have a marked effect on brain function. Both the vagus nerve and modulation of systemic tryptophan levels are strongly implicated in relaying the influence of the

understand the mechanisms involved in the direct interactions of the microbiota with the enteric nervous system itself and the immune response (O'Mahony et al. 2015) and the likely consequences for GI dysfunction in ASD patients.

9.11 Role of Other ASD Risk Factors

Valproic acid (VPA) is a pharmacological agent linked to ASD (Christianson et al. 1994; Grabrucker 2012). In particular, prenatal exposure to VPA, a histone deacetylase inhibitor, has been associated with an elevated incidence of ASD in children. VPA is used in the treatment of epilepsy, mania and migraine. However, VPA also displays teratogenic effects. VPA exposure and the increased occurrence of ASD were first reported in 1994 (Christianson et al. 1994) followed by multiple confirmatory studies.

Indeed, exposure of rodents to VPA in utero results in impaired social interaction (Lucchina and Depino 2014), increased repetitive/stereotypic-like activity, increased nociceptive thresholds, enhanced anxiety and increased fear memory in the offspring (Iwata et al. 2010). However, only male offspring show the full spectrum of behavioural abnormalities associated with ASD. Intriguingly, male VPA-exposed rats display immune system abnormalities such as increased basal levels of corticosterone and a lower interferon (IFN)-γ/IL-10 ratio. In addition, male offspring prenatally exposed to VPA showed disturbances not only in the brain but additionally in the GI tract with epithelial loss, increased neutrophilic inflammation and decreased levels of 5-HT in the ileum and altered microbiota (de Theije et al. 2014).

Similarly, to the gender bias seen in VPA-exposed animals, the experience of prenatal stress produces male-specific behavioural abnormalities in rats. For example, prenatal stress produces decreased social interaction and impaired spatial and reversal learning in males (Szuran et al. 2000; Mueller and Bale 2007). Intriguingly, stress also affects the immune system, including inflammation and the modulation of the gut microbiome. Indeed, perinatal stress has been suggested to act as a proinflammatory agent during pregnancy, leading to an increase in the levels of IL-6 and TNFα (Coussons-Read et al. 2005; Marques et al. 2014; Haroon et al. 2012).

Several other so-called environmental factors have been associated with the development of ASD (Grabrucker 2012). However, research in this field is limited by the availability of animal models or the lack of investigation of present models

Fig. 9.3 (continued) gut microbiota to the brain. In addition, short-chain fatty acids (SCFAs) are neuroactive bacterial metabolites of dietary fibres that can also modulate brain and behaviour. Harnessing such pathways may provide a novel approach to treat various brain disorders (with permission from Cryan and Dinan 2015)

for a possible ASD-like phenotype. For example, maternal diabetes has been proposed as risk factor for the development of ASD. Several rodent models exhibiting diabetes during pregnancy have been generated (Damasceno et al. 2013), but a detailed behavioural analysis of the offspring regarding ASD-like behaviour is rare. Indeed, rats from mothers with streptozotocin-induced diabetes during pregnancy were reported to display increased anxiety in the elevated plus-maze and a social interaction test and hyperactivity that was more pronounced in male offspring (Van Lieshout and Voruganti 2008).

Other risk factors include the prenatal exposure to certain toxins such as organophosphate and organochlorine pesticides. In addition, abnormal melatonin synthesis possibly affecting the circadian rhythm, immune response and synaptic plasticity has been reported in ASD (Grabrucker 2012). However, many of these factors are not exclusive to ASD but also occur as risk factors for schizophrenia and learning disabilities.

9.12 Conclusions

In recent years, much progress has been made in the identification of risk factors for ASD. In particular, advances in genetics provided new insights into molecular and cellular mechanisms of the pathology. However, at the same time, a need to understand additive or multiplicative effects to bridge the gap between genotype and phenotype has been recognised. To that end, the genetic heterogeneity of ASD but also gene versus environment interactions needs to be better understood.

A range of factors including genetic mutations, environmental factors such as maternal infection, the composition of the microbiota and the availability of trace metals (in particular zinc) likely contribute to the susceptibility and heterogeneous phenotypes observed in ASD patients. These influences are intimately associated with the regulatory systems of neural development and the correct functioning of the immune system (Fig. 9.4). For example, mice haploinsufficient for the tuberous sclerosis complex 2 (*Tsc2*) gene only display abnormal social approach behaviour when exposed to maternal immune activation (Ehninger et al. 2012). In humans, mutations in *TSC2* lead to tuberous sclerosis, a disorder tightly associated with the occurrence of ASD.

Whether genetic or environmental factors act independently to trigger the initial progression of ASD in individual cases is unknown. In general, several potential mechanisms by which genetic, environmental and epigenetic factors may act together to either trigger or modify the severity and phenotypic diversity of ASD may exist. For example, environmental factors may cause genetic damage in germ cells, affect signalling pathways that are important during brain development and act through epigenetic modifications. However, an environmental factor may only affect some genetic subgroups.

Thus, in order to fully understand the etiology of ASD, it is necessary to understand impairments in each of these areas in the context of a broader systems

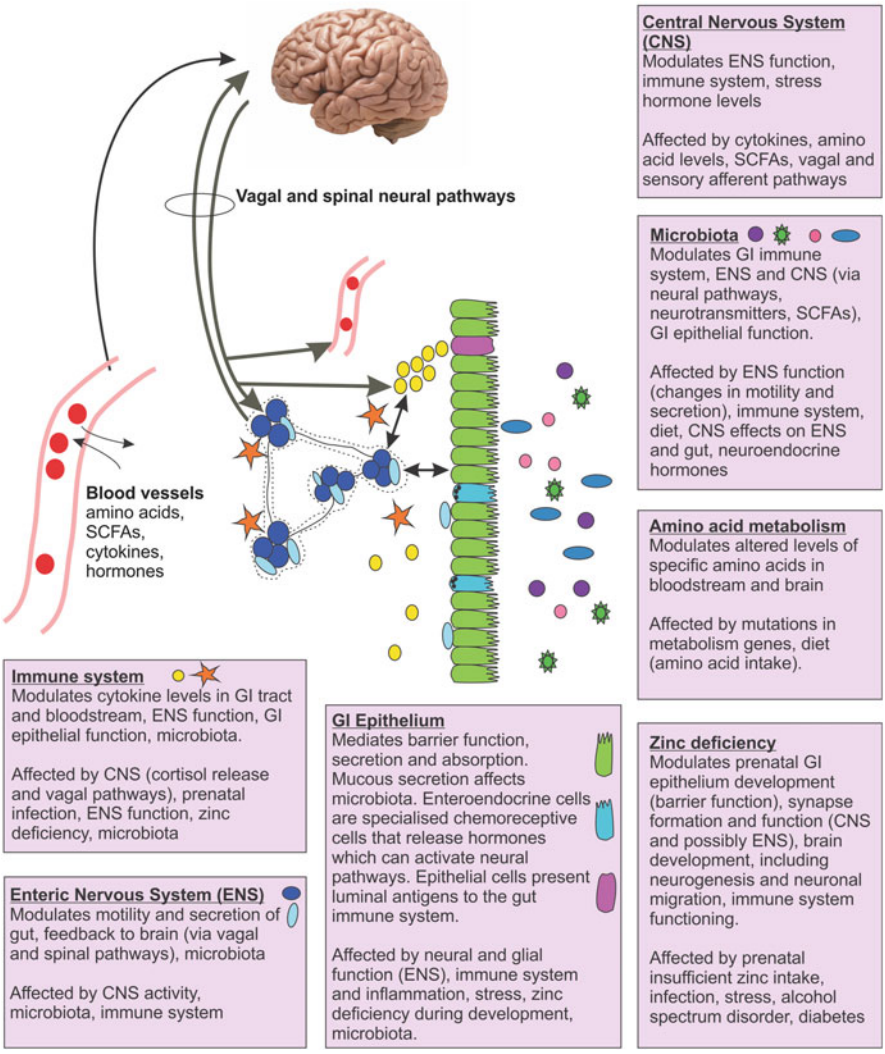

Fig. 9.4 Extracerebral dysfunction can modify CNS physiology via multiple pathways in ASD. Cytokine levels, the availability of essential trace metals and amino acids and afferent input via the vagal nerve all likely modify brain development and synapse function. However, the origin of these influences may involve peripheral systems including the GI tract, which harbours a complex microbiome. Alterations in the microbiome modulate both behaviour and the gut immune system. Additionally, immune system dysfunction and inflammation alter cytokine levels and brain function. However, it is important to note that both GI tract and immune system are influenced by the ENS. Similarly, the availability of sufficient zinc ions influences inflammatory processes, GI development and brain function directly. Amino acid metabolism impacts various systems, including brain function. Peripheral mechanisms, including inflammation, ENS function, amino acid metabolism, zinc deficiency and gut epithelial integrity, interact via complex feedback pathways. Thus, these peripheral influences are not separate entities but highly interdependent in both healthy and diseased individuals. *CNS* central nervous system, *ENS* enteric nervous system, *GI* gastrointestinal tract, *SCFAs* short-chain fatty acids

view. To this end, the use of conditional mutant models of ASD, germ-free mice and the modulation of inflammatory factors hold strong potential to shed light on causal mechanisms in relevant animal studies. However, significant areas remain that require clarification; for example, the complex nature of interactions of the gut-brain-microbiome axis with genetically determined traits of the host are yet to be fully understood. In addition, influences such as inflammatory insults and dietary changes must be studied to achieve a level of mechanistic understanding enabling personalised medicine approaches to be pursued. Nevertheless, it is likely that many environmental factors converge upon common biological targets previously identified through genetic associations, with many of these exerting effects extracerebrally in addition to altering CNS function in ASD.

Thus, the idea that extracerebral systems are both essential in ASD and influenced by environmental factors opens avenues for new treatment options to prevent disease or reduce its severity, with important implications for public health.

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Chapter 10
Genetic and Pharmacological Reversibility of Phenotypes in Mouse Models of Autism Spectrum Disorder

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10.1 Introduction

Neural development is characterised by several periods that occur in a precise sequence. During early stages, intrinsic genetic programmes drive neurogenesis, axonal outgrowth and initial connectivity. At later stages, neuronal activity induces activity-dependent anatomical and molecular changes; these changes are the basis for the refinement of neural circuits. The correct temporal sequence of all these events is essential to obtain a properly functioning brain.

Many studies suggest the existence of critical developmental periods, implying that once a critical window is closed, it might be difficult or impossible to develop certain skills (Hensch 2005; Espinosa and Stryker 2012). This suggests that if a gene is required for the accomplishment of specific developmental stages, restoring its function at later time points might not be sufficient to overcome the consequences of its early loss.

Autism spectrum disorder (ASD) is generally regarded as a group of neurodevelopmental conditions caused by abnormalities in one or more of the

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aforementioned developmental stages. As such ASD has been considered irreversible for a long time (Myers et al. 2007). In the last few years, however, an increasing number of studies have shown that in mice, ASD-like phenotypes are in some instances reversible. These remarkable results might change our way of thinking and encourage reasoning that correction of the underlying defects both during defined periods of postnatal development or even in the adult brain is an option to ameliorate or even reverse molecular, cellular and behavioural phenotypes of ASD.

Thus, accumulating data from the last decade encourage a revision of the historical irreversibility paradigm of ASD pathogenesis and warrant studies headed towards a better understanding of the underlying mechanisms. Here, we will focus on mouse models of ASD and review both genetic and pharmacological reversibility.

10.2 Genetic Reversibility

10.2.1 Fmr1

Fragile X syndrome (FXS), a monogenic syndromic neurodevelopmental disorder caused by disruption of the X-linked *FMR1* gene, is regarded as the most frequent genetic cause of intellectual disability (ID) and an identified cause of ASD. The first study on genetic reversibility in *Fmr1* KO, a widely used mammalian FXS disease model with strong construct and face validity, was published in 2000. Specifically, the authors reported that after breeding the *Fmr1* mutant mice with a YAC transgenic line carrying the full-length human *FMR1* gene, some of the behavioural phenotypes of the KO animals, such as hyperactivity or decreased anxiety, were even overcorrected, resulting in hypoactive animals with increased anxiety (Peier et al. 2000). Further studies using similar transgene strategies showed that increased audiogenic seizure susceptibility, abnormal sensorimotor gating and impaired social behaviour could be restored in *Fmr1* KO (Musumeci et al. 2007; Paylor et al. 2008; Spencer et al. 2008). Based on the mGluR5 theory of Fragile X proposing exaggerated metabotropic glutamate receptor 5 (mGluR5) function and exaggerated synaptic protein synthesis as the key molecular mechanisms in FXS (Bear et al. 2004), Dölen et al. generated *Fmr1* KO mice with a 50% reduction of mGluR5 and reported a significant rescue of behavioural and neurobiological phenotypes in these mutants (Dölen et al. 2007). Several studies with a similar approach followed to dissect the disrupted molecular machinery underlying the *Fmr1* KO phenotype (Auerbach et al. 2011; Bhattacharya et al. 2012; Ronesi et al. 2012; Busquets-Garcia et al. 2013; Udagawa et al. 2013; Gkogkas et al. 2014; Sidhu et al. 2014; Gross et al. 2015a, b). These studies collectively show that reduced expression or complete deletion of genes encoding for selected signalling or translational control molecules, including Homer1a, PI3K, PIKE, TSC2, S6K1, MNK1, MNK2, CPEB, MMP9 and CB1R, was each sufficient to reset translational

homeostasis and to rescue various phenotypes in *Fmr1* KO mice (Richter et al. 2015). Finally, a recent study further showed that both repetitive behaviours and social dominance deficits could be rescued in *Fmr1* KO mice after neonatal intracerebroventricular injection of a neuron-specific Fmr1-expressing adenovirus (Gholizadeh et al. 2014). However, no study has yet focused on genetically induced reversibility in *Fmr1* KO at later developmental stages or after symptom onset.

10.2.2 Mecp2

Mutations and copy number variations in the X-linked *MECP2* gene lead to neurodevelopmental disorders showing a strong overlap with ASD. The MECP2 protein is a DNA methylation reader that binds methylated CpG dinucleotides and acts as a transcriptional repressor. *MECP2* gene loss-of-function mutations lead to Rett syndrome (RTT), a neurological disease manifested in females as loss of acquired language and motor skills, ID, epileptic seizures, autistic behaviours and metabolic problems; males with RTT rarely survive infancy. Conversely, *MECP2* duplication, one of the most common genetic rearrangements in males, features ASD, ID, motor deficits, anxiety, epilepsy, recurrent respiratory tract infections and early death.

Deletion of Mecp2 in mice leads to a RTT-like neurological phenotype, albeit with reduced severity. *Mecp2* KO females remain healthy into adulthood, while *Mecp2* KO males show phenotypic onset around 3–8 weeks of age and die at 6–10 weeks of age. The first two studies attempting genetic rescue in *Mecp2* KO were published in 2004. Both reported that transgenic expression of *Mecp2* in *Mecp2* KO resulted in the rescue of several RTT-associated phenotypes (Collins et al. 2004; Luikenhuis et al. 2004). Collins et al. (2004) focused on characterising a mouse model for *MECP2* duplication syndrome ($Mecp2^{Tg1}$) and discovered that when crossing these mice with a Mecp2 KO line ($Mecp2^{bird/y}$), the resulting double mutants ($Mecp2^{bird/y}$; $Mecp2^{Tg1}$) have a normal phenotype, indistinguishable from wild types. Moreover, Luikenhuis et al. (2004) demonstrated that expression of *Mecp2* in postmitotic neurons of *Mecp2* KO mice rescues the RTT-like symptomatology and prolongs survival to more than 6.5 months.

These first studies, however, concentrated on inducing genetic rescue at early developmental stages, leaving open the question about the possibility to achieve phenotypic reversibility at later time points. In 2007, however, two breakthrough studies reported an amelioration of phenotypes in juvenile (Giacometti et al. 2007) and adult *Mecp2* KO (Guy et al. 2007) following reactivation of *Mecp2* gene expression. Importantly, the latter study was one of the first reporting that defects caused by a neurodevelopmental disorder can be rescued in adult animals, after symptom onset. Subsequent analyses complemented this study by additional behavioural and morphological data (Robinson et al. 2012; Lang et al. 2014) and/or used isoform- or cell-type-specific *Mecp2* transgene expression in *Mecp2* KO or other *Mecp2* mutants to identify isoform-specific effects and neuronal

populations involved in this genetic reversibility (Alvarez-Saavedra et al. 2007; Jugloff et al. 2008; Ward et al. 2011; Kerr et al. 2012; Lang et al. 2013; Pitcher et al. 2015; Ure et al. 2016). These studies intriguingly revealed that most RTT-like phenotypes are primarily rescued by restoration of Mecp2 protein levels either in neurons originating from the brainstem or in GABAergic neurons (Ward et al. 2011; Lang et al. 2013; Ure et al. 2016). In addition, both Mecp2 isoforms, which are alternatively spliced at the N-terminus, were able to exert a rescue effect, while gene dosage effects were observed (Kerr et al. 2012). Interestingly, genetic ablation of the NMDA receptor (NMDAR) subunit NR2A was further shown to rescue inhibitory hyperconnectivity in the visual cortex and the resulting deficits in visual processing of *Mecp2* KO mice, thus implying a molecular interplay of Mecp2 and NMDAR function in RTT pathogenesis (Durand et al. 2012). With respect to AAV-mediated *Mecp2* expression, it was shown that both intracranial injection at the neonatal (Gadalla et al. 2013) and intravenous systemic delivery at the adult stage (Garg et al. 2013) resulted in correction of RTT-like phenotypes in *Mecp2* KO.

Phenotypic reversibility has recently been demonstrated also in mice modelling *MECP2* duplication syndrome. In a *Mecp2*-overexpressing mouse model, genetically reducing *Mecp2* gene dosage even after symptom onset results in protein level restoration and reversal of both behavioural and molecular phenotypes in the adult animal (Sztainberg et al. 2015). Importantly, this study revealed that antisense oligonucleotides, designed to hybridise with and silence mRNA transcribed from *Mecp2*, promoted a broad phenotypic rescue of adult mutants. Treatment consisted of stereotaxic infusion of antisense oligonucleotides into the brains of 7–8-week-old transgenic animals, using microosmotic pumps. Six to seven weeks after treatment initiation, only some motor skills were restored, whereas by 10–11 weeks of therapy, the hypoactivity, anxiety-like behaviour and social deficits were reversed. Moreover, the treatment eliminated electroencephalogram abnormalities and behavioural seizures, which would have been apparent by transgenic mice of 25–35 weeks (Sztainberg et al. 2015).

10.2.3 Ube3a

In most cases, Angelman syndrome, a syndromic neurodevelopmental disorder including features of ASD, is caused by a 15q11–q13 deletion of the maternal chromosome encompassing the *UBE3A* gene, a maternally imprinted gene which encodes an E3 ubiquitin ligase. *Ube3a* KO mice, modelling Angelman syndrome, present with motor deficits, repetitive behaviours, seizures and cognitive dysfunction, along with hippocampal long-term potentiation (LTP) deficits. Among the molecular signatures of Ube3a deletion, particular interest bears the overall reduced activity of the calcium/calmodulin-dependent protein kinase II (CaMKII), which is due to elevated αCaMKII phosphorylation at the autophosphorylation sites Thr^{286}

and $Thr^{305/306}$. In 2007 it was shown that introduction of a single genetic mutation into *CamKII* gene, preventing its inhibitory autophosphorylation, was sufficient to rescue both major synaptic and behavioural phenotypes of *Ube3a* KO mice (van Woerden et al. 2007). A subsequent study reported an improvement of cognitive deficits after AAV-mediated increase of hippocampal *Ube3a* expression in adult *Ube3a* KO mice, thus implicating a therapeutic potential even after symptom onset (Daily et al. 2011). However, an extensive study on genetic reversibility in *Ube3a* KO mice during distinct developmental windows showed that most of the ASD- and anxiety-related behavioural phenotypes were only rescued by embryonic reactivation of *Ube3a*—except for the impairment in motor coordination, which could still be restored in juveniles. Interestingly, hippocampal LTP deficits were fully recovered by this treatment at any age, suggesting that cognitive deficits might be more amenable to therapy initiated also at later developmental stages (Silva-Santos et al. 2015).

Angelman syndrome patients present with deleterious mutations of the maternal allele, while the paternal allele is intact, but silenced by a nuclear-localised long non-coding RNA, the *UBE3A* antisense transcript (ATS). Because reduction of the murine *Ube3a* ATS had been proven to increase expression of the paternal allele, Meng and colleagues (2015) developed an antisense oligonucleotide-based treatment, which specifically diminished *Ube3a* ATS and unsilenced paternal *Ube3a* in neurons in vitro and in vivo. This targeted therapy was delivered acutely, via stereotaxic microinjection, and proved efficient in counteracting cognitive deficits of adult transgenic animals. However, motor deficits, repetitive behaviours and anxiety-like phenotypes were not corrected, which may indicate that complete recovery is only attainable by administering treatment within a critical developmental window (Meng et al. 2015).

10.2.4 Nrxn-Nlgn-Shank

Genetic reversibility has not only been addressed in mice modelling syndromic ASD, but recently also in mice modelling nonsyndromic ASD. Similar to the affected human individuals, these mutant lines harbour mutations either in the presynaptic cell adhesion molecule (CAM) *Nrxn1*, the postsynaptic CAM *Nlgn3* or *Shank3*, a synaptic scaffolding protein. Interestingly, *SHANK3* deletions are also causative for 22q13.3 deletion or Phelan-McDermid syndrome in humans, a syndromic ASD variant (Huguet et al. 2013; Bourgeron 2015).

First, regarding the correction of *Nrxn1* mutation-dependent ASD-like phenotypes, one study demonstrated that transgenic expression of a dominant-negative form of Neurexin-1β in CaMKII-expressing neurons in the third postnatal week results in synaptic deficits and ASD-like behaviours in mice (Rabaneda et al. 2014). Importantly, the behavioural phenotype could be rescued both in juvenile and adult animals by inactivation of transgene expression (Rabaneda et al. 2014).

Second, valuable insight on the potential reversibility of ASD-like features in mice arises from two recent reports in *Nlgn3* mutants (Baudouin et al. 2012; Rothwell et al. 2014). These studies demonstrate that successful reversal of phenotypes can be achieved not only by *Nlgn3* re-expression in the adult animal but also by inducing genetic reactivation in specific neuronal subpopulations within neuroanatomically defined circuits. In particular, Baudouin and colleagues showed that several molecular, morphological and behavioural phenotypes of *Nlgn3* KO animals, including increased cerebellar mGluR1 levels, extensive ectopic synapse formation between climbing fibres and Purkinje cell dendrites and motor coordination deficits, could be normalised by Purkinje cell-specific reactivation of *Nlgn3* expression in adult mice (Baudouin et al. 2012). In addition, Rothwell and colleagues revealed that AAV-mediated re-expression of *Nlgn3* in the nucleus accumbens of adult *D1-Cre-Nlgn3-cKO* mutants, which lack Nlgn3 selectively in D1 dopamine receptor-expressing medium spiny neurons, rescued the enhanced repetitive motor routine of these animals (Rothwell et al. 2014).

Finally, Shank3 deficiency, underlying the ASD-like features of *Shank3* KO mice, is also partially amenable to phenotypic rescue. Accordingly it was recently demonstrated that restoration of *Shank3* expression in adult *Shank3* KO mice is sufficient to correct social impairment, repetitive behaviours and striatal synaptic deficits (Mei et al. 2016). However, hypoactivity, anxiety and motor coordination problems could only be rescued by either germline or early re-expression of the *Shank3* gene (Mei et al. 2016). Conversely, increased locomotor activity and increased startle response of Shank3-overexpressing mice can be reversed by reducing *Shank3* gene dosage after crossing them with *Shank3* KO (Han et al. 2013).

10.2.5 Syngap1

Damaging mutations in *SYNGAP1*, which encodes a critical regulator of dendritic spine function, are among the most frequent genetic causes of nonsyndromic ID—commonly overlapping with ASD and epilepsy. Heterozygous *Syngap1* mutants—the mouse model for human *SYNGAP1* haploinsufficiency—exhibit accelerated spine maturation and excitation/inhibition imbalance during neural circuit development leading to profound cognitive dysfunction. Importantly, these behavioural phenotypes can only be rescued by reactivation of *Syngap1* expression during embryonic development and not at later stages of postnatal development (Clement et al. 2012; Ozkan et al. 2014; Aceti et al. 2015).

Table 10.1 summarises the seminal studies that provided striking data for genetic reversibility of phenotypes in the adult mouse even after onset of ASD-like symptoms.

Table 10.1 Summary of studies providing data on genetic reversibility of phenotypes in ASD mouse models at the adult stage after symptom onset

Model	Sex	Treatment beginning	Treatment duration	Start of analysis	Rescue effects	References
Mecp2 KO	♂	12 wk	5 wk Tmx	Progressively from start of treatment	Inertia, hind-limb clasping, tremor, irregular breathing, survival, weight, hippocampal LTP (only ♀ were tested here)	Guy et al. (2007)
	♀	20 wk	5 wk Tmx	Progressively from start of treatment		Guy et al. (2007)
	♂	8 wk	4 wk Tmx	13–14 wk	Breathing pattern, sensory-motor performance	Robinson et al. (2012)
	♂	12 wk	4 wk Tmx	20–22 wk	Thickness of motor cortex, layer 2/3 pyramidal cell soma size, dendritic length and complexity, spine density	Robinson et al. (2012)
	♂	8 wk	5 d Tmx	>14 wk	Survival, anxiety, motor coordination, locomotion, growth rate, nest building, cortical epileptiform activity, peak theta frequency, hippocampal gamma band power, home cage activity	Lang et al. (2014)
	♀	39–46 wk	5 d Tmx	>47–54 wk		
	♀	33–49 wk	1x AAV		Epilepsy, hind-limb clasping, tremor, locomotion, gait, motor performance, neuronal morphology, survival	Garg et al. (2013)
Mecp2 dup	♂/♀	8–9 wk	4 wk Tmx	14–16 wk	Locomotion, anxiety, motor performance, social behaviour, mRNA expression patterns, hippocampal LTP	Sztainberg et al. (2015)
Ube3a KO	♂/♀	Adult	1x AAV		Anxiety, spatial memory, hippocampal LTP	Daily et al. (2011)
	♂/♀	14 wk	1 wk Tmx	28 wk	Hippocampal LTP	Silva-Santos et al. (2015)
Nrxn1β ΔC	♂	2–4 mo/7–9 mo	2 wk Dox	Right after treatment	Social interaction, repetitive behaviour	Rabaneda et al. (2014)
Shank3 KO	♂	2–4,5 mo	5 d Tmx 2 wk rest 5 d Tmx	2 wk after end of treatment	Social interaction, repetitive behaviour, striatal neurotransmission, striatal PSD protein composition	Mei et al. (2016)

wk weeks, *Tmx* tamoxifen, *Dox* doxycycline, *AAV* adeno-associated virus, *LTP* long-term potentiation, *PSD* postsynaptic density

10.3 Pharmacological Reversibility

Genetic reversibility of ASD-associated phenotypes subsequently calls for the identification of pharmacological agents that are to be proven effective in the appropriate model systems and to be further used for future translational treatment trials. Major examples will be discussed in the following section.

10.3.1 Negative and Positive Allosteric Modulators of Group I mGluRs

The prevailing idea of FXS pathogenesis is that loss of FMRP, a translational repressor of several specific mRNAs, leads to exaggerated synaptic protein synthesis and boosting mGluR5-mediated pathways thus enhancing mGluR5-dependent long-term depression (LTD) and promoting dendritic spine abnormalities (Huber et al. 2002; Bear et al. 2004; Osterweil et al. 2010; Krueger and Bear 2011). Hence, inhibitors of mGluR5 signalling have been used in several studies to assess their capability of reversing FXS phenotypes in *Fmr1* KO.

The most widely used agent is 2-methyl-6-(phenylethynyl)-pyridine (MPEP), which acts as a negative allosteric modulator (NAM) of mGluR5 (IC_{50} of 36 nM) having no activity at mGluR1 up to 30 μM (Gasparini et al. 1999). Other mGluR5 NAMs with improved selectivity, bioavailability and effectiveness include 3-((2-methyl-4-thiazolyl)ethynyl)-pyridine (MTEP), fenobam, 2-chloro-4-((2,5-dimethyl-1-(4-(trifluoromethoxy)phenyl)-1H-imidazol-4-yl)ethynyl)-pyridine (CTEP), mavoglurant, basimglurant and dipraglurant (Scharf et al. 2015). Pharmacological inhibition of mGluR5 has been repeatedly shown to improve various phenotypes in *Fmr1* mutants (Yan et al. 2005; de Vrij et al. 2008; Qiu et al. 2009; Suvrathan et al. 2010; Gross et al. 2011; Levenga et al. 2011; Meredith et al. 2011; Su et al. 2011; Michalon et al. 2012, 2014; Thomas et al. 2012; Vinueza Veloz et al. 2012; Gantois et al. 2013; Gandhi et al. 2014; Pop et al. 2014; Wang et al. 2014; de Esch et al. 2015; Zhao et al. 2015). In this context, one study needs to be highlighted as it was the first to show phenotypic rescue of young adult *Fmr1* KO after symptom onset (Michalon et al. 2012). In this study, *Fmr1* KO mice received chronic treatment with the long-acting mGluR5 inhibitor CTEP starting after phenotype onset at approximately 5 weeks of age. A 4-week-long CTEP administration was sufficient to fully correct phenotypes such as increased dendritic spine density and protein synthesis rate, aberrant synaptic plasticity, learning and memory deficits and sensitivity to audiogenic seizures, while the elevated locomotor activity was corrected after 17 weeks of chronic treatment. FXS appears therefore to reflect a rather reversible disruption of brain development. Noteworthy, long-term, uninterrupted pharmacological inhibition of mGluR5 is probably key for adequate therapeutic results, since several of the mouse phenotypes could not be corrected by single-dose applications of mGluR5 NAMs.

As studies on genetic reversibility of phenotypes in adult *Fmr1* KO are still lacking, these data for the first time demonstrated effective intervention in *Fmr1* KO even after completion of neurodevelopment.

Importantly, mGluR5 NAMs were also used to successfully antagonise phenotypes in other mouse models of ASD. In human chromosome 16p11.2 microdeletion syndrome, the most common copy number variation (CNV) in autism, four deleted genes are FMRP targets, and disruption of several additional genes in this region is predicted to affect mGluR5 signalling or protein turnover. Not surprisingly, 16p11.2 microdeletion mice present with a phenotype partially overlapping with that of the *Fmr1* KO animals, especially with respect to the protein synthesis dependence of mGluR5-dependent LTD. Accordingly, cognitive deficits in these mutants were reversed by 4-week-long treatment with CTEP, initiated in young adult mice (at 4–6 weeks of age) (Tian et al. 2015).

In adult BTBR T+tf/J mice, an inbred, genetically unaltered, mouse strain exhibiting face validity for ASD diagnostic symptoms (e.g. deficits in reciprocal social interactions and social approach, unusual patterns of ultrasonic vocalisation, abnormal cognition and high levels of repetitive self-grooming), single acute administration of MPEP, 30 min before testing, was shown to reverse excessive self-grooming (Silverman et al. 2010), impaired long-term object location memory and elevated phosphorylated ERK1/ERK2 levels at hippocampal CA1 postsynaptic densities (Seese et al. 2014). Similarly, another mGluR5 NAM, GRN-529, administered in single dose, has been shown to be successful in restoring repetitive behaviour and social deficits in adult BTBR mice (Silverman et al. 2012). Since the background genes responsible for autism-relevant behavioural traits in BTBR mice have remained elusive, this ASD mouse model only presents face validity, closely relating to human idiopathic ASD, and pharmacological rescue of these phenotypic features, by inhibiting mGluR5 function in adulthood, is especially remarkable.

Prenatal exposure to valproic acid (VPA) is a risk factor of ASD in humans and also used to generate another mouse model of ASD, VPA mice (Roullet et al. 2013). In adult VPA animals, single MPEP injection prior to testing was shown to ameliorate both repetitive and anxiety-related behaviour (Mehta et al. 2011), as well as ASD-like endophenotypes, such as deficits in prepulse inhibition and reduced auditory-evoked gamma frequency synchrony (Gandal et al. 2010).

Based on the pathomechanistic hypothesis of exaggerated mRNA translation in ASD, a mutant mouse line with a deletion of the eukaryotic initiation factor 4E-binding protein 2 (Eif4ebp2), a negative regulator of mRNA translation initiation, has recently been generated (Gkogkas et al. 2013). *Eif4ebp2* adult KO mice indeed show ASD-like electrophysiological and behavioural alterations, which could be rescued by both mGluR5 (Fenobam) and mGluR1 (JNJ16259285) antagonists, each administered acutely (Aguilar-Valles et al. 2015).

Collectively, the results of these studies strongly support the idea that group I mGluR NAMs are robust pharmacological candidates for ASD treatment, particularly in cases where aberrant regulation of synaptic protein translation is mechanistically documented.

Interestingly, not only negative but also positive allosteric modulation of group I mGluRs was shown to revert ASD-like phenotypes in mice. In a mouse model of tuberous sclerosis (TSC), *Tsc2*$^{+/-}$, hippocampal mGluR-LTD was deficient and hippocampal protein synthesis decreased. Because this phenotype appeared opposite to that of *Fmr1* KO, correction of the features was attempted using pharmacological agents with agonistic activity at mGluR5. Indeed, these plasticity and protein synthesis defects as well as the associated cognitive deficits observed in the mutant animals could be rescued by acute administration of 3-cyano-N-(1,3-diphenyl-1H-pyrazol-5-yl)benzamide (CDPPB), an mGluR5-positive allosteric modulator (Auerbach et al. 2011).

In addition, CDPPB has been successfully used to rescue both synaptic physiology and ASD-like behaviour in *Shank2* (Won et al. 2012) and *Shank3* mutants (Vicidomini et al. 2016; Wang et al. 2016).

The Shanks are multidomain scaffolding proteins postsynaptically enriched at excitatory neuronal synapses, and humans with *SHANK2* or *SHANK3* mutations present with ASD and ID. *Shank2* KO lacking exons 6 and 7 of *Shank2*, for example, present with impaired synaptic plasticity (LTP severely reduced and LTD abolished) at hippocampal CA3-CA1 synapses, apparently due to NMDAR hypofunction. On the behavioural level, these mice exhibit impairments of social interaction and communication, spatial memory and learning, pup retrieval and nest building, repetitive jumping, hyperactivity and anxiety-like features. CDPPB treatment was considered because of its ability to increase mGluR5 responsiveness to glutamate and enhance NMDAR function. Accordingly, a single acute injection of CDPPB corrected the electrophysiological deficits of *Shank2* KO and selectively rescued social interaction abnormalities (Won et al. 2012).

Shank3Δ11 KO lacking exon 11 of Shank3 and therefore failing to express isoforms a, b and c are molecularly characterised by diminished mGluR5 activity due to inadequate recruitment of Homer1b/c to the PSD in striatum and cortex. The associated behavioural defects range from elevated self-grooming behaviour, diminished nest building activity, increased aggressiveness, impaired social interaction/recognition and resistance to change an acquired pattern of behaviour. Acute CDPPB treatment normalised mGluR5 function and downstream molecular pathways, as well as self-grooming activity, cognitive rigidity, social recognition and social interaction scores (Vicidomini et al. 2016).

Interestingly, the *Shank3Δ4–22* KO model lacking exons 4–22 and thus failing to express any Shank3 isoform presents with excessive self-grooming and other repetitive behaviours, normal social interest but persistence in unsuccessful efforts to engage the social partner, reduced ultrasonic vocalisations in pups and adults, motor impairments and hypoactivity, anxiety-like behaviours, enhanced reactivity to new environments, mild defects in hippocampal spatial memory and severe impairment of striatal learning. This behavioural phenotype correlates with reduced synaptic association of mGluR5 with Homer scaffolds, functional perturbations of striatal synapses (enhanced excitability of medium spiny neurons; reduced frequency of spontaneous excitatory postsynaptic currents and decreased LTD) and tonical hyperactivity of the cortical-striatal-thalamic circuit, but hypoactivity

during social behaviour. Because of the bimodal alterations found in the brain circuits of this mutant, and especially because mGluR5 function appeared enhanced at baseline, but the ligand-dependent response of mGluR5 attenuated in the striatum, both positive and negative mGluR5 allosteric modulators were considered for phenotypic rescue. The mGluR5 antagonist MPEP normalised activity in the open field and suppressed the abnormally increased self-grooming behaviour. Conversely, the mGluR5-positive allosteric modulator CDPPB had no corrective effect on mutant hypoactivity, but further enhanced the already elevated self-grooming behaviour, indicating that the latter was a result of excessive mGluR5 activity in the *Shank3Δ4–22* KO striatum. Nonetheless, CDPPB treatment partially rescued instrumental learning and restored the impaired LTD (but no other synaptic defects) (Wang et al. 2016).

Importantly, the two *Shank3* KO models used in these studies and the differential effects of CDPPB treatment argue towards careful consideration of mechanistic features and personalised therapy in ASD.

A chronic treatment, however, was not attempted in a majority of these studies. Based on the results of Michalon et al. (2012), it is reasonable to argue that mGluR5 allosteric modulation in ASD may become therapeutically effective in counteracting a wider range of symptoms, and to a larger degree, after prolonged therapy.

Recently, *Mecp2* KO mice, modelling RTT, responded well to mGluR5-positive allosteric modulation (Gogliotti et al. 2016). Symptomatic *Mecp2* KO mice at postnatal days 55–60 present with impaired mGluR5-dependent LTD in the hippocampus, when chemically induced by application of mGluR1/5 agonist dihydroxyphenylglycine (DHPG, 100 μM, 10 min). The LTD defect was corrected by a novel mGluR5-positive allosteric modulator, VU0462807. Chronic administration of VU0462807 in adult animals also improved motor performance, rescued repetitive clasping behaviour and cued fear-conditioning defects. However, treatment initiated at P24 and continued until the death end point did not improve survival (Gogliotti et al. 2016). Interestingly, also mGluR5-negative allosteric modulation has been attempted in *Mecp2* KO mice (Tao et al. 2016). The rationale of this approach was that when mGluR5-dependent LTD was tested in the 30–35 days old, presymptomatic animals, using a smaller concentration (50 μM) and shorter perfusion time of DHPG (5 min), LTD magnitude appeared similar in the mutants when compared with wild types (Tao et al. 2016). Nonetheless, in contrast with the controls, mGluR5-dependent LTD was insensitive to protein synthesis inhibitors in RTT mutants (Tao et al. 2016), a phenotype resembling mGluR5-LTD defects reported in *Fmr1* KO (Auerbach et al. 2011) and the 16p11.2 microdeletion mouse model (Tian et al. 2015). CTEP treatment of adult *Mecp2* KO mice, initiated at P30 and continued until P60, had modest outcomes, being restricted to lifespan prolongation, mild amelioration of cognitive deficits and the rescue of reduced soma size of hippocampal CA1 neurons (Tao et al. 2016). Comparing the studies of Gogliotti et al. (2016) and Tao et al. (2016), it is evident that LTD impairments in *Mecp2* KO mice may be masked at early adult stages (perisymptomatic mice) and milder induction protocols, possibly due to biphasic changes in mGluR5 function,

as well as exacerbation of phenotype with disease progression. Many aspects of RTT appear to be an ideal fit for mGluR5 positive allosteric modulators, as confirmed by the results of Gogliotti et al. (2016). However, the robust penetration of seizures in this patient population argues against this therapeutic approach and rather favours the use of mGluR5 negative allosteric modulators, which can actually prolong lifespan, as shown by Tao et al. (2016).

Careful consideration of aetiologies in early and late stage RTT should be addressed throughout the therapeutic development process, as inappropriate timing could interfere with treatment efficacy and adverse effect liability.

Autistic features seen in humans with *SHANK3* gene mutations have also been modelled in mice by selectively inducing Shank3 deficiency in the ventral tegmental area, using an shRNA approach (Bariselli et al. 2016). Neonatal mice (<P6) stereotaxically injected with an AAV expressing an shRNA targeting *Shank3* mRNA presented with defective maturation of excitatory (AMPA receptor mediated) transmission at ventral tegmental area dopaminergic and GABAergic neurons. Chronic administration of the mGluR1-positive allosteric modulator Ro 677476 to these mice, during the period of synaptic maturation (before P18), rescued the electrophysiological defects of the dopaminergic neurons and the associated social preference deficits, an effect that persisted into adulthood (Bariselli et al. 2016).

10.3.2 Agonists and Antagonists of the NMDAR

Genetic studies have identified variants of the gene encoding the NMDAR subunit GluN2B in individuals with ASD (O'Roak et al. 2011, 2012a, b; Tarabeux et al. 2011). In addition, mice with reduced NMDAR expression exhibit behavioural phenotypes reminiscent of ASD (Moy et al. 2008; Gandal et al. 2012; Saunders et al. 2013). Importantly, both NMDAR hypo- and hyperfunction have been reported in mouse models of ASD thus making the NMDAR another molecular target in translational treatment studies for ASD (Lee et al. 2015).

In particular, treatment with D-cycloserine (DCS), a partial agonist of the glycine-binding site of NMDAR (single administration), rapidly normalised electrophysiological defects and improved social deficits in the adult *Shank2* KO model described above (lacking exons 6 and 7 of *Shank2*) (Won et al. 2012). In contrast, mice lacking exon 7 of *Shank2* exhibit NMDAR hyperfunction in the hippocampus (Schmeisser et al. 2012), although pharmacological reversibility of behavioural phenotypes in these mutants by NMDAR antagonism has not been demonstrated yet. Acute treatment with DCS has further been successfully used to antagonise social impairment and repetitive behaviour in both BTBR (Burket et al. 2013) and Balb/c mice, another inbred mouse strain exhibiting ASD-like behaviour (Deutsch et al. 2011, 2012; Jacome et al. 2011; Benson et al. 2013). However, data on NMDAR hypofunction are still lacking in either model.

NMDAR hyperfunction in the VPA model of autism (Rinaldi et al. 2007; Kim et al. 2014) served as basis for the use of NMDAR antagonist as therapy for the

VPA mice. Indeed, acute treatment with the NMDAR blocker memantine rescued both social impairment and repetitive behaviour in this ASD mouse model (Kang and Kim 2015). In addition, prolonged treatment of both young and adult animals with a low dose of the NMDAR antagonist ketamine significantly improved cortical processing and ameliorated RTT-like phenotypes and survival in *Mecp2* KO mice (Patrizi et al. 2016).

10.3.3 Inhibitors of mTOR

The serine/threonine protein kinase mammalian target of rapamycin (mTOR), a signalling molecule present in mTOR complex 1 (mTORC1) and mTORC2, is involved in the regulation of synaptic protein biosynthesis, neuronal morphology and synaptic plasticity (Troca-Marín et al. 2012; Graber et al. 2013). Several lines of evidence suggest a link between abnormal mTOR signalling and ASD (Ehninger and Silva 2011; Huber et al. 2015).

A significant amount of individuals with tuberous sclerosis (TSC), a monogenic disorder caused by heterozygous loss of either *TSC1* or *TSC2*, presents with syndromic autism. Heterozygous *Tsc1* and *Tsc2* mutants serve as model systems and present with a wide range of TSC phenotypes including ASD-like behaviours and elevated mTOR signalling (Tsai and Sahin 2011; Ehninger 2013). Similarly, mutations in *PTEN* encoding the protein phosphatase and tensin homolog, another negative regulator of mTOR signalling, can result in ASD in humans and ASD-like behaviour in mice (Zhou and Parada 2012). The immunosuppressant rapamycin, which inhibits mTOR signalling primarily via inhibition of mTORC1, has been successfully used to revert TSC-relevant phenotypes in both *Tsc* and *Pten* mutant animals. In mice with ablation of *Tsc1* in astrocytes, for example, chronic rapamycin treatment before (P14) and after (6 weeks of age) onset of neurological abnormalities was able to prevent seizures and prolong survival (Zeng et al. 2008).

The beneficial outcomes of rapamycin administration were achieved both by initiating the treatment early in development (phenotype prevention) and with adult therapy (phenotype reversal). For instance, chronic treatment of neuron-specific *Tsc1* mutants with rapamycin and everolimus (another mTOR inhibitor), started at P7 and continued until P30, significantly improved several neuronal abnormalities and markedly increased survival (Meikle et al. 2008). In adulthood, deficits of synaptic plasticity and learning observed in adult germline $Tsc2^{+/-}$ mutants could be corrected by short-term daily rapamycin treatment (Ehninger et al. 2008).

More recent studies focused specifically on both origin and reversibility of ASD-like phenotypes in *Tsc* mutants. Intriguingly, loss of either *Tsc1* or *Tsc2* in cerebellar Purkinje cells was sufficient to induce ASD-like phenotypes in mice, and chronic rapamycin treatment initiated at P10 (Reith et al. 2013) or at P7 (Tsai et al. 2012) prevented the appearance of sociability defects (Tsai et al. 2012; Reith et al. 2013), cognitive deficits and neurological abnormalities and significantly ameliorated the motor problems (Tsai et al. 2012).

In addition, behavioural characterisation of both *Tsc1* and *Tsc2* heterozygous mutants revealed social deficits in both lines that could be reverted by a 2-day-long rapamycin treatment of adult animals (Sato et al. 2012).

Importantly, in adult forebrain-specific *Pten* mutants, rapamycin was administered for 1–1.5 months, beginning either before (5–6-week-old mice) or after (10–12-week-old animals) symptom onset, resulting in the correction of macrocephaly in both paradigms (Zhou et al. 2009). In young adult animals, prevention of neuronal cell hypertrophy, anxiety-like behaviour and social deficits was recorded, while in older mice the treatment reversed dentate gyrus enlargement and seizures (Zhou et al. 2009).

A 4-day-long rapamycin treatment further improved several measures of sociability in the BTBR strain while having no effect on stereotypic behaviours (Burket et al. 2014).

In an Angelman syndrome mouse model, a semi-chronic rapamycin treatment normalised LTP and actin polymerisation in hippocampal slices, as well as spine morphology and fear-conditioning learning (Sun et al. 2016).

10.3.4 Insulin-Like Growth Factor I

Although the underlying molecular mechanisms are still largely unexplored, insulin-like growth factor I (Igf-1) or its derivates have been successfully used to antagonise RTT-like phenotypes in *Mecp2* KO, FXS-like phenotypes in *Fmr1* KO and several abnormalities in heterozygous *Shank3* mutants serving as a mouse model for Phelan-McDermid syndrome.

In 2009, an active peptide fragment of human IGF-1, (1–3)IGF-1, was shown to improve both behavioural and synaptic phenotypes in *Mecp2* KO (Tropea et al. 2009). The treatment was administered daily, beginning with the second week of age and resulted in increased survival, improved locomotor activity, breathing pattern and heart rhythm and showed beneficial effects on brain weight, neuronal abnormalities and cortical plasticity in the mutant animals (Tropea et al. 2009). Subsequent studies with either polyethylene glycol-coupled IGF-1 (PEG-IGF-1) or full-length recombinant human IGF-1 (rhIGF-1) were successful in antagonising several RTT-like phenotypes in *Mecp2* mutants. A fact that requires increased attention is, in this case, appropriate dosage, since IGF-1 appears to have a tight therapeutic window in RTT-like mice. Chronic treatment with low doses of PEG-IGF-1, initiated in young animals (P28), was beneficial, in that it improved lifespan and heart rate of *Mecp2*$^{-/y}$ animals, while treatment with high doses of PEG-IGF-1 decreased lifespan and aggravated the metabolic phenotype (Pitcher et al. 2013). Moreover, both chronic application of rhIGF-1 to male *Mecp2*$^{-/y}$ at P14 and acute application of rhIGF-1 to adult symptomatic female *Mecp2*$^{-/+}$ prevented and, respectively, rescued several metabolic and behavioural RTT-like abnormalities in these mutants (Castro et al. 2014).

In *Fmr1* KO animals, a 28-day-long treatment with NNZ-2566, a synthetic analogue of (1–3)IGF-1, was able to reverse various FXS-like phenotypes in these mutants including cognitive and social impairment, hyperactivity and macroorchidism (Deacon et al. 2015).

Finally, a 2-week-long systemic administration of both (1–3)IGF-1 and rhIGF-1 was sufficient to rescue the hippocampal LTP deficits and rhIGF-1 and also the motor coordination deficits of heterozygous *Shank3* mutants (Bozdagi et al. 2013).

10.3.5 Oxytocin

The neuropeptide oxytocin is crucially involved in both reproductive and social behaviour in mammals (Ross and Young 2009; Insel 2010). Since animal models with altered oxytocin signalling display social deficits and oxytocin can be readily administered intranasally (Modi and Young 2012), several recent studies have investigated the effect of oxytocin on ASD-like phenotypes in mice. Interestingly, one of the first studies in this context reported that intranasal application of oxytocin had no major effect on ASD-like behaviours in BTBR mice (Bales et al. 2014). In contrast, another study showed that subchronic, but not acute intraperitoneal application of oxytocin to juvenile C58/J and BALB/c mice, two inbred strains exhibiting behavioural phenotypes reminiscent of ASD, had significant effects on sociability. In addition, motor stereotypies in C58/J mice could be ameliorated by acute treatment with oxytocin (Teng et al. 2013). A follow-up study reported that subchronic oxytocin administration promotes similar prosocial effects in adult C58/J and in adult *Grin1* knockdown mice, in which the ASD-like phenotypes are ascribable to NMDAR hypofunction (Teng et al. 2016). Furthermore, both intranasal and intraperitoneal acute administration of oxytocin to juvenile (4–6 weeks old) young adult mice (6–8 weeks old) lacking the contactin-associated protein-like 2 (Cntnap2), a genetic model of ASD, selectively rescued abnormal social behaviour. Remarkably, targeted stimulation of hypothalamic oxytocin producing neurons in these mutants exerted the same rescue effect (Peñagarikano et al. 2015).

Figure 10.1 provides a schematic overview of the primarily synaptic pathways targeted by the pharmacological agents discussed in this review.

10.4 Conclusions

Despite the fact that ASD is still regarded as a neurodevelopmental condition, studies of genetic reversibility in defined mouse models have provided clear evidence that phenotypes can be ameliorated at postnatal developmental stages and sometimes even in adulthood. However, successful reversibility seems to depend on several factors (e.g. the nature of the underlying genetic mutation, the

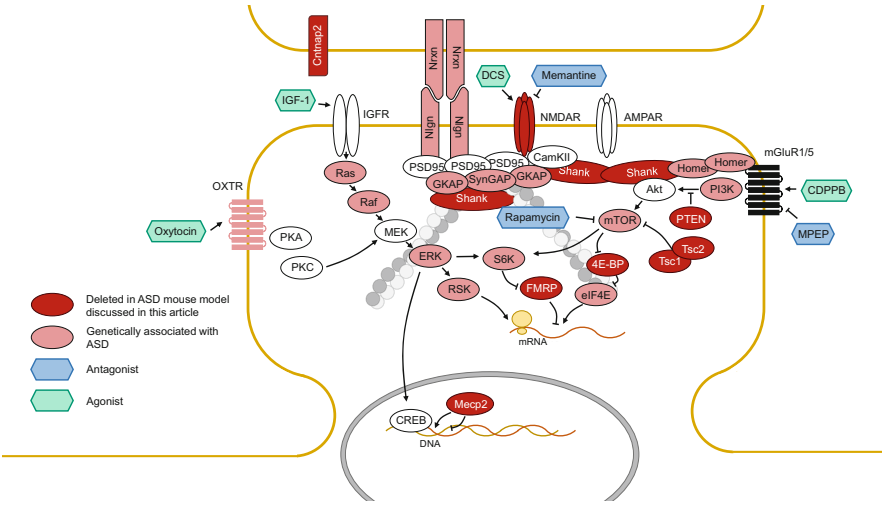

Fig. 10.1 Molecular targets of pharmacological agents mediating reversibility of ASD-like phenotypes in mice. The schematic diagram shows primarily synaptic pathways that can be modulated by pharmacological agents discussed in this article mediating reversibility of ASD-like phenotypes in the appropriate mouse models. Most of the proteins depicted are genetically associated with ASD according to the SFARI autism gene database (www.sfari.org)

molecular pathways affected or critical developmental temporal windows) that are to be studied in more detail.

In addition, pharmacological intervention in ASD mouse models thus far revealed that phenotypic reversibility may be strongly pathway specific. Thereafter, it will be essential in the future to establish good criteria to stratify ASD patients that can participate in clinical trials. This will require detailed molecular workup of each individual but may lead to potentially much more effective treatment development.

Taken together, the studies discussed in this article strongly suggest that curative intervention in ASD might be attainable—in some cases even after symptom onset. However, the translation of these findings from mice to humans remains a major challenge that continuously needs to be addressed.

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