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Marisa R. Boyd
Editor

Alzheimer's Disease Diagnosis and Treatments

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ALZHEIMER'S DISEASE DIAGNOSIS AND TREATMENTS

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NEUROLOGY – LABORATORY AND CLINICAL RESEARCH DEVELOPMENTS

ALZHEIMER'S DISEASE DIAGNOSIS AND TREATMENTS

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Nova Science Publishers, Inc.
New York

LIBRARY OF CONGRESS CATALOGING-IN-PUBLICATION DATA
Alzheimer's disease diagnosis and treatments / editor, Marisa R. Boyd.
p. ; cm.
Includes bibliographical references and index.
ISBN 978-1-61122-586-0 (eBook)
1. Alzheimer's disease--Diagnosis. 2. Alzheimer's disease--Treatment. I.
Boyd, Marisa R.
[DNLM: 1. Alzheimer Disease--diagnosis. 2. Alzheimer Disease--therapy.
WT 155]
RC523.A397596 2010
616.8'31--dc22
2010036435

Published by Nova Science Publishers, Inc. † New York

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PREFACE

Dementia is a brain disorder that seriously affects a person's ability to carry out daily activities. The most common form of dementia among older people is Alzheimer's Disease (AD), which involves the parts of the brain that control thought, memory, and language. Age is the most important known risk factor for AD. The course the disease takes and the speed at which changes occur vary from person to person. On average, AD patients live from 8 to 10 years after they are diagnosed, though the disease can last for as many as 20 years. This book presents research in the study of Alzheimer's Disease, including diagnosis, testing and treatment of this condition.

Chapter 1 - In November 1906, in Tubingen, Germany, Alois Alzheimer (1864-1915) first described his laboratory's clinical and neuropathological findings on a then novel neurological disorder in one of his female cases named Auguste D. Institutionalized by her concerned family at the age of 51, Alzheimer's first patient died of a progressive dementia just four years later. Although the clinical features of this '*disease of the aged*' were long known since ancient times, and often referred to as a '*senile psychosis*', '*age-related madness*' or '*old-timer's disease*', Alzheimer was probably the first to correlate senile plaque (*miliary foci*) and neurofibrillary tangle (*fibrils*) propensity within the association neocortex with disease diagnosis and severity. It is perhaps less well known that Alzheimer also associated cerebrovascular involvement and angiogenesis with his first description of Alzheimer's disease neuropathology, features that he termed '*focal lesions in the endothelium*' and '*new vessel formation*' in the diseased brain .

Chapter 2 - Over the last two decades studies of patients with Alzheimer's disease (AD) have made a significant contribution in helping to elucidate the neurological and cognitive bases for controlled and automatic forms of retrieval from long-term memory. These studies show that AD patients demonstrate severe deficits on tasks that involve controlled processes. In contrast, their performance on tasks involving automatic processes is variable. This article reviews experimental studies that have revealed dissociations between controlled and automatic memory processing in AD, and discusses evidence from functional neuroimaging studies which indicate that different forms of retrieval represent distinct aspects of brain activity. Attention is given to the assumption that memory retrieval reflects the operation of a single form of processing (automatic or controlled). The implications of adopting this assumption are discussed within the context of contemporary theoretical perspectives, and recent attempts to understand memory processing in AD and normal ageing by using the process-dissociation approach to memory are described. Finally, the importance of

understanding the status of controlled and automatic memory processing for the diagnosis and management of AD is considered.

Chapter 3 - Clinical presenting symptoms of Alzheimer's disease vary with age, complicating diagnosis in patients with atypical early onset of disease (age < 65 years). Patients with early onset of Alzheimer's disease symptoms have greater impairment in language, working memory and visuospatial abilities, and relatively less episodic and semantic memory impairment compared to those with the more typical later onset Alzheimer's disease. These differences suggest greater involvement of the parietal lobes in patients with early onset Alzheimer's disease, compared to greater early involvement of the hippocampus and medial temporal lobes in those with later onset Alzheimer's disease. Neuroimaging studies show greater areas of atrophy and decreased brain activity in the parietal lobes, precuneus regions, and posterior cingulate corticies in early onset patients compared to greater temporal lobe and hippocampal atrophy in those with later onset Alzheimer's disease. Patients with very late onset of Alzheimer's disease (age > 84 years) present with greater deficit of frontal lobe functions, consistent with the hypothesis of increased vulnerability of the frontal lobes and frontal-subcortical circuits to decline with age. Comparing the clinical findings of patients with Alzheimer's disease according to their age of onset highlights the complex relationship between the pathology of Alzheimer's disease and typical aging related changes that occur in the brain, and may aid clinicians in diagnosing this disease in patients at all ages on onset.

Chapter 4 - The presence of Aβ-positive neuritic plaques with dense cores is considered an essential pathological marker of Alzheimer's disease (AD). However, there are atypical cases that have abundant non-cored plaques with surrounding minor dystrophic neurites. The atypical plaques are called 'cotton wool plaques', and AD with cotton wool plaques is thought to be one of the variants of AD. Cotton wool plaques are usually large, round, and eosinophilic, and appear to displace surrounding normal structures. Inflammatory glial response is mild. We here describe two autopsy cases of early-onset dementia with abundant eosinophilic non-cored Aβ plaques, the histopathological features of which are different. Patient 1 was a 44-year-old man at the time of death, with a clinical course of 8 years. Nine relatives in three generations had died in their thirties to forties, and some of them were verified to have had dementia. The proband presented clinically with spastic paraparesis at age 36 prior to the development of dementia. The brain weight was 1330 g. Macroscopically, only mild atrophy was found in the frontal, temporal, and parietal cortices. Histopathological examination revealed abundant, large, eosinophilic, non-cored plaques having the typical appearance of cotton wool plaques. The plaques were more strongly immunopositive for Aβ42 than Aβ40. A moderate number of neurofibrillary changes were found in the hippocampus and parahippocampal gyrus, but only a few in other anatomical regions, including the neocortex. The pyramidal tract was degenerated. Although moderate neuronal loss was found in the insular and entorhinal cortices, the other cerebral cortices were relatively spared. Genetic analysis demonstrated the G384A presenilin-1 gene mutation. Patient 2 was a 46-year-old man at the time of death, with a clinical course of 8 years. His family had no history of neurological diseases in the previous three generations. He presented with memory impairment at the age of 39. Subsequently, he showed disinhibition, impulsiveness, and paranoid ideation, but no neurological abnormality. The brain weight was 1700 g. Macroscopically, neither brain atrophy nor edema was observed. Histopathological

examination disclosed abundant eosinophilic non-cored plaques in all cerebral cortices. The diameters were 40-100 μm, including plaques smaller than those in patient 1. The plaques showed little tendency to displace normal structures, but did not contain neurons. Intriguingly, the plaques in this case were were more strongly immunoreactive for Aβ40 than for Aβ42. In the cerebral cortex including the hippocampus, neurons were well preserved, and glial response was slight. In the pyramidal tract, glial proliferation was evident, although loss of myelin was not noted. No tau-positive lesions were found in any region. No mutations in presenilin-1, presenilin-2, or amyloid precursor genes were revealed by genetic analysis using formalin-fixed paraffin-embedded tissue. These findings suggest that factors besides neuritic plaques, neurofibrillary tangles, and severe neuronal loss play a pivotal role in the occurrence of cognitive decline in AD patients.

Chapter 5 - Protein aggregation is the basis for many of the common human neurodegenerative diseases such as Alzheimer's disease (AD), Parkinson's disease and a family of disorders that includes Huntington's disease. In AD the aggregatory species is termed amyloid β (Aβ), a peptide derived from the proteolytic cleavage of amyloid precursor protein (APP), a ubiquitous transmembrane protein. The aggregatory properties of Aβ are determined by variations in the position of the proteolytic cleavage that generates the C-terminus. In healthy elderly individuals the ratio of the 40 amino acid peptide ($A\beta_{1-40}$) to the 42 amino acid species ($A\beta_{1-42}$) favours the less aggregatory $A\beta_{1-40}$ resulting in effective clearance of the peptide from the brain. In contrast, individuals who go on to develop the common sporadic form of AD have elevated $A\beta_{1-42}$ concentrations, or have a molar ratio of $A\beta_{1-40}$ to $A\beta_{1-42}$ that favours aggregation. In the five percent of AD cases that are inherited as an autosomal dominant trait all the causal mutations have been shown to favour Aβ aggregation, mostly by altering APP processing, either increasing $A\beta_{1-42}$ in absolute terms or in comparison to $A\beta_{1-40}$. In rare examples, where $A\beta_{1-42}$ levels are not elevated, mutations are found within the Aβ sequence that accelerate the intrinsic rate of peptide aggregation and stabilise particularly toxic subpopulations of aggregates, a clear example of this is the Arctic APP mutation.

In the context of cognitive decline, the demonstration of Aβ deposition in the brain in combination with intraneuronal aggregates of a microtubule-associated protein, tau, comprise the diagnostic criteria for AD. Mature deposits of Aβ are composed of ordered amyloid fibrils and it is their distinctive microscopic appearance and their affinity for dyes such as Congo red that favoured their early characterisation. However there is a poor correlation between the burden of amyloid plaques and the degree of cognitive impairment, indeed elderly individuals may have many plaques without showing signs of cognitive impairment. In contrast, it is the intracellular tau pathology that has been shown to correlate more closely with clinical deficits. The location and progression of the tau lesions correlates well with the brain areas, such as the hippocampus, that are particularly impaired in AD.

The poor correlation between extracellular amyloid plaques and dementia has been used to detract from the significance of Aβ in the pathogenesis of AD. However recent evidence has clarified the situation, emphasising the toxic role of small Aβ aggregates rather than the amyloid fibrils. The finding that soluble Aβ correlates better with synaptic changes and cognitive deficits than plaque count has prompted the investigation of soluble aggregates of Aβ. These small aggregates can be purified by column chromatography and are composed of as few as 4 or as many as 180 Aβ molecules. When applied to cell cultures the oligomers are

toxic whereas in most cases amyloid fibrils and Aβ monomers are not. When oligomers are visualised under electron or atomic force microscopes they are heterogeneous, including spheres, beads-on-a-string and doughnuts, but it seems that the spherical species are most toxic. Toxic oligomers may also be specifically detected, *in vitro* and *in vivo*, using rabbit antisera raised against Aβ immobilised on gold beads. The antiserum, described by Kayed and colleagues, binds specifically to small toxic aggregates of Aβ and neutralises their toxicity, in contrast the serum fails to detect monomeric or fibrillar forms of Aβ. Subsequent work has shown that the antiserum recognises an epitope on Aβ oligomers that is common to the oligomeric aggregates of a range of pathological proteins. The interesting corollary of this observation is that a common structural motif predicts a common mechanism of toxicity. This prediction is supported by work by Bucciantini *et al*. showing that oligomeric aggregates of a non disease related protein can elicit toxicity similar to that of Aβ oligomers in cell culture. Further work done in cell culture by Demuro and colleagues has shown that a shared ability to disturb membrane conductivity may underlie at least part of the toxicity of soluble protein aggregates.

However the hypothesis that soluble aggregates of Aβ represent a stable neurotoxic species has had to be reconsidered in the light of recent work showing that it is the ongoing *process* of aggregation that is toxic. It seems now that the soluble aggregates may simply be an efficient seed that can promote further addition of Aβ monomers. In their recent study, Wogulis and colleagues showed that, as expected, neither monomeric nor fibrillar Aβ were toxic to human or rat neuronal cell cultures. Their novel observation was that pre-treatment of cells with fibrillar Aβ, followed by a wash to remove unbound fibrils, primed the cells to die when they were subsequently treated with monomeric Aβ. The stability of the interaction of the fibrils with the cells was a surprise; following exposure to fibrils for only one hour the cells were still sensitized to the toxic effects of monomeric Aβ one week later.

With emphasis being placed on the oligomeric aggregates and the initial stages of the aggregation process, the mature plaques and tangles are increasingly being viewed as tombstones of pathological protein aggregation. Indeed there is evidence from cell-based models of Parkinson's disease that inclusions may be protective, reducing the rate of apoptosis possibly by providing a sink for the disposal of toxic oligomers.

Chapter 6 - The spatial patterns of β-amyloid (Aβ) deposits and neurofibrillary tangles (NFT) were studied in areas of the cerebral cortex in 16 patients with the late-onset, sporadic form of Alzheimer's disease (AD). Diffuse, primitive, and classic Aβ deposits and NFT were aggregated into clusters; the clusters being regularly distributed parallel to the pia mater in many areas. In a significant proportion of regions, the sizes of the regularly distributed clusters approximated to those of the cells of origin of the cortico-cortical projections. The diffuse and primitive Aβ deposits exhibited a similar range of spatial patterns but the classic Aβ deposits occurred less frequently in large clusters >6400μm. In addition, the NFT often occurred in larger regularly distributed clusters than the Aβ deposits. The location, size, and distribution of the clusters of Aβ deposits and NFT supports the hypothesis that AD is a 'disconnection syndrome' in which degeneration of specific cortico-cortical and cortico-hippocampal pathways results in synaptic disconnection and the formation of clusters of NFT and Aβ deposits.

Chapter 7 - Disorder of consciousness is not an all-or-none phenomenon but it rather represents a continuum. Alzheimer's disease (AD) is the most common cause of dementia

among people aged 65 and older, and patients are frequently unaware of the importance of their cognitive deficits. Vegetative state (VS) is a clinical entity with a complete lack of behavioural signs of awareness, but preserved arousal. Both clinical entities share a certain level of consciousness alteration, and a certain similarity in brain metabolic impairment. Here, we review differences and similarities in brain function between these two types of disorders of consciousness, as revealed by functional neuroimaging studies.

Chapter 8 - Mild Cognitive Impairment (MCI) describes older adults whose cognitive and functional status is considered in-between normal cognitive aging and dementia. MCI is an heterogeneous entity with a number of subtypes each with a different neuropsychological profile. The MCI amnestic type is the better known of the subtypes and many patients with this clinical and cognitive profile will develop Alzheimer's disease. Although the amnestic MCI concept emphasizes memory loss, other cognitive functions are frequently affected, namely semantic fluency, attention/executive functions, visuo-spatial abilities and language comprehension.

MCI criteria make use of scores in delayed recall of episodic memory tasks to establish the presence of memory impairment. Poor delayed recall can, however, reflect deficits in distinct memory processes. Difficulties in the learning process of MCI patients have also been documented. During the acquisition of semantically structured lists of words, these patients employ less semantic clustering strategies than controls. However, if attention is called to the semantic structure, they can make use of it on subsequent trials in order to improve learning.

Detailed knowledge of the memory processes disturbed in MCI should contribute to the understanding of the pathophysiology of MCI, allow a more precise identification of patients with high probability of progression, and help to delineate future rehabilitation interventions in these patients.

Chapter 9 - There is substantial evidence of morphological, biochemical and molecular abnormalities in mitochondria of patients with neurodegenerative disorders, including Alzheimer's disease (AD). The functions and properties of mitochondria might render subsets of selectively vulnerable neurons intrinsically susceptible to cellular aging and stress. However, the question "is mitochondrial dysfunction a necessary step in neurodegeneration?" is still unanswered.

This chapter presents how malfunctioning mitochondria might contribute to neuronal death in AD. Moreover, we will investigate the cause and effect relationships between mitochondria and the pathological mechanisms thought to be involved in the disease.

Chapter 10 - The improper regulation of calcium levels in neurons is proposed as a primary regulatory impairment that underlies the onset of Alzheimer's Disease (AD). Calmodulin is a primary target of calcium ions in all human cells but has essentially been ignored as a downstream target in the onset of AD. Our lab previously has theoretically implicated calmodulin as an interacting protein for of a number of upstream proteins involved in the production of amyloid-beta peptide (Aβ), a pathogenic marker of Alzheimer's disease (AD) and the primary element of the "amyloid hypothesis". The first enzyme in the proteolytic processing of amyloid precursor protein (APP1) into Aβ is β-secretase (β site-amyloid converting enzyme 1 or BACE1) which was one of the enzymes identified as a putative calmodulin-binding protein. In this study we tested the effects of calmodulin, calcium and calmodulin antagonists on the *in vitro* activity of BACE1 to determine if it is potentially regulated by calmodulin. BACE1 enzyme activity was dose-dependently increased

by calmodulin reaching a maximum ~2.5-fold increase at 3μM calmodulin. Calcium (1.0mM) enhanced BACE1 activity while the calcium-chelator EGTA (10mM) inhibited it supporting a role for calcium in regulating BACE1 activity. In keeping the role of calmodulin as a regulator of BACE1 activity, five different calmodulin antagonists (trifluoperazine, W7, W5, W12, W13) each differentially inhibited BACE1 activity *in vitro*. The binding of BACE1 to calmodulin-agarose in the presence of calcium ions but not EGTA further supports the concept of BACE1 as a potential calcium-dependent calmodulin-binding protein.

Chapter 11 - As a result of advances in molecular biological techniques, the first mice overexpressing mutated genes associated with familial Alzheimer's disease (AD) were engineered ten years ago. Most of the transgenic murine models replicate one key neuropathological sign of AD, namely cerebral amyloidosis consisting of parenchymal accumulation of amyloid-beta (Aβ) peptides that subsequently form plaques. Major research efforts today focus on the use of sophisticated transgenic approaches to discover and validate drugs aimed at reducing the brain amyloid load (eg recent immunotherapeutical attempts).

However, since the initial publications, the limitations associated with classic transgenic (APP and APP/PS1) models have become apparent. First, induction of AD-related brain lesions in genetically modified mice mimics, through parallel causal mechanisms, the physiopathogeny of familial forms of AD; however, the relevance of such transgenic mice in modeling the most prevalent forms (sporadic late-onset) of AD remains largely uncertain. Second, the neuropathological phenotype of mice bearing human mutated transgenes is largely incomplete. In particular, neurofibrillary alterations (tangles) are not reported in these models.

Transgenic mice nonetheless provide a unique opportunity to address different questions regarding AD pathology. Since these models do not replicate classic neurofibrillary lesions they can be used to specifically investigate and isolate the impact of the remaining brain injuries (Aβ deposition) on different aspects of the mouse phenotype. In addition, comparisons can be made between Aβ-induced alterations in mice and known features of the human pathology.

The present review questions the specific impact of Aβ brain lesions at different levels. First we describe macroscopic and microscopic neuropathological alterations (neuritic dystrophy, inflammation, neuronal loss) associated with amyloid deposits in transgenic mice. Then, modifications of the behavioral phenotype of these animals are listed to illustrate the functional consequences of Aβ accumulation. Next we describe the non-invasive methods that are used to follow the course of cerebral alterations. Finally, we discuss the usefulness of these models to preclinical research through examples of therapeutical trials involving AD drug candidates.

Chapter 12 - Cyclooxygenase 2 (COX-2) is one of the main enzymes involved in inflammation and a major player in prostaglandin synthesis. There exists data that suggest a potential role of COX-2 in Alzheimer's disease (AD) pathogenesis. AD is the most prevalent form of dementia affecting 10% of individuals over the age of 65 and 50% of individuals over 85 years of age and is characterized by the presence of beta-amyloid (Aβ) deposits and neurofibrillary tangles (NFT) comprising of hyperphosphorylated tau. Aβ peptides have been shown to trigger inflammation and to stimulate COX-2 activity in various cell types including neurons, glia (microglia and astrocytes) and cerebrovascular cells. Several epidemiological studies have shown that the use of non-selective COX inhibitors are associated with reduced

risk of developing AD. COX-2 inhibitors have also been shown to alter AD pathology and ameliorate some behavioral impairment in transgenic mouse models of AD. Furthermore, in these mouse models, it has been shown that COX-2 inhibitors may influence APP processing. More studies are required to determine whether COX-2 inhibitors have beneficial or detrimental effects on the treatment of AD.

Chapter 13 - Abnormalities of brain metal homeostasis in Alzheimer's disease (AD) could contribute to set up chemical conditions where β-amyloid (Aβ) toxicity and deposition are promoted. Recent studies, some also *in vivo*, have shown the possible implication of copper in AD pathogenesis. In particular, evidence collected in the last five years showed that abnormalities in copper distribution deriving from blood stream variations, or as a consequence of aging, correlate with functional or anatomical deficits in AD. Serum copper increases specifically in AD and its assessment may help to non-invasively discriminate AD from normalcy and vascular dementia. Moreover, changes in distribution of the serum copper components, consisting of an increase of a copper fraction not related to ceruloplasmin, seem to be characteristic of AD and possibly implicated in the pathogenesis of the disease.

Chapter 14 - Neurodegenerative diseases, such as Alzheimer's disease (AD) and prion diseases (PDs), are among the most serious threats to human health. Although the pathogenetic mechanisms of these diseases are not very clear, it is widely accepted that transition metal ions (*e.g.*, copper ions) and reactive oxidative species (ROS) are implicated in the pathogenesis of AD and PDs. As a result, there is growing interest in using metal chelators and antioxidants to combat both diseases. Some metal chelators have showed promising preventive effects on AD and PDs. For instance, desferrioxamine, clioquinol and D-(-)-penicillamine are effective to prevent AD *in vitro* and/or *in vivo* and D-(-)-penicillamine can delay the onset of PD in mice. As to antioxidants' effects, although convincing clinical evidence is still lacking, some modest therapeutic effects on AD and PDs have been observed for antioxidant combinations.

Considering the preliminary success of metal chelators in treating AD and PDs and the fact that some superoxide dismutase (SOD) mimics are metal chelates, we proposed a new strategy to combat these diseases. That is, using SOD-mimetic ligands to chelate copper ions, then the chelates will hold radical-scavenging potential, which may lead to better clinical effects than pure metal chelators. It is interesting to note that this strategy is supported by recent *in vitro* experimental findings that copper chelators whose copper complexes have high SOD-like activity are potential anti-prion drug candidates. To evaluate the potential of existing copper chelators as anti-Alzheimer and anti-prion drug candidates, we attempted to compare the copper-binding ability and SOD-like activity of various chelators and derived chelates by theoretical calculations. The results may help screen new anti-Alzheimer and anti-prion drugs.

Chapter 15 - Psychosis and behavioral problems are very common in patients with dementia and the burden this causes caregivers cannot be overstated. Behavioral problems in dementia are the leading reason that families place dementia patients in facility settings, yet facilities themselves are often overwhelmed by such behaviors. No less important, patients suffer when they feel agitated, psychotic or combative and the humane treatment of dementia patients includes treating their symptoms for quality of life.

Currently, there are no FDA approved treatments for dementia with psychosis or behavioral disturbance. Atypical antipsychotics have been prescribed for these behaviors. They had been considered to have a better side effect profile compared with typical

antipsychotics, with lower rates of adverse effects such tardive dyskinesia, extrapyramidal symptoms and orthostasis. However, recent concerns including increased risk of cerebrovascular adverse events and death have resulted in an FDA warning, bringing into question their use in the demented population.

However, the research examining efficacy and safety of treatment of such patients has been fraught with difficulty. The main problem is that dementia with psychosis and behavioral disturbance is a heterogeneous group of patients, not a single disorder. Treating dementia patients with behavioral problems as if they have a single diagnosis that can all be treated by a single type of medicine is a mistake. Unfortunately, most studies examining treatment of behavioral disturbance in dementia have been designed in this way.

Chapter 16 - Immunization strategies which aid in the clearance of beta-amyloid (Aβ) plaques have raised new hopes for the treatment of Alzheimer's disease (AD). Two particularly promising passive immunization therapies currently being investigated include intravenous immunoglobulins (IVIG) containing Aβ antibodies and specifically developed monoclonal antibodies for Aβ. These Aβ antibodies may reduce amyloid accumulation in the brain by binding to the amyloid peptide and drawing it in through the blood-brain barrier for subsequent removal from the capillaries. However, as this strategy aims at removing extracellular amyloid through cerebral vessels, a redistribution of amyloid pathology may manifest as increased cerebral amyloid angiopathy (CAA). CAA occurs when Aβ becomes embedded in the walls of cerebral vessels associated with weakening of the vessel walls. Antibody mediated Aβ clearance from the parenchyma could significantly increase the Aβ burden in the vessel lumen and wall, therefore increasing the risk of vessel rupture and hemorrhage. This chapter will review the current literature on Aβ immunotherapy for AD and explore the mechanisms as well as possible risks of amyloid clearance treatment, particularly cerebral amyloid angiopathy.

Chapter 17 - *Aims:* The objective of the study was to provide observational clinical data on psychotropic drugs used in older people with mental illness.

Method: This was an observational, single-centre, one-week prevalence study of psychiatric symptoms, disorders and psychotropic/antidepressant drug use in older people with mental illness cared for by the South West people Yorkshire Mental Health NHS Trust (Wakefield Locality), UK. The clinical assessment included completion of the Psychosis Evaluation Tool for Common use by Caregivers.

Results: A total of 593/660 older patients with mental illness (mean±SD age, 76±8.1 years) were assessed). 44.5% had dementia (excluding vascular dementia) and 33.7% had a mood disorder. Of the total, 20.4% did not receive CNS active medication and 46.2% of patients were prescribed an antidepressant. Antidepressants were commonly prescribed where the primary diagnosis was not depression including vascular dementia (31%), dementia (26.1%), schizophrenia and related disorders (26.2%) and anxiety disorders (51.5%). SSRIs were the most commonly prescribed drugs (63.2%) followed by TCAs (22.4%), venlafaxine (9%), mirtazapine (3.2%), reboxetine (1.8%) and phenelzine (0.36%). The single most commonly prescribed drug was paroxetine (n=77) which accounted for 27.7% of all prescriptions. Medications were well tolerated but some patients prescribed a TCA received relatively small doses. Patients with non-vascular dementia received a significantly lower dose of paroxetine compared with other diagnostic groups ($F=3.14$, $p<0.02$) though this was still within the recommended/therapeutic range.

Conclusions: Antidepressants are commonly used in older people with mental illness including dementia, schizophrenia and anxiety disorders as well as for patients with a primary diagnosis of depression. Antidepressants are generally well-tolerated and patients were broadly satisfied with their medication. The evidence for the use of low dose TCAs in older people remains controversial and further work is needed in this area.

Declaration of interest: None.

Chapter 18 - In the brain cystatin C is synthesized by the choroid plexus and leptomeningeal cells, and it is localized in glial cells and in neurons. Its physiological high concentration in the cerebrospinal fluid (CSF) of the central nervous system and its proliferative effect on neural rat stem cells strongly suggest that cystatin C could exert a trophic function in the brain. Acute and chronic neurodegenerative processes induce an increase of cystatin C expression levels, mainly in activated glial cells. In brains from Alzheimer disease (AD) patients neuronal concentration of cystatin C protein is increased and its association to beta-amyloid peptide (A-beta) was revealed. A direct interaction of cystatin C and A-beta, resulting in an inhibition of amyloid formation, was demonstrated. An involvement of cystatin C in the pathogenesis of AD was further suggested by genetic studies in which the allelic haplotype B in cystatin C gene (CST3), determining an Ala25Thr substitution in the signal peptide, was associated with risk to develop late-onset AD. The B/B haplotype is specifically associated to highly reduced levels of extracellular cystatin C. In this view, the molecular correlate of the genetic risk conferred by cystatin C B variant could be the reduction in cystatin C secretion, which may result in A-beta formation and deposition. Alternatively, a reduced secretion of this protein could cause an impairment in neuroregeneration in response to brain damage.

Chapter 19 - One century has passed since the discovery of Alzheimer’s disease (AD), however, there has been no effective therapeutics to the disease. Since multiple factors are involved in the pathogenesis of AD, finding multipotent agents that can hit the multiple targets implicated in the disease is attracting more and more attention. Recently, accumulating evidence indicated that quercetin, a flavonoid abundant in fruits and vegetables, is a multipotent anti-AD agent. It can block Aβ- or τ-aggregation with IC_{50}s of < 1 μM and inhibit monoamine oxidases A and B (MAO A and MAO B) with IC_{50}s of 0.01 μM and 10.89 μM, respectively. Besides, quercetin is an efficient inhibitor for butyrylcholinesterase (BChE, a recently recognized potential target for treating AD) with an IC_{50} of 1 μM. Of course, quercetin is also an excellent antioxidant, both as reactive oxygen species (ROS) scavenger and transition metal chelator. As quercetin is highly bioavailable and can pass through the blood-brain barrier (BBB), it is highly possible to be responsible for the benefits of fruit and vegetable juices to AD. However, considering the fact that the current strategy in the fight against AD depends largely on inhibiting acetylcholinesterase (AChE), it is of interest to explore the AChE- inhibitory potential of quercetin.

Chapter 20 - Alzheimer's disease (AD) is a group of disorders involving the areas of the brain that control thought, memory, and language. AD is the most common form of dementia among the elderly. Almost four million Americans and eight million more worldwide suffer from AD; after the age of 65, the incidence of the disease doubles every five years and, by the age of 85, it affects nearly half of the population. Currently approved Alzheimer's therapies primarily treat the disease symptoms but do not reverse or slow down the disease progression. The increasing awareness of the diverse factors involved in the onset of AD has outlined new

paths of research for prevention and pharmacological treatments. A pivot clinical trial using Abeta1-42 immunization (AN1792) on AD patients showed a possible therapeutic effect, in line with previous experiments using animal models; however, the trial was interrupted because of meningoencephalitis probably due to the activation of T-cells and microglia, in 6% of participants. Although no significant amelioration of cognitive dysfunction was observed, CSF tau decreased in anti-AN1792 antibody responder patients. A MRI study on AD patients with immunotherapy demonstrated decreased volume of neuronal tissue including hippocampus, which is unrelated to worsening cognitive dysfunction; this shows a possible amyloid removal by immunotherapy. Another approach to observe the decrease of Abeta-associated amyloidogenesis is the inhibition of Abeta aggregation and its clearance.

In this commentary, the Authors express their opinion regarding the *Questio* of AZD-103 (scyllo-cyclohexanehexol) and AD concomitantly with the publication of the paper by McLaurin J et al. The findings in the *Nature Medicine* publication show that oral treatment of AZD-103 (scyllo-cyclohexanehexol) reduces accumulation of amyloid beta and amyloid beta plaques in the brain, and it also reduces, or eliminates, learning deficits in an AD transgenic mouse model. Transition Therapeutics Inc. (Canada) is pursuing the clinical drug development of AZD-103 in an expedited manner and it has also announced that dosing with AZD-103 has commenced in Phase I clinical trial. The Phase I trial is a single blind, randomized, placebo controlled study in which healthy volunteers will receive placebo or increasing acute doses of AZD-103. The primary aim of the trial is to evaluate AZD-103 safety, tolerability, and pharmacokinetics.

Chapter 21 - Brain inflammation is an underlying factor in the pathogenesis of Alzheimer's disease (AD) and epidemiological studies indicate that sustained use of non-steroidal anti-inflammatory drugs (NSAIDs) reduces the risk of AD and may delay its onset or slow its progression. Nevertheless, recent clinical trials have shown that NSAIDs do not alter the progression of AD. Neuroinflammation occurs in vulnerable regions of the AD brain where highly insoluble β-amyloid (Aβ) peptide deposits and neurofibrillary tangles, as well as damaged neurons and neurites, provide stimuli for inflammation. To elucidate the complex role of inflammation in neurodegenerative processes and the efficacy of NSAIDs in AD we developed an animal model of neuroinflammation/neurodegeneration *in vivo*. An "artificial plaque" was formed by injecting aggregated ß-amyloid peptide (Aβ(1-40) or Aβ(1-42)) into the nucleus basalis magnocellularis (NBM) of rats. We investigated several aspects of the neuroinflammatory reaction around the "artificial plaque" such as microglia and astrocyte activation, production of proinflammatory compounds, activation of cyclooxigenase-2 (COX-2), p38 Mitogen Activated Protein Kinase (p38MAPK) and induction of inducible Nitric Oxide Synthase (iNOS). Finally, degeneration of cortically projecting cholinergic neurons was also evaluated by means of immunohistochemistry and microdialysis. We examined whether the attenuation of brain inflammatory reaction by NSAIDs and NO-donors may protect neurons against neurodegeneration. The data reported in this review show that in *in vivo* model of brain inflammation and neurodegeneration, the administration of NSAIDs and NO-donors prevent not only the inflammatory reaction, but also the cholinergic hypofunction. Our data may help elucidating the role of neuroinflammation in the pathogenesis of AD and the ability of anti-inflammatory agents to reduce the risk of developing AD and to slow its progression.

Versions of these chapters were also published in *Alzheimer's Disease Research Journal, Volume 1*, published by Nova Science Publishers, Inc. They were submitted for appropriate modifications to encourage wider dissemination of research.

In: Alzheimer's Disease Diagnosis and Treatments ISBN: 978-1-61122-064-3
Editor: Marisa R. Boyd

Chapter 1

ALZHEIMER'S DISEASE – 100 YEARS OF RESEARCH A HISTORICAL PERSPECTIVE AND COMMENTARY

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In November 1906, in Tubingen, Germany, Alois Alzheimer (1864-1915) first described his laboratory's clinical and neuropathological findings on a then novel neurological disorder in one of his female cases named Auguste D. Institutionalized by her concerned family at the age of 51, Alzheimer's first patient died of a progressive dementia just four years later [1]. Although the clinical features of this '*disease of the aged*' were long known since ancient times, and often referred to as a '*senile psychosis*', '*age-related madness*' or '*old-timer's disease*', Alzheimer was probably the first to correlate senile plaque (*miliary foci*) and neurofibrillary tangle (*fibrils*) propensity within the association neocortex with disease diagnosis and severity [1-5]. It is perhaps less well known that Alzheimer also associated cerebrovascular involvement and angiogenesis with his first description of Alzheimer's disease neuropathology, features that he termed '*focal lesions in the endothelium*' and '*new vessel formation*' in the diseased brain [1].

Alzheimer's advances in linking the clinical symptoms with novel neuropatholgical findings in his disease were enabled by the concurrent development of highly sensitive silver-staining, and related techniques, applied to human brain tissue sections pioneered by Maxwell Bielchowsky (1845-1928), Franz Nissl (1860-1926), Gaetano Perusini (1879-1915), Rudolph Virchow (1821-1902), Camillo Golgi (1844-1926), Santiago Ramon y Cajal (1852-1934), and others. Interestingly, Alzheimer's and his colleagues' description of a biophysical and ultrastructural basis for '*senile psychosis*' and Alzheimer's disease pathology was highly controversial for that time, and at odds with other theories for these kinds of afflictions, such as the series of psychoanalytical hypotheses proposed by Sigmund Freud (1856-1939) and his colleagues [6]. Although 'Alzheimer's disease', an eponym for this now common affliction,

was given by Alzheimer's colleague and mentor Emil Kraepilin (1856-1926) in about 1910, Alzheimer's findings remained somewhat of a medical and neurological curiosity for many years, and it was not until almost 6 decades later that any new significant developments were reported that further expanded our clinical, neuropathological and biological understanding of this now common neurological disorder.

The modern era of Alzheimer's disease research really began with a series of important ground-breaking observations in the middle-to-late sixties by Kidd, Terry, Gonatas and Weiss on ultrastructural aspects of Alzheimer's disease lesions. In these studies neurofibrillary tangles were discovered to be twisted paired helical filaments, and complex amyloid-containing structures were found to be a major component of the insoluble, heterogenous senile 'miliary' plaques [7-10]. Shortly thereafter, Blessed, Tomlinson and Roth were the first to realize that microscopic examination of autopsied brains from the vast majority of all senile dementia/senile psychosis cases actually contained pathological features consistent with a diagnosis of the neuopathogical entity which Alzheimer had originally described [11-12]. Hence, it has only been about 4 or 5 decades since these discoveries have gradually brought interest in Alzheimer's disease to the medical research forefront.

During this later period, the 'amyloid beta (Aβ) peptide cascade hypothesis', that beta-amyloid precursor protein (βAPP) and Aβ peptide production, morphology, speciation, trafficking, deposition and it's neuropathological consequences, such as being a trigger for brain-specific oxidative and neuron-inflammatory processes, lies at the very heart of the Alzheimer process, has progressed to the extent that the neurobiology and genetics of βAPP and Aβ peptides are now probably one of the most thoroughly studied entities in all of modern cell biology [3-5,13-15]. Despite massive research efforts, however, the precise role of βAPP and Aβ peptides in the initiation and progression of Alzheimer's disease remains unresolved, and open to serious question; the biological role of neurofibrillary tangle formation has received considerably less research attention [2,15,16]. Key to the 'Aβ peptide cascade hypothesis' are the genetics and molecular interactions amongst the membrane associated proteins that define the '*gamma-secretase complex*' of neural cells in degenerative disease. These include βAPP, nicastrin, sortilin (SORL1), beta-amyloid cleavage enzyme 1 (BACE1), presenilin-1 and -2 (PS1, PS2) membrane proteins, and the related catalytic and structural complexes of the cholesterol enriched lipid-raft domains in which they reside. Significant advances in Alzheimer's disease and amyloid research are the subject of several excellent comprehensive reviews, this being the 100th anniversary of the first description of Alzheimer's disease, and interested readers are encouraged to study them along with the references contained therein [2-5,13-15].

Interestingly, several of these recent reviews have come to the common and gloomy conclusion that currently, a very great deal of work remains to be done in the second century of Alzheimer's disease research, and that if Auguste D. were alive today, '*her sad prognosis would be pretty much the same as in 1906*' [3].

The socioeconomic costs of Alzheimer's senile dementia are a very serious and growing concern as our elderly currently represent the fastest growing segment of Western populations. Recent epidemiological studies show that globally, about 25 million people today have senile dementia, with approximately 5 million new cases of dementia occurring every year, and with one new case being reported every 7 seconds [17-19]. Worldwide, the total number of people affected by dementia is expected to double every 20 years to at least

81 million by 2040 with Western civilizations and developing countries at particular risk [17,18]. In the United States alone, the average annual cost of Alzheimer's disease patient care is soaring out of control, and has been currently estimated to be about 100 billion dollars [17-19]. Our deeper understanding of the neurobiology of aging, basic Alzheimer's disease molecular-genetic mechanisms, and the prevention of Alzheimer's disease, remains a primary medical research concern, and effective drugs to treat Alzheimer's disease are an urgent pharmacological objective [18-22]. Unfortunately, while many primary clinical trials for Alzheimer's disease drugs are ongoing, *no primary pharmacological-based prevention trial has yet successfully delayed the development of this prevalent neurological disorder* [19]. Whether our basic understanding or modeling of the Alzheimer's disease process is severely flawed, or if longer clinical trials, or alternative pharmacological strategies are required, is open to question and debate.

What is not controversial is that, without question, new understanding, novel research methodologies and new clinical treatments are essential to more effectively address the escalating incidence of Alzheimer's disease. Alternative treatment approaches such as those involving the use of omega-3 fatty acid supplementation, docosahexanoic acid (DHA) and it's oxygenated derivatives such as neuroprotectin D1 (NPD1), cholesterol reducing statins, novel antioxidants and neurotoxic metal chelators, natural herbal treatments from the extensive Asian pharmacopeia, and many others, are currently receiving a lot of research attention [19-24]. Western medical approaches to age-related diseases more often than not favor 'quick fix' strategies for healing chronic and progressive diseases that take many decades to develop. Significant non-pharmaceutical-based protection against Alzheimer's disease can be clearly obtained from life-long lifestyle changes. Diets reduced in saturated and trans-fat, enriched in omega-3-fatty acids and antioxidants, appropriate weight maintenance, the cessation of smoking, regular physical activity and Alzheimer health care education collectively represent statistically significant and highly cost effective long-term therapeutic solutions [17-21]. It is unfortunate that in the recent "Perfect Storm" of dwindling National Institutes of Health research support, and at a time of most critical need, when opportunities for scientific progress and advances in Alzheimer's disease research have never been greater, that significantly less federal funding is available to support the eager battalions of highly qualified and research-ready Alzheimer's disease investigators and their medical and graduate students whose research will ultimately define the future progress and treatment of this expanding health care problem [25-27].

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In: Alzheimer's Disease Diagnosis and Treatments ISBN: 978-1-61122-064-3
Editor: Marisa R. Boyd

Chapter 2

CONTROLLED AND AUTOMATIC MEMORY PROCESSING IN ALZHEIMER'S DISEASE

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ABSTRACT

Over the last two decades studies of patients with Alzheimer's disease (AD) have made a significant contribution in helping to elucidate the neurological and cognitive bases for controlled and automatic forms of retrieval from long-term memory. These studies show that AD patients demonstrate severe deficits on tasks that involve controlled processes. In contrast, their performance on tasks involving automatic processes is variable. This article reviews experimental studies that have revealed dissociations between controlled and automatic memory processing in AD, and discusses evidence from functional neuroimaging studies which indicate that different forms of retrieval represent distinct aspects of brain activity. Attention is given to the assumption that memory retrieval reflects the operation of a single form of processing (automatic or controlled). The implications of adopting this assumption are discussed within the context of contemporary theoretical perspectives, and recent attempts to understand memory processing in AD and normal ageing by using the process-dissociation approach to memory are described. Finally, the importance of understanding the status of controlled and automatic memory processing for the diagnosis and management of AD is considered.

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INTRODUCTION

It is well established that the capacity to recall previous experiences from memory declines with normal ageing (Craik, 1977) and more significantly so in pathological conditions such as Alzheimer's disease (AD) (Morris and Kopelman, 1986). This article considers the cognitive and neurological basis of the memory retrieval impairment in AD and discusses how these changes in memory processing differ from those associated with normal ageing.

Memory retrieval is not a unitary process; contemporary models of cognition make an important distinction between retrieval processes that are controlled from those processes which are automatic (Jacoby, 1991). Controlled retrieval refers to an intentional or deliberate effort to remember an experienced event; and is measured by performance on direct (explicit, declarative) memory tasks. In contrast, automatic retrieval is revealed when prior exposure to an event influences behaviour without the intention to remember, and is traditionally associated with performance on indirect (implicit, nondeclarative) memory tasks. Experimental dissociations between direct and indirect tasks suggest that the two forms of retrieval are functionally independent (see Roediger and McDermott, 1993 for a review). For example, manipulations of attention (Parkin and Russo, 1990) and levels of processing (Jacoby and Dallas, 1981) have a much greater influence on direct tasks than indirect tasks, whereas the reverse pattern is found for manipulations of surface features (Craik et al., 1994). Moreover, convergent evidence from neuropsychological and brain imaging research (see Henson, 2003; Moscovitch et al., 1993 for reviews) indicate that direct and indirect tasks are subserved by dissociable neural structures. For example, amnesic patients with lesions focused to the hippocampus and related structures within the medial temporal lobe (MTL), who characteristically demonstrate impaired performance on direct memory tasks, typically perform within normal limits on indirect tasks (see Kopelman, 2002 for a review).This finding implies that the integrity of MTL structures appear to be more important for controlled than for automatic retrieval processes.

The pattern of neuropathology in AD extends beyond the MTL and proliferates to temporal, parietal and frontal association cortices (Arriagada et al., 1992). Consequently the performance of patients with AD on direct and indirect tasks is not the same as that found in focal amnesia. This article reviews experimental studies of memory retrieval in AD, and considers whether the changes in memory processing observed in patients with AD are merely quantitatively or actually qualitatively different to those found in normal ageing. These issues are discussed in relation to contrasting theoretical perspectives and evidence from neuroimaging studies.

TASK DISSOCIATION APPOACHES

Direct and indirect tasks are defined in terms of the retrieval instructions which subjects are asked to follow. Direct tasks, such as free recall, cued recall and recognition, request subjects to intentionally recollect a previously experienced event. In contrast, indirect retrieval is revealed when prior exposure to an event facilitates performance without intent. For example, if participants are asked to complete the word fragment 's-a--' with the first

word that comes to mind, prior exposure to a compatible target word (e.g. spade) will increase the probability that it is produced compared to other compatible completions that have not been exposed (e.g. stale, snack, stamp). The increased probability of a subject selecting, or responding faster to a target stimulus under indirect conditions is referred to as repetition priming. AD is characterized by profound impairments on all direct tests of memory which is assumed to reflect a deficit in the use of controlled memory processes (Jorm, 1986; Morris and Kopelman, 1986; Salmon, 2000). However, the performance of AD patients on indirect tasks is much more varied, although some studies report normal repetition priming others report that priming is impaired (see Flesichman and Gabrieli, 1998; Meiran and Jelicic, 1995 for reviews). Because of these inconsistent findings the status of automatic processing in AD is controversial.

Processing Theories

It has been postulated that the key factor that distinguishes between intact and impaired memory performance in AD is the processing characteristics of the task and not whether retrieval is intentional or not. Processing theorists (e.g. Blaxton, 1992) emphasize the distinction between perceptual and conceptual processing. Whereas perceptual tasks use study and test stimuli that share perceptual features (e.g. study: 'spade'; test: 's-a--'); the study and test stimuli used for conceptual tasks are related on the basis of meaning (e.g. study: 'spade'; test: 'name a tool'). According to this view, indirect tasks are considered to be perceptually driven, whereas direct tasks are conceptually driven.

The processing account accommodates a range of experimental observations involving neurotypical individuals (Roediger and McDermott, 1993); with regard to memory impaired individuals, conceptual processing is postulated to be impaired, whereas perceptual processing remains intact. Indeed there is some support for this contention, a number of studies indicate that AD patients in the early to middle stages of the disease demonstrate normal perceptual priming on tasks such as; the identification of structurally degraded words (Keane et al., 1991), nonwords (Keane et al., 1994) and pictures (Park et al., 1998; Gabrieli et al., 1994); lexical decision (Ober and Shenaut, 1988; Ober et al., 1991) and novel nonassociative information (Keane et al., 1994). In contrast, performance on conceptual priming tasks such as category exemplar generation (Monti et al., 1995) and word association (Carlesimo et al., 1995; Salmon et al., 1988) is generally impaired in AD.

However, a number of neuropsychological studies report dissociations between direct and indirect memory performance and not between perceptual and conceptual processing. For instance, focal amnesic patients show normal or near to normal performance on a wide range of indirect memory tasks regardless of whether the task is mediated by perceptual or conceptual processing (Graf and Schacter, 1985; Graf et al., 1984). Moreover, there are reports of a patient with bilateral occipital lesions exhibiting impaired perceptual processing on an indirect task, and intact perceptual processing on a direct task (see below, Gabrieli et al., 1995). These findings are inconsistent with a processing account of memory retrieval.

System Theories

In contrast to the processing approach, the systems view posits that direct and indirect retrieval are subserved by separable neural systems (e.g. Tulving and Schacter, 1990). According to this view, direct memory is subserved by MTL and diencephalic structures - regions that are damaged in both focal amnesia and AD; whereas indirect retrieval is subserved by neocortical areas – regions that are affected by AD but not focal amnesia. The system view accommodates the finding that both groups are impaired on direct retrieval tasks and also explains why indirect retrieval is intact in amnesia. The approach further maintains that the reported dissociation between intact perceptual priming and impaired conceptual priming in AD arises because the two forms of priming reflect the operation of distinct memory systems that are differentially affected by the pattern of neuropathological encroachment in AD (Arnold et al., 1991; Arriagada et al., 1992). It is proposed that perceptual priming is mediated by a structural-perceptual memory system localized within occipital structures – regions minimally affected in the early stages of AD; whereas conceptual priming is mediated by a lexical-semantic memory system localized to fronto-temporo-parietal association cortices – regions that endure major neuronal alterations throughout the course of the disease.

Initial postulations for distinct memory systems were based primarily on single dissociations, that is, patients with focal amnesia performing normally on indirect but not on direct retrieval tasks (Carlesimo, 1995; Graf et al., 1985); and patients with AD performing normally on indirect perceptual tasks (Gabrieli et al., 1994; Keane et al., 1991, 1994; Ober and Shenaut, 1988; Ober et al., 1991; Park et al., 1998) and poorly on indirect conceptual tasks (Carlesimo et al., 1995; Gabrieli et al.,1999; Monti et al., 1995; Salmon et al., 1988). However, statements of localization of function based upon evidence derived from single dissociations need to be treated cautiously. Rather than indicating the participation of separate neural systems on two different tasks, it is plausible that both tasks are subserved by a single system. Damage to a single system may disrupt performance on a more challenging task (e.g. direct memory or conceptual priming) but spare performance on a task that is undemanding (e.g. indirect memory or perceptual priming). Stronger evidence for the existence of distinct systems is obtained through double dissociations (Teuber, 1955). Double dissociation are demonstrated, if the reverse pattern of spared (e.g. direct memory or conceptual priming) and impaired (e.g. indirect memory or perceptual priming) performance is obtained from a different neuropsychological patient/group - such observations cannot be explained by task complexity.

A study by Keane et al. (1995) directly examined this issue, they tested the amnesic patient H.M and a patient with bilateral occipital lobe lesions L.H on a direct task (recognition) and on both perceptual (identification) and conceptual (category exemplar generation) indirect tasks. Their results showed a double dissociation between recognition (intact in L.H and impaired in H.M) and perceptual priming (intact in H.M and impaired in L.H). Moreover, despite impaired perceptual priming L.H demonstrated normal conceptual priming – the reverse dissociation which is found in AD. Collectively, these results support the view that conceptual priming is subserved by neocortical regions (impaired in AD but intact in L.H and in focal amnesia), whereas occipital structures (intact in AD and impaired in L.H) subserve perceptual priming.

PROCESS DISSOCIATION APPROACHES

The perceptual-conceptual distinction explains the variable performance of AD patients on many indirect tasks of memory retrieval; however the distinction does not so readily account for word stem completion performance in AD (e.g. study: 'spade'; test: 'complete the following stem with the first word that comes to mind: spa--'). Word stem completion is the most widely administered indirect task in AD studies, and although it is defined as a perceptual priming task (Rajaram and Roediger, 1993) and should therefore be intact in AD, both normal (Deweer et al., 1994; Grosse et al., 1990; Partridge et al., 1990) and impaired (Carlesimo et al., 1995; Fleischman and Gabrieli, 1998; Meiran and Jelicic, 1995; Russo and Spinnler, 1994) word stem completion rates have been reported. These discordant findings are not readily explainable; in part they may reflect differences in methodology (Ostergaard, 1994) or dementia severity (Gabrieli et al., 1994).

One problem with the majority of studies that examine memory retrieval in AD is that they adopt a task dissociation premise, which assumes task performance represents the operation of a specific memory process or memory system. That is, direct tasks only invoke controlled memory processes/system, and indirect tasks only invoke automatic memory processes/system. This assumption however, fails to accommodate instances of contamination which can occur when performance on an indirect task is facilitated by controlled uses of memory (Toth et al., 1994), or direct task performance is facilitated by automatic uses of memory (Jacoby et al., 1993). Moreover, task dissociation designs are not able to establish whether the contribution of controlled and automatic memory processes to either a direct or indirect task is comparable between patients with AD and neurologically healthy individuals. Healthy control subjects have the potential of approaching a word stem completion task in the same manner as a cued recall task. Therefore, rather than completing a stem with the first word that comes to mind, they may intentionally try to recall target word completions (Randolph et al., 1995). This strategy is not available to AD patients since cued recall is severely impaired in this group (Carlesimo et al., 1995; Partridge et al., 1990). It is therefore possible that impaired word stem completion in AD, may not reflect a deficit in automatic memory processes per se, but reflect the capacity of neurologically intact individuals to use controlled memory processes (Vaidya et al., 1996).

Test awareness on indirect tasks including word stem completion has been demonstrated in a number of studies that have used post-test questionnaires to examine whether subjects knew of the relationship between studied items and test cues (Bowers and Schacter, 1990). Though awareness itself does not necessarily constitute conscious contamination (Richardson-Klavehn et al., 1996), word stem completion rates have been shown to be larger in subjects who are test-aware (Bowers and Schacter, 1990). A recent review by Mitchell and Bruss (2003) of 12 studies that used self-report measures of awareness found that in each case a greater proportion of younger adults reported test awareness than older adults. Moreover, age-related decrements in word stem completion have been shown to diminish when awareness is controlled (Light and Albertson, 1989; Park and Shaw, 1992). There are however general problems of testing awareness with self-report measures. First, the method is retrospective - assessing awareness after the test phase may not be a reliable indicator of an individual's prior phenomenological status. Second, there is a question over what constitutes awareness - subjective reports may well differ from operational definitions (Eriksen, 1960).

Process-Dissociation Procedure

The process-dissociation procedure is one technique that has been developed to overcome the problem of contamination. The procedure (Jacoby, 1991, 1998; Jacoby et al., 1993; Toth et al., 1994; Reingold and Toth, 1996) is an oppositional methodology that contrasts performance from inclusion and exclusion conditions in order to derive uncontaminated estimates of memory processes within the same task. Using stem completion as an example, subjects performing under inclusion conditions are directed to use the stem as a cue to recall a studied word and use that word to complete the stem, if they are unable to recall a studied word they are required to complete the stem with the first word that comes to mind. Therefore, the inclusion condition is the same as a traditional cued-recall task. Under inclusion conditions controlled (C) and automatic (A) memory processes operate in concert; thus, the probability of completing a stem with a target word is the additive probabilities of controlled memory processing (C) and of the word automatically coming to mind (A) when recollection fails (1 – C). Therefore inclusion = C + A (1 – C).

Under exclusion conditions, subjects are again required to use the stem as a cue to recall a studied word, but this time they are asked to avoid using a studied word to complete the stem, if they are unable to recall a studied word they are again required to complete the stem with the first word that comes to mind. The probability of completing a stem with a studied word under exclusion conditions depends on automatic processes and the failure of controlled memory processes. Therefore exclusion = A (1 – C).

Estimates of controlled processes are derived by subtracting the probability of completing a stem with a target word in the exclusion condition from the probability of using a target word completion in the inclusion condition. Therefore C = inclusion – exclusion. Automatic influences of memory are estimated by the equation: A = exclusion/(1 – C).

The process-dissociation approach has been applied to examine the effects that a wide range of experimental manipulations (Debner and Jacoby, 1994; Jacoby, 1991; Jacoby et al., 1993; Toth et al., 1994) and clinical phenomena (Grattan and Vogel-Sprott, 2001; Hirshman et al., 2003; Stapleton and Andrade, 2000) have on controlled and automatic memory processes. More pertinently, it has been deployed in a number of neuropsychological studies, including those involving patients with epilepsy (Del Vecchio et al., 2004), multiple sclerosis (Seinela et al., 2002), Parkinson's disease (Hay et al., 2002), frontal lobe lesions (Kopelman and Stanhope, 1997) and schizophrenia (Linscott and Knight, 2001). In the next section, studies that have used the process-dissociation to examine memory processing in AD will be discussed.

Processing Dissociations in Alzheimer's Disease

The process-dissociation procedure enables comparisons between memory processes to be made, without relying on the assumption that performance on a given task is a pure measure of a certain cognitive process, or that the processes that subserve task performance for a healthy group of subjects are identical to those processes which support performance by a memory impaired group. A few studies have used the procedure to quantify the contribution of controlled and automatic processes to memory retrieval in AD. Three studies that used a similar stem completion task reported a common pattern for inclusion and exclusion

conditions (Hudson and Robertson, 2007; Knight, 1998; Koivisto et al., 1998). In each of these cases (see table 1), elderly age-matched controls demonstrated significantly higher stem completion rates under inclusion conditions than patients with AD - a finding consistent with other studies that have examined cued-recall in AD (Carlesimo et al., 1995; Partridge et al., 1990).

Nevertheless, it is notable that patients with AD in these three studies did in fact complete more stems with target words than unstudied/baseline words under inclusion conditions. Thus it might be plausible to conclude that the AD patients did actually demonstrate some capacity for controlled retrieval. However, the performance of the AD groups under exclusion conditions indicates that this conclusion is likely to be invalid. Under exclusion conditions, the aim of the task is to recall studied words then complete the test stem with an alternative word. Therefore target word completions that are produced under exclusion conditions represent an automatic form of retrieval, since conscious recollection would result in target words being withheld. In these studies, the AD groups completed significantly more stems with target words under exclusion conditions than elderly control subjects. Indeed, for the AD patient groups in Hudson and Robertson (2007) and Koivisto et al. (1998), volition made no difference to the probability of producing a target word completion, as inclusion and exclusion performance was actually invariant. That is, regardless of whether they tried to use a target word or tried to avoid using a target word, the probability of AD patients actually producing a target word was exactly the same.

Table 1 Proportion of stems completed with target items under inclusion and exclusion condition by patients with Alzheimer's disease and elderly controls from Hudson and Robertson (2007), Knight (1998) and Koivisto et al. (1998)

Study	Condition	Alzheimer Patients	Elderly Controls
Hudson and Robertson (2007)	Inclusion	0.42	0.73
	Exclusion	0.42	0.29
Knight (1998)	Inclusion	0.47	0.86
	Exclusion	0.28	0.13
Koivisto et al. (1998)	Inclusion	0.43	0.62
	Exclusion	0.43	0.35

Estimates derived from the process-dissociation calculations indeed confirmed that the AD patients demonstrated profound deficits in controlled memory processing (see also Adam et al., 2001). Moreover, in the studies of Hudson and Robertson (2007) and Knight (1998) patients with AD also showed a significant decline in automatic memory processing (see also Grafman et al., 1990; Smith and Knight, 2002). However, whereas there was no significant overlap between patient and control groups in the estimates of controlled processing, there was overlap in the estimates of automatic processes. Indicating that either automatic processes are less sensitive to the effects of AD, or automatic memory processes can be vulnerable to the effects of normal ageing. This finding is additionally important because it may explain why patients with AD tend to display lower stem completion rates than amnesic patients (Gabrieli et al., 1994). Studies that have used variants of the process-dissociation procedure concur that automatic memory processing is unimpaired in amnesia (Cermak et al., 1992; Ste-Marie et al., 1996).

Processing Dissociations in Normal Ageing and Alzheimer's Disease

From both a theoretical and clinical standpoint it is important to determine whether the alterations in memory processing associated with AD is qualitatively different from those observed in normal ageing (Albert and Killiany, 2001). Alternatively, ageing may produce deficits in both controlled and automatic uses of memory on stem completion that only represent quantitative differences to those found in AD (Huppert, 1994). This is a pertinent issue given that the performance of AD patients relative to elderly control subjects on tasks of word stem completion and cued recall parallels the performance of healthy older adults relative to younger adults. For example, similar to AD, normal ageing has been shown to produce reliable deficits on cued recall tasks (Chiarello and Hoyer, 1988; Clarys et al., 2000; Fleischman et al., 1999; Mitchell and Bruss, 2003; Ryan et al., 2001), therefore suggesting an impairment in controlled uses of memory in the healthy elderly. Again, similar to AD, the findings for word stem completion are less consistent with some studies reporting normal (Clarys et al., 2000; Mitchell and Bruss, 2003; Park and Shaw, 1992) and others reporting impaired stem completion rates (Chiarello and Hoyer 1988; Davis et al., 1990; Hultsch et al., 1991).

Moreover, just as conscious contamination can be a potential confound in AD studies, younger adults are more likely to deploy controlled uses of memory during word stem completion than older adults are. Indeed, the magnitude of the difference between older and younger subjects decreases when the potential for conscious contamination is reduced through manipulations of retention interval (Chiarello and Hoyer, 1988), exposure duration (Mitchell and Bruss, 2003), modality and levels of processing (Habib et al., 1996).

Variations of the process-dissociation paradigm have been used in a few studies to examine whether age has independent effects on controlled and automatic forms of retrieval. For example, on a source recognition task, Jennings and Jacoby (1993) found that relative to younger subjects in a full attention condition, estimates of controlled uses of memory were significantly reduced in older adults in a full attention condition and in younger adults studying under divided attention conditions. In contrast, neither a division of attention nor age was found to reduce the deployment of automatic memory processes. Titov and Knight (1997) compared the contribution of automatic and controlled processes to a source

recognition task for words, similar to Jennings and Jacoby (1993) there was no main effect of age on automatic processes, but age was found to impair controlled uses of memory. A similar pattern has also emerged from studies using the word stem based process-dissociation paradigm (Salthouse et al., 1997; Schmitter-Edgecombe, 1999; Zelazo et al., 2004).

Most attempts to understand the difference between changes in memory processing that arise from normal ageing and those produced pathologically by AD have primarily relied on conclusions drawn from different studies. These studies have deployed different methods and materials and it is therefore possible that these variations are confounding. Recently, Hudson (2008) examined the contribution of automatic and controlled uses of memory to stem completion across three adult age ranges – young (19-39), middle-aged (40-59) and old (60-78), and compared these scores with data obtained from AD patients who had performed exactly the same task (Hudson and Robertson, 2007). The results from this study showed a steady age-related decline in controlled memory processing that was marked in middle age (see figure 2). In contrast, the estimates of automatic memory processing remained unchanged across the three age groups. These results were different to those found in AD, where the capacity for both controlled and automatic memory processing was found to be reduced. Therefore the nature of the decline in memory retrieval observed in AD appears to be qualitatively different to that observed in normal ageing.

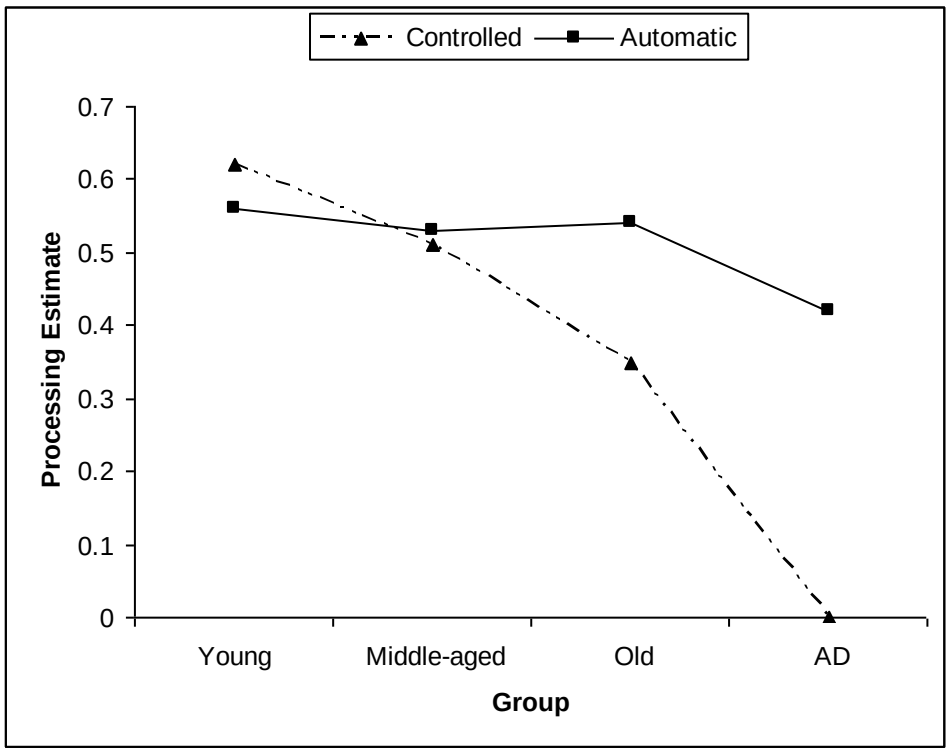

Figure 1. Changes in controlled and automatic memory processes in different age groups (Hudson, 2008) and in patients with Alzheimer's disease (Hudson and Roberton, 2007).

Evidence from Brain Imaging Studies

The age-related decline in controlled memory processes, and the dissociation between intact automatic processes in normal ageing and impaired automatic processing in AD, may prove to be an important pattern for helping to discriminate between normal and pathological ageing. Evidence from functional neuroimaging studies indicate that distinct aspects of brain activity are associated with the independent contribution of automatic and controlled memory processes to stem completion. Using positron emission tomography, Squire and colleagues (Squire et al., 1992) measured regional blood flow changes in young adults performing word stem completion and cued recall. Relative to baseline measures, cued recall selectively increased blood flow in the frontal cortex, whereas both tasks were found to increase blood flow in the right hippocampal region and decrease blood flow in the right extrastriate occipital cortex. Given the common characteristics that the tasks share, the overlap in activation changes associated with each task is not surprising and is consistent with the view that memory tasks seldom represent the operation of a single process or system (Jacoby et al., 1993).

The reduction in occipital activation has been interpreted as representing a neural mechanism for perceptual priming based upon repetition suppression (Wiggs and Martin, 1998), and it is possible that this property represents a neural base for the automatic contribution to stem completion. Although this view is tentative, it does concur with the finding that older adults also exhibit similar reductions in extrastriate occipital cortical activations during word stem completion (Bäckman et al., 1997), and as demonstrated by Hudson (2008) and Zelazo et al. (2004) are unimpaired in the capacity to deploy automatic memory processes. Furthermore, patients with AD have a reduced capacity for automatic memory processing (Hudson and Robertson, 2007; Knight, 1998) and exhibit abnormal occipital functioning during word stem completion. Bäckman and colleagues (Bäckman et al., 2000) found that in contrast to the reduction in occipital activation that has been reported in normal old and younger adults during word stem completion, patients with AD showed increased activity in this area, postulated to arise from a compensatory neuronal response due to inadequate stimulus encoding.

One interpretation of the increased hippocampal activation observed on both tasks by Squire et al. (1992) is that it represents the use of an explicit retrieval strategy for cued recall, and conscious contamination of word stem completion. Therefore based on this assumption it would appear that hippocampal structures subserve a controlled component to stem completion. Indeed, this conclusion would concur with the age-related deficit in controlled memory processing since reductions in hippocampal volume have been reported in normal ageing (Raz, 2000). However, when Schacter et al. (1996) used similar methodology to Squire et al. (1992) but additionally manipulated recall difficulty, they found increased hippocampal activity was not related to controlled uses of memory but was associated with successful recollection; in contrast deploying controlled memory processes was found to robustly increase activation of the frontal cortex. The frontal lobes have long been associated with executive functioning (Fuster, 1989; Luria, 1973), and it may be reasonable to assume that the deployment of controlled memory processes involves executive operations and therefore depends on the integrity of the frontal lobes. In the study by Zelazo and colleagues (Zelazo et al., 2004) regression analyses indicated that the process-dissociation estimates of controlled memory processing was related to performance on a visual sorting task of

executive function. Notably, impairments in executive function have been widely reported in older adults compared to younger adults (Kray and Lindenberger, 2000; Mayr and Kliegl,1993), and in patients with AD compared to age-matched controls (Collette et al., 1999; Perry and Hodges, 1999), with both normal ageing (Albert and Killiany, 2001; Fuster 1989) and AD producing marked pathological changes to the frontal lobes (DeKosky and Scheff, 1990).

FUTURE DIRECTIONS

In order to further understand the relationship between the neuropathology of AD and decline in memory processing, there is a need for future research to directly focus upon the brain-behaviour bases for controlled and automatic memory processing. As discussed above, inferences can be drawn from the numerous neuroimaging studies that have been designed to detail the networks of brain activity that mediate performance on direct and indirect retrieval tasks. However, these inferences are based on task dissociation methods. Imaging research involving both healthy individuals and patients with AD performing process-dissociation tasks is needed to clarify the relationship between memory processing and brain dysfunction in AD.

The process-dissociation approach was originally designed to compare estimates of controlled and automatic memory processes in neurotypical subjects with measures obtained from traditional direct and indirect tests of memory following a wide range of experimental manipulations. Although growing numbers of studies have adopted process-dissociation tasks to investigate memory performance in neuropsychological populations, in relation to ageing and AD the theoretical and clinical utility of the procedure has not been fully explored. Further research using process-dissociation methodology with larger AD samples is greatly needed to examine the reliability of the results reported so far. It is important to see whether the pattern of processing dissociations that have been observed generalize across tasks with different processing constraints. Additionally, it is important for studies to include AD patients with varying degrees of dementia severity, and to examine how the changes in memory processing that occur in AD differs from those found in other dementing disorders (e.g. vascular dementia and frontotemporal dementia).

Obtaining uncontaminated measures of controlled and automatic memory processes is an essential step towards understanding the normal progression of age-related changes in memory processing, and for discriminating between normal and abnormal changes. A chief problem in diagnosing AD is that the behavioral changes that occur in the early stages of the disease, such as slowed cognitive processing, inattentiveness and emotional withdrawal are also evident in the elderly who present with depression. Some studies now concur that decrements in automatic memory processes are present in AD but do not to appear to be a part of normal ageing, nor are they apparent in depression (Hertel and Milan, 1994). If this pattern is shown to be reliable, tasks that involve process-dissociation procedures may prove to be useful for discriminating between patients with early course AD and other memory impaired groups who have reversible symptoms. Indeed, a recent large-scale study of nondemented elderly subjects by Spaan et al. (2005) suggests that deficits on both direct and also indirect retrieval tasks are significant prodromal markers of developing AD within a

period of 2 years. An important direction for future longitudinal studies of the healthy elderly would be to examine how process-dissociation estimates of memory functioning compare to task dissociation measures in predicting subsequent AD.

The progression of changes in memory status is indeed a vital index not only for identifying individuals who are vulnerable of developing AD, but also for monitoring patients in whom AD is suspected, and research is needed to determine longitudinally the stability of memory processing. Estimates derived from the process-dissociation calculations could be used to indicate whether the breakdown in memory processing is gradual or sudden, and indicate the extent to which controlled and automatic forms of retrieval remain available throughout the course of the disease. For example, if automatic retrieval is preserved in the early stages of AD, there is some evidence that it can be utilized by cognitive rehabilitation programmes. For example Clare et al (2002) used errorless learning principles with AD patients to relearn face-name associations, over 80% of these patients showed clear gains which were largely maintained after 6 months. The process-dissociation approach could prove to be helpful for identifying patients who are likely to benefit from this form of intervention.

Furthermore, the consequences of behavioral (e.g. exercise, diet, cognitive training) and pharmacological interventions upon memory processing can only be accurately assessed if separate indices of controlled and automatic memory processing are obtained. For example, a given intervention might be assumed to have had no cognitive benefit if a significant change in overt task performance is not observed. However, null effects between pre and post-intervention performance does not necessarily equate to there being no variation in the covert processes that subserve that performance. It is plausible that an intervention produces gains in the use of controlled memory processes and a reduction in the use of automatic memory processes, whilst not having an overall effect on task performance per se. Without uncontaminated estimates of memory processes the true efficacy of an intervention cannot be gauged.

CONCLUSION

Attempts to understand memory processing in AD have mostly been based on studies that have deployed task dissociation methods. These studies show that patients with AD have characteristic deficits on tests that involve direct forms of retrieval but their performance on tests involving indirect retrieval is variable. Task dissociation approaches preclude understanding whether controlled and automatic memory processes are differentially affected by AD because on traditional tests of memory both processes are facilitative. Recently, the process-dissociation procedure has been deployed to examine the contribution of automatic and controlled uses of memory to stem completion tasks in AD. This research indicates that AD can compromise the deployment of both automatic and controlled memory processes, whereas normal ageing has been shown to only compromise the deployment of controlled memory processes.

Drawing upon neuroimaging evidence a tentative interpretation of the qualitative difference between normal ageing and AD is that the controlled contribution to stem completion is mediated by neural substrates within the frontal lobes, the functioning of which is reduced in older adults relative to younger adults, and in AD relative to the normal elderly.

Secondly, the automatic contribution to stem completion may involve the operation of the occipital cortex, the functioning of which is unaffected by normal ageing but is abnormal in AD.

Obtaining uncontaminated estimates of memory processing is central to understanding the theoretical and neurological basis of memory retrieval. Moreover, it is equally important for the neuropsychological assessment of patients with AD. Neuropsychological assessment plays a pivotal role in the diagnosis of AD, and is the only objective means for monitoring the behavioral consequences of pharmacological and psychological interventions. The process-dissociation procedure has so far proven to be a valuable methodological tool for conducting basic research; it may prove to be an equally valuable assessment tool for measuring the status of controlled and automatic memory processing in normal ageing and in patients with AD.

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In: Alzheimer's Disease Diagnosis and Treatments ISBN: 978-1-61122-064-3
Editor: Marisa R. Boyd

Chapter 3

AGE OF ONSET RELATED DIFFERENCES IN CLINICAL AND NEUROPSYCHOLOGICAL FEATURES OF ALZHEIMER'S DISEASE

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ABSTRACT

Clinical presenting symptoms of Alzheimer's disease vary with age, complicating diagnosis in patients with atypical early onset of disease (age < 65 years). Patients with early onset of Alzheimer's disease symptoms have greater impairment in language, working memory and visuospatial abilities, and relatively less episodic and semantic memory impairment compared to those with the more typical later onset Alzheimer's disease. These differences suggest greater involvement of the parietal lobes in patients with early onset Alzheimer's disease, compared to greater early involvement of the hippocampus and medial temporal lobes in those with later onset Alzheimer's disease. Neuroimaging studies show greater areas of atrophy and decreased brain activity in the parietal lobes, precuneus regions, and posterior cingulate corticies in early onset patients compared to greater temporal lobe and hippocampal atrophy in those with later onset Alzheimer's disease. Patients with very late onset of Alzheimer's disease (age > 84 years) present with greater deficit of frontal lobe functions, consistent with the hypothesis of increased vulnerability of the frontal lobes and frontal-subcortical circuits to decline with age. Comparing the clinical findings of patients with Alzheimer's disease according to their age of onset highlights the complex relationship between the pathology of

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Alzheimer's disease and typical aging related changes that occur in the brain, and may aid clinicians in diagnosing this disease in patients at all ages on onset.

Keywords: Alzheimer's Disease, Presenting Symptoms, Magnetic Resonance Imaging

INTRODUCTION

Onset of Alzheimer's disease (AD) typically occurs at elderly ages (age > 65 years), and patients with an early onset of symptoms in the middle ages (ages 40 to 65 years) are inherently atypical and therefore more difficult to diagnose. Although onset of AD during middle ages is generally rare with a prevalence of less than 1% at ages 60-64 [2], patients with familial or autosomal dominant forms of AD often experience an early onset of symptoms in their forties and fifties [1]. The prevalence then increases exponentially with age, roughly doubling with every 5 years of additional age up to 26-45% among individuals aged 85 years or older [3].

Due to the relatively high prevalence at older ages, dementia continues to impose a significant economic burden on society with an estimated worldwide cost of US$315.4 billion in 2005 [4]. As the most common etiology of dementia (47-66% of all degenerative dementias), AD accounts for a considerable proportion of these costs [3]. The most obvious risk factor for AD is advancing age; however, other important risk factors include family history of dementia, presence of the ApoE4 allele, presenilin mutations and abnormal amyloid precursor protein gene, Down's syndrome, female sex, elevated serum homocysteine, elevated cholesterol, head trauma, low educational and lifelong occupational attainment, and small head size [3]. Recent studies have also suggested an association between AD and the metabolic syndrome and related vascular risk factors such as diabetes mellitus, insulin resistance, hypercholesterolemia, hypertension, atherosclerosis, coronary heart disease, smoking, reduced exercise, and obesity [2, 5].

The need for diagnostic accuracy may be greatest in those with early onset of AD (EAD, age < 65 years), as optimal management of patients with AD requires early diagnosis and treatment [3]. However, clinicians are more likely to misdiagnose EAD patients than those presenting at more typical elderly ages [6, 7], possibly as a result of clinicians being more familiar with the clinical characteristics of the more common later onset AD (LAD) [7]. Currently there is no definitive diagnostic test or biomarker for AD and diagnosis depends solely on clinical findings with pathological confirmation available only after death. Even after death, diagnostic certainty is not always possible as patients may have contradictory clinical and histologic diagnoses [1]. Consequently, it is important for clinicians who care for patients with dementia to have a thorough knowledge of the presenting clinical features of AD across the age of onset spectrum.

The clinical evaluation of dementia should include a detailed history, physical and neurological examination, quantified assessment of cognitive function, as well as ancillary tests. Two commonly used sets of clinical criteria for AD include the criteria developed by the National Institute of Neurologic and Communicative Disorders and Stroke and the AD and Related Disorders Association Work Group (NINCDS-ADRDA) [9], and the American Psychiatric Association's *Diagnostic and Statistical Manual-IV* (DSM-IV) [10]. It is

important to note that both sets of clinical criteria are largely based on clinical features, the expression of which may be affected by age of onset.

RELATIONSHIP BETWEEN AGE OF ONSET AND CLINICAL AND NEUROPSYCHOLOGICAL FEATURES OF ALZHEIMER'S DISEASE

Historically, AD was a diagnosis restricted to patients experiencing early onset of the disorder, and patients presenting with onset later in life were considered to have a distinct "senile dementia." While the discovery of similar neuropathology for both EAD and LAD eliminated this distinction, many studies have continued to demonstrate the existence of differences in clinical features between patients with EAD and LAD. This is important because EAD may be more common than previously believed, although the majority of studies characterizing clinical features in AD have focused on patients with more typical later ages of onset [11-13]. Understanding clinical features unique to EAD is also important given that this disorder often affects people of working-age, resulting in significant economic consequences to these patients and their families.

Typical Clinical and Neuropsychological Features of Alzheimer's Disease

Episodic memory deficits are often the first presenting symptom in patients with AD and can precede other clinical signs and symptoms of dementia by many years [3]. Impairment in visuospatial functions may also occur relatively early in the disease course, including difficulties in becoming oriented to the surrounding environment as well as problems drawing and copying simple and complex figures [3]. Other symptoms that may develop early in the disease course include visual agnosia which manifests as difficulty recognizing objects, impairment of frontal-executive functions such as planning and goal-oriented behavior as well as understanding abstract concepts and impaired judgment and reasoning abilities [3]. Some patients may also experience language difficulties while in the early stages of dementia. Common language difficulties include problems with word finding, naming and comprehension. As the dementia progresses, these language difficulties tend to worsen and the patient may begin to develop whole word substitutions resulting in verbal paraphasias [3]. Speech, in contrast, remains relatively preserved throughout much of the disease course for AD and is usually unaffected until the later stages [3].

The behavioral symptoms that occur in AD are typically mild during the early stages of dementia, allowing many patients to remain in their own homes under the care of their spouse or family and not require nursing home placement until later on in the disease course. Apathy, agitation, verbal and even physical aggression, as well as poor impulse control, disinhibition, and wandering are all common behavioral symptoms that can occur at later stages [3]. Neuropsychiatric symptoms including depression, delusions, and hallucinations may also occur and can be troubling to those caring for the patient. Delusions may have paranoid characteristics, often due to patients misinterpreting or misunderstanding an event or person [3]. In early stages of AD, the gross-neurologic examination is usually considered normal; however, as the disease progresses many patients will develop extrapyramidal symptoms

including parkinsonian rigidity, gegenhalten, and spasticity [3]. Seizures may occur late in the disease course, and when they do occur are most frequently generalized tonic-clonic convulsions.

Age of Onset Related Differences in Clinical and Neuropsychological Features of Alzheimer's Disease

The most widely reported and consistent difference identified between patients with EAD and LAD is a greater frequency and severity of language dysfunction among those with early onset of disease [15-18]. Several studies found more frequent impairment in spontaneous speech among patients with disease onset before age 65 years [15-17]. A 1993 study by Sevush et al. used factor analysis of cognitive scores from 150 patients with AD of all onset ages to demonstrate that EAD patients tend to have lower performance on an orthogonal factor comprised mostly of language related measures, including tests of spontaneous speech, repetition, comprehension, reading, and writing, as well as digit span and left/right discrimination [18]. Patients with late-onset disease did not exhibit the same performance patterns, scoring lower on another factor which included tests examining cognitive areas more traditionally considered to be impaired in AD, such as tests of memory, orientation, object naming, and abstraction. However, other studies have not detected differences in the extent of language dysfunction between early and late onset AD patients after adjusting for age and differences in attention/concentration [19, 20].

In addition to language dysfunction, patients with EAD exhibit poorer performance on neuropsychological tests of working memory and visuospatial functions, including forward and backward digit and visual spans, visual counting, copying Rey complex figure, and block design tasks [21]. Earlier age of disease onset is also associated with significantly more impairment on tests of attention span and working memory (digit span), graphomotor function (copy loops) and apraxia [22]. Finally, an analysis of WAIS subtests also found that subjects with EAD performed more poorly on age-adjusted measures of sustained concentration and mental tracking [16].

Patients with EAD are reported to experience a lesser degree of semantic memory impairment early in the disease course and may not differ on measures of episodic memory compared to those with LAD [23, 24]. In 1994, Jacobs et al. found that subjects with LAD had significantly poorer baseline performance for memory and naming of items [24]. Additionally, Grosse et al. demonstrated that while early onset and late onset patients did not differ in performance on measures of episodic memory, LAD patients were more impaired on measures of semantic memory [23].

A recent study by Licht et al. highlights the relationship between the extremes of the age of onset spectrum and clinical characteristics. Apparently in contrast to earlier reports, Licht et al. did not identify differences in language or memory functions between patients with early and late disease onset in their study population at a large Veteran's Affairs clinic [19]. EAD and LAD patients also did not differ in visuospatial or other cortical cognitive deficits, although differences in performance on tasks of category verbal fluency and frontal-executive functions were found with patients with LAD performing more poorly than patients with EAD. Specifically, patients with LAD performed significantly worse on animal list generation but not on "F" word list generation, as well as on three motor tests of frontal-

executive functions: the Luria Hand Sequence, Go/No Go and the Luria Alternating Programs.

The findings of this study are partially explained by the age of the study participants. In order to highlight age of onset related differences in clinical features, the age of the study participants with LAD (85.6 ± 2.2 years) exceeded that typically reported by other studies [15, 16], and consequently the study compared patients presenting at the extremes of the age of onset spectrum. The results of the study also suggest regional age-related vulnerabilities of the brain to the AD pathophysiologic process, with the frontal-lobes and/or frontal-subcortical circuits possibly being particularly vulnerable at very older ages of AD onset [3]. Thus, patients with very late ages of AD onset may be considered to be clinically distinct not only from those with EAD but also from those with more typical ages of onset in their 60's and 70's.

In addition to differences in presenting features, prior studies have suggested that patients with EAD experience a more rapid disease progression and decreased survival time relative to patients with LAD [24, 25]. In 1994 Jacobs et al. reported that early age of AD onset predicted a more rapid decline in cognitive function as measured by performance on the Mini-Mental State Examination (MMSE) and the Blessed Dementia Rating Scale-Part 1 [24]. This study also identified differences in patterns of performance on the MMSE, with EAD subjects performing more poorly on items relating to attention at baseline and follow-up, and those with LAD experiencing poorer performance on items testing verbal memory and naming at baseline, although these differences disappeared at follow-up. The faster progression of cognitive decline in patients with EAD has been confirmed by a separate investigation which concluded that age of onset was inversely related to the progression of cognitive impairment [25]. Patient age at disease onset also modifies predictors of institutionalization or death, with higher rates of institutionalization or death among younger versus older patients even after controlling for degree of cognitive impairment [26].

While some inconsistencies exist between study results, these are likely due to variability in methodological choices such as selection of diagnostic criteria and neuropsychological tests, and limitations such as sample size [14]. Overall, reported differences in cognitive impairment between patients with EAD and LAD suggest that patients with EAD experience greater parietal lobe involvement early in the disease course, compared to greater temporal lobe involvement among those with LAD [2, 14, 21, 22]. In addition, as shown by Licht et al., the relatively greater deficits in frontal lobe functions among patients with very late onset AD support the idea that specific brain regions may have age-related vulnerabilities to AD neuropathology [19]. Finally, studies on the rate of cognitive decline and survival also suggest age-related clinical differences, demonstrating that patients with an early onset of symptoms may have more rapid disease progression which may manifest as decreased duration of survival from time of diagnosis.

NEUROIMAGING AND AGE OF ONSET RELATED DIFFERENCES IN CLINICAL FEATURES OF ALZHEIMER'S DISEASE

Findings from studies using neuroimaging techniques to compare brain structure and function between patients with EAD and LAD support the localization of the reported clinical

differences. By identifying the structural and functional variations associated with the clinical features of AD at different ages of onset, neuroimaging studies can help elucidate the underlying etiology of these differences.

Structural Neuroimaging Findings

Several studies have used MRI and voxel-based-morphometry to compare whole brain and gray matter volumes between patients with early and later onset AD and demonstrate that different patterns of cortical atrophy are associated with age of disease onset. Using voxel-based morphometry, Ishii et al. compared brain MRI scans of 60 patients, 30 with EAD and 30 with LAD, and found that patients with early onset disease had relatively greater atrophy in the parietotemporal and posterior cingulate areas [27]. This finding was replicated by Shiino et al. in 2006 [28]. In 2007, a study by Frisoni et al. reported that early onset AD patients had relatively greater neocortical gray matter loss in all brain regions compared to later onset patients [29]. Finally, Karas et al, identified the precuneus region as an additional specific area showing increased atrophy in early onset AD patients compared to those with later onset AD [30].

Functional Neuroimaging Findings

Functional neuroimaging studies using positron emission tomography (PET) have corroborated the findings of the structural MRI studies by demonstrating regional differences in cortical metabolism consistent with areas of increased volume loss or atrophy in patients with EAD compared to those with LAD. In general, patients with LAD show greater hypometabolism of the hippocampi and medial temporal regions bilaterally, while those with EAD more frequently have hypometabolism in the precuneus region as well as the parietal lobes and cingulate cortices [31-34]. These studies also provided evidence of functional correlation between the regional anatomic differences and performance on cognitive tests, including performance on the Mini Mental Status Exam, full scale IQ score and intrusions in free recall with metabolic changes in the right superior frontal gyrus, as well as verbal and non-verbal semantic memory impairments with decreased left sided metabolism in the temporal, parietal, and occipital lobes [35-37].

Additional differences in cortical metabolism between patients with early onset AD compared to those with later onset of disease include more frequent left hemisphere and frontal lobe involvement [15, 16, 32]. The finding of more frequent left hemisphere involvement in patients with EAD is in agreement with clinical studies that have identified greater impairment in performance on tests of language functions such as verbal IQ, measures of word discrimination and writing ability among patients with early onset of symptoms [15, 16, 32]. In contrast, patients with LAD are found to have greater hypometabolism of the hippocampi and medial temporal regions bilaterally compared to those with EAD, which is consistent with reports of greater early memory impairment on neuropsychological testing in patients with LAD [31, 33, 34].

CONCLUSION

Age of onset is associated with important differences in clinical and neuropsychological features of AD suggesting the possibility of age-related vulnerabilities of specific cortical regions to AD pathophysiology. As a result, studies of subjects presenting only with typical AD may not be generalizable to patients at other ages of onset, either early or very late. Studies of age related clinical differences in AD seek to improve diagnostic accuracy at all ages of onset and facilitate the possibility of early intervention and optimal treatment benefit. It is important to remember that the neuropathological changes of AD do not occur independent from the aging process and more research is needed to identify potential interactions between the effects of typical aging and AD.

ACKNOWLEDGMENT

This work is supported by the University of Hawaii Ph.D. in Clinical Research Program - 1K07GM072A84.

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In: Alzheimer's Disease Diagnosis and Treatments ISBN: 978-1-61122-064-3
Editor: Marisa R. Boyd

Chapter 4

EARLY ONSET DEMENTIA WITH ABUNDANT NON-NEURITIC Aβ PLAQUES AND WITHOUT SIGNIFICANT NEURONAL LOSS: REPORT OF TWO JAPANESE AUTOPSY CASES

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ABSTRACT

The presence of Aβ-positive neuritic plaques with dense cores is considered an essential pathological marker of Alzheimer's disease (AD). However, there are atypical cases that have abundant non-cored plaques with surrounding minor dystrophic neurites. The atypical plaques are called 'cotton wool plaques', and AD with cotton wool plaques is thought to be one of the variants of AD. Cotton wool plaques are usually large, round, and eosinophilic, and appear to displace surrounding normal structures. Inflammatory glial response is mild. We here describe two autopsy cases of early-onset dementia with abundant eosinophilic non-cored Aβ plaques, the histopathological features of which are different. Patient 1 was a 44-year-old man at the time of death, with a clinical course of 8 years. Nine relatives in three generations had died in their thirties to forties, and some of them were verified to have had dementia. The proband presented clinically with spastic

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paraparesis at age 36 prior to the development of dementia. The brain weight was 1330 g. Macroscopically, only mild atrophy was found in the frontal, temporal, and parietal cortices. Histopathological examination revealed abundant, large, eosinophilic, non-cored plaques having the typical appearance of cotton wool plaques. The plaques were more strongly immunopositive for Aβ42 than Aβ40. A moderate number of neurofibrillary changes were found in the hippocampus and parahippocampal gyrus, but only a few in other anatomical regions, including the neocortex. The pyramidal tract was degenerated. Although moderate neuronal loss was found in the insular and entorhinal cortices, the other cerebral cortices were relatively spared. Genetic analysis demonstrated the G384A presenilin-1 gene mutation. Patient 2 was a 46-year-old man at the time of death, with a clinical course of 8 years. His family had no history of neurological diseases in the previous three generations. He presented with memory impairment at the age of 39. Subsequently, he showed disinhibition, impulsiveness, and paranoid ideation, but no neurological abnormality. The brain weight was 1700 g. Macroscopically, neither brain atrophy nor edema was observed. Histopathological examination disclosed abundant eosinophilic non-cored plaques in all cerebral cortices. The diameters were 40-100 μm, including plaques smaller than those in patient 1. The plaques showed little tendency to displace normal structures, but did not contain neurons. Intriguingly, the plaques in this case were were more strongly immunoreactive for Aβ40 than for Aβ42. In the cerebral cortex including the hippocampus, neurons were well preserved, and glial response was slight. In the pyramidal tract, glial proliferation was evident, although loss of myelin was not noted. No tau-positive lesions were found in any region. No mutations in presenilin-1, presenilin-2, or amyloid precursor genes were revealed by genetic analysis using formalin-fixed paraffin-embedded tissue. These findings suggest that factors besides neuritic plaques, neurofibrillary tangles, and severe neuronal loss play a pivotal role in the occurrence of cognitive decline in AD patients.

INTRODUCTION

The presence of Aβ-positive senile plaques is considered to be an essential pathological marker of Alzheimer's disease (AD). Senile plaques are classified into several morphological subtypes. The most representative subtype is a classic mature plaque with a dense amyloid core and surrounding dystrophic neurites with reactive glial cells. They are often called neuritic plaques, and the presence of neuritic plaques is an essential criterion for pathological diagnosis of AD [1, 2]. Another pivotal subtype is the diffuse plaque, which is visualized with Aβ immunostaining but not hematoxylin-eosin (HandE) staining. Diffuse plaques are ill-defined and lack inflammatory glial response. In addition to the formation of senile plaques, variable degrees of neurofibrillary changes usually develop in AD cases. The severity of neurofibrillary changes rather than that of senile plaques correlates with the severity of AD [3]. However, because the presence of senile plaques rather than that of neurofibrillary changes is more specific for AD, the formation of senile plaques is considered to be more closely related to the pathogenesis of AD.

In 1998, Crook et al. [4] reported an unusual variant AD bearing deletion of exon 9 of the presenilin-1 (PS1) gene. This variant was clinically characterized by dementia with spastic paraparesis, and pathologically, by the occurrence of large non-cored plaques with minor neuritic changes. Thereafter, familial cases with different PS1 gene mutations or deletions, as well as a few sporadic cases, were also reported. The atypical plaques in this variant were large, round, well-defined, and eosinophilic, and were called cotton wool plaques (CWPs).

They had few dystrophic neurites and little surrounding glial response, and appeared to displace surrounding normal structures. This variant AD with CWPs (CWP-AD) raised the question of how the atypical plaques resulted in the neurodegeneration of CWP-AD, and whether the presence of mature plaques with dystrophic neurites is really necessary in the development of dementia in AD.

We here describe two autopsy cases of early-onset dementia with abundant eosinophilic non-cored Aβ plaques. Case 1 had abundant large Aβ plaques having morphological features consistent with those of CWPs reported previously. This case also had neurofibrillary changes corresponding to Braak stage V. Neurons in the superficial layer in the cerebral cortex were slightly reduced in number. Case 2 also had abundant eosinophilic Aβ-positive plaques. These plaques varied in size from 40-100 μm, including plaques of rather small size. They were often ill-defined and lacked the tendency to displace surrounding normal structures. In contrast to the remarkable Aβ deposition, neither amyloid angiopathy nor glial response was observed in the cerebral cortex. Furthermore, intriguingly, no tau-positive lesions were noted in any anatomical region. Neurons and the laminar structure of the cerebral cortex were well preserved despite the presence of dementia.

MATERIALS AND METHODS

Brains tissue samples from both subjects were fixed post mortem with 10% formaldehyde and embedded in paraffin. Ten-μm-thick sections from the frontal, temporal, parietal, occipital, insular, and cingulate cortices, hippocampus, amygdala, basal ganglia, midbrain, pons, medulla oblongata, cerebellum, and spinal cord were prepared. These sections were stained by the hematoxylin-eosin (HandE), Klüver-Barrera (KB), Holzer, periodic acid-Schiff stain (PAS), methenamine silver, Bodian, modified Bielschowsky silver, and Gallyas-Braak silver methods.

Sections from the various regions in the cerebrum, brainstem, and spinal cord were examined immunohistochemically using anti-Aβ42 antibody (rabbit, polyclonal, 1:750, FCA3542, courtesy of Dr. F. Checler [5]), anti-Aβ40 antibody (rabbit, polyclonal, 1:750, FCA3340, kindly provided by Dr. F. Checler [5]), anti-Aβ antibody (against Aβ a.a.17-24, mouse, monoclonal, 1:2000, 4G8, Senetek), anti-Aβ antibody (against Aβ a.a.1-17, mouse, monoclonal, 1:2000, 6E10, Senetek), phosphorylated tau (AT8, mouse, monoclonal, 1:3,000, Innogenetics), phosphorylated α-synuclein (pSyn#64, mouse, monoclonal, 1:1,000, Wako), prion protein (3F4, mouse, monoclonal, 1:1000, Dako), and glial fibrillary acidic protein (GFAP, rabbit, polyclonal, 1:5,000, Dako). Deparaffinized sections were incubated with 1% H_2O_2 in methanol for 20 min to eliminate endogenous peroxidase activity in the tissue. Sections were treated with 0.2% TritonX-100 for 5 min and washed in phosphate-buffered saline (PBS, pH 7.4). Sections were pretreated with formic acid (99%, 5 min; Sigma) for antigen retrieval when using anti-Aβ, anti-α-synuclein, and anti-prion protein antibodies. After blocking with 10% normal serum, sections were incubated 72 hours at 4°C with one of the primary antibodies in 0.05 M Tris-HCl buffer, pH 7.2, containing 0.1% Tween and 15 mM NaN_3. After three 10-min washes in PBS, sections were incubated in biotinylated anti-rabbit or anti-mouse secondary antibody for 1 h, and then in avidin-biotinylated horseradish

peroxidase complex (ABC Elite kit, Vector) for 1 h. The peroxidase labeling was visualized with 0.2% 3,3'-diaminobenzidine (DAB) or diaminobenzidine-nickel as the chromogen.

Neuronal loss and gliosis in representative regions were semiquantitatively evaluated. In the cerebral cortex, the degree of degeneration was assessed on HandE, KB-, and GFAP-stained sections according to the grading system employed in our previous studies [6-11]: -, no histopathological alteration is observed; +, slight neuronal loss and gliosis are observed only in the superficial layers; ++, obvious neuronal loss and gliosis are found in cortical layers II and III, often accompanied by status spongiosis and relative preservation of neurons in layers V and VI; +++, pronounced neuronal loss with gliosis is found in all cortical layers with prominent fibrous gliosis exhibited in adjacent subcortical white matter. In the basal ganglia and brainstem nuclei, the degree of neuronal loss and gliosis was assessed on HandE, KB-, and GFAP-stained sections according to the following grading system: -, neither neuronal loss nor gliosis is observed; ±, mild gliosis is observed on HandE-stained sections or GFAP-immunostained sections, but neurons are not reduced in number; +, mild gliosis and mild neuronal loss are present; ++, neuronal loss and gliosis are moderate, but tissue rarefaction is absent; +++, severe neuronal loss, severe fibrous gliosis, and tissue rarefaction are observed. The degeneration of the corticospinal tract was assessed based on the loss of myelin, glial proliferation, and appearance of macrophages at the level of the medulla oblongata and spinal cord, and indicated as + or –.

Genomic DNA was extracted from paraffin-embedded brain sections. The regions encoding exons 3-12 of PS1, exons 3-12 of PS2, exons 16 and 17 of APP, and the regions including codons 112 and 158 of the apo E gene were amplified by PCR using unique primer sets according to previously described procedures [12, 13]. Designation of the number of each exon of the PS1 follows Hutton's nomenclature [12]. PCR products of PS1 were directly sequenced. The apo E genotype was determined by restriction fragment length polymorphism analysis using *Cfo*I.

CASE REPORTS

Case 1

Clinical Course

This man was 44 years old at the time of death. Figure 1 shows the pedigree of this case (IV.6). At least nine relatives in three generations suffered from dementia. The age at onset in all affected members was in the 30s, and the age at death was in the 40s [14]. A part of the clinical information and the pathological findings of this case have been reported in Japanese [15, 16]. This patient had a history of polyneuropathy at the age of 33, but without aftereffect. At the age of 36, in 1979, he initially complained of numbness in the legs. In 1981, his stance became wide-based, and he walked in a forward-bent posture. In addition, his dysarthria and forgetfulness were first noticed by his wife. Reduction of spontaneity, dysarthria, and gait instability developed subsequently. In 1983, neurological examination disclosed dysarthria, pyramidal signs, cerebellar signs, and memory impairment. Deep tendon reflexes were exaggerated in all four extremities, and Babinski signs were bilaterally positive. Swallowing function was not disturbed. Mild dysmetria in the left arm and left side-predominant bilateral

adiadochokinesis were noted. His score on the Hasegawa Dementia Scale (HDS), which is most frequently employed to assess cognitive function of people with dementia in Japan and correlates well with the score of the Mini Mental State examination, was 28.5 points (full score 32.5 points), indicating normal cognitive function. He obtained a verbal IQ score of 100, a performance IQ score of 86, and a full scale IQ of 99 on the Wechsler Adult Intelligence Scale-revised (WAIS-R). Cerebrospinal fluid examination demonstrated a mild increase in protein concentration.

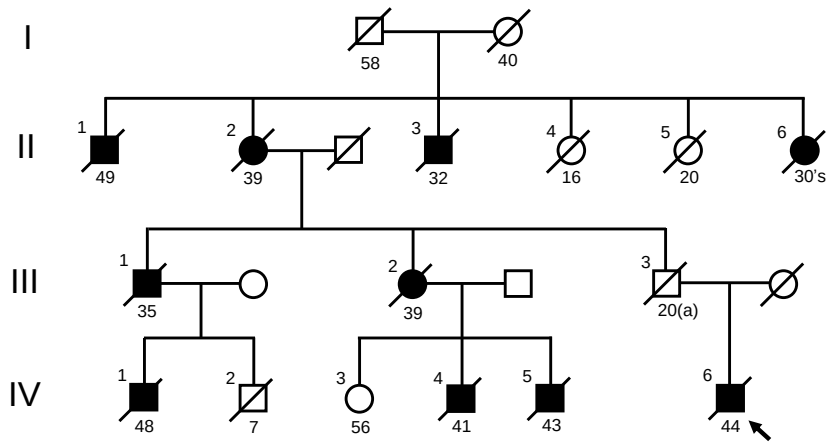

Figure 1. Family tree of case 1 (arrow). Squares = males; circles = females; filled symbols = affected; slashes = deceased. G384A presenilin-1 mutation was found in cases IV.1 and IV.6. (a) Killed in the World War II.

One year later, at age 40, neurological reexamination revealed progression of memory impairment, dysarthria, ataxia, and gait disturbance. Both verbal IQ and performance IQ were under 60 on the WAIS-R. Ocular movement was not restricted but saccadic. Rigidity was found in the upper extremities, and rigospasticity in the lower extremities. Deep tendon reflexes were apparently increased. Babinski signs, palmomental reflexes, and snout reflex were positive. Clonus was not found. Diadochokinesis was bilaterally impaired. Cerebellar ataxia was also noted. Muscle atrophy, weakness, or involuntary movements were not noted. Bladder and erectile functions were impaired. Blood and urine examinations were within normal limits. Cerebrospinal fluid examination again demonstrated a mild elevation of protein concentration. He was clinically suspected of having Gerstmann-Sträussler-Scheinker syndrome. He was admitted to a psychiatric hospital at age 41. On admission, he presented with dementia, severe memory impairment, dysarthria, and gait disturbance. His score on the Hasegawa Dementia Scale Revised (HDS-R: full score 30 points, cutoff 19/20) was 10 points, indicating moderate dementia. Baseline examinations of blood, urine, and cerebrospinal fluid were within normal limits. Electroencephalography registered much diffuse slow wave activity. Head computed tomography (CT) showed mild dilation of the Sylvian fissures and lateral ventricles. Cortical atrophy in the cerebrum was not evident. The cerebellum was unremarkable. One year later, CT revealed mild atrophy in the frontal and temporal cortices, and ventricular enlargement and atrophy in the brainstem were evident. At age 43, he walked

dragging his legs while extending his knees. His gait was wide-based but not ataxic. He could not comprehend simple neurological examination instructions such as the finger-nose test and eye-tracking test. Pyramidal signs, pseudobulbar sign, and slight cerebellar ataxia were observed. Parkinsonism or primitive reflexes were not found. CT revealed moderate cerebral atrophy; however, the severity was far milder than expected from severe dementia. The atrophy in the brainstem progressed. At age 44, he could not walk without support. Spontaneity was increasingly reduced, and emaciation became evident. He finally became bedridden, and he died of pneumonia at age 44 in 1988, 8 years after the disease onset. No respiratory support was given throughout the clinical course. The final neurological diagnosis was unclassified presenile dementia.

Pathological Findings

The brain weight was 1330 g after fixation. Macroscopically, mild cerebral atrophy in the frontal, temporal, and parietal lobes was found. The bilateral anterior horns of the lateral ventricles were dilated. The substantia nigra and locus coeruleus showed mild depigmentation.

Microscopically, the most striking feature was the occurrence of numerous atypical senile plaques. They were large, round, and eosinophilic, and distributed throughout the cerebral cortex (Figure 2a) and limbic system. Almost all of the plaques lacked cores, and surrounding neuritic changes were scarce. These morphological features were consistent with those of CWPs [4]. The non-cored plaques were most frequently encountered in cortical layers II and III, but they were also found in the deep layers, although in rather small numbers. The plaques in the upper cortical layers were larger than those in the deep layers. The plaques were easily detected on HandE-stained sections (Figures 2a, 4a, 4b). On KB-stained sections also, the plaques were visible, but the intensity of the staining was weak compared with that of myelin (Figures 2b, 4c, 4d). The plaques were stained with Bodian stain (Figures 2c, 5a). The plaques showed argyrophilia on methenamine and Bielschowsky silver stains (Figures 3a, 3b, 5b). On Gallyas-Braak silver-stained sections, the plaques were weakly argyrophilic (Figures 3c, 5c). The diameters of the plaques were often over 100 μm, and some of the plaques had a glial nucleus in the central portion (Figures 4b, 5a, 5b, 5c). Silver stains revealed minor neuritic changes surrounding the plaques (Figure 5c). On the other hand, only a few mature plaques with amyloid cores and surrounding neuritic changes were seen. Glial response surrounding non-cored plaques was almost totally lacking, in contrast to the evident proliferation of reactive astrocytes associated with classic cored plaques (Figure 5d). In the cerebellum, HandE stain revealed a few cored plaques in the molecular layer. Moderate numbers of neurofibrillary tangles (NFTs) were observed in the hippocampus and parahippocampal gyrus. NFTs were encountered in the middle and deep cortical layers in the temporal and frontal cortices (Figure 3c). NFTs were also found in the nucleus basalis of Meynert, caudate nucleus, putamen, and hippocampal dentate gyrus. Although the number of NFTs was small, the distribution fits Braak stage V.

In contrast to the abundant CWPs, the degree of neuronal loss in the cerebral cortex was generally mild (Table). Severe neuronal loss involving all cortical layers was not found in any region. Moderate neuronal loss with preservation of the cortical layers V and VI was found only in the entorhinal cortex and the ventral portion of the insular cortex.

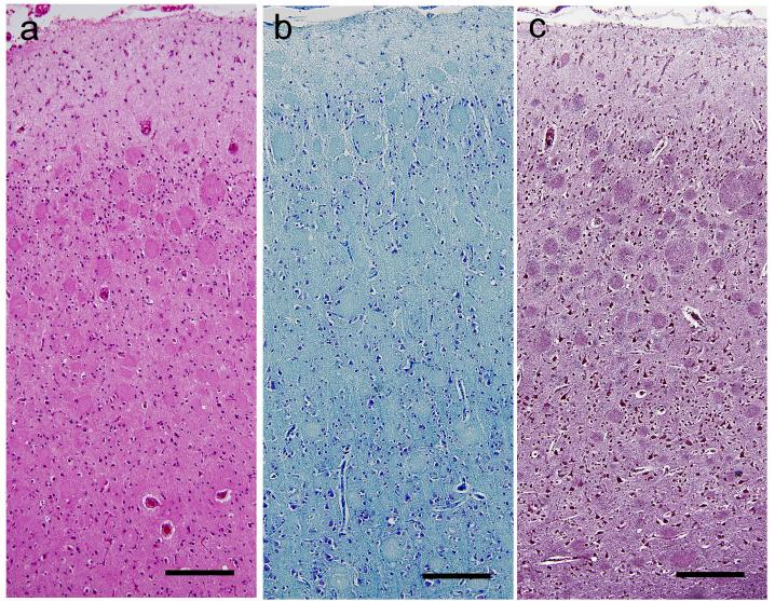

Figure 2. The temporal cortex in case 1. (a) Hematoxylin-eosin stain. Many large round eosinophilic cotton wool plaques are easily detected in the cortical layers II and III and layers V and VI. (b) Klüver-Barrera stain also reveals cotton wool plaques. In the deep cortical layers, the neuropil is more intensely stained rather than the cotton wool plaques. (c) Bodian stain. Many cotton wool plaques are easily detected. The plaques are mainly distributed in the deep and upper layers. All scale bars = 200 μm.

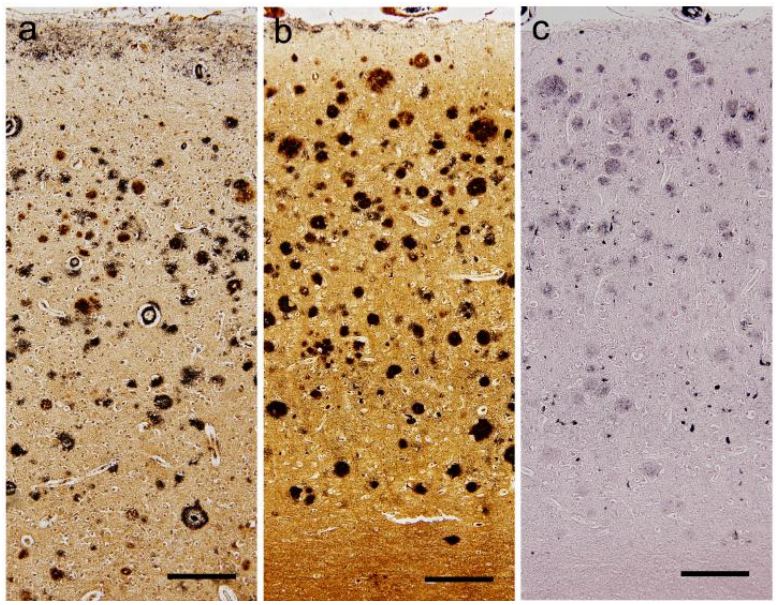

Figure 3. The temporal cortex in case 1. (a) Methenamine silver stain. Many cotton wool plaques and a small number of cored plaques are seen. (b) Modified Bielschowsky silver stain also reveals cotton wool plaques. (c) Gallyas-Braak silver stain. Cotton wool plaques are faintly argyrophilic. Neurofibrillary changes are found in the deep cortical layers. All scale bars = 200 μm.

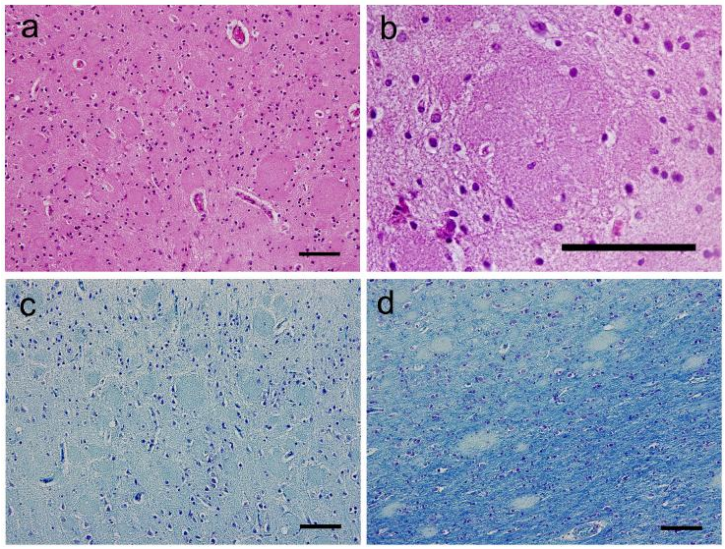

Figure 4. Cotton wool plaques in the temporal cortex in case 1 at higher magnification. (a) Hematoxylin-eosin stain. Many round plaques appear to displace surrounding normal structures. Inflammatory response is not evident. (b) Hematoxylin-eosin stain. A large cotton wool plaque of over 100 μm in diameter. A glial nucleus, but not an amyloid core, is present in the central portion. Surrounding glial response is not evident. (c) Klüver-Barrera stain. Neurons are displaced by many cotton wool plaques, but relatively preserved. (d) Cotton wool plaques in the white matter of the temporal lobe. Klüver-Barrera stain. All scale bars = 100 μm.

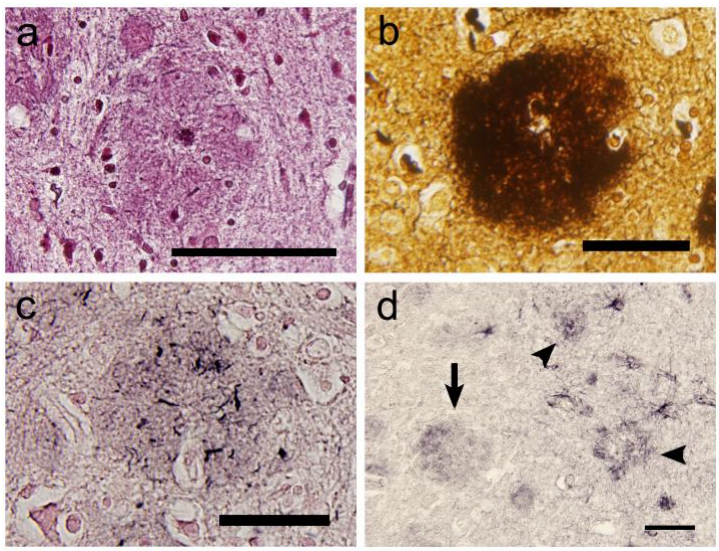

Figure 5. Cotton wool plaques in case 1. (a) Bodian stain. A plaque without neuritic changes in the temporal cortex. A glial nucleus is seen in the central portion. There is no amyloid core. (b) Modified Bielschowsky stain. A plaque without neuritic changes in the temporal cortex. (c) Gallyas-Braak silver stain. A plaque with a few neuritic changes in the temporal cortex. (d) GFAP immunohistochemistry. Reactive astrocytes are found around classic cored plaques (arrowheads). By contrast, a cotton wool plaque (arrow) lacks them. Scale bars = (a) 100 μm, (b) 50 μm, (c) 50 μm, (d) 50 μm.

Table. Distribution of neuronal loss and astrocytosis in the present cases

	Case 1	Case 2
Superior frontal gyrus	+	+
Medial frontal gyrus	+	-
Inferior frontal gyrus	+	-
Orbital gyrus	+	-
Primary motor cortex	+	+
Superior temporal gyrus	+	+
Medial temporal gyrus	+	+
Inferior temporal gyrus	+	+
Insular cortex	++	+
Cingulate gyrus	+	+
Amygdala	±	n.a.
Ambient gyrus	+	n.a.
CA1 in hippocampus	-	-
Hippocampal dentate gyrus	-	-
Subiculum	-	-
Entorhinal cortex	++	n.a.
Parahippocampal gyrus	+	+
Caudate nucleus	-	-
Putamen	-	-
Globus pallidus	-	-
Thalamus	±	-
Subthalamic nucleus	-	-
Nucleus basalis of Meynert	++	±
Dentate nucleus of cerebellum	-	±
Trochlear nucleus	n.a.	-
Oculomotor nucleus	±	n.a.
Substantia nigra	±	-
Red nucleus	-	n.a.
Locus coeruleus	+	n.a.
Pontine nucleus	-	-
Dorsal vagal nucleus	±	-
Hypoglossal nucleus	±	-
Inferior olivary nucleus	±	±
Corticospinal tract	+	+ (a)
Anterior horn	n.a.	n.a.

The severity of degeneration in the cerebral cortex: -, no histopathological alteration; +, slight neuronal loss and gliosis only in the superficial layers; ++, obvious neuronal loss and gliosis in cortical layers II and III, often accompanied by status spongiosis and relative preservation of neurons in layers V and VI; +++, pronounced neuronal loss with gliosis in all cortical layers with prominent fibrous gliosis in adjacent subcortical white matter. The severity of neuronal loss and gliosis in the basal ganglia and brainstem nuclei: -, neither neuronal loss nor gliosis; ±, mild gliosis on HandE- and GFAP-immunostained sections, but neurons e preserved; +, mild gliosis and mild neuronal loss; ++, neuronal loss and gliosis, but tissue rarefaction absent; +++, severe neuronal loss, severe gliosis, and tissue rarefaction. The degeneration of the corticospinal tract was assessed at the level of the medulla oblongata or spinal cord, and the existence of degeneration was indicated as + or - . n.a., not available. (a) Although loss of myelin was not evident, glial proliferation was observed.

Slight neuronal loss involving the superficial cortical layer alone was noted in the cingulate, frontal, and temporal cortices, prarahippocampal gyrus, and ambient gyrus. In the primary motor cortex, mild neuronal loss involving the upper cortical layers alone were found; however, loss of Betz cells, with small groupings of lipofuscin-laden macrophages in the holes from which the Betz cells had presumably disappeared, was found in this site. Neurons in the hippocampus and subiculum were not reduced in number. The number of neurons in the nucleus basalis of Meynert and locus coeruleus was also reduced. The pyramidal tract was involved at the levels of the midbrain and medulla oblongata (Figures 6a, 6b). The lower motor neurons in the hypoglossal nuclei were preserved in number.

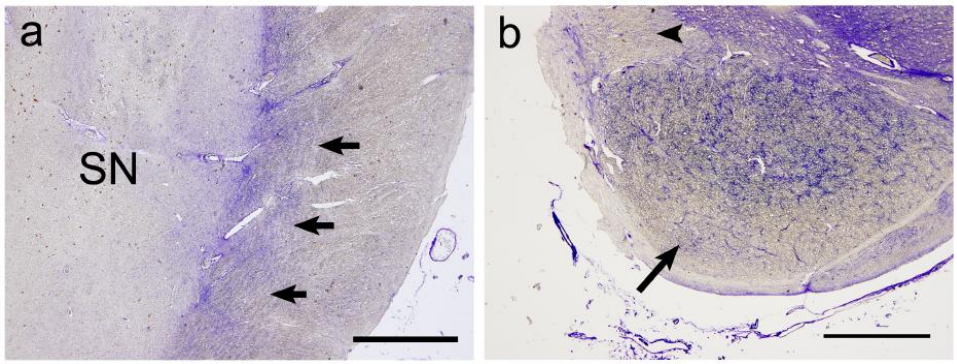

Figure 6. Pyramidal tract involvement in case 1. (a) Evident gliosis in the pyramidal tract at the level of the midbrain (arrows). SN indicates the substantia nigra. (b) Gliosis in the pyramidal tract at the level of the medulla oblongata (arrow) is also evident in comparison with the medial lemniscus (arrowhead). Holzer stain. All scale bars = 1 mm.

Cotton wool plaques were immunostained with anti-Aβ antibodies (Figures 7a, 7b, 7c). They were immunolabeled with anti-Aβ42 more strongly than Aβ40 antibodies. The plaques were found in all cortical layers, but mainly distributed in the upper and deep layers. Aβ deposits in the subpial region were often observed (Figure 7c). Cerebral amyloid angiopathy was labeled with anti-Aβ40 antibody (Figure 7b). Some CWPs were slightly tau-positive; however, there were few tau-positive neuritic changes in CWPs. Tau-positive NFTs were scattered mainly in the deep cortical layers (Figure 7d). Neither α-synuclein-positive nor prion protein-positive lesions were present in any region.

Genetic analysis using paraffin-embedded brain sections revealed a G384A mutation in the PS1 gene. The Apo E genotype was not examined.

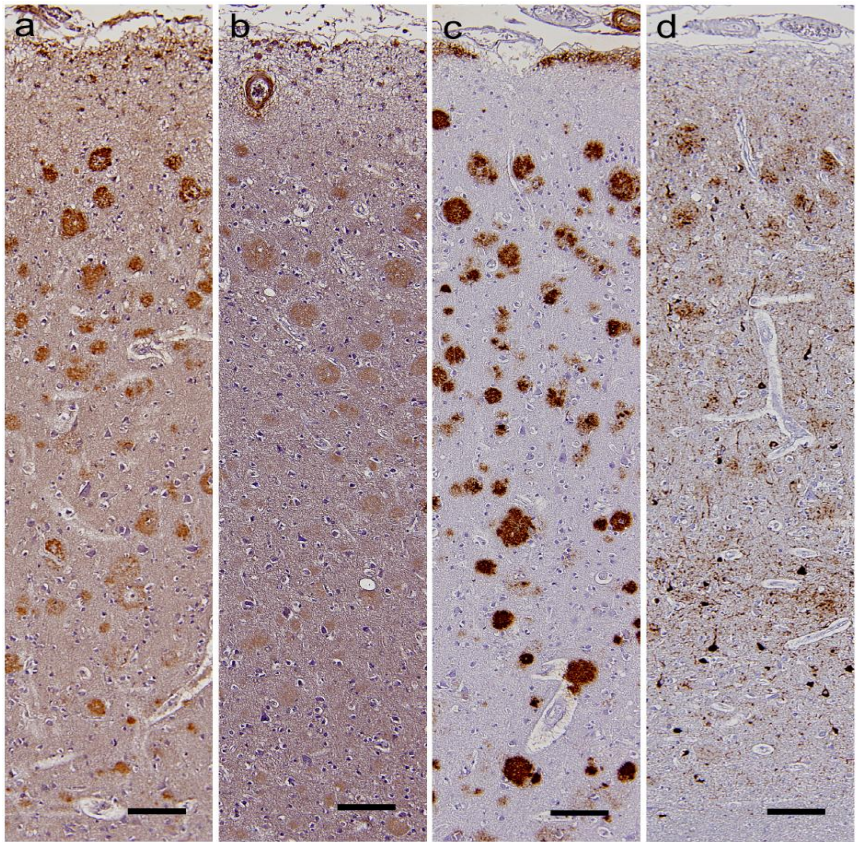

Figure 7. Aβ and tau immunohistochemistry in case 1. (a) Aβ42-positive cotton wool plaques. (b) Aβ40-positive plaques. Aβ42 rather than Aβ40 is predominantly deposited in the cerebellum. Amyloid angiopathy is also found. (c) Cotton wool plaques are intensely labeled with 4G8. (d) Some of the plaques are faintly tau positive. However, few tau-positive dystrophic neurites are noted in the cotton wool plaques. (a) FCA3542 (Aβ42) immunostain, (b) FCA3340 (Aβ40) immunostain, (c) 4G8 immunostain, (d) AT-8 immunostain. All scale bars = 200 μm.

Case 2

Clinical Course

This was a 46-year-old man at the time of death with a clinical course of 8 years. The detailed clinical course and conventional pathological findings of this case were first described by Hayashi et al. in Japanese in 1967 [17], and a clinical summary and additional immunohistochemical findings were reported recently [18]. This patient was initially aware of forgetfulness and a reduction of spontaneity at the age of 39 in 1958. The first neurological examination at a university hospital in 1960 revealed memory impairment, but no other neurological abnormalities. He had no family history of neurological disorders in the previous three generations. He was clinically diagnosed as having Alzheimer's disease. Subsequently, disinhibition, disorientation in time and place, restlessness, and indifference occurred, and he admitted to a psychiatric hospital in 1963. His restlessness, irritability, anxiety, and a tendency to exaggerate things were remarkable. Recent memory, but not remote memory, was

severely impaired. He often lost his way in the ward. Calculation ability was impaired. He obtained an IQ score of 58 on the Wechsler-Bellevue Intelligence Scale. Parkinsonism was not found. Baseline examinations of blood and urine were within normal limits. Electroencephalogram showed a basic activity of 9-11 Hz and a high voltage slow wave in the frontal and parietal regions. His appetite gradually declined, and seizures and prolonged coma developed. He died of an unknown cause in 1965. Repeated neurological examinations during the clinical course did not disclose parkinsonism, pyramidal signs, or cerebellar signs. Genetic analysis using paraffin-embedded brain sections did not reveal any mutation in exons 3 to 12 of the PS1 gene, exons 3-12 of the PS2 gene, or exons 16 and 17 of the APP gene, which included all coding regions. The Apo E genotype was ε3/ε4.

Pathological Findings

Brain weight was 1700 g. Macroscopically, neither brain atrophy nor edema was found. Arteriosclerosis was not noted in the basilar artery. The bilateral mamillary bodies were atrophic. The cerebellum and brainstem were unremarkable. Coronal sections showed only slight dilation of the third ventricle and bilateral lateral ventricles, but not cortical atrophy (Figures 8a, 8b).

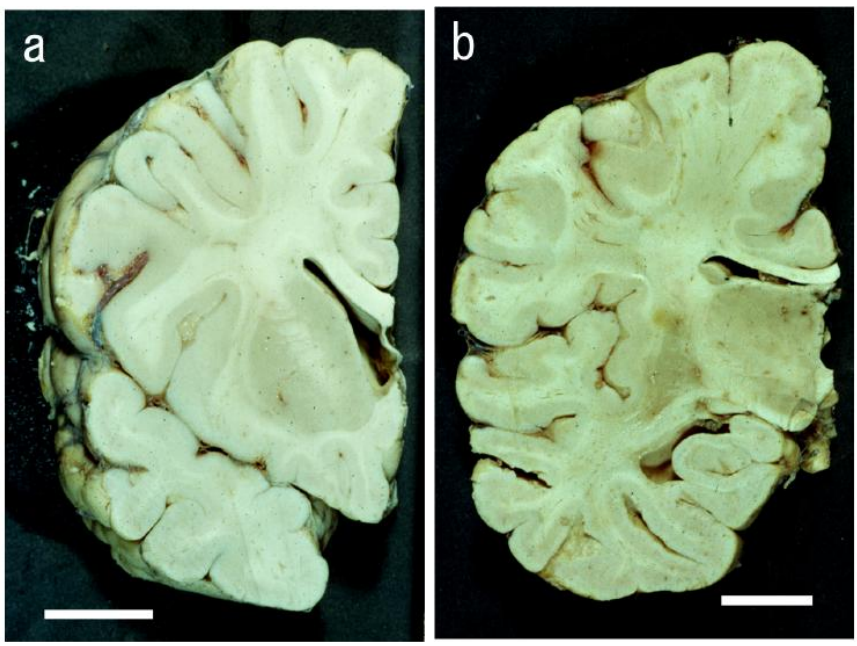

Figure 8. Coronal sections of case 2. (a) The frontal and temporal lobes and basal ganglia are unremarkable. (b) The hippocampal region is not atrophic. All scale bars = 2 cm.

Microscopically, the most remarkable finding was the occurrence of abundant atypical plaques, easily visible on HandE-stained sections (Figures 9a, 11a). The eosinophilic plaques were distributed in all cortical layers. The sizes of the plaques were variable, with diameters of 40-100 μm. These plaques often showed ill-defined boundaries. These plaques lacked cores, and no typical senile plaque with an amyloid core was found. The plaques did not appear to displace surrounding normal structures. The plaques were hardly stained with KB

stain (Figure 9b), but easily visible with Bodian (Figures 9c, 11b), Gallyas-Braak silver (Figures 10b, 11d), and PAS stains (Figure 10c). The plaques showed no argyrophilia on methenamine silver-stained sections, and slight argyrophilia on modified Bielschowsky silver-stained sections (Figures 10a, 11c). No dystrophic neurites were revealed by any silver stain (Figures 12a, 12b, 12c). In contrast to the existence of numerous plaques, neurons and laminar structure in the cerebral cortex were well spared (Figure 9b). In the cerebellum, large eosinophilic plaques associated with the blood vessels were frequently encountered (Figure 15a).

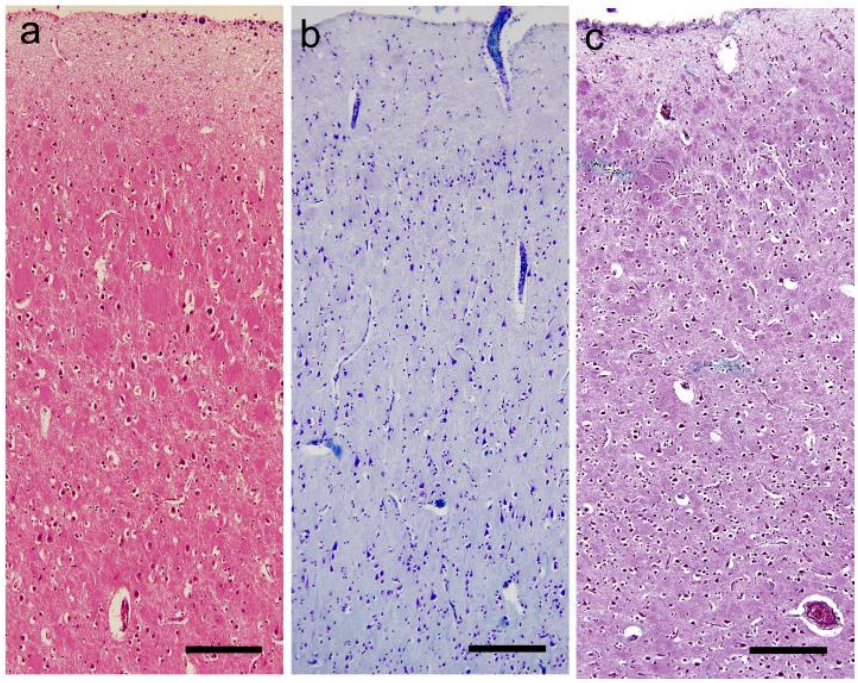

Figure 9. The temporal cortex in case 2. (a) Hematoxylin-eosin stain shows abundant irregularly shaped plaques in all cortical layers. (b) Klüver-Barrera stain. The cortical laminar structure is relatively well preserved, although neurons in the superficial layer are reduced in number. (c) Bodian stain also shows many slight argyrophilic plaques in all cortical layers. All scale bars = 200 μm.

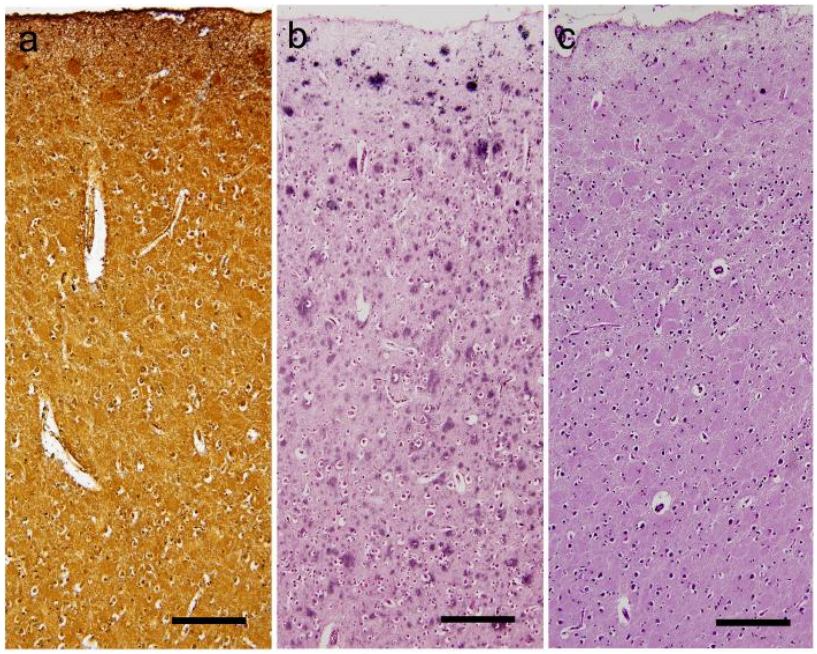

Figure 10. The temporal cortex in case 2. (a) Bielschowsky silver stain. Plaques are faintly argyrophilic. (b) Gallyas-Braak silver stain reveals many small plaques, but no neuritic changes are found. (c) PAS stain. Many plaques are found in all cortical layers. All scale bars = 200 μm.

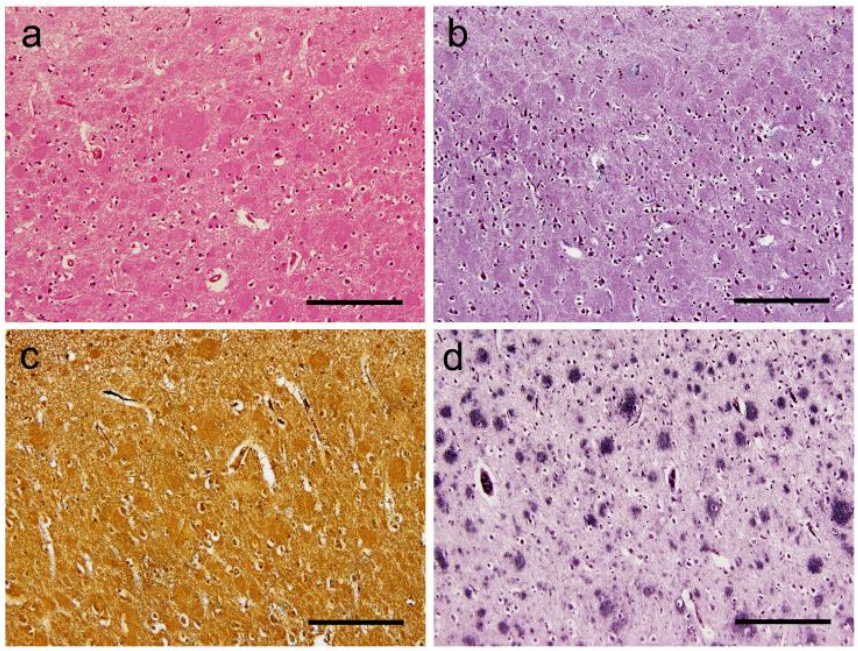

Figure 11. The temporal cortex in case 2 at higher magnification. (a) Many irregularly shaped plaques with ill-defined boundaries are found on hematoxylin-eosin stained sections. Variable sizes of plaques are also found on (b) Bodian, (c) Bielschowsky, and (d) Gallyas-Braak silver stains. All scale bars = 200 μm.

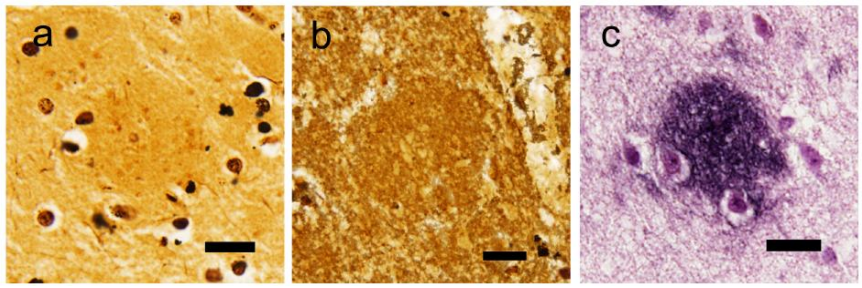

Figure 12. Plaques in the temporal cortex in case 2. Core and dystrophic neurites are not revealed with (a) methenamine, (b) Bielschowsky, or (c) Gallyas-Braak stains. All scale bars = 20 μm.

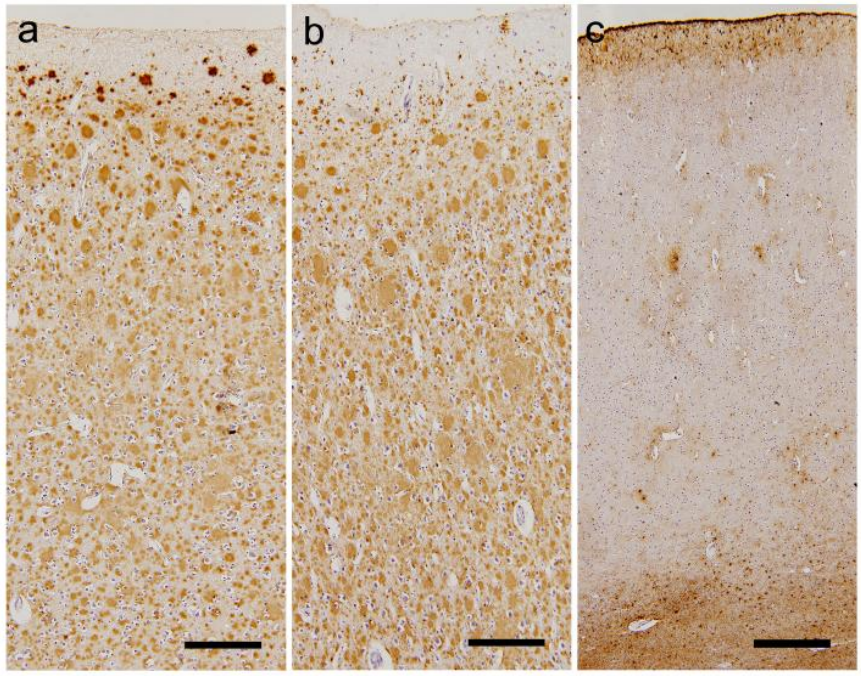

Figure 13. Aβ and GFAP immunohistochemistry in the temporal cortex in case 2. Abundant plaques with variable size are visualized with (a) 6E10 and (b) 4G8. Aβ deposits are not found in the subpial region. (c) GFAP immunohistochemistry reveals a few reactive astrocytes in the cortex, in contrast to the evident gliosis in the subpial region and subcortical white matter. Scale bars = (a, b) 200 μm, (c) 500 μm.

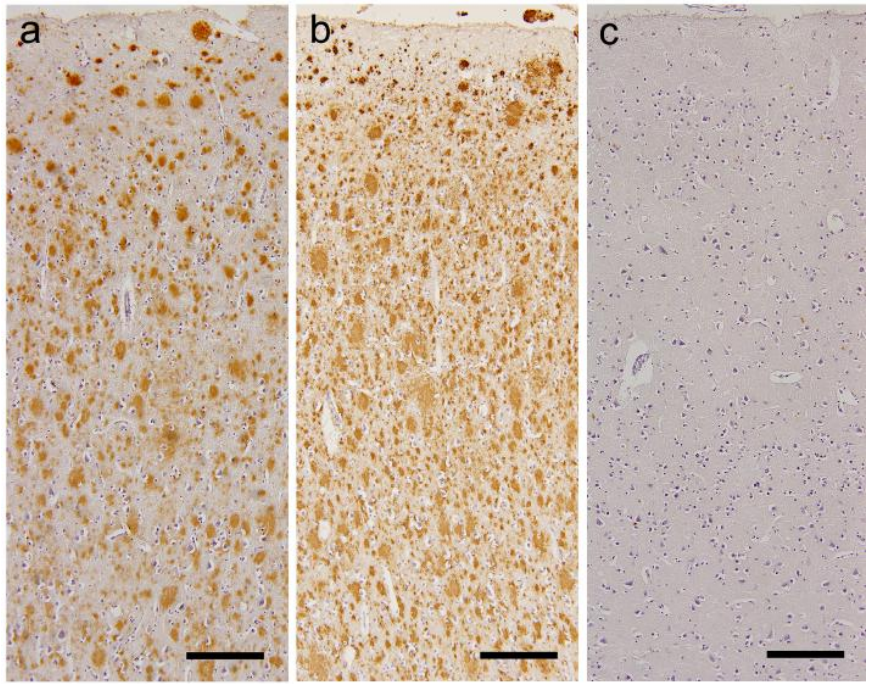

Figure 14. Aβ40 and Aβ42 immunohistochemistry in the temporal cortex in case 2. (a) Aβ42-positive plaques. (b) Aβ40-positive plaques. Aβ40 rather than Aβ42 is predominantly deposited in the cerebrum. (c) There are no tau-positive lesions. (a) FCA3542 (Aβ42) immunostain, (b) FCA3340 (Aβ40) immunostain, (c) AT-8 immunostain. All scale bars = 200 μm.

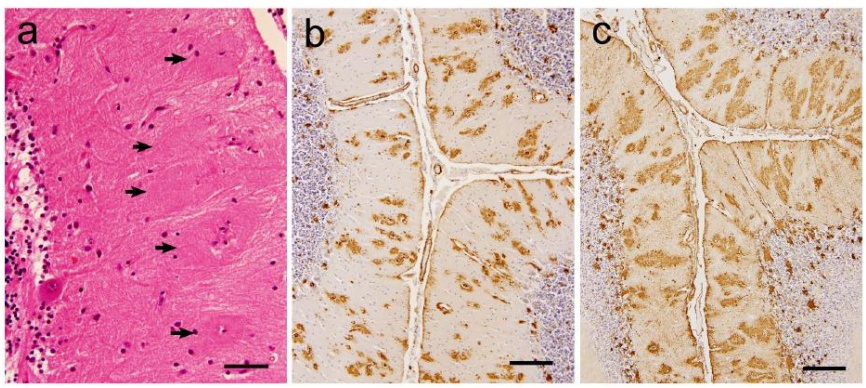

Figure 15. Plaques in the cerebellum in case 2. (a) Many homogeneous non-cored plaques visible with hematoxylin-eosin stain (arrows). (b) Aβ42-positive deposits in the cerebellum. (c) Aβ40-positive deposits in the cerebellum. Aβ40 rather than Aβ42 is predominantly deposited in the cerebellum, as well as in the cerebral cortex. (a) Hematoxylin-eosin stain, (b) FCA3542 (Aβ42) immunostain, (c) FCA3340 (Aβ40) immunostain. Scale bars = (a) 50 μm, (b, c) 200 μm.

Neurons in the cerebral cortex, basal ganglia, and brainstem nuclei were surprisingly well preserved in number despite the occurrence of abundant plaques (Table). In the cerebral cortex, slight loss of neurons in the superficial layers was noted in a part of the frontal and temporal cortices. In the basal ganglia, neurons in the nucleus basalis of Meynert were spared

in number, although glial proliferation was evident. The other sites in the basal ganglia and brainstem nuclei were not affected by neuronal loss. In the pyramidal tract, although loss of myelin was not noted, glial proliferation was evident. Lower motor neurons in the hypoglossal nuclei were not reduced in number.

Aβ immunohistochemistry demonstrated abundant Aβ deposits in the entire cerebral cortex (Figures 13a, 13b). The Aβ deposits were distributed in all cortical layers; however, they were not noted in the subpial region. Most of the plaques were irregularly round or oval, and the boundary was often ill-defined. The plaques in the cerebral cortex were not associated with blood vessels. The diameter of the plaques was 40-100 μm. Most of the plaques were smaller than those in case 1; however, they often conglomerated and formed large plaques. Unusually, Aβ40 rather than Aβ42 was predominantly deposited in these plaques (Figures 14a, 14b). In addition, no amyloid angiopathy was encountered in any region in the cerebral cortex. In the cerebellum, Aβ was severely deposited in the molecular layer, and to a lesser degree, in the Purkinje cell layer and granular cell layer. As in the cerebral cortex, Aβ40 rather than Aβ42 was predominantly accumulated in these plaques (Figures 15b, 15c). In contrast to the plaques in the cerebral cortex, Aβ deposits in the molecular layer in the cerebellum frequently surrounded the blood vessels. Neither α-synuclein-positive nor prion protein-positive lesions were noted in any regions.

Another intriguing feature was the almost complete absence of inflammatory response in the cerebral cortex. Few reactive astrocytes immunopositive for GFAP were found in the cortex in contrast to abundant Aβ deposits (Figure 13c). By contrast, astrocytic proliferation was evident only in the superficial cortical layer and subcortical white matter. Furthermore, no tau-positive lesion was encountered in any region (Figure 14c).

Genetic analysis using paraffin-embedded brain sections revealed no mutation in exons 3 to 12 of the PS1 gene, exons 3-12 of PS2 gene, or exons 16 and 17 of the APP gene, which included all coding regions. Apo E genotype was ε3/ε4.

DISCUSSION

Since a report of CWP-AD due to the deletion of exon 9 of the PS1 gene in 1998 [4], about 20 novel mutations of the PS1 gene associated with CWP-AD have been reported [19-32]. In addition, several cases of CWP-AD lacking an obvious family history have been reported. Cases of CWP-AD with PS1 mutation usually present with initial symptoms in their thirties to fifties. Further, although rare, there are early-onset cases that develop their first symptoms at under 30 years of age [20, 27, 33]. On the other hand, some of the sporadic CWP-AD cases are late onset [18, 34]. Dementia and spastic paraparesis are the most representative symptoms of CWP-AD [4, 21], but the presentation of clinical phenotype of CWP-AD is not uniform. Indeed, the order of the development of dementia and spastic paraparesis is not necessarily consistent between cases with a same PS1 mutation in the same pedigree [4]. Further, there are cases that exhibit only dementia or only spastic paraparesis in one pedigree [23, 31]. Sporadic CWP-AD cases often showed dementia but not spastic paraparesis [18, 34]. In addition, several uncommon symptoms in CWP-AD have been described: morbid jealousy [31], severely stooped posture and kyphoscoliosis [28], low back pain (especially as an onset symptom) [35], hyperorality [28], diplopia and ocular movement

disturbance [24], facial palsy [35], and ataxia [14, 24, 33, 35]. Likewise, histopathological phenotypes showed some variation between cases even when they were from the same pedigree. For example, some cases carrying a mutation associated with CWPs had cored plaques but not CWPs [23]. The quantity of CWPs often differs between cases with the same mutation [23]. Also in the pedigree of case 1 presented in this paper, a demented relative with the PS1 gene mutation (case IV.1, see Figure 1) had many neuritic plaques in addition to a few CWPs that were found only in the hippocampus and insular cortex (personal communication from Dr. Kawakatsu, Department of Neuropsychiatry, Yamagata University). Coexistence of Lewy bodies with CWPs was described in at least two previous cases [18, 32]. One of the cases reported by our group is a woman who was 45 years old at the time of death. This case initially developed personality change with disinhibition at age 34. Dementia and L-dopa-responsive parkinsonism were observed 5 years after the onset [18]. This case was clinically diagnosed as having Parkinson's disease with dementia. Postmortem examination disclosed numerous CWPs widespread in the cerebral cortex and Lewy bodies in the neocortex and substantia nigra. Another case was a 52-year-old man with a deletion in exon 12 of the PS1 gene [32]. The onset symptom was L-dopa-responsive parkinsonism, and dementia occurred subsequently. This case had CWPs, corticospinal tract involvement, and Lewy body pathology widespread in the cerebral cortex. The occurrence of NFTs was not severe, corresponding to Braak stage IV. At present, the frequency and significance of coexistence of Lewy bodies with CWPs are unclear.

To the best of our knowledge, the first description of CWPs is the 'plaque-like bodies' reported by Matsuoka et al. in Japanese in 1967 [35]. Recently, this case was reexamined with conventional histopathological and immunohistochemical methods by several researchers including our group, and the features of the plaques were verified to be consistent with those of what are now called CWPs [18, 37]. In summary, this was a 36-year-old woman at the time of death with a clinical course of 8 years. Her initial symptom was dysarthria, hypersalivation, and pain in the left leg at age 28. Pain and motor disturbance occurred in the right leg also, and she could not walk at age 31. Thereafter, irritability, impulsivity, double vision, impairment of ocular movement, left facial palsy, muscle atrophy in the tongue, dysarthria, hearing loss on the right side, pyramidal signs, and cerebellar signs also occurred. Fasciculation, aphasia, apraxia, and agnosia were not noted. She showed dementia, and her IQ was 30 on the Wechsler-Bellevue Intelligence Scale. Brain weight was 1185g. Macroscopically, the bilateral frontal lobes, base of the pons, corpus callosum, and cerebral peduncle were atrophic. Microscopically, numerous CWPs were widespread in the cerebral cortex, basal ganglia, brainstem, cerebellum, and spinal cord. Many NFTs, corticospinal tract degeneration, and amyloid angiopathy were also encountered. We did not find any mutations of the PS1, presenilin-2, and amyloid precursor protein genes using paraffin-embedded brain sections [18, 37], although the possibility that the present cases had a deletion or other mutation could not be excluded because neither frozen tissue nor lymphoblast lines was available. The apo E genotype was ε3/ε4. Interestingly, a concordant monozygotic sibling of this case also showed nystagmus, dysarthria, gait disturbance, increased deep tendon reflexes, and cerebellar ataxia at age 32. Pathological examination was not done. Her brother had motor disturbance in the bilateral lower extremities, but the details are not clear. Other early cases we know include a case reported by Mizushima et al. in 1974 (unfortunately, only a Japanese abstract was published in Shineki Kenkyu no Shinpo, volume 18, pages 206-7) and a case reported by Fukatsu et al. in 1980 [36]. The case reported by Mizushima et al. was a

37-year-old man at the time of death, with a clinical course of 11 years. The onset symptom was bradykinesia. Thereafter, memory impairment, masked face, disorientation, rigidity, and gait disturbance were observed. The family history was not described. CWPs, amyloid angiopathy, and pyramidal tract degeneration were revealed by recent postmortem reexamination [38]. Another case reported by Fukatsu et al. [36] was a 42-year-old man at the time of death, with a disease duration of 2.5 years. The initial symptom was low back pain and pain in the left leg. Reduced spontaneity, irritability, bradykinesia, memory impairment, and gait disturbance also developed during the course. Pathologically, numerous lesions, the now-called CWPs, widespread amyloid angiopathy, and NFT formation were disclosed.

Although many conventional histopathological and immunohistochemical characteristics of CWPs have been accumulated [22, 39, 40], we consider that the trend that cortical neurons and laminar structure are relatively better preserved in CWP-AD than in common AD may be a significant characteristic to understand the pathomechanism leading to CWP formation and cognitive impairment. Cortical neurons in CWP-AD are often considerably well preserved, being disproportional to the occurrence of abundant CWPs. The preservation of the cortical laminar structure may be associated with the minimal glial response in CWP-AD [4, 34, 40, 41]. These features had been already noticed in 1967 by Matsuoka et al. [35] and Hayashi et al. [17], and Fujisawa also emphasized them in 1980 in association with a paper by Fukatsu et al. [36]. Although CWPs tend to displace surrounding normal structures, which was often noted in recent papers [34, 40], the tendency may be associated with a well preserved laminar structure in the cerebral cortex. Only limited data concerning neuronal loss in CWP-AD are available. Ishikawa et al. [32] systematically examined neuronal loss in a case of CWP-AD associated with a PS1 mutation. This case had mild to moderate neuronal loss and severe amyloid angiopathy in the neocortex. Tau pathology was not severe, corresponding to Braak stage IV. On the other hand, severe neuronal loss has also been described in several previous cases of CWP-AD. Takao et al. [28] reported two CWP-AD cases with a G217D PS1 mutation that had severe neuronal loss in extensive regions, including the frontal, temporal, and cingulate cortices, amygdala, hippocampus, subiculum, entorhinal cortex, caudate nucleus, substantia nigra, and locus coeruleus. This case had numerous CWPs in the cerebral cortex and basal ganglia, as well as severe NFT formation, severe amyloid angiopathy, and a relatively small number of neuritic plaques throughout the cerebral cortex. Likewise, among six cases of CWP-AD described by Brooks et al. [31], one had severe neuronal loss in the cerebral cortex and hippocampus.

Although the factors determining the loss of neurons in the cerebral cortex in CWP-AD remain unclear, it is possible that the quantity of neuritic plaques and/or NFTs is associated with its severity. For example, Smith et al. [23] reported a PS1-linked pedigree that included cases having variable degrees of neuronal loss and CWPs. Among three cases for which detailed microscopic findings were described, widespread and marked neuronal loss was observed only in one case with severe NFT formation and frequent cored plaques. This case lacked CWP formation. Another case bearing CWPs, cored plaques (fewer than CWPs), and sparse NFTs showed less marked neuronal loss compared with the prior case. Interestingly, the last case with many CWPs but lacking cored plaques and significant NFTs showed no significant neuronal loss. This case presented with spastic paraparesis but not dementia. These findings are consistent with those in our cases presented in this paper. Namely, our case 1 had more neuritic changes including NFTs compared with case 2, and the neuronal loss in case 1 was more severe than that in case 2 (Table). It should be also emphasized that both our cases

presented with severe dementia despite only slight neuronal loss in the cerebrum. Furthermore, it is noteworthy that one of our cases lacked tau pathology. These findings suggest the possibility that neuritic plaques, NFTs, and severe neuronal loss are not always necessary for the occurrence of cognitive decline in AD patients. Some researchers have demonstrated that intracellular Aβ accumulation is associated with the pathogenesis of AD including apoptosis [42-45]. Further accumulation of findings regarding other factors is awaited to explore potential therapeutic targets in the pathogenesis of AD.

ACKNOWLEDGMENT

We would like to thank Mr. Y. Shoda and Ms. K. Suzuki (Tokyo Institute of Psychiatry) and Ms. M. Onbe (Department of Neuropsychiatry, Okayama University Graduate School of Medicine, Dentistry and Pharmaceutical Sciences) for their excellent technical assistance and Mr. A. Sasaki for help with the production of the manuscript. This work was supported by a grant-in-aid for scientific research from the Ministry of Education, Culture, Sports, Science and Technology (14570957) and a research grant from the Zikei Institute of Psychiatry.

This work was supported by a grant-in-aid for scientific research from the Ministry of Education, Culture, Sports, Science and Technology (14570957) and a research grant from the Zikei Institute of Psychiatry.

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In: Alzheimer's Disease Diagnosis and Treatments ISBN: 978-1-61122-064-3
Editor: Marisa R. Boyd

Chapter 5

HOW AND WHERE DOES Aβ EXERT ITS TOXIC EFFECTS IN ALZHEIMER'S DISEASE?

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INTRODUCTION

Protein aggregation is the basis for many of the common human neurodegenerative diseases such as Alzheimer's disease (AD), Parkinson's disease and a family of disorders that includes Huntington's disease. In AD the aggregatory species is termed amyloid β (Aβ), a peptide derived from the proteolytic cleavage of amyloid precursor protein (APP), a ubiquitous transmembrane protein. The aggregatory properties of Aβ are determined by variations in the position of the proteolytic cleavage that generates the C-terminus. In healthy elderly individuals the ratio of the 40 amino acid peptide ($A\beta_{1\text{-}40}$) to the 42 amino acid species ($A\beta_{1\text{-}42}$) favours the less aggregatory $A\beta_{1\text{-}40}$ resulting in effective clearance of the peptide from the brain. In contrast, individuals who go on to develop the common sporadic form of AD have elevated $A\beta_{1\text{-}42}$ concentrations, or have a molar ratio of $A\beta_{1\text{-}40}$ to $A\beta_{1\text{-}42}$ that favours aggregation. In the five percent of AD cases that are inherited as an autosomal dominant trait all the causal mutations have been shown to favour Aβ aggregation, mostly by altering APP processing, either increasing $A\beta_{1\text{-}42}$ in absolute terms or in comparison to $A\beta_{1\text{-}40}$. In rare examples, where $A\beta_{1\text{-}42}$ levels are not elevated, mutations are found within the Aβ sequence

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that accelerate the intrinsic rate of peptide aggregation and stabilise particularly toxic subpopulations of aggregates, a clear example of this is the Arctic APP mutation [1, 2].

In the context of cognitive decline, the demonstration of Aβ deposition in the brain in combination with intraneuronal aggregates of a microtubule-associated protein, tau, comprise the diagnostic criteria for AD. Mature deposits of Aβ are composed of ordered amyloid fibrils and it is their distinctive microscopic appearance and their affinity for dyes such as Congo red that favoured their early characterisation. However there is a poor correlation between the burden of amyloid plaques and the degree of cognitive impairment, indeed elderly individuals may have many plaques without showing signs of cognitive impairment [3-5]. In contrast, it is the intracellular tau pathology that has been shown to correlate more closely with clinical deficits. The location and progression of the tau lesions correlates well with the brain areas, such as the hippocampus, that are particularly impaired in AD [6].

The poor correlation between extracellular amyloid plaques and dementia has been used to detract from the significance of Aβ in the pathogenesis of AD. However recent evidence has clarified the situation, emphasising the toxic role of small Aβ aggregates rather than the amyloid fibrils. The finding that soluble Aβ correlates better with synaptic changes and cognitive deficits than plaque count [7-9] has prompted the investigation of soluble aggregates of Aβ. These small aggregates can be purified by column chromatography and are composed of as few as 4 [10] or as many as 180 [2] Aβ molecules. When applied to cell cultures the oligomers are toxic whereas in most cases amyloid fibrils and Aβ monomers are not [11, 12]. When oligomers are visualised under electron or atomic force microscopes they are heterogeneous, including spheres, beads-on-a-string and doughnuts [2], but it seems that the spherical species are most toxic [13]. Toxic oligomers may also be specifically detected, *in vitro* and *in vivo*, using rabbit antisera raised against Aβ immobilised on gold beads. The antiserum, described by Kayed and colleagues [14], binds specifically to small toxic aggregates of Aβ and neutralises their toxicity, in contrast the serum fails to detect monomeric or fibrillar forms of Aβ. Subsequent work has shown that the antiserum recognises an epitope on Aβ oligomers that is common to the oligomeric aggregates of a range of pathological proteins. The interesting corollary of this observation is that a common structural motif predicts a common mechanism of toxicity. This prediction is supported by work by Bucciantini *et al*. showing that oligomeric aggregates of a non disease related protein can elicit toxicity similar to that of Aβ oligomers in cell culture [15]. Further work done in cell culture by Demuro and colleagues [16] has shown that a shared ability to disturb membrane conductivity may underlie at least part of the toxicity of soluble protein aggregates.

However the hypothesis that soluble aggregates of Aβ represent a stable neurotoxic species has had to be reconsidered in the light of recent work showing that it is the ongoing *process* of aggregation that is toxic. It seems now that the soluble aggregates may simply be an efficient seed that can promote further addition of Aβ monomers. In their recent study, Wogulis and colleagues showed that, as expected, neither monomeric nor fibrillar Aβ were toxic to human or rat neuronal cell cultures. Their novel observation was that pre-treatment of cells with fibrillar Aβ, followed by a wash to remove unbound fibrils, primed the cells to die when they were subsequently treated with monomeric Aβ. The stability of the interaction of the fibrils with the cells was a surprise; following exposure to fibrils for only one hour the cells were still sensitized to the toxic effects of monomeric Aβ one week later [17].

With emphasis being placed on the oligomeric aggregates and the initial stages of the aggregation process, the mature plaques and tangles are increasingly being viewed as tombstones of pathological protein aggregation. Indeed there is evidence from cell-based models of Parkinson's disease that inclusions may be protective, reducing the rate of apoptosis [18] possibly by providing a sink for the disposal of toxic oligomers.

INTRANEURONAL $A\beta_{1-42}$ ACCUMULATION AND AGGREGATION

The classical view of APP processing is that Aβ is generated and released at the cell surface, resulting in extracellular amyloid plaque deposition and neurotoxicity. However it well documented that the machinery for generating Aβ exists intracellularly [19-22]. Some investigators have emphasised the importance of the secretory pathway in generating Aβ by showing that the treatment of cells with inhibitors of vesicular transport that effectively block APP export from the endoplasmic reticulum [20] or trans-Golgi network [21] do not abolish Aβ generation. Moreover APP processing in the endoplasmic reticulum preferentially yields $A\beta_{1-42}$ [22-24] that remains intracellular, whereas $A\beta_{1-40}$ is preferentially generated in the trans-Golgi network and packaged into secretory vesicles. There is also evidence that APP is processed after it has reached the plasma membrane and that endocytosis is important for the generation of Aβ [25]. This is of particular note because the low pH of the endosomes/lysosomes compartment will predictably favour oligomer formation [26, 27]. However there is the possibility that intracellular Aβ exerts at least part of its toxicity, not from the aqueous environment of vesicle lumen, but from within the membrane itself. There is evidence that Aβ peptides are sequestered in membranes predominantly as dimers [28] and some workers have proposed that specific intramembraneous protein-protein interactions may mediate some of the toxic effects of Aβ [29].

Aβ oligomerisation has been shown to start intracellularly in cell culture [30] and oligomers are present in the brains of patients with AD [10]. Clinical specimens have also shown that Aβ is intracellular during the early stages of AD but becomes predominantly extracellular as the patient develops advanced disease [31]. It may be that intracellular Aβ disappears with time because heavily-burdened neurones die, releasing their aggregates; indeed studies looking at the distribution and morphology of amyloid plaques suggest that each amyloid plaque is the result of a single neuronal lysis event [32].

The history of AD research has shown that good animal models have helped enormously to accelerate our understanding. The most recent animal models of Alzheimer's disease are providing strong support for the role of intracellular Aβ in generating the earliest symptoms of Alzheimer's disease [33, 34]. Triple transgenic mice that express disease-causing mutants of human APP, presenilin-1 and tau demonstrate clearly that intraneuronal accumulation of Aβ is sufficient to cause the earliest cognitive deficits [35]. At an age of four months the mice exhibit impaired long-term memory retention at a stage when plaques, tau pathology and neuronal death are entirely absent but intracellular Aβ is present. The presence of intracellular accumulation of Aβ may also explain why these triple transgenic mice are the first to show convincing neuronal loss as well as dysfunction [34]. This work in mouse models is supported by recent *Drosophila* models of AD that demonstrate non-amyloid intracellular Aβ aggregates are sufficient to cause locomotor deficits before extracellular Aβ deposits or cell

death are seen [36]. Treating model organisms with anti-aggregatory compounds [36] or antibodies to Aβ [35] can ameliorate or even reverse the neuronal dysfunction that results from intraneuronal Aβ.

WHAT IS THE ROLE OF EXTRACELLULAR Aβ?

Extracellular Aβ has a wide range of effects that can be divided into two main categories. Firstly, Aβ has sequence-specific interactions with other proteins; notable is the binding of Aβ to receptors that are normally involved in the clearance of the peptide from the brain. Aβ also interacts specifically with receptors involved in neurotransmission and may cause some of the early, potentially reversible, symptoms of AD. Secondly, Aβ has biophysical effects on the electrical properties of membranes and also promotes oxidative stress, both of which contribute to the neuronal death seen in established cases of AD.

THE INTERACTION OF EXTRACELLULAR Aβ WITH THE CLEARANCE PATHWAYS

The concentration of Aβ in the brain depends both on the rate of production of the peptide and on the efficiency of the clearance mechanisms. Although the bulk of human genetic evidence points to the primary importance of Aβ synthesis in causing familial disease (presenilin 1 and 2 and APP mutations), there is some evidence that polymorphisms in genes related to clearance, such as the degrading enzyme neprilysin [37, 38], may influence an individual's risk of AD. It is thought that plaques may be cleared by the phagocytic activity of microglia and it is known that Aβ binds specifically to the plasma membranes of both microglia and neurones. On microglia a receptor complex has been identified that mediates the binding to Aβ fibrils [39]. Components of this receptor complex includes the B-class scavenger receptor CD36, the integrin-associated protein/CD47, and the alpha(6)beta(1)-integrin. It has also been reported that the receptor for advanced glycosylation end-products (RAGE) and FPRL1 (formyl peptide receptorlike 1) are able to bind both the monomeric and fibrillar forms of Aβ [40]. Microglia are found around neuritic plaques in the brains of patients with AD and the binding of Aβ to the receptors may stimulate an inflammatory response and mediate peptide clearance. Although these receptors may have a purely beneficial role in delivering peptide to the endosomes for degradation, however in the light of the discussion above, the internalisation of Aβ may in fact result in enhanced aggregation and toxicity.

Aβ AND LONG TERM POTENTIATION

LTP is a phenomenon whereby stimulus-dependent enhancement of synaptic efficacy may encode memories. The role of LTP in the memory deficits of AD has been studied in transgenic mice that express the AD-causing Swedish mutant of human APP. Experiments in brain slices showed loss of LTP in the absence of neuronal death; moreover the loss of LTP

correlated with deteriorating performance in behavioural tests of learning [41]. Similar loss of LTP was seen in rat brain slices treated with soluble Aβ aggregates of laboratory-synthesised peptides [42]. Walsh and colleagues have gone on to show that the oligomeric Aβ, secreted from CHO cells expressing a disease-causing APP mutant, can interfere with LTP in intact rat hippocampus. Intracerebroventricular injection of the conditioned medium containing Aβ oligomers was shown to completely abolish LTP, an effect that was not seen when control conditioned medium was injected [43]. Fractionation of Aβ species in the conditioned media into monomers and monomer-free oligomers demonstrated that it was the oligomers that were responsible for the learning deficits in rats following intracerebroventricular injection [44]. The straightforward explanation for the effects on LTP are that extracellular application of Aβ results in an effect mediated at the plasma membrane. However the demonstration of Aβ aggregates in endosomes implies that an intracellular mechanism remains a possibility [43].

Aβ AND NICOTINIC NEUROTRANSMISSION

The disruption of cholinergic neurotransmission occurs early in AD and the elevation of synaptic acetylcholine (ACh) concentrations remains the main therapeutic approach in the clinic. Wang and colleagues have shown that Aβ binds with high affinity to alpha7n Ach receptors [45] resulting in the inhibition of receptor-dependent calcium signalling and acetylcholine release, two processes critically involved in neurotransmission and synaptic plasticity. The binding interaction between exogenous Aβ and the alpha 7 receptor may well facilitate the internalization and intracellular accumulation of Aβ in Alzheimer's disease brains. Indeed intracellular accumulation of Aβ in neurons has been shown in a cell culture model to correlate with the level of this receptor [46] and Aβ internalization can be blocked by alpha-bungarotoxin, an alpha 7 receptor antagonist. Moreover the high levels of the alpha 7 receptor found in the hippocampus and cortex [47] may account for the early involvement of these brain regions in AD. Although nicotinic stimulation has traditionally been seen as beneficial in AD (reviewed by Buccafusco *et al*. [48]) because of improved LTP and reduced amyloid deposition [49, 50] there is concern that up regulation of ACh receptors in smokers [51] may increase the proportion of the total Aβ that is soluble [50] and available for internalisation. This may account for the exacerbation of alpha 7 receptor-mediated tau phosphorylation [52] in transgenic mice treated with nicotine [53].

Aβ AND ELECTRICAL PROPERTIES OF MEMBRANES

The electrical integrity of biological membranes is particularly important for the correct functioning and survival of neurones. Disruption of the resting potential across the plasma membrane may contribute to the toxic effects of Aβ as described in the preceding sections, namely LTP deficits and aberrant neurotransmission. In a similar way, disruption of the mitochondrial membrane may result in the oxidative stress [54] component of AD pathogenesis. There is however conflicting data about how oligomers cause membrane disruption; some groups have shown pore-like Aβ aggregates that insert into membranes;

others have evidence that membrane conductivity is increased but in the absence of discrete ion channel activity [55].

Pore-like aggregates of Aβ have a doughnut-shaped appearance, [56] being composed of 30-60 peptides monomers [2, 57] and resembling pore-forming bacterial toxins. These pore-like aggregates are proposed to insert into membranes forming a pathological ion channel, causing depolarisation of membranes and possibly calcium influx into the cell. In support of this hypothesis Lin and colleagues can demonstrate that pore-like aggregates have discrete ion channel activity that can be inhibited by zinc ions [56].

However, Aβ may share a channel-independent mechanism of membrane disruption with other aggregation-prone proteins. A recent study using fluorescently-loaded SHSY5Y cells showed that application of $A\beta_{1-42}$ and other oligomeric aggregates elevated intracellular Ca^{2+}, an effect that persisted even after depletion of intracellular Ca^{2+} stores [16]. The fact that the potent Ca^{2+} channel blocker cobalt failed to affect this response combined with the rapid leakage of anionic fluorescent dyes, point to a generalized increase in membrane permeability. This study provided evidence that the unregulated flux of ions across "leaky" membranes may provide a common mechanism for oligomer-mediated toxicity in many amyloid diseases. The dysregulation of calcium metabolism is likely to play an important role [58] because of the strong transmembrane concentration gradient and the involvement of calcium in intracellular signalling.

THE ROLE OF OXIDATIVE STRESS

It is well established that oxidative stress, as measured by oxidation products of proteins [59-61], lipids [62-64] and nucleic acids [65-68], has an important role in the pathogenesis of AD. Many of these studies have been performed in clinical specimens from late disease, however recent work has shown that isoprostane levels, a biochemical marker of lipid oxidation, are elevated even at the earliest stages of clinical AD [69].

How the reactive oxygen species are generated is hotly debated, however it is known that Aβ peptides are able to bind copper ions (Cu(II)) and reduce them (to Cu(I)), releasing hydrogen peroxide. Others metals may also be involved in similar reactions with aluminium and iron being possible candidates. Other workers have suggested that mitochondrial membrane disruption may release oxidative species. Interestingly, in concordance with the idea that it is the process of Aβ aggregation that is toxic, Tabner and colleagues have shown that, in the absence of redox-active metal ions, the earliest stages of Aβ aggregation can generate a brief burst of hydrogen peroxide [70].

CONCLUSION

There is an ongoing debate as to how Aβ causes neuronal dysfunction and death. The focus has recently been on small, soluble aggregates of Aβ, as the pathogenic agent however recent work suggest that the process of aggregation may be toxic, perhaps by generating oxidative species. A better understanding of the significance of intracellular Aβ may provide us with new therapeutic strategies for Alzheimer's disease.

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In: Alzheimer's Disease Diagnosis and Treatments
Editor: Marisa R. Boyd
ISBN: 978-1-61122-064-3

Chapter 6

THE SPATIAL PATTERNS OF β-AMYLOID (Aβ) DEPOSITS AND NEUROFIBRILLARY TANGLES (NFT) IN LATE-ONSET SPORADIC ALZHEIMER'S DISEASE

Richard A. Armstrong*

ABSTRACT

The spatial patterns of β-amyloid (Aβ) deposits and neurofibrillary tangles (NFT) were studied in areas of the cerebral cortex in 16 patients with the late-onset, sporadic form of Alzheimer's disease (AD). Diffuse, primitive, and classic Aβ deposits and NFT were aggregated into clusters; the clusters being regularly distributed parallel to the pia mater in many areas. In a significant proportion of regions, the sizes of the regularly distributed clusters approximated to those of the cells of origin of the cortico-cortical projections. The diffuse and primitive Aβ deposits exhibited a similar range of spatial patterns but the classic Aβ deposits occurred less frequently in large clusters >6400μm. In addition, the NFT often occurred in larger regularly distributed clusters than the Aβ deposits. The location, size, and distribution of the clusters of Aβ deposits and NFT supports the hypothesis that AD is a 'disconnection syndrome' in which degeneration of specific cortico-cortical and cortico-hippocampal pathways results in synaptic disconnection and the formation of clusters of NFT and Aβ deposits.

Keywords: Alzheimer's disease, β-amyloid (Aβ) deposits, neurofibrillary tangles (NFT), spatial patterns, clustering, cortico-cortical projections.

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INTRODUCTION

Discrete lesions in the form of β-amyloid (Aβ) deposits and neurofibrillary tangles (NFT) are the hallmark pathological features of Alzheimer's disease (AD). Several types of Aβ deposit have been described in the brains of patients with AD, but the majority can be classified into three morphological subtypes (Deleare et al., 1991, Armstrong, 1998): 1) diffuse deposits, in which most of the Aβ peptide is not aggregated into fibrils and dystrophic neurites and paired helical filaments (PHF) are infrequent or absent; 2) primitive deposits, in which the Aβ is aggregated into amyloid and dystrophic neurites and PHF are present, and 3) classic deposits, in which Aβ is highly aggregated to form a central amyloid core surrounded by a 'ring' of dystrophic neurites.

Aβ is a 4-kDa peptide arising as a result of cleavage of a larger trans-membrane amyloid precursor protein (APP) found in most brain cells. A variety of Aβ peptides are present within Aβ deposits in AD (Delaere et al., 1991; Greenberg, 1995; Armstrong, 1998). The most common of these peptides is $A\beta_{42/43}$ found largely in Aβ deposits, whereas the more soluble $A\beta_{40}$ is also found in association with blood vessels (Miller et al., 1993; Roher et al., 1993) and may develop later in the disease (Delacourte et al., 2002). In addition, Aβ deposits have a variety of 'secondary' constituents including acute-phase proteins such as α-antichymotrypsin and α_2-macroglobulin (Eikelenboom et al., 1994), intercellular adhesion molecules such as cell adhesion molecule 1 (CAM1) (Eikelenboom et al., 1994), apolipoprotein E (Apo E) (Yamaguchi et al., 1994) and D (Apo D) (Desai et al., 2005), the heterodimeric glycoprotein clusterin, vibronectin, the complement proteins C1q, C4 and C3 (Verga et al., 1989), blood proteins such as amyloid P, and the sulphated glycosaminoglycans such as heparin sulphate proteoglycan (HSPG).

The most important molecular constituent of the NFT is the microtubule associated protein (MAP) tau which is involved in the assembly and stabilization of the microtubules and therefore, establishes and maintains neuronal morphology (Lee et al., 1988; Roder et al., 1993). In normal neurons, tau is soluble and binds reversibly to microtubules with a rapid turnover (Caputo et al., 1992). In disorders such as AD, tau does not bind to the microtubules but collects as insoluble aggregates to form PHF which resist proteolysis and ultimately accumulate in the cell body as NFT. There is a single gene for tau and the different isoforms, i.e., tau 55, 64 and 69 result from alternative splicing and post-transcriptional changes (Dustin et al., 1992). Tau extracted from AD brains consists of both soluble and insoluble forms with, in the latter, the tau present in an abnormally phosphorylated isoform (Hanger et al., 1991).

In patients with AD, Aβ deposits and NFT are not randomly distributed in a brain region but exhibit a spatial pattern, i.e., a departure from randomness towards clustering and regularity. Analysis of these spatial patterns may contribute to an understanding of the pathogenesis of Aβ deposits and NFT and therefore, of AD itself. Hence, this article reports a study of the spatial patterns of Aβ deposits and NFT in 16 cases of sporadic AD and examines: 1) the relationships between the spatial patterns of the three subtypes of Aβ deposit, 2) the relationship between the Aβ deposits and NFT, and 3) the factors that may determine the spatial pattern of these lesions.

MATERIALS AND METHODS

Cases

Sixteen cases of late-onset, sporadic AD (details in table 1) were obtained from the Brain Bank, Department of Neuropathology, Institute of Psychiatry. Informed consent was given for the removal of all tissue and followed the principles embodied in the 1964 Helsinki declaration (as modified Edinburgh, 2000). Post-mortem (PM) delay was less than 20 hours in each case. The AD cases were clinically assessed and all fulfilled the 'National Institute of Neurological and Communicative Disorders and Stroke and Alzheimer's Disease and Related Disorders Association' (NINCDS/ADRDA) criteria for probable AD (Tierney et al., 1988). The histological diagnosis of AD was established by the presence of widespread neocortical senile plaques (SP) consistent with the 'Consortium to Establish a Registry of Alzheimer's Disease' (CERAD) criteria (Mirra et al., 1991) In addition, NFT were abundant in the cerebral cortex and hippocampus of each case.

Table 1. Demographic data and cause of death of the cases studied

Patient	Gender	Age	Onset	Cause of death
A	M	82	78	Bronchopneumonia
B	M	73	66	Bronchopneumonia
C	F	87	82	Myocardial infarction
D	F	82	75	Bronchopneumonia
E	F	66	59	NA
F	F	70	64	Bronchopneumonia
G	F	85	80	Bronchopneumonia
H	F	70	NA	Ischaemic heart disease
I	M	80	77	Bronchopneumonia
J	M	88	72	Bronchopneumonia
K	F	93	91	NA
L	F	91	85	Bronchopneumonia
M	F	86	83	Bronchopneumonia
N	F	88	72	Bronchopneumonia
0	F	81	77	Bronchopneumonia
P	F	70	64	Bronchopneumona

M = Male, F = Female, NA = data not available

Tissue Preparation

Blocks of the superior frontal lobe (SFL) and medial temporal lobe (MTL) were taken at the level of the genu of the corpus callosum and lateral geniculate body respectively. The MTL block included the lateral occipitotemporal gyrus (LOT), the parahippocampal gyrus (PHG), and hippocampus. Tissue was fixed in 10% phosphate buffered formal-saline and

embedded in paraffin wax and adjacent 7µm coronal sections cut from each block. One section was immunostained with a rabbit polyclonal antibody (Gift of Prof. B.H. Anderton) raised to the 12-28 amino acid sequence of the Aβ protein (Spargo et al., 1990) which clearly distinguishes the major types of Aβ deposit. Aβ deposit subtypes were identified using morphological criteria as follows (Armstrong,1998): 1) diffuse deposits were irregularly shaped, weakly stained, and poorly demarcated; 2) primitive deposits were more symmetrical in shape, strongly stained, and well demarcated, and 3) classic deposits had a distinct ring and central core. The adjacent sections from each block were stained with the Gallyas silver impregnation method (Gallyas, 1971) which reveals the cellular NFT particularly clearly. Sections were counterstained with haematoxylin to reveal the neuronal and glial cytoarchitecture.

Morphometric Methods

The density of the Aβ deposits and cellular NFT was estimated in the upper 1mm of the cortex by manually counting all discrete lesions in 64 to 128, 1000 x 200µm fields, arranged contiguously, with the short dimension aligned with the pia mater. The upper region of the cortex often contains the maximum density of Aβ deposits and NFT in AD cases. A micrometer grid, aligned with the pia mater, was used as the sample field. Counts were separately made of the diffuse, primitive, and classic deposits and the NFT in each sample field. In the hippocampus, the sample fields were aligned first, with the alveus and the pyramidal cell layer from area CA1 to CA3 was sampled. Sampling was continued into area CA4 using a guideline marked on the slide which ceased approximately 400µm from the DG granular cell layer. In the DG, the sample field was aligned with the upper edge of the granular cell layer to sample the Aβ deposits in the molecular layer.

Data Analysis

If a lesion is randomly distributed among the sample fields, the frequency of samples which contain 0, 1, 2, ..., n lesions is described by the Poisson distribution (Pearson et al., 1985). In a Poisson distribution, the variance is equal to the mean and therefore, if a lesion is randomly distributed, V/M will approximate to unity. A V/M ratio less than unity indicates a regular distribution and greater than unity a clumped or clustered distribution. If a lesion is clustered, it may be important to determine the sizes of the clusters and whether the clusters are themselves randomly or regularly distributed within the tissue. Hence, counts of lesions in adjacent sample fields are added together successively to provide data for increasing field sizes, e.g., 200 x 1000µm, 400 x 1000µm, 800 x 1000µm etc., up to a size limited by the length of the strip sampled. V/M is calculated at each stage and plotted against the field size. A V/M peak indicates the presence of regularly spaced clusters while an increase in V/M to an asymptotic level suggests the presence of randomly distributed clusters (Armstrong, 1993a). The field size corresponding either to the peak or to the point at which the V/M ratio reaches its asymptote indicates the cluster size. The statistical significance of the V/M peak can be tested using the 't' distribution (Brower et al., 1990). A limitation of the V/M method is

that the number of degrees of freedom (DF) decreases with increasing field size as a result of combining adjacent samples. Hence, smaller peaks will reach statistical significance at small compared with large field sizes.

RESULTS

Examples of the spatial pattern of Aβ deposits in the cerebral cortex in AD are shown in figure 1. The V/M ratio of the classic Aβ deposits in the frontal cortex did not deviate significantly from unity at any field size indicating a random distribution. The V/M of the primitive deposits in the parahippocampal gyrus (PHG) exhibited a peak at field size 16 suggesting the presence of clusters of, 3200µm in diameter, regularly distributed parallel to the pia mater. The V/M ratio of the diffuse Aβ deposits in the PHG increased with field size without reaching a peak suggesting the presence of a large cluster of deposits of at least 6400µm in diameter.

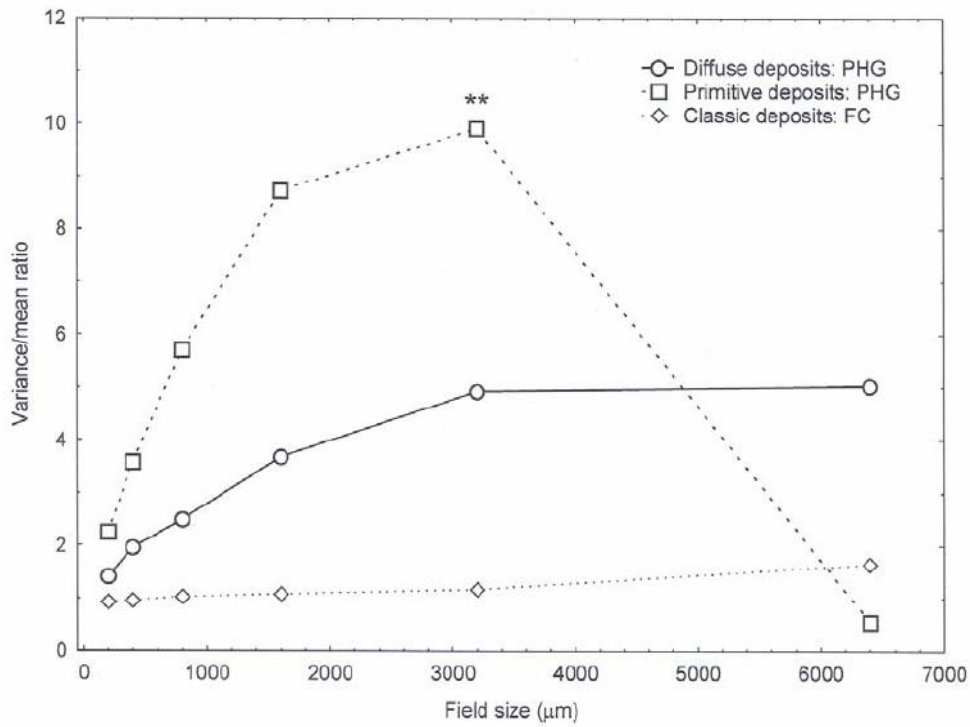

Figure 1. Pattern analysis plots by the V/M method showing examples of the spatial patterns exhibited by β-amyloid (Aβ) deposits in the cerebral cortex of patients with Alzheimer's disease. ** indicate the presence of significant V/M peaks.

A summary of the spatial patterns in the cerebral cortex in the16 AD cases as a whole is summarised in table 2. Randomly distributed lesions were rare and occurred in only 3/159 (1.8%) of analyses of Aβ deposits and 1/63 (1.6%) of analyses of NFT. Regularly distributed lesions were also rare occurring in 6/159 (3.8%) of analyses of Aβ deposits and 1/63 (1.6%)

of analyses of NFT. The most common spatial pattern observed was aggregation of the lesions into clusters. Clusters which were randomly distributed, however, were rarely observed and in the majority of cortical regions, there were two types of clustering. First, the most common type of pattern was of clusters, 100 to 3200µm in diameter, which were regularly distributed parallel to the pia mater. In most patients, this pattern occurred in between 51% and 68% of cortical areas investigated depending on the type of lesion. Second, lesions occurred in very large clusters (>6400µm), relative to the size of the area sampled, without evidence for regular spacing and were present in 57/159 (35.8%) of analyses of Aβ deposits and 19/63 (30.2%) of analyses of NFT.

Comparison of the frequencies of the different types of spatial patterns exhibited by the Aβ deposits and NFT revealed both similarities and differences. First, all types of lesion exhibited clustering with the regularly distributed cluster as the single most common type of spatial pattern. Second, the diffuse and primitive Aβ deposits exhibited a similar range of spatial patterns but these two types of deposit were more likely to occur in larger clusters than the classic deposits. Third, the spatial patterns of the NFT did not differ significantly from those shown by the diffuse Aβ deposits but were distributed less often in large clusters than the primitive deposits and more often in large, regularly distributed clusters than the classic deposits.

DISCUSSION

Despite differences in morphology and in molecular determinants, the different subtypes of Aβ deposit and NFT exhibited essentially similar spatial patterns within the frontal and temporal cortex in AD; the commonest single type of spatial pattern observed being the regularly distributed cluster of lesions. There are several possible explanations for the clustering patterns of the Aβ deposits and NFT. First, a lesion may develop in relation to a specific neuroanatomical feature, e.g., blood vessels (Bell and Ball, 1990; Armstrong, 1995), the cells of origin of an anatomical pathway (Pearson et al., 1985, De Lacoste and White, 1993) or neuronal populations which use a particular neurotransmitter (Kowall and Beal, 1988).

The relationship between Aβ deposits and blood vessels has been controversial with some studies suggesting that the association is a chance effect due to the abundance of lesions and blood vessels in the AD brain (Kawai et al., 1990, Luthert et al., 1991). In the cerebral cortex, the arterioles penetrate the pia mater at intervals and then extend vertically through the laminae before reaching a maximum density in cortical lamina IV (Bell and Ball, 1990). The result is that the larger diameter arterioles are distributed in a fairly regular pattern in the upper cortical laminae (Armstrong, 1995). Hence, regularity in the distribution of blood vessels could explain the regular clustering of lesions. Previous data (Armstrong, 1995; 2006) suggested that there was a positive correlation between the clusters of the diffuse Aβ deposits and blood vessels and a negative correlation between the clusters of primitive deposits and blood vessels in a number of cases. The clusters of the classic deposits, however, coincided with the clusters of the larger diameter arterioles in all cases examined (Armstrong, 1995). Hence, the different types of Aβ deposit may differ in their spatial relationship to blood

vessels, the data suggesting a more direct and specific role for the larger arterioles in the formation of the classic deposits.

Second, the regular clustering of lesions in AD could reflect their development in relation to cells associated with specific neuroanatomical pathways. This hypothesis is supported by the observation that NFT are highly area specific, lamina-specific, and cell-type specific (Braak and Braak, 1992) and that neurons affected by NFT are functionally related suggesting the spread of degeneration across normal synaptic boundaries (Saper et al., 1987). In addition, loss of synaptic markers has been observed in AD, especially in laminae III and V of the cortex which are likely to contain the cells of origin of the long and short cortico-cortical projections (Scheff and Price, 1993). Although the pattern of distribution of cells in the cortex is complex, the cells of origin of the long and short cortico-cortical projections are clustered and occur in bands which are distributed along the cortical strip. In the primate brain, individual bands of cells associated with a particular projection are 500-800μm in width and traverse the cortical laminae (Hiorns et al., 1991). There is a regular distribution of these bands along the cortex although there is also a complex pattern of branching and rejoining of adjacent groups of cells. The spaces between the bands of cells are occupied by afferent or efferent connections with different cortical sites or with subcortical regions. In a proportion of cortical strips examined, varying from between 21% and 58% in those brain areas which exhibit regular clustering of lesions, the estimated width of the Aβ deposit and NFT clusters and their distribution along the cortex is consistent with their development in relation to these cell clusters. Hence, one possible explanation for the regularly distributed clusters observed in the cerebral cortex in AD is that the lesions are associated with the cortico-cortical pathways.

Third, if the clusters of two different lesions are spatially correlated, the pathogenesis of one lesion may be related to the other. For example, the formation of NFT clusters could be related to the formation of Aβ deposits (Armstrong, 1993b). In addition, the spatial pattern of the different subtypes of Aβ deposit could themselves be interrelated. The present data suggest that the clusters of the diffuse and primitive Aβ deposits are negatively correlated in approximately 50% of cortical areas analysed. By contrast, in the majority of brain areas, clusters of the diffuse and classic deposits were distributed independently while the distribution of clusters of primitive and classic deposits were positively correlated in approximately 50% of analyses. Hence, in a proportion of cortical areas, clusters of the diffuse and primitive deposits appeared to alternate along the cortical strip. One possible explanation for this pattern is that the primitive and diffuse deposits may represent distinct types of Aβ deposit which develop in relation to alternating groups of cells (Armstrong, 1998). Alternatively, diffuse deposits may be the first type to be formed in AD and a proportion of these diffuse deposits may evolve into primitive deposits giving rise to an alternating pattern (Mann and Esiri, 1989; Mann et al., 1992).

The formation of the clusters of NFT could be related to the development of Aβ deposits as predicted by the 'Amyloid Cascade Hypothesis' (Hardy and Higgins, 1992). The hypothesis proposes that the deposition of Aβ is sufficient cause to produce NFT, cell loss, and vascular damage in the brain. In a study of six AD patients (Armstrong et al., 1993), however, evidence for a positive correlation between the clusters of Aβ deposits and NFT was found in only 4/33 (12%) of cortical areas, i.e., in the majority of areas the clusters of plaques and tangles in the upper cortical laminae were distributed independently of each other. Dissociation between Aβ and tau pathologies in AD has been reported in a number of studies.

For example, Duyckaerts et al. (1997) studied a piece of the frontal cortex in an AD patient disconnected from adjacent tissues due to a tumor removal 27 years earlier. Although Aβ deposits and NFT were observed throughout the cortex, within the disconnected piece, Aβ deposits were present but tau positive lesions were absent. Nevertheless, it is possible that there is an association between NFT and Aβ deposits in brain areas which are anatomically connected (Pearson et al., 1985; De Lacoste and White, 1993). In the medial temporal lobe, for example, the density of NFT at a site is positively correlated with the sum of the Aβ deposits present at the projection sites of these neurons (Armstrong, 1993b) consistent with this hypothesis. The formation of Aβ in association with the axon terminals of neurons in a brain region could induce the formation of NFT within the cells of origin of these projections according to the 'Amyloid Cascade hypothesis'. Another possibility, however, is that the secretion of Aβ at axon terminals and the formation of NFT within perikarya (Tabaton et al., 1991; Masliah et al., 1992; Wisniewski et al., 1995) both occur as a consequence of the degeneration of neurons. This hypothesis is supported by the observations that SP in the dentate gyrus are positively correlated with thioflavin positive NFT in the entorhinal cortex (Senut et al., 1991) and that experimental lesions made in the subcortical nuclei of the rat result in APP synthesis at the axon terminals in the cortex (Wallace et al., 1993).

The study of the spatial patterns of Aβ deposits and NFT suggests a hypothesis of how the pathological changes may develop in the frontal and temporal cortex in AD. The size, location, and distribution of the clusters of lesions suggests they are associated primarily with the cortico-cortical projections (Pearson et al., 1985; De Lacoste and White, 1993; Armstrong and Slaven, 1994). The degeneration of the cortico-cortical pathways leads to the accumulation of PHF in cell bodies and their processes (Tabaton et al., 1991; Masliah et al., 1992) and the secretion of Aβ at their axon terminals (Regland and Gottfries, 1992). There is evidence that in the earliest stages of the pathology, secreted Aβ is in the form of large clusters of diffuse deposits (Mann and Esiri, 1989; Mann et al., 1992). Within the clusters of diffuse Aβ deposits, in regions of cortex affected by clusters of NFT, a proportion of diffuse deposits evolve into primitive deposits giving rise to an alternating pattern of diffuse and primitive deposit clusters. An alternative hypothesis is that alternating clusters of cells are involved in the formation of morphologically dissimilar Aβ deposits (Armstrong, 1998). Finally, in regions of cortex affected by neuritic degeneration, and which are close to the vertically penetrating arterioles, there is the formation of the cored classic-type deposits.

CONCLUSION

In the cerebral cortex of patients with AD, Aβ deposits and NFT exhibit essentially similar types of spatial pattern. The commonest single type of spatial pattern observed is a regular distribution of clusters parallel to the pia mater. The size, location, and distribution of the clusters suggest that the lesions reflect the degeneration of specific cortical pathways. Furthermore, blood vessels may be involved in the development of the classic-type of Aβ deposit. Data from the study of spatial patterns of lesions in AD are consistent with the hypothesis that AD is a 'disconnection syndrome' in which degeneration of specific cortical pathways results in the formation of clusters of Aβ deposits and NFT.

Table 2. A summary of the spatial patterns exhibited by β-amyloid (Aβ) deposits and neurofibrillary tangles (NFT) in the cerebral cortex of 16 patients with Alzheimer's disease. The data represent the frequency of the different types of spatial pattern in a range of cortical areas. The percentages in data row 5 are the proportion of brain areas which exhibit regular clustering in which the cluster size was between 400 and 800 µm. (N= Number of brain areas analysed)

	Pathological lesion			
Spatial pattern	Diffuse Aβ	Primitive Aβ	Classic Aβ	NFT
(Number of analyses)	59	62	38	63
Random	2	0	1	1
Regular	0	0	6	1
Regular clusters <400µm	3	11	2	6
Regular clusters 400-800µm	12 (40%)	16 (43%)	15 (58%)	9 (21%)
Regular clusters >800µm	15	10	9	27
Single cluster > 6400µm	27	25	5	19

Comparison between groups (χ^2 contingency tests): Diffuse v Primitive $\chi^2 = 8.15$ (4DF, $P > 0.05$), Diffuse v Classic $\chi^2 = 19.88$ (5DF, $P < 0.01$), Primitive v Classic $\chi^2 = 22.16$ (5DF, $P < 0.001$), Diffuse v NFT $\chi^2 = 7.45$ (5DF, $P > 0.05$), Primitive v NFT $\chi^2 = 14.05$ (5DF, $P < 0.05$), Classic v NFT $\chi^2 = 19.23$ (5DF, $P > 0.01$).

ACKNOWLEDGMENTS

The studies described in this article utilised brain material provided and processed by the Brain Bank, Institute of Psychiatry, King's College, London and their assistance is gratefully acknowledged.

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In: Alzheimer's Disease Diagnosis and Treatments ISBN: 978-1-61122-064-3
Editor: Marisa R. Boyd

Chapter 7

BRAIN FUNCTION IN ALTERED STATES OF CONSCIOUSNESS: COMPARISON BETWEEN ALZHEIMER DEMENTIA AND VEGETATIVE STATE

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ABSTRACT

Disorder of consciousness is not an all-or-none phenomenon but it rather represents a continuum. Alzheimer's disease (AD) is the most common cause of dementia among people aged 65 and older, and patients are frequently unaware of the importance of their cognitive deficits (Derouesne *et al.*, 1999). Vegetative state (VS) is a clinical entity with a complete lack of behavioural signs of awareness, but preserved arousal (ANA Committee on Ethical Affairs, 1993; The Multi-Society Task Force on PVS, 1994). Both clinical entities share a certain level of consciousness alteration, and a certain similarity in brain metabolic impairment. Here, we review differences and similarities in brain function between these two types of disorders of consciousness, as revealed by functional neuroimaging studies.

Keywords: vegetative state – Alzheimer dementia – functional brain imaging – consciousness.

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INTRODUCTION

Consciousness is a multifaceted concept that can be divided into two main components: arousal (i.e., wakefulness, or vigilance) and awareness (e.g., awareness of the self and the environment) (Plum and Posner, 1983; Zeman *et al.*, 1997). Arousal is supported by several brainstem neuron populations that directly project to both thalamic and cortical neurons (Steriade *et al.*, 1997). Awareness is thought to be dependent upon the functional integrity of the cerebral cortex and its sub-cortical connections; each of its many parts are located, to some extent, in anatomically defined regions in the brain (Dehaene and Naccache, 2001; Zeman, 2001). Usually, consciousness is assessed in neurologically disabled patients by the presence of voluntary or meaningful behaviours (in contrast to automatic, reflexive reactions) in response to various external stimulations.

"CONSCIOUSNESS" IN AD

AD is clinically characterized by a dementia syndrome. Current criteria for dementia include a deterioration in cognitive functions, sufficient to impair daily living activities, with mood and behaviour disturbances (APA, 1987). Dementia stages may be defined using a Mini-Mental State Examination (MMSE) score range (Folstein *et al.*, 1975). Usually, a mild stage corresponds to a MMSE score greater than 20, dementia is moderate between scores 20 and 12, and the deficit is severe when MMSE value is below 12.

There are different aspects of impaired consciousness in AD. An episodic memory trouble is the most frequent early clinical symptom (Perry and Hodges, 1996; Fleischman and Gabrieli, 1999). Episodic memory refers to personal episodes that can be associated to precise contextual information, concerning time and place for example. AD patients cannot vividly recollect and re-experience a number of episodes of their (recent) life, in which they were however deeply involved. Such an impairment in processing contextual characteristics of an episode that make it unique for a subject corresponds to a decrease of autonoetic consciousness (Wheeler *et al.*, 1997; Levine *et al.*, 1998; Tulving, 2002).

More generally, AD is characterised by a deficit of controlled cognitive processes that require conscious processing of information. Neuropsychological studies have demonstrated that AD patients show impairment in controlled processes during memory and executive tasks, while automatic activities may be more preserved (Fabrigoule *et al.*, 1998). It is frequently observed that AD patients fail to consciously recollect information whereas they provide target memories in implicit conditions. Several neuropsychological data show that AD patients, because of diminished control capacities, base their daily functioning upon automatic processes (Adam *et al.*, 2005). They remain efficient in routine situations, but are not able to face unexpected situations.

Beside cognitive impairment, dementia symptoms include personality change and altered judgment (APA, 1987). Behavioural and psychological impairments are well described in AD (Cummings *et al.*, 1994; Neary *et al.*, 1998). Importantly, there is an early lack of awareness for self-cognitive or self-behavioural difficulties (an anosognosia for deterioration) in the disease (Kalbe *et al.*, 2005b). AD patients may show different degrees of awareness in different domains (e.g., reasoning, memory, affect; Figure 1), and there happens to be a

continuum in levels of anosognosia concerning the cognitive or behavioural deficits in the disease. (Damasio, 1994; Salmon *et al.*, 2005a). The level of anosognosia has been related to many clinical variables, and not always to dementia severity (Salmon *et al*., 2005b).

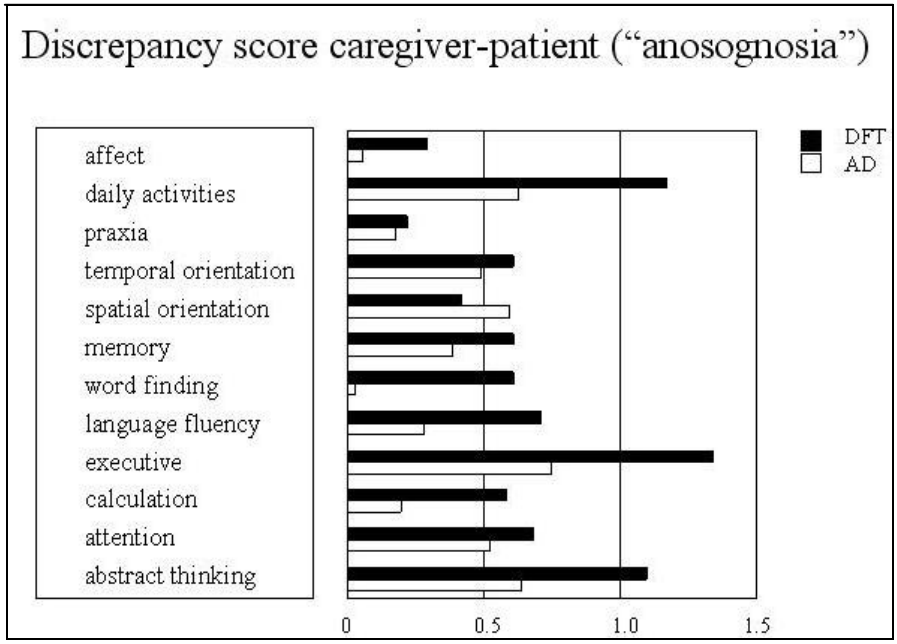

Figure 1. Anosognosia levels in several aspects of cognitive impairment in demented patients, as measured by the discrepancy score between caregivers and patients judgment in Alzheimer disease (AD) and fronto-temporal dementia (DFT)

As dementia develops, patients lose the awareness of time, place and other people (Fishback, 1977). Progression of AD gradually decreases the ability of patients to interact with their environment. Patients may develop aphasia, become unable to recognize friends and family members, and eventually lose the ability to maintain eye contact with their caregivers (Volicer *et al.*, 1987, 1997). As mental oblivion intervenes, the last thing the patient forgets is his/her own name (Fishback, 1977). In later stages, awareness completely disappears, marking the start of a sort of vegetative state (The Multi-Society Task Force on PVS, 1994).

Vegetative State

In 1972, Jennet and Plum defined the vegetative state as a clinical condition of 'wakefulness without awareness'(Jennett and Plum, 1972). These patients have preserved sleep-wake cycles but they do not show clinical signs of awareness of self or environment. They usually present reflex or spontaneous eye opening and breathing, they occasionally move their limbs or trunk in a meaningless way. They may be aroused by painful or prominent stimuli, opening their eyes if they are closed, increasing their respiratory rate, heart rate and blood pressure and occasionally grimacing or moving. Vegetative state patients can make a range of spontaneous movements including chewing, teeth-grinding and swallowing.

More distressingly, they can even show rage, cry, grunt, moan, scream or smile reactions spontaneously or to non-specific stimulation. Their head and eyes sometimes, inconsistently, turn fleeting towards new sounds or sights (Laureys *et al.*, 2000, 2002, 2004). Emergence of vegetative state is defined by the minimally conscious state (MCS). MCS patients show minimal but definite evidence of self or environment awareness but are unable to communicate (Giacino *et al.*, 2002).

Degenerative disorders such as AD are considered as a classical aetiology of vegetative state (The Multi-Society Task Force on PVS, 1994). In patients with degenerative diseases, a persistent vegetative state usually evolves over a period of several months or years (Walshe and Leonard, 1985). Those who remain in a vegetative state may die of superimposed infection or illness. Those who survive such an illness remain in a vegetative state or go into a coma (The Multi-Society Task Force on PVS, 1994). End-of-life questions and ethical debates are similar for VS and bed-ridden, latest stage AD. The question is what action is consistent with the ethical principles of proportionality in balancing the benefits and burdens of medical intervention, and how best to respect the autonomy and self-determination of the patient (Jennett, 1972). It is always difficult to know demented patients' awareness of the end of life. It is also really difficult to accompany these patients, with whom communication is essentially nonverbal (Michel *et al.*, 2002). Many patients with dementia lose the ability to feed themselves in the advanced stages of the disease (McNamara and Kennedy, 2001). Once a patient is considered permanently vegetative, the ethical debate revolves largely around the decision about continuing or withdrawing artificial nutrition and hydration (Jennett, 2005).

GLOBAL CEREBRAL METABOLISM IN VS AND IN AD

Since the beginnings of positron emission tomography (PET) imaging, quantified studies of regional consumption of oxygen and glucose have been performed in AD. They showed a global diminution of cerebral activity (Demetriades, 2002). This diminution of metabolism was proportional to dementia severity (Figure 2; Cutler et al., 1985), being 20% decreased in patients with mild to moderate dementia, and 40% decreased in patients with severe dementia, compared to normal age-matched controls (Frackowiak et al., 1981).

PET has also shown a substantial reduction in global brain metabolism in vegetative patients. Studies of our group and others have shown a 50 to 60% decrease in brain metabolism in vegetative state of different aetiology and duration (Figure 3; Levy et al., 1987; Momose et al., 1989; De Volder et al., 1990; Beuret et al., 1993; Tommasino et al., 1995; Laureys et al., 1999a,b, Rudolf et al., 1999, 2002; Schiff et al., 2002; Edgren et al., 2003). In "permanent" vegetative state (i.e., 12 months after a trauma or 3 months after non-traumatic brain damage), brain metabolism values drop to 30–40% of the normal mean activity (Rudolf et al., 1999, 2002).

A global decrease in cerebral metabolism is not specific to AD or VS. When different anaesthetics are titrated to the point of unresponsiveness, the reduction in brain metabolism is similar to that in comatose patients (Alkire et al., 1995, 1997, 1999). The lowest values of brain metabolism have been reported during propofol anaesthesia (to 28% of the normal range). (Alkire et al., 1995) A transient decrease in brain metabolism also takes place during

deep sleep (stage III and IV), (Maquet et al., 1996, 1997, 1999) where cortical cerebral metabolism can drop to nearly 40% of the normal range of values.

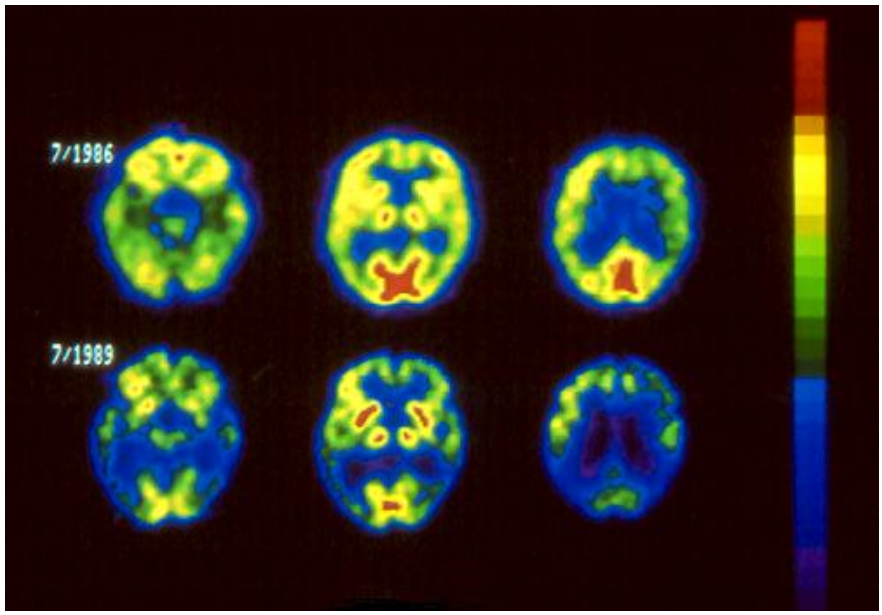

Figure 2. Quantified brain glucose metabolism in a single patient examined twice while in mild dementia stage (1986, upper panel) then in moderate dementia (1989, lower panel). This figure illustrate progressive brain metabolism reduction correlated with the clinical evolution of the Alzheimer disease

Regional Metabolic Distribution

Neuroimaging studies in AD revealed a characteristic regional distribution of decreased activity, showing consistent impairment of metabolism in temporo-parietal cortices, postero-medial regions (posterior cingulate cortex, precuneus), and lateral frontal associative cortices (Salmon, 2002). There is, however, in these patients a relative preservation of primary neo-cortical structures, such as the sensori-motor and primary visual cortex, and also of subcortical structures, like the basal ganglia, brainstem, and thalamus (Herholz *et al.*, 2002). Beside hypometabolism in cortical associative regions, a significant functional impairment of medial temporal regions was also reported in AD (Cutler *et al.*, 1985). However, when voxel-based analysis were used, medial temporal regions were found less diminished than associative cortical regions in these patients (Minoshima *et al.*, 1997). This is even more striking given that brain morphometric studies (voxel-based morphometry) show a major structural temporal medial atrophy (Busatto *et al.*, 2003), and that correlation studies showed a relationship between diminution of amnesic performances and alteration of medial temporal cortex activity (Lekeu *et al.*, 2003). Noteworthy, the involvement of medial temporal structures in autonoetic consciousness remains a matter of debate. It has been suggested that after a stage of diminished hippocampal and entorhinal activity, in the first stages of AD, complexity of medial temporal circuitry would explain local maintenance of activity despite atrophy severity and neurofibrillary histological lesions. One can recognize in AD different

'pathological poles' involving (1) medial posterior regions, often in relationship with frontal and parietal lateral associative regions, or (2) medial temporal regions, but also (3) medial frontal regions, like the anterior cingulate cortex. These poles would be involved variably depending on individuals, and this variability could explain individual clinical profiles (Salmon, 2002).

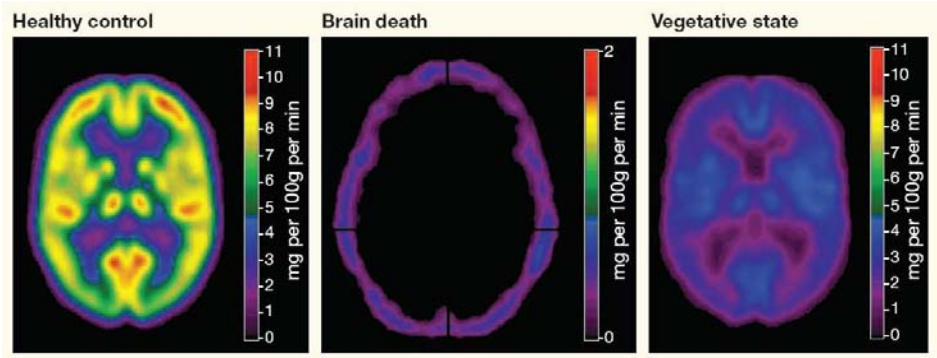

Figure 3. Illustration of the differences in resting brain metabolism measured in brain death and in the vegetative state, compared with controls. The image in patients with brain death shows a clear-cut 'hollow-skull sign', which is tantamount to a 'functional decapitation'. This is markedly different from the situation seen in patients in a vegetative state, in whom cerebral metabolism is massively and globally decreased (to 50% of normal value) but not absent. The colour scale shows the amount of glucose metabolized per 100 g of brain tissue per minute (reproduced with permission from Nature Reviews Neuroscience (Laureys, 2005) copyright 2005 Macmillan Magazines Ltd.)

In AD patients, reduction of metabolism in associative cortical regions is correlated to dementia severity (Salmon *et al.*, 2000, 2005b). This severity is evaluated by means of cognitive capacities scales, like the Mini Mental State Exam or of daily functionality, like the Instrumental Activity of Daily Living scale (Lawton and Brody, 1969). Lower scores mainly reflect lower (consciously) controlled capacities, related to lower metabolism in the first pathological pole just described. Longitudinal PET studies in AD patients showed an expansion as well as an increased severity of hypometabolism in association cortical areas and subcortical structures (Mielke *et al.*, 1996), and a close correlation between progressive metabolic reduction and impaired cognitive performance has been shown (Minoshima *et al.*, 1997; Demetriades, 2002). Some of the fronto-posterior regions included in the first AD pathological pole have been linked, in healthy volunteers, to autonoetic information retrieval, i.e. a remembering with full awareness of re-lived events, which is particularly impaired in AD patients (Whalen and Liberman, 1987).

On the other hand, the metabolic activity of the temporo-parietal junction is in relationship with a measure of anosognosia, i.e. the differential score between a relative and a patient evaluation of cognitive capacities of this patient (Salmon, *et al***.,** 2005b). Thus, the anosognosia, or 'decreased of awareness of cognitive impairment' that happens very early in AD reflects a lack of conscious access to daily reality in AD patients, and is related to the activity in a portion of the fronto-posterior associative pathological pole (Kalbe *et al.*, 2005b).

The hallmark of the vegetative state is a complete loss of awareness and a systematic impairment of metabolism in the polymodal associative cortices (bilateral prefrontal regions, Broca's area, parieto-temporal, posterior parietal areas and precuneus and posterior cingulate)

(Laureys *et al.*, 2002). These regions are important in various functions that are necessary for consciousness, such as attention, memory, and language (Baars *et al.*, 2003). On the other hand, VS patients show a relative preservation of metabolism in brainstem, thalamus and posterior hypothalamic regions. In rare cases where patients in a vegetative state recover awareness of self and environment, PET shows a functional recovery of metabolism in these same cortical regions (Laureys *et al.*, 1999b).

The pattern of metabolic impairment in fronto-parietal associative areas found in VS is quite similar to that found in advanced AD (Figure 4). In AD as in VS, the medial posterior cortex has received great attention. The posterior cingulate, retrosplenial cortex and precuneus have all been involved in early stages of AD (Neunzig and Kunze, 1987; Minoshima *et al.*, 1997). In AD, posterior cingulate cortex metabolism is inversely correlated to score reduction in MMSE examination (Figure 5; Salmon *et al.*, 2000). Similarly, the medial parietal cortex (precuneus) and adjacent posterior cingulate cortex seem to be the brain regions that differentiate patients in minimally conscious state from those in vegetative state (Laureys *et al.*, 2004). Interestingly, these areas are among the most active brain regions in conscious waking (Maquet *et al.*, 1996; Gusnard and Raichle, 2001) and are among the least active regions in altered states of consciousness such as halothane (Alkire *et al.*, 1999) or propofol (Fiset *et al.*, 1999) -induced general anaesthesia, sleep (Maquet *et al.*, 1996, 1999), hypnotic state (Maquet *et al.*, 1997; Rainville *et al.*, 1999), and also in Wernicke–Korsakoff's or postanoxic amnesia (Aupee *et al.*, 2001). In a recent study, Vogt et al. suggested that the ventral portion of the posterior cingulate cortex would be related to processing of events for their self/emotional significance (Vogt *et al.*, 2005).

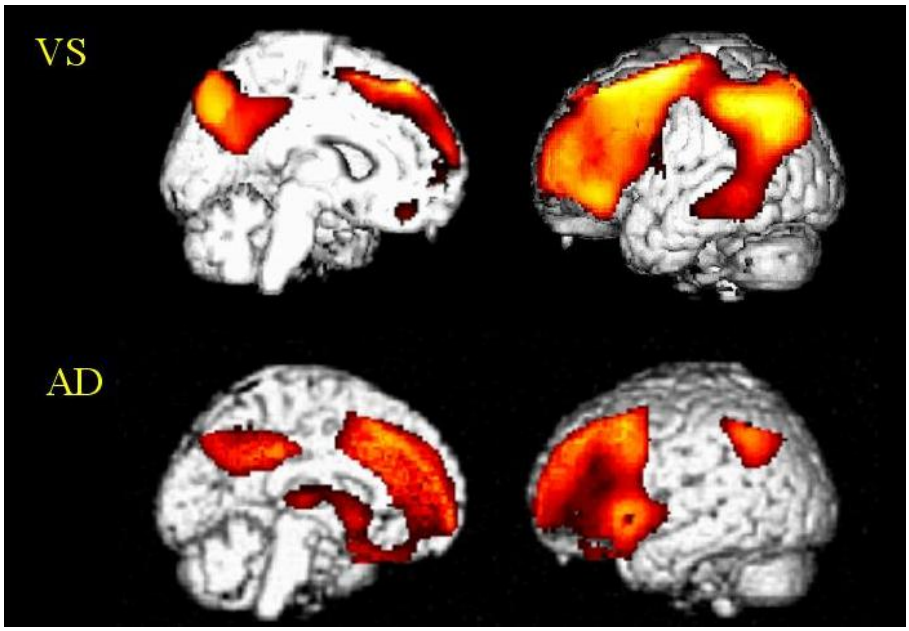

Figure 4. Regional metabolic alteration as compared to healthy subjects in patients in vegetative state (VS, upper panel) and Alzheimer demented patients (AD, lower panel) reveals a striking similarity in medial and lateral fronto-parietal associative areas impairment between both populations

Other PET and fMRI studies showed involvement of precuneus in reflective self-awareness (Kjaer *et al.*, 2002) and processing of one's own name compared to other names (Vogeley *et al.*, 2004; Perrin *et al.*, 2005). Precuneus and posterior cingulate cortex may thus be part of the neural network subserving human consciousness (Baars *et al.*, 2003), especially of a midline parieto-frontal core involved in self-awareness (Lou *et al.*, 2005).

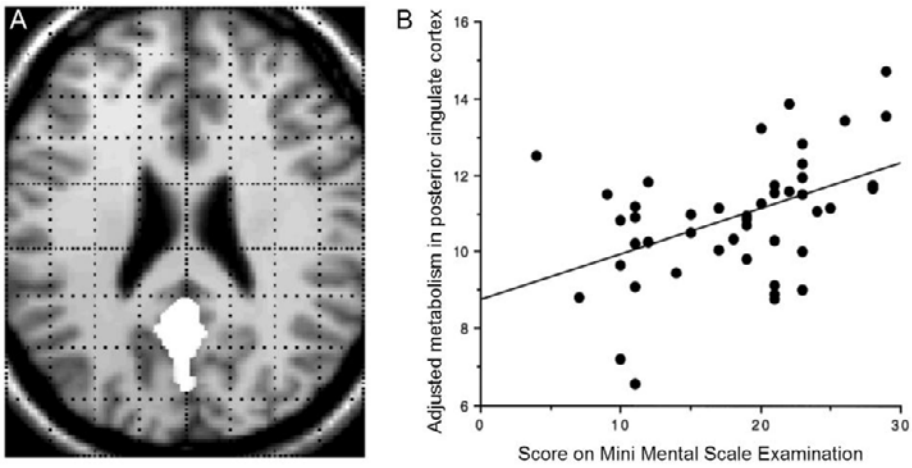

Figure 5. A. Early decrease of activity in posteromedial areas (encompassing posterior cingulate cortex and precuneus) in patients with Alzheimer's disease. B. Linear correlation between dementia severity (score on mini mental state examination) and metabolism in posterior cingulate cortex (area 31, coordinates: x = 0, y =-56, z = 28 mm) in Alzheimer's disease. (From Salmon et al., 2000, reprinted with permission of Wiley-Liss, Inc., a subsidiary of John Wiley and Sons, Inc)

FUNCTIONAL CONNECTIVITY

In AD, some studies showed a functional disconnection between certain pathological poles, i.e. a diminished functional correlation between parahippocampal and frontal regions (Lekeu *et al.*, 2003), or between prefrontal and other associative brain areas involved in short term memory tasks (Grady *et al.*, 2001). EEG studies also showed decreased interhemispheric EEG coherence in AD, reflecting both a lower mean level of functional connectivity as well as diminished fluctuations in the level of synchronization (Stam *et al.*, 2005). Other studies show a decreased complexity of EEG patterns and reduced information transmission among cortical areas in AD (Jeong, 2004). Diffusion tensor magnetic resonance imaging was also used to compare the integrity of several white matter fibre tracts in patients with probable AD (Rose *et al.*, 2004; Bozzali *et al.*, 2002). Relative to normal controls, patients with probable AD showed a highly significant reduction in the integrity of the association white matter fibre tracts, such as the splenium of the corpus callosum, superior longitudinal fasciculus, and cingulum. By contrast, pyramidal tract integrity seemed unchanged (Rose *et al.*, 2000). In another study, strong correlations were found between the mini mental state examination score and the average overall white matter integrity (Bozzali *et al.*, 2002). All these data are in line with the hypothesis that AD is a disconnection pathology, linked to the distribution of histological lesions in the different cortical layers (Knowles *et al.*, 1999). Activation studies

in AD have broadly showed two types of patterns. In some memory studies, there is a decrease of activation in pathological poles such as the medial temporal area, probably related to impaired performances in memory tasks. In very early cases, it is however possible to observe a "normal" hippocampal activation, that might be related to compensatory functioning. In most reports, AD patients show activation in more cortical areas than their controls. The hypotheses are (1) that supplementary activation correspond to recruitment of further resources to reach the current performance or (2) that a non-efficient and useless activation occurs because selection and inhibition processes are deficient.

In vivo brain imaging data show multifocal brain atrophy (Juengling *et al.*, 2005) and impaired functional connectivity in patients in VS (Laureys *et al.*, 1999a, 2000). The resumption of long range functional connectivity between different associative cortices (Laureys *et al.*, 2004) and between some of these and the intralaminar thalamic nuclei parallels the restoration of the functional integrity of these patients (Laureys *et al.*, 2000b). An EEG study in a single VS patient showed markedly asymmetrically reduced EEG coherence in relationship with subcortical structural damage (Davey *et al.*, 2000). In cohort studies of patients unambiguously meeting the clinical diagnosis of vegetative state, simple noxious somatosensory (Laureys *et al.*, 2002) and auditory (Laureys *et al.*, 2000a; Boly *et al.*, 2004) stimuli have shown systematic activation of primary sensory cortices and lack of activation in higher order associative cortices from which they were functionally disconnected. High intensity noxious electrical stimulation activated midbrain, contralateral thalamus, and primary somatosensory cortex (Laureys *et al.*, 2002). However, secondary somatosensory, insular, posterior parietal, and anterior cingulate cortices, which were activated in all control individuals, did not show significant activation in any patient. Moreover, in patients in a vegetative state, the activated primary somatosensory cortex was functionally disconnected from higher-order associative cortices of the pain matrix (Laureys *et al.*, 2002). Similarly, although simple auditory stimuli activated bilateral primary auditory cortices, higher-order multimodal association cortices were not activated. A cascade of functional disconnections were also observed along the auditory cortical pathways, from primary auditory areas to multimodal and limbic areas (Laureys *et al.*, 2000a), suggesting that the observed residual cortical processing in the vegetative state does not lead to integrative processes, which are thought to be necessary for awareness. In contrary, functional connectivity analysis for similar simple auditory stimuli, performed in patients in a minimally conscious state, showed preserved functional connections between secondary auditory cortex and a large set of cortical areas (encompassing frontal and temporal association cortices) compared to VS patients (Boly *et al.*, 2004).

CONCLUSIONS

Disorder of consciousness should be considered as a continuum, not as an all-or-none phenomenon. Our review of correlation and longitudinal studies suggests that this is true in dementia as well as in severely brain injured patients like in vegetative and minimally conscious states. However, progression to VS is rarely present in AD patients (Volicer *et al.*, 1997). Even in the end stage dementia, some behavioural signs of consciousness remain in most cases. Experienced caregivers can detect a discomfort even in patients with very

advanced dementia who are unable to maintain their posture in a chair and are mute (Hurley *et al.*, 1992). Late-stage patients are in fact quite different from one another and in most cases continue to interact with their environment (Boller *et al.*, 2002).

Looking at functional imaging data, both patients' populations show diminished global metabolism, diminished regional metabolism in associative areas with a relative preservation of brainstem, thalamus and primary sensory cortices, and impaired functional connectivity. These similarities in brain activity impairment patterns could reflect the general mechanisms of alteration of consciousness present in both clinical conditions, with impairment of the parieto-frontal associative network thought to be necessary to reach conscious perception (global workspace theory (Baars *et al.*, 2003)).

The level of metabolic alteration in the hippocampus and its role in autonoetic consciousness impairment in AD patients remain a matter of debate, even if its structural atrophy was related to episodic memory impairment early in the evolution of AD. Medial temporal regions seem not to be part of the more metabolically impaired regions in VS and were not related to impaired consciousness in this population of patients. A direct comparisons of metabolic disturbances in both conditions might add to the discussion on altered consciousness in neurological diseases and its physiopathological correlations.

ACKNOWLEDGMENTS

The work in the Cyclotron Research Centre in Liege was made possible by grants from the National Fund for Scientific Research (FNRS), Fondation Medical Reine Elisabeth, a 5th European Framework project (NEST-DD) and InterUniversity Attraction Pole 5/04 – Belgian Science Policy. SL is also supported by FNRS grants 3.4517.04 and by grants from the Centre Hospitalier Universitaire Sart Tilman, the University of Liège, and the Mind Science Foundation. MB and SL are respectively Research Fellow and Research Associate at the Fonds National de la Recherche Scientifique (FNRS).

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In: Alzheimer's Disease Diagnosis and Treatments ISBN: 978-1-61122-064-3
Editor: Marisa R. Boyd

Chapter 8

COGNITIVE DEFICITS IN MILD COGNITIVE IMPAIRMENT

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ABSTRACT

Mild Cognitive Impairment (MCI) describes older adults whose cognitive and functional status is considered in-between normal cognitive aging and dementia. MCI is an heterogeneous entity with a number of subtypes each with a different neuropsychological profile. The MCI amnestic type is the better known of the subtypes and many patients with this clinical and cognitive profile will develop Alzheimer's disease. Although the amnestic MCI concept emphasizes memory loss, other cognitive functions are frequently affected, namely semantic fluency, attention/executive functions, visuo-spatial abilities and language comprehension.

MCI criteria make use of scores in delayed recall of episodic memory tasks to establish the presence of memory impairment. Poor delayed recall can, however, reflect deficits in distinct memory processes. Difficulties in the learning process of MCI patients have also been documented. During the acquisition of semantically structured lists of words, these patients employ less semantic clustering strategies than controls. However, if attention is called to the semantic structure, they can make use of it on subsequent trials in order to improve learning.

Detailed knowledge of the memory processes disturbed in MCI should contribute to the understanding of the pathophysiology of MCI, allow a more precise identification of patients with high probability of progression, and help to delineate future rehabilitation interventions in these patients.

DEFINITION OF MCI

The term Mild Cognitive Impairment (MCI) has been developed to describe older adults whose cognitive and functional status is considered in-between normal cognitive aging and dementia [1]. Currently, the term MCI is used to refer to non-demented older subjects with memory or other cognitive impairments that cannot be explained solely by age or a known medical condition [2].

The most widely used definition of MCI is based on criteria put forward by the Mayo Clinic group in 1999 [3]. Petersen proposed the MCI concept to classify subjects who complain about their memory and show an abnormal memory performance for age, have a normal general cognitive function, maintain their activities of daily living and are not demented [3]. The American Academy of Neurology used the MCI concept designed by the Mayo Clinic when looking into early detection of dementia. The approved guidelines recommend the evaluation and clinical monitoring of MCI patients using dementia screening tests and neuropsychological batteries [4]. Since the initial formulations, the MCI concept emphasizes memory loss. In the first place, a memory complaint is necessary and, if possible, should be corroborated by an informant. Additionally, a deficit in memory functions, specifically in episodic memory, should be demonstrated by neuropsychological testing. Although the criteria require that the general cognitive function is preserved, the presence of mild deficits in other cognitive domains is not specifically excluded. In fact, several studies have shown that the MCI patients diagnosed according to Petersen's criteria [3] frequently have other cognitive deficits beyond episodic memory. In a series of 116 consecutive patients who met Petersen's criteria for amnestic MCI, we found that as much as 68.7% of the patients had deficits in temporal orientation, 30.2% in semantic fluency, 33.7 % in verbal comprehension (on the Token test), 23.4 % in calculation, and 23.9% in motor initiative [5]. Deficits on semantic fluency tasks were also found in other studies with MCI amnestic patients [6,7,8]. The cognitive functions most frequently affected in MCI patients, besides episodic memory, were semantic fluency, attention/executive functions, visuo-spatial abilities and language comprehension [5,6,8]. Thus, purely amnesic MCI patients are less frequent than originally thought, either in population studies [9] or in clinical studies. Actually, Alladi and coworkers found that only 30% of the MCI patients in a memory clinic were purely amnesic [6]. If detailed neuropsychological testing is performed, the majority of MCI patients defined by the original amnestic MCI criteria will have deficits in cognitive domains other than memory.

The numerous studies of cognitively impaired, non-demented older subjects, revealed a complex picture. Besides the purely amnesic MCI patients and the amnesic MCI patients with other cognitive deficits, researchers also found subjects with cognitive impairments in non-amnesic abilities whose episodic memory was unimpaired [10].

In 2003, the International Working Group on Mild Cognitive Impairment, in a symposium held in Stockholm, discussed the most recent issues on MCI criteria, management and characteristics. According to consensus reached, the recommendations for the general MCI criteria are as follows [11]:

a. the person is neither normal nor demented;
b. there is evidence of cognitive deterioration shown by either objectively measured decline over time and/or subjective report of decline by self and/or informant in conjunction with objective cognitive deficits;
c. basic activities of daily living are preserved and instrumental activities are either intact or minimally impaired.

After a MCI diagnosis, the clinician can further classify the patient in one of the MCI subtypes according to the clinical and cognitive profile [11]. For this classification, a comprehensive neuropsychological assessment is necessary, although no specific instruments were recommended. Clinical subtypes of MCI have thus been proposed to include subjects with cognitive impairments in areas other than memory, who are also not demented. Two primary clinical subtypes of MCI were proposed: amnestic-MCI and non-amnestic-MCI. Each subtype can be further subdivided in multiple domains or single domain subtypes according to the neuropsychological profile. The amnestic-MCI concept corresponds to the original Petersen´s MCI definition of patients with a primary memory impairment purely isolated (single domain) or with other cognitive impairments (multiple domains) [3].

PREVALENCE OF MCI

The prevalence of the MCI syndrome varies between studies as a result of differences in the diagnostic criteria concerning the sample recruitment and the neuropsychological assessment tools. Hence, for MCI a range of different values can be found in the literature: 3.2 % for subjects over 59 years old [12], 6% in the Cardiovascular Health Study (CHS) Cognition Study [13] and 15% in people over 74 years old [14]. This disparity occurs mainly on population based studies using different diagnostic criteria. However, an effort made in several studies to use the same MCI criteria, operationalized in a similar way, resulted in a more homogeneous picture. Thus, the prevalence values estimated for MCI defined using Mayo Clinic criteria have been consistent over different studies and populations: 3.2 % in the abovementioned population-based French study [12] and in the Italian Longitudinal Study on Aging [15] and 3.1% in the Leipzig Longitudinal Study of the Aged [16]. Recently, Artero, Petersen, Touchon and Ritchie [17], in a population-based study, used the Stockholm revised criteria for MCI diagnosis [11] and found a MCI prevalence of 16.6%.

In clinical settings, the prevalence is expected to be higher than in population-based studies. In one of the few referral-based samples studies reporting MCI frequency, Wahlund, Pihlstrand and Jönhagen [18] investigated the occurrence of MCI in a population referred to a memory clinic during one year, and found that as much as 37% of the patients examined for memory complaints had MCI.

PROGNOSIS OF MCI

Several studies have shown increased rates of progression to dementia in MCI subjects compared to subjects without cognitive impairment. Progression rates to Alzheimer's disease

(AD) of 10% to 15% per year have been found for MCI patients in clinical settings [3], whereas the progression rates observed in a normal population with the same age are between 1 and 2% [19]. In the aforementioned study using the Stockholm revised criteria, the authors found a better prediction of conversion to dementia than with the previous Petersen's criteria [17].

Knowledge of the prevalence values and rates of progression to dementia for the different MCI subtypes is currently an important topic of research. Results from a cohort of consecutive patients referred to a Dutch memory clinic showed no differences in the 10 years risk of dementia for the MCI pure amnestic type compared with the amnestic multiple domain MCI patients [20]. In contrast, Alexopoulos, Grimmer, Perneczky, Domes and Kurz [21] found almost twofold higher rates of conversion for the amnestic multiple domain MCI than for those with pure amnestic MCI.

As recently pointed out, progression is probably not linear as has been assumed in the majority of the studies. In the Leipzig Longitudinal Study of the Aged, Busse, Angermeyer and Riedel-Heller [22] found that the progression from MCI to dementia was time dependent, being higher during the first 18 months (about 20% annual rate) and then progressively reduced to a 10% annual rate of conversion.

MEMORY DISTURBANCES IN MCI

As previously mentioned, the diagnostic criteria of amnestic-MCI include the presence of an objective memory impairment, and the detection of this impairment requires a neuropsychological assessment of memory. Episodic memory tests for delayed recall of verbal material were found to be a sensitive and selective measure for distinguishing normal aging from early AD [23]. Therefore, verbal learning and recall tests are commonly used to assess episodic memory when a suspicion of MCI exists. There are many verbal learning tests and two of the most common categories, stories and word lists, were recommended for MCI assessment [11]. Stories, like the Logical Memory subtest of the Wechsler Memory Scale (WMS) [24], are small narratives (with around 20 items or ideas), read out loud by the examiner, and which the subject is meant to recall immediately after the reading. In most protocols, after an interference period varying from 15 to 30 minutes, the subject is requested to recall the story once again (delayed recall).

Word lists are also frequent tests of verbal learning. Larger lists with a number of words exceeding the capacity of immediate memory are called supraspan lists. Word lists, usually with 10 to 16 items, are read and then recalled through several trials to characterize the learning process. Both the Rey Auditory Verbal Learning Test (RAVLT) [25] and the California Verbal Learning Test (CVLT) [26], use supraspan word lists. The CVLT is a five trial shopping list learning test with immediate and delayed recall, both free and semantically cued. The list consists of 16 words from four semantically distinct categories arranged in a way such that adjacent words on the list are from different categories. The semantic structure of the CVLT list allows for the assessment of learning strategies that would not be possible with lists of unrelated words [27]. The CVLT also includes a recognition task at the end of the protocol.

Deficits in any of the memory processes, encoding, consolidation and retrieval can be responsible for poor delayed recall of learned material. The contribution of these processes to the observed deficit is still being investigated [28]. In order to characterize the MCI memory profile, we used the California Verbal Learning Test [26] in a recent study [29]. Learning across the 5 trials, A1 through A5, was compared among the 3 groups using ANOVA for repeated measures. MCI patients scored lower than controls, and higher than AD patients. To detail the learning difficulties we drew learning curves. Our data were best fitted by a quadratic model $Y=A+Bx+Cx^2$ ($r^2>0.98$ for the 3 learning curves of controls, MCI and AD subjects). In this equation, the coefficient B represents the rate of acquisition and the coefficient C the rate of deceleration of learning [30]. Curve estimated parameters showed differences among the 3 groups for the B coefficient (mutually exclusive 95% CIs), with control subjects learning faster than MCI patients and these faster than AD patients. For the C coefficient significant differences were found between control and the 2 patient groups. The greater deceleration of learning seen in the controls suggests that this group reaches a maximum list learning capacity faster than the patients groups [29]. This results are in agreement with the ones by Greenaway *et al*., who also found that MCI subjects had CVLT learning scores in an intermediate position between normal controls and AD patients [31].

Taking advantage of the semantic structure of the material to learn, when this structure does exist, is an automatic behaviour in healthy subjects [32]. The use of the semantic structure of the list during the learning process is revealed by the analysis of the semantic clusters. Although MCI patients could use some clustering, they employed less semantic clustering strategies than the control group [29]. MCI spontaneous use of those strategies was low, but if attention was called to the semantic structure of the list, the patients could make use of it on subsequent trials. The observation that California Verbal Learning test scores and indices in MCI patients were generally intermediate between the values in controls and in patients with AD is consistent with the notion that amnestic MCI may correspond to a very initial phase of AD [33,34]. A recent study, with an experimental memory battery using two lists, one with semantically related words and the other with unrelated ones, also revealed MCI deficits in list learning and ineffective use of the list semantic structure when it existed [35].

Memory difficulties in MCI subjects are not confined to episodic memory. Deficits in semantic memory were also detected. Semantic knowledge of famous people was found to be impaired in MCI patients whether it was assessed with naming or with recognition tasks [36,37]. Since the first research studies in MCI patients [3], non-verbal memory impairments, measured with the visual reproduction task from the WMS, were recognized as part of the MCI neuropsychological profile. In addition, low scores in a visual memory test were associated with high risk of developing AD later in life [38]. The importance of using verbal and non verbal tasks was further emphasized by the work from Alladi and coworkers [6]. They used one verbal memory test (RAVLT) [25] and two visual memory tests, the Rey complex figure test [39] and a modified version of the Paired Associates Learning (PAL) test [40] to assess episodic memory in a sample of 166 subjects with memory complaints. They found that 43% of the subjects fulfilled amnestic MCI criteria using the verbal memory score to document abnormal memory function. Interestingly, when examining all the memory tests, they found that 18 subjects with normal scores on the verbal memory test showed impaired performances on non verbal tests. The precise implications of these data can only be known

by following up these subjects, but it appears evident that a significant number of cognitively impaired subjects could be left undiagnosed if only verbal tests were used [6].

Episodic memory assessment can be done both with recall or recognition tasks. Free delayed recall of newly acquired information is known as a sensitive measure of the memory deficits typical of AD and MCI. However, free recall is also a demanding task in terms of executive resources. Deficits in executive resources can be responsible for recall impairments exaggerating an existent memory impairment. Executive deficits have already been described in MCI patients [41], and were considered useful in predicting those who will progress to AD [42]. Cued recall and recognition tasks facilitate retrieval of learned information minimizing eventual executive deficits, thus allowing for a easier assessment of memory abilities in MCI patients. A new cued recall test, the RI48, is similar to the Selective Reminding Test [43] but includes four times more items to minimize ceiling effects. The diagnostic validity of this test for MCI and dementia was determined in a prospective, longitudinal study performed in a clinical setting. The RI48 showed good diagnostic validity for MCI and was also a good predictor of the MCI patients outcome, demonstrating the utility of a cued recall test in MCI diagnosis [44]. Recognition tasks tend to be even less demanding than cued recall ones and can also be successfully applied to discriminate between MCI patients and normal subjects in verbal and nonverbal tests [45,46]. The use of these cued recall and recognition tasks may be important if we want to minimize the influence of possible executive deficits on episodic memory test scores in MCI patients.

Most MCI patients spontaneously complain about their memory failures. These complaints are often related to everyday tasks such as remembering appointments, remembering where they left their belongings, learning and remembering other people's names and finding their way in new locations. The Rivermead Behavioral Memory Test (RBMT) [47] was developed to provide measures related to the effects of memory loss in those everyday tasks involving memory. Everyday memory, assessed with the RBMT, was impaired in MCI patients and the sensitivity and specificity of the total score were high enough to differentiate MCI patients from normal controls [48]. More than 90% of the MCI patients failed in RBMT tasks of prospective memory such as remembering to ask for a hidden belonging, to ask about an appointment and to deliver a message [48]. Everyday memory tests seem promising as tools for detecting early memory changes with an impact on functionality in MCI patients.

Other memory domains in which further research will help us understanding MCI neuropsychological profile are nondeclarative memories such as priming and emotional memory. The relevance of examining memory deficits in areas other than declarative memory, by using verbal and non-verbal priming tests, was already pointed out in an extensive study of the memory profile of MCI patients [35].

IMPLICATIONS FOR COGNITIVE REHABILITATION

Detailed knowledge of the memory processes disturbed in MCI should contribute to the understanding of the pathophysiology of MCI, allow a more precise identification of patients with high probability of progression, and help to delineate future rehabilitation interventions in these patients.

A preliminary study on the efficacy of cognitive rehabilitation on patients with MCI was reported, but this study enrolled a small number of subjects, and did not define a priori efficacy variables [49]. The Cochrane Collaboration produced a review, selecting 6 studies that focused on the use of cognitive interventions in early stages of Alzheimer's disease, however, none of these studies specifically included MCI patients or designed an individualized approach to cognitive rehabilitation [50]. Recently, a cognitive intervention program focusing on teaching episodic memory strategies showed improvement of memory performance in patients with MCI [51].

It will be important to gather more scientific evidence about the efficacy of cognitive rehabilitation in patients with MCI. If cognitive rehabilitation improves cognitive performance, and particularly the functional abilities of patients with MCI, this may help keeping old subjects with memory complaints longer in the community, thus having a great impact for the patients and the society.

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In: Alzheimer's Disease Diagnosis and Treatments ISBN: 978-1-61122-064-3
Editor: Marisa R. Boyd

Chapter 9

MITOCHONDRIAL PATHOLOGY AND ALZHEIMER'S DISEASE

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ABSTRACT

There is substantial evidence of morphological, biochemical and molecular abnormalities in mitochondria of patients with neurodegenerative disorders, including Alzheimer's disease (AD). The functions and properties of mitochondria might render subsets of selectively vulnerable neurons intrinsically susceptible to cellular aging and stress. However, the question "is mitochondrial dysfunction a necessary step in neurodegeneration?" is still unanswered.

This chapter presents how malfunctioning mitochondria might contribute to neuronal death in AD. Moreover, we will investigate the cause and effect relationships between mitochondria and the pathological mechanisms thought to be involved in the disease.

Keywords: mitochondria, Alzheimer's disease, mtDNA.

INTRODUCTION

Alzheimer's disease (AD) is a late-onset, progressive, age-dependent neurodegenerative disorder, characterized clinically by progressive memory impairment, disordered cognitive function, altered behaviour, and a progressive decline in language function (Selkoe, 2001). Its pathological hallmarks are the presence of intracellular neurofibrillary tangles and extracellular beta amyloid (Aβ) plaques, a loss of neuronal subpopulations, synaptophysin

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immunoreactivity of presynaptic terminals, cholinergic fibers, and the proliferation of reactive astrocytes and microglia (Tanzi and Bertram, 2001).

AD occurs in both familial and sporadic forms. Familial AD can be caused by mutations in the Aβ protein precursor (*APP*) gene and the presenilin 1 and presenilin 2 genes, likely involved with APP processing, usually causing early-onset dementia with plaque formation (Goate et al, 1991; Levy-Lahad et al, 1995; Finch and Tanzi, 1997).

The main plaques component is the Aβ peptide that derived from the membrane bound APP which can be processed via two distinct processing pathways: the amyloidogenic pathway that liberates the Aβ peptide and the non-amyloidogenic pathway which precludes the formation of Aβ and instead generates a secreted form of APP, sAPPα. In the amyloid cascade hypothesis a dysregulation in APP processing results an increased production of the longer amyloid peptide Aβ1–42, which induces plaques core formation, tau aggregation and phosphorylation. How Aβ does induce the neurodegeneration is not clear (Chapman et al, 2001).

The causes of the much more frequently occurring sporadic AD (SAD) remain unknown: patients with SAD generally lack mutations of APP gene, presenilin 1 and presenilin 2; therefore, it is unclear what initiates plaque formation in such cases. Furthermore, plaques are a relatively common finding in the non-demented elderly (Davis et al, 1999; Snowdon, 2003). The major risk factor in SAD is the Apolipoprotein E genotype. Several studies reported that patients with the E4 allele are associated with an increased risk of developing SAD (Poirier et al, 1993; Roses, 1996; Schmechel et al, 1993). The mechanisms leading to neuronal death are still unclear. The Aβ cascade hypothesis remains the main pathogenetic model of AD (Mudher and Lovestone, 2000), however its role in the SAD is unclear (Swerdlow and Khan, 2004).

Several studies suggest that abnormalities in oxidative metabolism and specifically in mitochondria may play an important role in late-onset neurodegenerative disorders including, AD (Blass and Gibson, 1991; Beal, 2005).

This article focuses on the role of mitochondria and its metabolism in AD, and reviews some of the recent relevant genetic and biochemical data.

MITOCHONDRIA COMPARTMENT

Mitochondria are highly dynamic and pleomorphic organelles. They are composed of a smooth outer membrane surrounding an inner membrane of significantly larger surface area that, in turn, surrounds a protein-rich core, the matrix (Logan, 2007). Although mitochondria contain their own genome and protein synthesizing machinery (Leaver et al., 1983; Unseld et al., 1997; Gray et al., 1999), the majority of mitochondrial polypeptides are encoded in the nuclear genome, synthesized in the cytosol and imported into the mitochondria post-transcriptionally (Unseld et al., 1997; Whelan and Glaser, 1997; Duby and Boutry, 2002).

Human mitochondrial DNA (mtDNA) is a 16,569-kb circular, double-stranded molecule, which contains 37 genes: 2 rRNA genes, 22 tRNA genes, and 13 structural genes encoding subunits of the mitochondrial respiratory chain (DiMauro and Schon, 2003).

The main mitochondria role is the synthesis of ATP formed by oxidative phosphorylation (Saraste, 1999) but they are involved in other metabolic processes including the biosynthesis

of amino acids, vitamin cofactors, fatty acids, iron-sulphur clusters (Bowsher and Tobin, 2001), cell signalling (Logan and Knight, 2003) and programmed cell death (Youle and Karbowski, 2005).

ATP molecules are generated via glycolysis or by oxidation of glucose to ethanol or lactic acid. Electrons from oxidative substrates are transferred to oxygen, via a series of redox reactions, to generate water (Elston et al, 1998). In the process, protons are pumped from the matrix across the mitochondrial inner membrane through the electron transport chain (ETC), which consists of four multimeric complexes -I to IV- plus two small electron carriers, coenzyme Q -or ubiquinone- and cytochrome *c* (figure 1). This process creates an electrochemical proton gradient, which is utilized by complex V (ATP-synthase), which generates ATP flowing back as protons into the matrix (Noji and Yoshida, 2001).

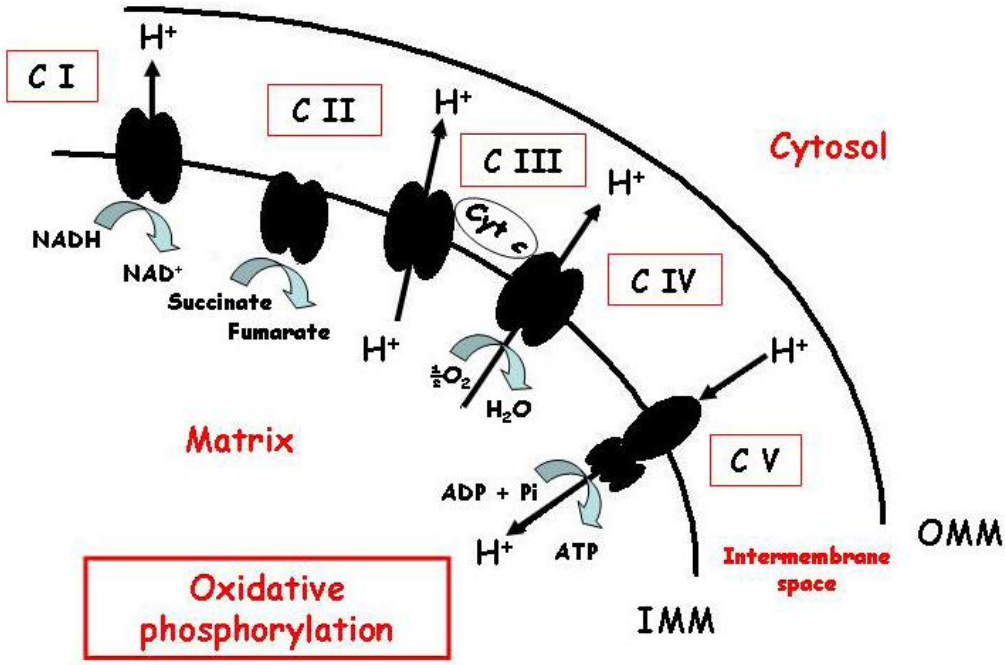

Figure 1. Cartoon of the mitochondrial respiratory chain. C= respiratory complex. IMM= inner mitochondrial membrane; OMM= outer mitochondrial membrane.

MITOCHONDRIA ARE MORPHOLOGICALLY ABNORMAL IN AD

Morphological and ultrastructural alterations in neuronal mitochondria in AD have been reported.

Several studies on the morphological and morphometric estimation of mitochondria in Alzheimer's disease, by electron microscopy, showed substantial morphological and morphometric changes in the neurons of the hippocampus, the acoustic cortex, the frontal cortex, the cerebellar cortex, the climbing fibers, the thalamus, the globus pallidus, the red nucleus and the locus coeruleus. The morphological alterations consisted of considerable changes of the mitochondrial cristae, accumulation of osmiophilic material, and decrease of

their size, in comparison with the normal controls. The majority of the mitochondria, small, round, or elongated, presented disruption of the cristae or osmiophilic inclusions (Baloyannis et al, 2004; Baloyannis, 2006).

Stewart and collegues (1992) in a morphometric studies of the mitochondria in AD revealed a significant reduction in mitochondria density in endothelial cells as well as in fibroblasts obtained from patients with AD. Furthermore, apparently normal dendrites from the frontal cortex of seven patients with AD showed mitochondria with increased-density matrices and paracrystalline inclusions in the intercristal space (Lovell et al, 1999).

MITOCHONDRIAL DYSFUNCTION IN AD

Several studies indicates that abnormalities of cerebral metabolism are common in neurodegenerative disease, including AD (Gibson et al, 2000). Decreased glucose metabolism in AD precedes clinical diagnosis and correlates closely with the clinical state (Minoshima et al 1997). The decline in the Mini-Mental State Examination scores in AD correlated highly to reductions in glucose metabolism as measured by positron emission tomography in the temporoparietal, frontal and occipital cortices (Mielke et al, 1994). This suggests that the clinical deterioration and metabolic impairment in AD are related closely.

Since mitochondria are the powerhouse of all cells, damage to mitochondria will inevitably impair energy metabolism. Measurement of mitochondrial enzyme activities indicate inherent damage to mitochondria in AD brain (Perry et al, 1980; Sorbi et al, 1983; Mastrogiacomo et al, 1994; Simonian and Hyman, 1994). In brain the main pathway for oxidation of glucose is the tricarboxylic acid (TCA) cycle (the Krebs' cycle), which takes place in the mitochondria. The oxidative decarboxylation of pyruvate, the product of glycolysis, by the pyruvate dehydrogenase complex (PDHC) provides acetyl CoA to initiate the TCA cycle, which includes eight different enzymes. Deficiency of two cycle TCA key enzyme has been documented in AD, suggesting defects in glucose metabolism in the AD brains (Elson et al, 2006; Sorbi et al, 1993): the pyruvate dehidrogenase complex (PDHC) and the α-Ketoglutarate dehydrogenase complex (KGDHC) (Bubber et al, 2005). PDHC catalyzes the reaction by which pyruvate, the product of glycolysis is converted to acetyl-CoA which then enters the TCA cycle (Sorbi et al, 1983). KGDHC catalyzes a critical reaction within the TCA cycle: the oxidation of α-Ketoglutarate to succinyl-CoA (Gibson et al 1988). KGDHC is also an important enzyme in glutamate metabolism; α-Ketoglutarate is readily interconvert with glutamic acid by transamination and is the product of glutamate oxidation by the glutamate dehydrogenase catalysed reaction (Blass, 1997). KGDHC activity is reduced in AD brain, in both histopathologically affected and unaffected areas (Gibson et al, 1988; Mastrogiacomo and Kish, 1994; Butterworth and Besnard, 1990). Activity of KGDHC has also been found to be reduced in cultured skin fibroblasts from SAD patients (Blass et al, 1997a) and in some (Sheu et al, 1994) but not all (Blass et al, 1997a) patients with presenilin-1 mutations.

Finally, these enzymes can also change secondarily to other pathologic events in AD, including oxidative stress. Evidence suggest that this secondary reduction may be part of critical cascade of events that lead to neurodegeneration (Gibson et al, 1998).

MITOCHONDRIAL RESPIRATORY CHAIN ENZYMES IN AD PATIENTS

Abnormal mitochondrial function in AD was first shown in 1992, when Kish et al demonstrated a marked reduction (-50%) of cytochrome oxidase activity (COX; complex IV) in platelets of AD patients. Several authors also evidenced a decrease of COX activity in different brain regions (Maurer et al, 2000; Mutsya et al, 1994; Simonian and Hyman et al 1994) as well as in platelets (Parker et al, 1990) and fibroblasts (Curti et al, 1997) of AD patients. Cardoso and collaborators (1997) found that COX activity is reduced in platelets of both early and early-onset SAD subjects, and that this defect is associated with increased reactive oxygen species (ROS) generation and ATP depletion. No differences were found in platelet membrane fluidity, suggesting that the impaired COX activity is not driven through peroxidation of the mitochondrial inner membrane. Moreover, our group evidenced a significantly increased blood resting levels of lactate in AD and a decreased of COX but not of F1F0-ATPase activity in hippocampus and platelets of sporadic AD cases (Mancuso et al 2003; Bosetti et al, 2002), with dysfunction of energy metabolism (Bosetti et al, 2002), particularly at the synapse level, where high metabolic activity is present (Cassarino and Bennett, 1999).

OXIDATIVE STRESS RELATED TO MITOCHONDRIAL DYSFUNCTION IN AD

Oxidative stress has been widely implicated in AD pathogenesis. The hypothesis of a relationship between oxidative stress and AD originally derived from the free radical hypothesis of aging, which speculates that the age-related accumulation of ROS could be responsible of damage to major cell components (Beal et al, 1995). ROS increase is consequent to mitochondrial dysfunction with age and mitochondrial dysfunction results to be accelerated in AD patients (figure 2). Environmental, metabolic, dietary, and/or genetic factors could also contribute to mitochondrial dysfunction in AD, with consequent energy metabolism impairment, oxidative stress and cell apoptosis (Atamna and Frey, 2007; Armstrong, 2006).

Praticò and Delanty (2000) evidenced that, in animal models of AD, oxidative damage occurs before Aβ peptide deposition and plaque formation. Autopsied brain tissue and peripheral cells, as fibroblasts, of AD patients presented evident signs of impaired energy metabolism and abnormalities of mitochondrial respiration (Bader Lange et al, 2007; Naderi et al, 2006).

Studies on AD patients' brain demonstrated elevated levels of protein carbonyls and nitration of tyrosine residues, markers of oxidised proteins (Butterfield and Stadtman, 1997).

An increase of malondialdehyde, 4-hydroxy-2-trans-nonenal and isoprostanes, markers of lipid peroxidation, was also showed (Butterfield and Lauderback, 2002), as well as the presence of 8-_hydroxy-2-deoxyguanosine (8OHdG) and 8-hydroxyguanosine (8OHG), substances derived from DNA/RNA oxidation (Lovell and Markesbery, 2001).

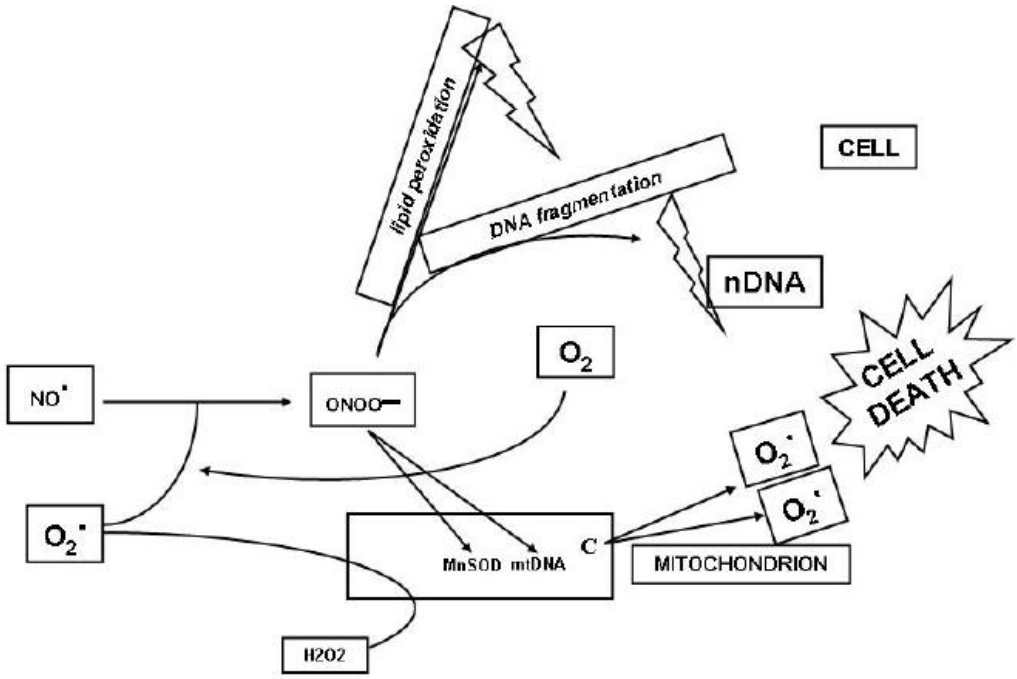

Figure 2. Mitochondrion, cell and oxidative stress. The cartoon represents a call under the attack of oxidative stress. NO•: nitric oxide; O2•: superoxide anion; ONOO•: peroxynitrite anion; MnSOD: Manganese-Superoxide-Dismutase; H2O2: hydrogen peroxide; mtDNA: mitochondrial DNA; O2: Oxygen; nDNA: nuclear DNA.

THE "CYBRID MODEL" OF AD

To define the origin of bioenergetic deficits in AD, particularly the deficient COX activity, in 1989 King and Attardi used exogenous mitochondria to repopulate two human cell lines (termed rho 0), which had been completely depleted of mtDNA. Transformants obtained with various mitochondrial donors exhibited a respiratory phenotype that was in most cases distinct from that of the rho 0 parent or the donor, indicating that the genotypes of the mitochondrial and nuclear genomes as well as their specific interactions play a role in the respiratory competence of a cell. Phenotypic differences among cybrid lines therefore derive from amplification of donor mtDNA and not from nuclear or environmental factors.

Swerdlow and co-workers (1997) developed a tissue culture system by which demonstrated that mitochondrial COX is defective in patients with sporadic AD. They depleted Ntera2/D1 (NT2) teratocarcinoma cells of endogenous mtDNA and repopulated them with platelet mtDNA from AD patients. COX activity was depressed in the resulting AD cytoplasmic hybrids (cybrids) compared with cybrids prepared with mtDNA from non-AD controls. ROS production and free radical scavenging enzyme activities were significantly elevated in AD cybrids.

Studies on cybrid cells made from mitochondrial DNA of non familial AD showed abnormalities of mitochondrial membrane potential, increased secretion of Aβ (1-40) and Aβ (1-42), increased intracellular Aβ (1-40), congo red-positive Aβ deposits, elevated

cytoplasmic cytochrome *c* and caspase-3 activities. The increased secretion of Aβ (1-40) in AD cybrid was normalized by inhibition of caspase-3 or secretase and reduced by treatment with the antioxidant S (-) pramipexole. Expression of AD mitochondrial genes in cybrid cells depresses COX activity and increases oxidative stress. Under stress, cells with AD mitochondrial genes are more likely to activate cell death pathways, which drive caspase 3-mediated Aβ peptide secretion and may account for increased Aβ deposition in the AD brain (Khan et al, 2000).

Trimmer et al (2004) demonstrated that cybrid cells derived from SAD, after specific metabolic selection, presented an increase of mitochondrial number, a reduction of mitochondrial size and an increase in morphologically abnormal mitochondria. They also evidenced an increase of mtDNA replication with relative worsening of bioenergetic function.

Finally, the cybrid models also permitted to detect a reduction of mitochondrial membrane potential variation (Thiffault and Bennett 2005) and a significantly reduction of mean velocity of mitochondrial movement, as well as of the percentage of moving mitochondria. The velocity of lysosomal movement was also reduced, suggesting that the axonal transport machinery is impaired in AD cybrid cells (Trimmer and Borland, 2005).

MITOCHONDRIAL GENETICS AND AD

Studies on cybrid cells supported the hypotheses that mtDNA gene(s) play a role in sporadic AD pathology. However no causative mtDNA mutations have been discovered in AD patients so far.

By analysing 321 very old subjects (>90-yrs old) and 489 middle-aged controls from Finland and Japan, Niemi and co-workers (2005) found that specific mtDNA polymorphisms were more frequent among the very old than among controls.

Zhang et al (2003) reported that a homoplasmic C150T transition occurs at a much higher frequency in leukocytes from centenarians and from twins than in leukocytes from the rest of the population. Evidence was obtained that this mutation causes a remodeling of the replication origin at position 151, and that both maternal inheritance and somatic events play a role in this phenomenon. However, no relationship of its occurrence to aging, longevity, or twin gestations has been reported.

Michikawa et al (1999) showed the presence of progressive damage to mtDNA during life, with high copy point mutations at specific positions in the control region for replication of human fibroblast mtDNA from normal old, but not young, individuals. In particularly, a heteroplasmic T414G transversion was found in high proportion in over 65-yrs old individuals, and absent in younger individuals.

Wang et al (2001) analysed skeletal muscle specimens of individuals affected by polyneuropathy, with no history of neuromuscular diseases; they have observed that older individuals exhibited at mtDNA replication control two age-related mtDNA point mutations, A189G and T408A, absent or marginally present in young people. The authors postulated that these changes could lead to impaired energy generation and to increased ROS production, with secondary cell damage and death.

Elson et al (2006) evidenced an increase of somatic mtDNA re-arrangements in AD brains. For instance, the mtDNA "common deletion" has been observed to be elevated about 15-fold in AD brains compared to controls.

Polymorphisms in mtDNA may cause subtle differences in the encoded proteins, in OXPHOS activity and in free radical production. Some polymorphism could predispose to an earlier onset of apoptotic processes or could be beneficial increasing OXPHOS activity and/or reducing ROS production. Common mtDNA polymorphisms determine classes of continent-specific genotypes, haplogroups, (H,I,J,K,T,U,V,W,X). For instance, 150T is a polymorphism in the mtDNA control region of individuals from Europe, Asia, and Africa (Mitomap, 2002) and it have been associated with longevity. Coskun et al (2004) investigated mtDNA obtained from AD brains and discovered that many patients harboured the T414G mutation, whereas this mutation was absent in controls. They also evidenced that AD patients presented an increase in heteroplasmic mtDNA mutations, preferentially in regulatory elements, and a reduction in the mtDNA transcripts and in the mtDNA-nuclear DNA ratio.

Other genetic studies focused their attention on allele ε4 of the nuclear APOE gene, a genetic risk factor for sporadic AD, and tried to evidence all the interactions between APOE and mtDNA mutations. In this contest, Carrieri et al (2001) hypothesized an interaction between APOE polymorphism and mtDNA inherited variability in the genetic susceptibility to SAD. They analyzed mtDNA haplogroups in a sample of AD patients genotyped for APOE and classified as APOE ε4 carriers and non-carriers and found that the frequency distribution of mtDNA haplogroups is different between ε4 carriers and non-carriers. The same analysis, carried out in two samples of healthy subjects showed independence between ε4 allele and mtDNA haplogroups. Therefore, the APOE/mtDNA interaction is restricted to AD and may affect susceptibility to the disease. In particular, some mtDNA haplogroups (K and U) seem to neutralize the harmful effect of the APOE ε4 allele.

Chinnery et al (2000) analyzed the relationship between APOE genotype and mtDNA sequence variants in AD and in Lewy bodies dementia (DLB) as compared to control subjects. They did not evidenced increased risk of AD in patients with specific mtDNA haplogroups or with A4336G mutation, while they showed an over-expression of mtDNA haplogroup H in the DLB patients.

Van der Walt et al (2004) demonstrated that males classified as haplogroup U showed an increase in AD risk as compared to the most common haplogroup H, while females demonstrated a significant decrease in risk with haplogroup U. These results were also independent of APOE genotype by suggesting that mtDNA associated with environmental exposures or nuclear proteins, different from APOE, could be involved in AD expression.

Other works did not evidenced any relationship between mtDNA and sporadic AD. For instance, Chinnery et al (2001) found no evidence of somatic mtDNA point mutations accumulate in the brains of normal elderly individuals or in the brains of individuals with neurodegenerative disease, as AD and DLB. Furthermore, our group also excluded any association between mtDNA haplogroups and AD and all kind of correlation between mtDNA haplogroups and gender, or between mtDNA haplogroups, ApoE alleles and clinical features (Mancuso et al, 2007).

In conclusion, all the recent studies fail to be an incentive to understand the importance of haplogroups and/or mtDNA point mutations in sporadic AD.

CONCLUSION

As reported in the previous paragraphs, mitochondrial impairment, oxidative stress and energy failure are common findings in AD pathology. Indeed, Aβ causes mtDNA damage, impairment of the mitochondrial respiration, and oxidative stress, in a vicious manner. The result is the activation of the mitochondrial permeability transition pore, the release of cytochrome *c*, and the induction of caspase-mediated apoptosis. These data suggest a strong link between primary mitochondrial respiratory chain defect and Aβ peptide. Amyloid plaques characteristic of AD consist of extracellular aggregates of the toxic Aβ peptide, but amyloid species may exert toxicity from within the cell. Oddo and collegues (2003) observed early intracellular aggregates of Aβ in mice overexpressing APP and that the formation of these aggregates correlate with cognitive impairment (Oddo et al, 2003). How intracellular aggregates of Aβ might cause cellular dysfunction remains unclear. However, Anandatheerthavarada and collaborators (2003) linked amyloid to the mitochondrion, which at that time was not yet widely recognized as a site of amyloid accumulation or toxicity. This study, conducted in transgenic mouse model for AD, showed that APP, by virtue of its chimeric NH2-terminal signal, is targeted to neuronal mitochondria, under some physiologic and pathologic conditions and that the mitochondrial APP exists in NH2-terminal inside transmembrane orientation and in contact with mitochondrial translocase proteins. This link causes the transmembrane arrest and consequent mitochondrial dysfunction with reduced COX activity, decreased ATP synthesis, and loss of the mitochondrial membrane potential. Recently, experiments from the same group (Devi et al, 2007) showed that nonglycosylated full-length and C-terminally–truncated APP was associated with mitochondria in samples from the brains of individuals with AD, but not with mitochondria in samples from non demented subjects and they confirmed that APP forms complexes with mitochondrial inner and outer membrane translocases (TOM40 and TIM23) in AD with consequent mitochondrial dysfunction. They concluded that the abnormal accumulation of APP across mitochondrial import channels, causing mitochondrial dysfunction, is a hallmark of human AD pathology.

Mitochondria are now at the center stage in human neurodegenerative diseases and ageing. In AD, mitochondria, APP, and Aβ metabolism might be interconnected in the cascade, leading to neurodegeneration and dementia. Their role in those processes is much more "realistic" than believed in the past years, and we should look forward to exciting developments in this field during the coming years. It will be important to develop a better understanding of the role of mitochondrial energy metabolism in AD, and its link with the amyloid hypothesis in aging and AD, since it may lead to the development of effective treatment strategies.

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In: Alzheimer's Disease Diagnosis and Treatments ISBN: 978-1-61122-064-3
Editor: Marisa R. Boyd

Chapter 10

CALMODULIN BINDS TO AND REGULATES THE ACTIVITY OF BETA-SECRETASE (BACE1)

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ABSTRACT

The improper regulation of calcium levels in neurons is proposed as a primary regulatory impairment that underlies the onset of Alzheimer's Disease (AD). Calmodulin is a primary target of calcium ions in all human cells but has essentially been ignored as a downstream target in the onset of AD. Our lab previously has theoretically implicated calmodulin as an interacting protein for of a number of upstream proteins involved in the production of amyloid-beta peptide (Aβ), a pathogenic marker of Alzheimer's disease (AD) and the primary element of the "amyloid hypothesis" (O'Day and Myre, 2004. Biochem. Biophys. Res. Commun 320: 1051-1054). The first enzyme in the proteolytic processing of amyloid precursor protein (APP1) into Aβ is β-secretase (β site-amyloid converting enzyme 1 or BACE1) which was one of the enzymes identified as a putative calmodulin-binding protein. In this study we tested the effects of calmodulin, calcium and calmodulin antagonists on the *in vitro* activity of BACE1 to determine if it is potentially regulated by calmodulin. BACE1 enzyme activity was dose-dependently increased by calmodulin reaching a maximum ~2.5-fold increase at 3μM calmodulin. Calcium (1.0mM) enhanced BACE1 activity while the calcium-chelator EGTA (10mM) inhibited it supporting a role for calcium in regulating BACE1 activity. In keeping the role of calmodulin as a regulator of BACE1 activity, five different calmodulin antagonists (trifluoperazine, W7, W5, W12, W13) each differentially inhibited BACE1 activity *in vitro*. The binding of BACE1 to calmodulin-agarose in the presence of calcium ions but not EGTA further supports the concept of BACE1 as a potential calcium-dependent calmodulin-binding protein.

Keywords: Calmodulin-binding, BACE1, enzyme regulation, trifluoperazine, W5, W7, Alzheimer's Disease.

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INTRODUCTION

Two major hypotheses based upon the types of protein aggregates in the brains of Alzheimer's patients serve to characterize the disease: the "amyloid hypothesis", based upon the presence of amyloid plaques and the "tau hypothesis", based upon neurofibrillary tangles. Amyloid plaques are fibrils of the amyloid-β peptide (Aβ) while neurofibrillary tangles are twisted filaments of hyperphosphorylated tau protein. Sequential proteolytic processing of amyloid-β precursor protein (APP) in the amyloidogenic pathway produces the small Aβ peptides. The amyloid hypothesis considers the Aβ peptide the initiator of a pathological cascade that leads to Alzheimer's disease (Hardy, 1997; Selkoe, 2001). Both genetically inherited early-onset and late-onset (usually after 60 years of age) Alzheimer's disease are characterized by similar Aβ-rich aggregates and neurofibrillary tangles in specific regions of the brain. Familial forms of Alzheimer's are caused by missense mutations in the *APP* gene, in the *presenilin 1* (*PS1*) gene, or in the *presenilin 2* (*PS2*) gene. These mutations lead to the increased production of Aβ peptides (Annaert and De Strooper, 2002). The literature is full of confusing evidence about the relationship between amyloid beta and cognitive impairment suggesting the relationship is not a simple one. However, a recent study in *Tg2576* mice has shown that memory loss is caused by the accumulation of soluble, extracellular 56kDa amyloid beta assemblies referred to as Aβ*56 (Lesne et al, 2006). When young rats are treated with Aβ*56 purified from old *Tg2576* mice their memory becomes impaired independently of neuronal loss or plaque formation. As a result, the amyloid hypothesis remains a dominant theory underlying research and the development of therapeutic strategies (De Strooper and König, 2001).

An overwhelming body of scientific literature details the importance of calcium homeostasis and signal transduction in neuronal function. Homeostatic intraneuronal levels of calcium are maintained through fluxes across the cell membrane, by uptake and release from intracellular stores (endoplasmic reticulum, mitochondria) and from various calcium-binding proteins. Neuronal stimulation leads to increases in intracellular calcium levels that, in turn, lead to controlled neurosecretion, among other things. The "Calcium Hypothesis" of Khachaturian (1989) proposed that sustained alterations to calcium homeostasis, or as LaFerla (2002) puts it "calcium dyshomeostasis", is the underlying cause of Alzheimer's Disease (AD). The accumulated evidence argues that, among other things, increased levels of intracellular calcium can drive the formation of some of the characteristic lesions of AD such as the accumulation of amyloid-β (Mattson and Chan, 2003). Long-term disruption of calcium homeostasis can lead to neuronal death (apoptosis). In keeping with this evidence exists that amyloid-β can in turn affect calcium homeostasis (Small et al, 2001). Treatment of AD patients with nitrendipine, a calcium channel antagonist, reduced the incidence of dementia by almost 50% providing further evidence for the significance of calcium in AD (Forette et al, 2002). Since calmodulin (CaM) is a primary calcium-binding protein and effector of calcium signaling, alterations in intracellular calcium levels will impact this essential protein and the targets (calmodulin binding proteins, CaMBPs) that it regulates.

Table 1. Identification and Classification of Putative Calmodulin Binding Domains in Some Proteins Linked to β-Amyloid Production in Alzheimer's Disease

[1]Calcium-Dependent Calmodulin-Binding Domains (CaMBDs)

Protein	Acc.#	Location	CaMBD
1-5-10 Motif			1 5 10
APH-1a	Q96BI3	78-97	-----VSVLLQE**V**FRF**A**YYKL**L**KKA
PSN-1	P49768	376-395	---------ERG**V**KLG**L**GDFI**F**YSVLVGK
PSN-2	101-121	101-121	----------VV**V**ATI**K**SVR**F**YTEKNGQLIY
1-14 Motif			1 14
PEN-2	Q9NZ42	8-28	NEEK**L**NLCRKYYLGGFA**F**LPF
1-5-8-14 Motif			1 5 8 14
BACE2	Q9Y5Z0	414-433	--G**F**YVI**F**DR**A**QKRVG**F**AASPC
PSN-1	P49768	376-395	ERG**V**KLG**L**GD**F**IFYSV**L**VGK

[2]Calcium-Independent Calmodulin-Binding Domains

IQ-Like Motif			IQxxx**R**xxxxxxxx
[3]APH-1a	Q96BI3	121-135	**L**QEVF**R**FAYYKLLK
[3]Nic	Q92542	427-441	**L**QRFL**R**ARNISGVV
[3]Nic	Q92542	533-547	**F**QSIL**R**QDLRSYLG

Hydrophobic amino acids are shown in bold. [1]Ca^{2+}-dependent calmodulin-binding domains (CaMBDs) identified through Calmodulin Database screening (http://calcium.uhnres.utoronto.ca/ctdb/no_flash.htm; Yap et al, 2000). [2]Putative Ca^{2+}-independent CaMBDs detected by visual motif scanning. APH-1a,b = Presenilin stabilization factor a or b; APP, Amyloid β Precursor Protein (A4); BACE, β-Secretase; Nic, Nicastrin; PEN-2 = Presenilin enhancer protein 2; PSN-1, -2 = Presenilin-1, -2. After O'Day and Myre (2004).

The mammalian brain has a large number of CaMBPs many of which remain to be identified (O'Day, 2003; O'Day et al, 2001). The presence of identified CaM-binding domains or motifs (CaMBDs) within the main proteins that lie upstream of beta-amyloid formation suggests that CaMBPs may play a key role in AD (O'Day and Myre, 2004). All of these proteins were found to possess one or more calcium-dependent CaMBDs while presenilin stabilizing factor a1 and nicastrin also have potential calcium-independent binding domains (table 1). Prior to evaluating the potential role of calmodulin in the onset of AD *in vivo*, it is critical to determine if the theoretical data is borne out and, if so, to gain some insight into agents that might be useful for *in vivo* studies and appropriate concentrations for their use. β-Secretase or BACE-1 (β-site APP-cleaving enzyme) is a type 1 transmembrane aspartyl protease that defines a subgroup of membrane-associated hydrolases associated with the pepsin family (Vassar, 2001). BACE-1 is the only protease with a well-defined β-secretase activity and BACE1 knockout mice appear to be normal. For these and other reasons, BACE1 is a primary target of research on AD. Since BACE1 is the initial enzyme upstream of beta-amyloid production and since we have identified it as a potential calmodulin binding protein, we carried out studies here on the *in vitro* regulation of BACE1 activity by calmodulin. Here we show that BACE1 activity is enhanced by calmodulin in a time and dose-dependent manner. In keeping with its potential regulation by calmodulin, BACE1

activity is inhibited by five different calmodulin antagonists and EGTA but stimulated by the presence of calcium ions. CaM-agarose binding analyses reveal that BACE1 binds in the presence of calcium but not EGTA further supporting BACE1 as a calcium-dependent CaM-binding protein.

MATERIAL AND METHODS

BACE1 Enzyme Analysis

The enzymatic assays were carried out following the instructions on the EnzoLyte™ 520 Beta-Secretase Assay Kit from AnaSpec (www.anaspec.com) using 10 units (~5 μM) of human recombinant β-Secretase (Cat. # S-4195, www.sigma-aldrich.com) and BACE1 inhibitor where appropriate. Various agents were added to the reaction mixture as appropriate to the specific experiment. Phosphodiesterase 3′,5′-cyclic nucleotide activator (Calmodulin, CaM), Calcium chloride ($CaCl_2$) and Ethylene glycol-bis β-aminoethylether-tetraacetic acid (EGTA) were from Sigma (Cat. # P-2277). Trifluoperazine dihydrochloride (TFP), N-(6-Aminohexyl)-1-naphthalenesulfonamide·HCl (W-5) and N-(6-Aminohexyl)-5-chloro-1-naphthalenesulfonamide·HCl (W-7) were from Alexis Biochemicals (www.axxora.com). Two additional naphthalenesulfonamide Ca^{2+}-calmodulin antagonists, W12 (0.4mM) and W13 (0.4mM) were also tested (Sigma). The assays were monitored using a Fluoromark microplate fluorometer from Bio-Rad (www.bio-rad.com) adjusted to measure wavelengths of 488 nm excitation and 520 nm emissions while keeping a constant temperature of 37° C.

CaM-Agarose Binding Assay

Ten units of human recombinant β-Secretase (~ 5 μM; Sigma Cat # S-4195) were used for the calmodulin-agarose binding assay with Calcineurin (CN, 5 μM; Sigma (Cat. # C-1907) as the positive control as described previously (Myre and O'Day, 2002). Calmodulin-agarose beads were purchased from Sigma (Cat. # 14-426). After binding 15% native gels were used: one was run in calcium containing CaM-binding buffer (20 mM Tris-HCl, pH 7.6, 100 mM KCl, 0.1 mM DTT, and 2 mm $CaCl_2$) and the other run in EGTA buffer (CaM-binding buffer lacking calcium chloride and containing 5 mM EGTA). Western blotting was carried out using rabbit anti-Human BACE1 from US Biological (1:500 dilution; Cat. # B0002-91, www.usbio.net), and peroxidase-conjugated AffiniPure Goat Anti-Rabbit IgG (H+L; 1:1000 dilution) from Cedarlane laboratories (Cat. # 111-035-045, www.cedarlanelabs.com) as detailed previously (Myre and O'Day, 2002). BioTrace PVDF membranes from PALL Gelman Laboratory and ECL Plus western blotting detection kit from Amersham Biosciences (www.amershambiosciences.com) were also used for the western blots. In all cases, a BenchMark™ prestained protein ladder from Invitrogen (www.invitrogen.com) was used to determine molecular weights.

RESULTS AND DISCUSSION

The EnzoLyte™ BACE1 Assay Kit uses a β-secretase-cleavable FRET peptide substrate that is designed for the screening of potential β-secretase inhibitors. In our hands the BACE1 assay yielded time-dependent results revealing typical enzyme kinetics in control experiments (figure 1). The company supplied β-secretase inhibitor was effective at inhibiting the enzyme as expected. The addition of calmodulin at 3μM enhanced the rate of enzyme activity over the full course of the reaction supporting the role for CaM as a regulator of BACE1 activity *in vitro* (figure 1). In keeping with this, subsequent studies showed that CaM dose-dependently enhanced BACE1 activity with an optimal increase in activity occurring at 3μM CaM (figure 2).

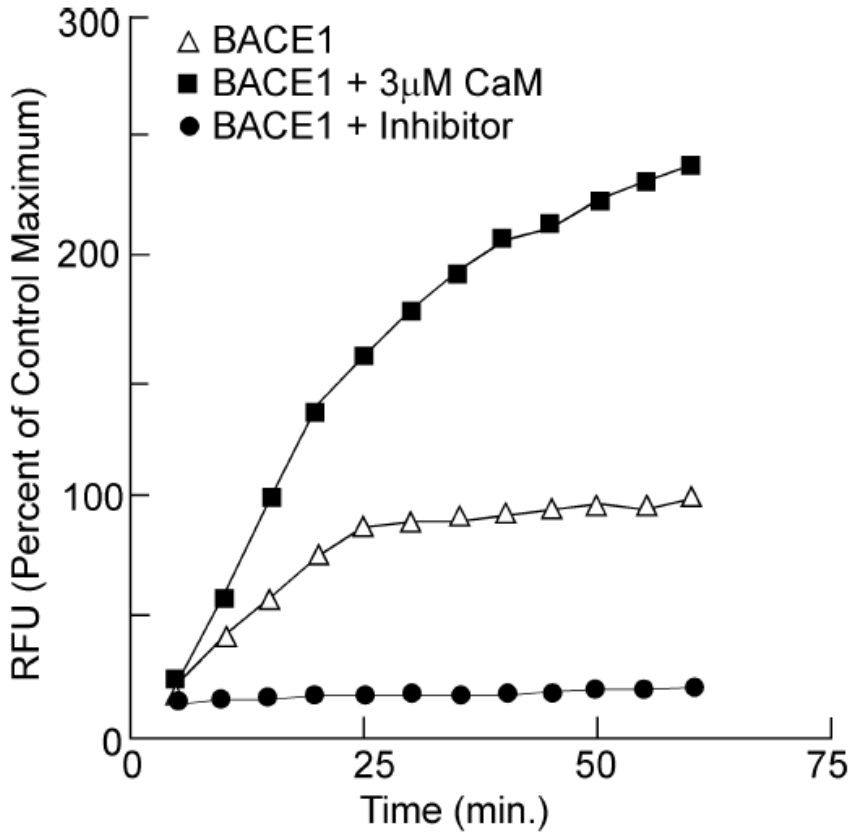

Figure 1. Enzyme kinetics for BACE1. BACE1 enzyme activity was measured using the EnzoLyte™ 520 Beta-Secretase Assay Kit from AnaSpec. The effects of added enzyme kit inhibitor and calmodulin were also tested as detailed in the Materials and Methods.

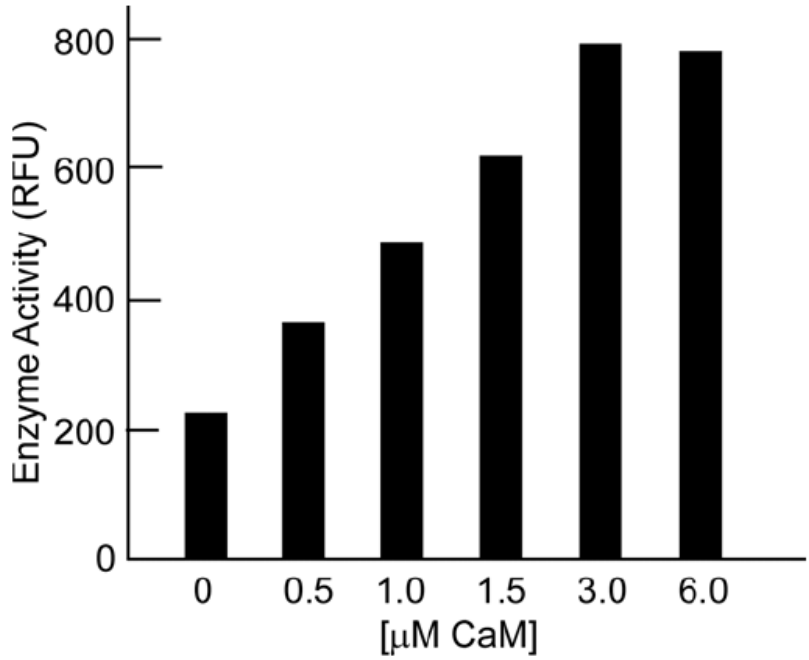

Figure 2. The effects of varying calmodulin concentration on BACE1 activity.

Calmodulin binding occurs through domains/motifs that regulate calcium-dependent or calcium independent binding (For review: Bahler and Rhoads, 2002; Hoeflich and Ikura, 2003). Our initial study, indicated that BACE1 would be a calcium-dependent calmodulin binding protein since it possesses a putative 1-16 calcium-dependent CaMBD with no evidence for a calcium-independent (IQ or IQ-like) CaMBD (O'Day and Myre, 2004; table 1). If BACE1 is regulated by CaM in a calcium-dependent manner, then removal of calcium ions should decrease the enzyme activity while addition of calcium should enhance it. In keeping with a role for calcium ions, the addition of 10mM EGTA inhibited BACE1 activity approximately 60% while the addition of 10mM calcium augmented BACE1 activity even in the absence of added CaM (figure 3, A). As before, the addition of calmodulin enhanced BACE1 activity over 2-fold but in the presence of EGTA this enhancement was abrogated in keeping with a calcium-dependent role for CaM. As a control to ensure CaM wasn't affecting BACE1 itself and not binding to the substrate, CaM was incubated alone with the substrate in the absence of BACE1. This combination indicated that there was no substrate-CaM binding that could have generated false results (CaM; figure 3, A). Finally, in the presence of the BACE1 inhibitor, CaM had minimal effect at restoring the enzyme activity indicating they likely are operating independently.

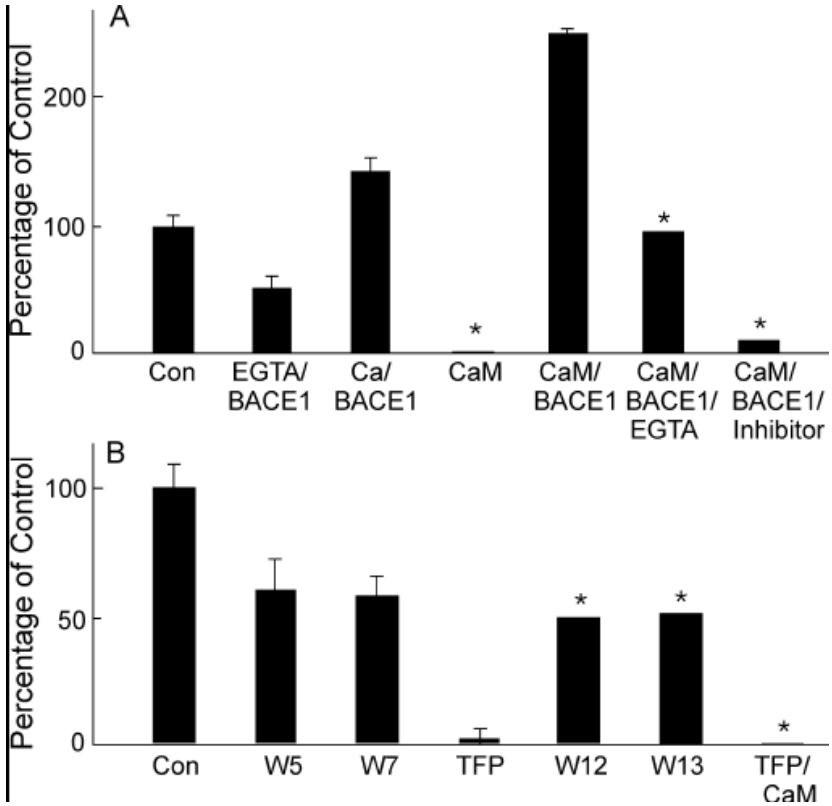

Figure 3. The effects of various agents on BACE1 activity. A. The effects on BACE1 activity of the addition of calmodulin (CaM/BACE1), calcium (10mM; Ca/BACE1) and the calcium chelator EGTA alone (10mM; EGTA/BACE1) or with calmodulin (CaM/BACE1/EGTA) were determined. As an additional control, calmodulin was incubated with the substrate in the absence of BACE1 (CaM). B. The effects of the calmodulin antagonists W5 (8μM), W7 (7μM), TFP (50μM) W12 (0.2mM) and W13 (0.2mM) on BACE1 activity. Experiments in which TFP and calmodulin were added together were also carried out (TFP/CaM). Con = control, BACE1 enzyme activity in the absence of exogenous agents. Bars = Standard error of the mean of 3 or more independent experiments. * = only two experiments were run.

To further verify if BACE1 is regulated by calmodulin, five different antagonists were used: W5 (8μM), W7 (7μM), W12 (0.2mM), W13 (0.2mM) and the classic inhibitor trifluoperazine (TFP, 50μM; figure 3B). W5 and W7 led to similar inhibitions each reducing BACE1 activity by about 40%. In the two experiments that were carried out with W12 and W13, they each inhibited BACE1 activity by about 50%. On the other hand, the antagonist TFP was the most effective at inhibiting BACE1 activity leading to an almost complete eradication of its activity. The addition of CaM in the presence of TFP could not rescue this inhibition of BACE1 activity (CaM/TFP; figure 3, B). Thus in vitro BACE1 activity is inhibited by calmodulin antagonists.

While these data supported a regulatory role for calmodulin in BACE1 activity, it was essential to verify that CaM actually binds to the BACE1 protein. To test the binding of BACE1 to CaM-agarose, a traditional method for the verification of calmodulin-binding, was carried out followed by western blotting. In the presence of calcium ions, BACE1 bound to CaM but in the presence of EGTA, which would chelate available calcium ions, it did not (figure 4). The specificity of this binding was verified by the binding of the well known

calcium-dependent CaMBP calcineurin (Cohen and Klee, 1988). As expected, calcineurin bound to CaM-agarose in the presence of calcium ions but not in the presence of EGTA (figure 4). These date support the contention that BACE1 is a true calcium-dependent calmodulin binding protein.

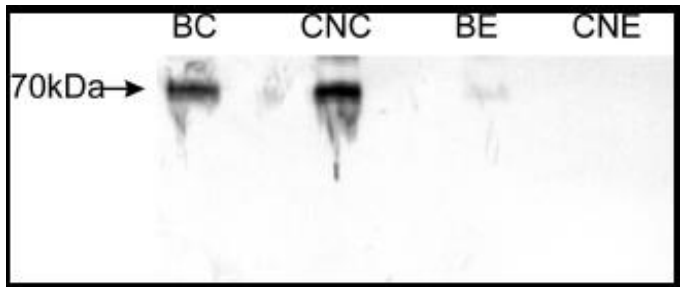

Figure 4. The binding of BACE1 and calcineurin to calmodulin agarose. BC = BACE1 plus 1mM calcium chloride; CNC = calcineurin plus 1mM calcium chloride; BE = BACE1 plus 10mM EGTA; CNE = calcineurin plus 10mM EGTA.

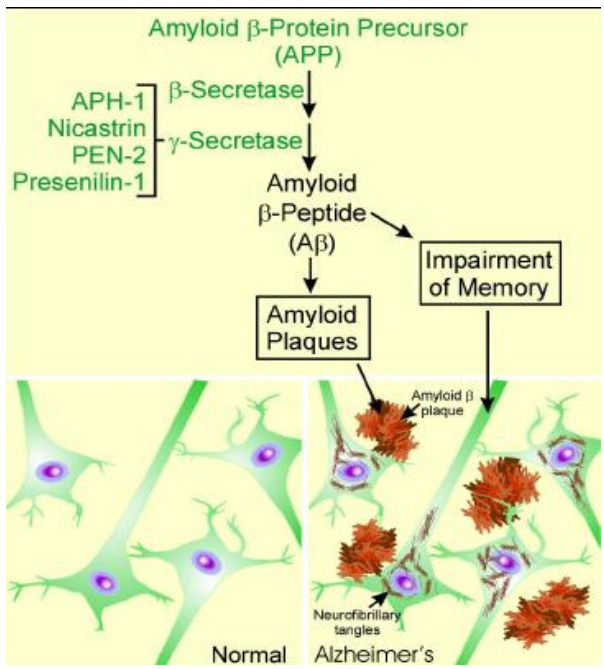

Figure 5. Major proteins leading to characteristic beta-amyloid deposits linked to early memory impairment and Alzheimer's disease. APH-1 = Presenilin stabilization factor a or b; BACE, β-Secretase; PEN-2 = Presenilin enhancer protein 2. Proteins with previously identified potential CaM-binding domains are shown in green (O'Day and Myre, 2004).

Previous work has implicated BACE1 as one of several proteins upstream of beta-amyloid production that are potential calmodulin binding proteins. Here we have shown that BACE1 activity is significantly increased in the presence of calcium and calmodulin *in vitro*. In addition, the removal of calcium and the presence of different types of camodulin antagonists each inhibit BACE1 activity while purified BACE1 binds to calmodulin in a

calcium-dependent manner. In total, these results implicate calmodulin as a potential of regulator of BACE1 activity *in vivo*.

CONCLUSION

In keeping with the calcium hypothesis and well-established models of calcium signal transduction, the increased cytosolic levels of Ca^{2+} in AD neurons would promote CaM binding to and regulation of available Ca^{2+}/CaM-dependent CaMBPs (O'Day and Myre, 2004). That study showed that the majority of the proteins that lie upstream of beta-amyloid production are likely to be calmodulin binding proteins. The work presented here reveals that in vitro BACE1 fits the role of a true calmodulin binding protein with its activity being significantly enhanced by the presence of calmodulin and calcium. As yet, the other the putative CaMBPs linked to beta-amyloid production remain to be studied (table 1). A summary of these proteins and their relationship to beta-amyloid production are shown in figure 5. While we've shown here that one direct effect of calmodulin in AD could be the regulation of enzyme activity, other scenarios for CaM remain in play. For example, the presence of CaM-binding motifs in each of the identified components of γ-secretase could affect the coalescence of the subunits and, subsequently, regulate the activity of the holoenzyme once it is formed. While upstream components in Aβ formation are primary targets for therapeutic intervention, recent work has focused on amyloid degrading enzymes (Turner et al, 2004). Analysis of the three metaloproteinases (neprilysin, endothelin converting enzyme(s), and insulin-degrading enzyme) cited in that article has revealed that all three possess one or more presumptive calcium-dependent calmodulin binding domains suggesting they too might all be CaMBPs (O'Day and Myre, 2007).

A new way of looking at the way Aβ production is regulated involves looking for mutations related to the receptor-mediated endocytosis of the APP protein. For example, the multifunctional endocytotic sortilin-related receptor (SORL1) is involved in APP recycling leading to internalization of APP which would direct this precursor protein away from Aβ formation (Jacobsen et al, 1996). Rogaeva et al (2007) have shown not only that inherited variants in neuronal SORL1 are linked to late onset AD but when SORL1 is under-expressed APP enters compartments directed towards Aβ formation. Because calmodulin is involved in various aspects of endocytosis and linked to other events of Aβ formation we scanned SORL1 for potential CaM-binding domains. One strong calcium-dependent and several weaker potential CaMBDs were identified again implicating calmodulin as a major player in Aβ processing (O'Day and Myre, 2007). Regardless of the final role of calmodulin in regulating amyloid-plaque formation, the discovery of a large number of potential CaMBDs in a number of central proteins upstream and downstream of beta-amyloid formation opens novel avenues for research into the study of memory impairment and the onset of AD.

ACKNOWLEDGMENTS

We thank Michael Myre for his comments on a draft of this manuscript. This project was supported by a grant from the Natural Sciences and Engineering Research Council of Canada.

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In: Alzheimer's Disease Diagnosis and Treatments
Editor: Marisa R. Boyd
ISBN: 978-1-61122-064-3

Chapter 11

TRANSGENIC MODELS OF ALZHEIMER'S PATHOLOGY: SUCCESS AND CAVEATS

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ABSTRACT

As a result of advances in molecular biological techniques, the first mice overexpressing mutated genes associated with familial Alzheimer's disease (AD) were engineered ten years ago. Most of the transgenic murine models replicate one key neuropathological sign of AD, namely cerebral amyloidosis consisting of parenchymal accumulation of amyloid-beta (Aβ) peptides that subsequently form plaques. Major research efforts today focus on the use of sophisticated transgenic approaches to discover and validate drugs aimed at reducing the brain amyloid load (eg recent immunotherapeutical attempts).

However, since the initial publications, the limitations associated with classic transgenic (APP and APP/PS1) models have become apparent. First, induction of AD-related brain lesions in genetically modified mice mimics, through parallel causal mechanisms, the physiopathogeny of familial forms of AD; however, the relevance of such transgenic mice in modeling the most prevalent forms (sporadic late-onset) of AD remains largely uncertain. Second, the neuropathological phenotype of mice bearing human mutated transgenes is largely incomplete. In particular, neurofibrillary alterations (tangles) are not reported in these models.

Transgenic mice nonetheless provide a unique opportunity to address different questions regarding AD pathology. Since these models do not replicate classic neurofibrillary lesions they can be used to specifically investigate and isolate the impact of the remaining brain injuries (Aβ deposition) on different aspects of the mouse phenotype. In addition, comparisons can be made between Aβ-induced alterations in mice and known features of the human pathology.

The present review questions the specific impact of Aβ brain lesions at different levels. First we describe macroscopic and microscopic neuropathological alterations (neuritic dystrophy, inflammation, neuronal loss) associated with amyloid deposits in transgenic mice. Then, modifications of the behavioral phenotype of these animals are listed to illustrate the functional consequences of Aβ accumulation. Next we describe the non-invasive methods that are used to follow the course of cerebral alterations. Finally, we discuss the usefulness of these models to preclinical research through examples of therapeutical trials involving AD drug candidates.

INTRODUCTION

Apart from dealing with the symptoms, pharmaceutical efforts to combat the onset and progression of Alzheimer's disease (AD) are largely guided by a dominant physiopathogenic hypothesis, the so-called amyloid cascade theory [Hardy 1992]. Regularly commented on and amended (eg [Sommer 2002]), this hypothesis places one of the histopathological hallmarks of the disease, the accumulation of amyloid-beta (Aβ) in the brain, as a key primary event that determines the onset of other brain alterations (e.g. cytoskeletal abnormalities, inflammation, synaptic and neuronal death), finally leading to the phenotypic demented stage. Strong support for the amyloid cascade hypothesis is the early-onset familial forms of AD (FAD) which are associated with mutations in different genes (Amyloid Precursor Protein (APP) and Presenilins 1&2, (PS1&2)) involved in the biosynthesis of Aβ. Dysfunction of these genes is logically thought to compromise the normal catabolism of APP resulting in exaggerated Aβ production. The definite in vivo demonstration of the neuropathological consequences of AD-linked gene mutations was shown 10 years ago by Games and collaborators using a transgenic approach [Games 1995]: an APP minigene bearing the human Indiana V717F mutation was inserted and overexpressed (driven by the PDGF promoter) in the genome of mice that subsequently developed neuropathological lesions (plaques, synaptic loss) reminiscent of those observed in the brain of AD patients. The same animals (PDAPP) were also found to develop behavioral disturbances when tested in learning and memory tasks (eg [Dodart 1999]). From the pivotal study of Games, dozen of different transgenic lines have been generated and tested (for recent review see [German 2004, Higgins 2003]; for an updated list of available research models see http://www.alzforum.org/res/com/tra/default.asp from the Alzheimer Research Forum; commercially models available for instance from the Jackson Laboratory: http://jaxmice.jax.org/library/models/ad.pdf). Models have evolved from single missense mutation, monogenic (APP) lines to the use of plurimutated, double- and triple-crossed transgenic mice. These models present histological and behavioral abnormalities that may vary from one line to the other [Higgins 2003], both in their onset and magnitude. The main (or at least most used) transgenic mouse models that overexpress mutant APP are the PDAPP ([Games 1995] - see above), Tg2576 ($APP_{695(K670N,M671L)}$ under the control of the hamster prion protein gene promoter [Hsiao 1996]), and APP23 ($APP_{751(K670N,M671L)}$ controlled by Thy-1 promoter [Sturchler-Pierrat 1997]) lines. These three models develop mature senile plaques before the age of one year. TgCRND8 mice ($APP695_{(K670N,M671L + V717F)}$ with prion promoter) have a more aggressive pathology with considerably high levels of cerebral Aβ peptides and an onset of plaques at only 3 months of age [Chishti 2001]. Also multi-mutated models such as those relying on both APP and PS1 transgenes (eg [Blanchard

2003, Holcomb 1998]) develop extensive neuropathological lesions from the first months of life. All these models that mimic some neuropathological and functional traits of human pathology are now currently used both for drug evaluations in preclinical studies and for academic research seeking for a better understanding of AD's physiopathogeny.

The transgenic line of attack undeniably derives from the amyloid cascade hypothesis and shows that genetically-induced increase of Aβ production leads to brain and behavioral alterations. The aim of the present chapter will not be to debate the relevance of any theoretical frame supporting the growing development of transgenic approaches for the study of AD (see [Lee 2004a] for alternative views). Criticisms have been repeatedly made about transgenic models, the most classical being that these models do not fully reproduce AD's neuropathology. In particular, standard neurofibrillary lesions (tangles) harbored by human patients with dementia are clearly not inducible by mutations of APP and related proteins (eg presenilins) expressed in mice. Paradoxically we do derive some benefits from the limitations of transgenic models. Mice developing plaques without neurofibrillary tangles give a unique opportunity to evaluate the specific impact of brain Aβ without major coexisting lesions. The individual effects of extracellular (amyloid deposits) and intracellular (tangles) alterations to explain AD phenotype are difficult to dissociate in human brains as the two lesions largely coexpress during the course of the disease. Animal models such as genetically modified mice can therefore provide a strategy to isolate one single variable of interest and to test its role as a putative pathogenic event with deleterious outcomes. Although direct stereotaxic intracerebral injections of Aβ [Davis 2003] may also help understanding the physiological effects of the peptide in targeted brain areas, the transgenic approach might be considered, for construct validity, as a more appropriate manner to investigate consequences of cerebral Aβ accumulation. Disparity of research models, in terms of neuropathological and behavioral phenotypes (see below), is derived from obvious differences between lines (with variables such as transgenes, number and nature of mutations, promoters with temporal/spatial specificities, genetic backgrounds used). However this problem should not preclude answering a key question: *what are the consequences of brain A overproduction in terms of AD pathology?* We will focus this review on APP and APP/PS1 models that, from the mechanistic and cartesian point of view, directly and solely tax Aβ metabolism dysfunctions. Recent mouse models developing both plaques (provoked by APP or APP/PS1 transgenes) and cytoskeletal alterations (induced by Tau transgenes; eg [Oddo 2003a]) will be addressed in order to assess the relationships between plaques and tangles lesions that constitute the core of the human disease. Single tau transgenic mice with neuronal pathology induced by mutated transgenes from human tauopathies (eg frontotemporal dementia linked to chromosome 17) are beyond the scope of this review and will not be addressed here (for recent review on the use of Tau transgenic mice, one could refer to [Lee 2005]).

We will first focus on the neuropathology developed by transgenic mice: what are the consequences of parenchymal Aβ accumulation on microscopic and macroscopic brain morphology? To what extent do these lesions evoke human pathology? The impact of cerebral amyloidosis on the behavioral phenotype will also be discussed. For example, do brain lesions developed by transgenic mice compromise normal learning and memory functions? In a third and last part, we will discuss the opportunity of using transgenic mouse models for applied research such as preclinical drug testing or methodological developments for non invasing brain imaging.

NEUROPATHOLOGY

The principal lesion developed by APP transgenic mice is the accumulation of Aβ positive deposits in the parenchyma and/or in blood vessels (cerebral amyloid angiopathy). We will, in this first part, also review the secondary macroscopic (eg atrophies) and microscopic (eg cytoskeletal alterations, neuronal loss) brain lesions developed by these models, in close association with cerebral amyloidosis.

At the Macroscopical Level

At the macroscopic level, brains from AD patients are characterized by a severe atrophy leading to dilation of the ventricular system and a widening of cortical sulci [Valk 2002]. In the early stages of the disease, the atrophy process affects mainly medial temporal areas including the hippocampal formation. The atrophy could be used as a marker of disease progression in clinical trials for new drugs [Albert 2005].

Most of the studies that have evaluated brain atrophy in transgenic mice have been carried out using the PDAPP model [Dodart 2000, Gonzalez-Lima 2001, Redwine 2003, Weiss 2002]. These investigations reported a reduction in hippocampal volume and a severe atrophy or agenesis of fiber tracts (fornix and corpus callosum). These alterations are already observed in young animals (3 months) and show no further deterioration in older mice [Dodart 2000, Gonzalez-Lima 2001, Redwine 2003, Weiss 2002]. Because of their early occurrence, these lesions might thus be viewed as a neurodevelopmental deficit rather than as an age-related brain shrinkage induced by progressive deposits of Aβ. Brain atrophy developed by young APP transgenic mice might be related to pleiotropic effects of APP expression [Herms 2004], that could be amplified in strains with specific genetic backgrounds [Magara 1999], or conversely to early alterations caused by pre-plaque Aβ oligomers that have been proved to be toxic. We recently carried out an in vivo (MRI) evaluation of brain atrophy in APP/PS1 mice (Double Thy1 APP751 SL (Swedish and London mutations) x HMG PS1 M146L) that were compared to plaque-free PS1 animals [Delatour In press]. No atrophy was detected in young APP/PS1 animals, as showed for example, by their normal brain, hippocampus or cerebrospinal fluid (CSF) volumes. Both genotypes showed continuous growth of the hippocampus during adulthood and hippocampal volumes were not affected by APP overexpression, regardless of age. However, an age-related atrophy process occurs in APP/PS1 mice as indicated by lower brain volumes and increased CSF volumes compared with PS1 controls. This atrophy process was mainly related to alterations in posterior brain regions and not to atrophy of cortical brain areas with high amyloid burden. More precisely, the locus of the atrophy was, at least in part, related to the midbrain region and to the internal capsule that both showed uninterrupted growth during adulthood in control PS1 mice and, on the contrary, did not increase in size in double transgenic mice. Some fiber tracts such as the corpus callosum and fornix had shrunk in aged APP/PS1 but not in PS1 mice. Notably, in this study the severity of atrophy process was not correlated with the amyloid load. This atrophy pattern, that involves white matter anomalies and largely spares the isocortex and hippocampus, is different from that reported in AD patients. It indicates that

overexpression of mutated APP is not invariably accompanied by AD-like brain atrophy in transgenic mice.

At the Microscopical Level

Core Neuropathological Lesion: Cerebral Amyloidosis

Expression of mutated hAPP in mice induces the formation of Aβ plaques in the extracellular space, associated, to varying degrees, with amyloid angiopathy (eg [Calhoun 1999]). These core lesions derived mechanically from genetically-induced Aβ oversynthesis and are observed in most of the transgenic lines created up to now (eg PDAPP, APP23, Tg2576 models) but with an onset, topography and burden intensity that may vary from one model to the other, presumably as a consequence of different genetic constructs, strain backgrounds, and levels of hAPP expression. Crossing PS1 mutated mice with APP transgenic mice dramatically increases Aβ pathology that is characterized by very early onset during the first months of life (eg PSAPP model [McGowan 1999]; APP/PS1 model [Blanchard 2003]).

Aβ deposits observed in transgenic mice resemble those depicted in human patients, showing classical immunoreactivity with specific anti-Aβ antibodies and also amyloid characteristics following histochemical stainings (green fluorescence with thioflavine-S and Congo red birefringence under polarized light). The intracellular accumulation of Aβ, described in human brains [Gouras 2000, Takahashi 2002], is also reported in transgenic lines [Langui 2004]. This preceeds plaque formation and decreases in intensity with progression of aging. These observations suggest initial neuronal accumulation of Aβ, especially in its pathogenic 42 amino acid isoform, and secondary secretion of the peptide outside the cell, a mechanism that could participate in plaque formation [Wirths 2001].

Some biochemical properties of the plaques, such as solubility, appeared different in mutated mice and AD patients [Kuo 2001]. The cellular microenvironment of Aβ deposits also varies somehow between human and transgenic tissue (see below and [Schwab 2004]). In addition the aggregated/amyloid nature of intracellular Aβ has been reported in one transgenic model [Casas 2004] but not in AD brains [Gouras 2000]. Another important question, although rarely addressed in the literature, concerns the topography and progression of the previously described lesions during aging. In humans, the hierarchical spreading of tau-positive neurofibrillary lesions from the medial temporal lobe to the entire cortical mantle has been decribed in details [Braak 1991, Braak 1996, Delacourte 1999] but until recently, no conclusive information concerning progression of Aβ deposits has been available (see however the ABC stages from [Braak 1991]). According to Thal and collaborators [Thal 2002], Aβ plaques originate from isocortical and allocortical areas and progressively invade deeper brain regions (diencephalon, brainstem nuclei). Similarly transgenic mice first develop plaques in cortical [Irizarry 1997a] and limbic archicortical [Blanchard 2003] areas. Plaque deposition in subcortical structures (eg thalamus, accumbens nucleus, septal nuclei, colliculi) are additionally depicted in transgenic animals but, to our knowledge, the exact progression of the disease during aging (from cortex to deep brain regions?) has not been addressed in details in these models. Interestingly Aβ plaques in the cerebellum are described as corresponding to a final neuropathological stage (stage V) of cerebral amyloidosis in human

brains [Thal 2002]; parallel observations have shown either non or very rare presence of plaques in the cerebellum of transgenic mice [McGowan 1999].

Vascular Alterations

Cerebral amyloid angiopathy (CAA) is another lesion widely described in the brain of Alzheimer's patients. It is characterized by Aβ deposition in the wall of cerebral blood vessels. In humans, it occurs mainly in small arteries of the leptomeninges and penetrating arteries of the cerebral cortex. Most of the APP transgenic mice also exhibit amyloid angiopathy. As in humans [Wisniewski 1994], its origin has been partly attributed to Aβ secretion by the smooth muscle cells [Frackowiak 2003]. However, mice such as the APP23 models, for which the mutated transgene is under the control of a neuron-specific Thy 1 promoter, also show CAA [Calhoun 1999]. This favors the hypothesis that CAA might involve the periarterial drainage of the interstitial fluid, as suggested by some human studies [Weller 1998]. Other vascular alterations have been reported in various transgenic mouse models. First, magnetic resonance angiography with a method sensitive to vascular flow has shown flow voids starting in the internal carotid arteries in 11 month old APP23 mice and then involving the large arteries of the circle of Willis in 20 month old animals [Beckmann 2003]. Vessel constrictions detected ex vivo on corrosion casts from vessel architecture of the same mice could partly account for these alterations.

Altered hemodynamic response has also been described in APP transgenic models. MRI studies highlighted an altered hemodynamic response detected after somatosensorial stimulation (electrical stimulation of the paw) in 25 month old animals that is not obvious in 13 month animals [Mueggler 2003]. Reduced hemodynamic responses have similarly been reported in transgenic mice after pharmacological stimulation with vasodilatators [Christie 2001a, Mueggler 2002, Niwa 2002]. Two main hypothesis could explain these altered responses. First, a direct link between functional alterations and amyloid angiopathy has been suggested by studies reporting that the two alterations start at the same time [Christie 2001a, Mueggler 2003, Mueggler 2002]. Aβ deposits in blood vessels might act by mechanistic constriction [Beckmann 2003, Christie 2001a, Mueggler 2003] or, alternatively, by disorganizing the arrangement of smooth muscle cells [Christie 2001a]. In Tg2576 mice, disruption of smooth muscle cells (without obvious vessel cell loss) occurs at 14 months, which is the same time as the reduction in response to vasodilatators [Christie 2001a]. In older animals from the same strain, a loss of smooth muscle cells is described and may be related to a more dramatic pattern of vascular alterations [Christie 2001a]. A second hypothesis has been suggested to explain the occurrence of altered vascular response or blood flow in regions free of amyloid angiopathy [Beckmann 2003] or before the start of amyloid deposits [Niwa 2002]. These alterations might be related to toxicity of Aβ peptides, particularly when they are in a soluble form. Such an effect would be mediated by reactive oxygen species that can be suppressed by superoxide dismutase activity [Iadecola 1999, Niwa 2002].

Neuronal Cytoskeletal Alterations

From the principle work of Alois Alzheimer [Alzheimer 1995], a characteristic, almost pathognomonic, histological lesion in AD brains was identified as argyrophilic neuronal filamentous inclusions. They correspond to neurofibrillary tangles, principally made of

aggregates of hyperphosphorylated tau proteins. They form paired helical filaments (PHF) at the ultrastructural level and compromise the cytoskeleton morphology and function.

To date similar lesions have not been described in the brain of any APP or crossed APP/PS1 mutants, although disorganization of microtubules/neurofilaments as well as tau hyperphosphorylation immunoreactivity can be observed in these models (see below). An early study from Kawabata and collaborators [Kawabata 1991] described neuronal tangles in transgenic mice overexpressing APP C-terminal fragments but the published paper was finally retracted a few months later. More recently Kurt and colleagues [Kurt 2003] reported EM-characterized "paired helical filament-like structures" in the hippocampus of APP/PS1 mice. The authors nonetheless subdued this observation by pointing out the fact that it was done in a single "dark neuron" (from one mouse) that accumulated both straight and paired filamentous material resembling AD's PHFs.

Lack of development of neurofibrillary tangles in APP or APP/PS1 mice is somewhat puzzling with regard to the amyloid cascade hypothesis but does not preclude any relationship between Aβ cerebral accumulation and the induction or potentiation of cytoskeletal abnormalities, for several reasons. First, Aβ deposits in APP mice is clearly associated with neuritic dystrophy and degenerescence showing the same immunohistochemical characteristics (hyperphosphorylated tau epitopes) as tangle-filled neurites of the human senile plaques (see next section). Secondly, several studies have emphasized the potent role of Aβ in the initiation or modulation of tau-positive lesions developed by single tau-mutants [Gotz 2001, Lewis 2001] or by triple transgenic mice where Aβ accumulation is described as preceeding and determining the onset of tau pathology [Oddo 2003b].

Senile Plaques

During the course of AD, disrupted cytoskeletal morphology is shown in the cell body of neurons as classical "flame-shaped" intracytoplasmic neurofibrillary tangles but also seen in neurites, taking the shape of tortuous (dendritic?) fibers and dystrophic axonal/dendritic elements surrounding amyloid plaques. The composite lesion made by the amyloid core and peripheral crown of dystrophic, degenerated neurites forms the so-called senile neuritic plaque.

There are good evidence that APP transgenic mice encompass similar neuritic degeneration in close contact with Aβ deposits (eg PDAPP model [Masliah 1996]; APP23 model [Sturchler-Pierrat 1997]; Tg2576 model [Irizarry 1997b]; double-crossed APPxPS1 lines [Blanchard 2003, Borchelt 1997]). Plaque-associated dystrophic neurites developed in genetically-modified mice have an immunohistochemical profile evocative of AD brain lesions (eg APP, ubiquitin and phospho-tau epitopes can be detected). The pathological neurites observed in transgenic mice also show abnormal morphology as described as bulbous, swollen structures, often grouped in clusters of enlarged varicosities around plaques. However they lack the classical ultrastructure (PHFs) reported in the human disease.

Synaptic and Neuronal Loss

Synaptic loss occurs to varying degrees in the brain of AD patients [Honer 2003] and has been described by some authors as an important correlate of dementia (eg [Terry 1991]). Density of synapses in transgenic mice has not been systematically assessed and, to date, this has led to contradictory results, such as in studies in which synaptophysin-immunoreactivity

has been investigated ([King 2002b] versus [Dodart 2000]). Cholinergic networks, largely disrupted in AD, have been the major focus of research in transgenic animals; considering this particular system, several studies have demonstrated decreased cholinergic terminals in APP [German 2003] or APP/PS1 [Wong 1999] transgenic mice (see however [Diez 2000] for mixed results). The effect of Aβ parenchymal deposition on axonal degeneration and synaptic loss has been experimentally proven with in vivo neuroanatomical tracing [Delatour 2004, Phinney 1999] and confocal multiphoton approaches [Tsai 2004]. These studies indicate that Aβ (1) promotes neuritic dystrophy, affecting cortico-cortical connections and even misrouting axonal projections to ectopic targets. (2) Induces spine loss and dendritic shaft atrophy, therefore potentiating synaptic pathology on the postsynaptic side.

Neuronal loss associated with brain macroscopic atrophy, is also described in AD brains. Decreased cell number, quantified by means of unbiased stereological methods, affect both cortical and subcortical brain areas and is particularly prominent in the hippocampal CA1 field where the difference in neuronal counts between AD patients and age-matched controls can reach almost 60% [West 2000]. Cell loss in transgenic mice is still a matter of debate, particularly with respect to studies reporting paradoxically increased numbers of cortical neurons in young transgenic mice [Bondolfi 2002]. Cell loss is absent in archicortical (including CA1) and isocortical brain regions of PDAPP [Irizarry 1997a] and Tg2576 mice [Irizarry 1997b] but is reported, although not to a great extent, in the hippocampal pyramidal cell layer of APP23 transgenic mice [Calhoun 1998]. Loss of neurons in the APP23 line might be due to the fact that these mice develop a very high density of fibrillar, potentially toxic, amyloid deposits in comparison to other transgenic lines. Strikingly, cell loss affecting basal forebrain cholinergic areas that is classically depicted in people with AD, has not to our knowledge been reported in transgenic mice (reviewed in[German 2004]). Recent use of double mutants with aggressive cerebral Aβ amyloidosis has revealed some more extensive cell loss in multiple transgenic mice. Urbanc and collaborators [Urbanc 2002] have reported focal neuronal loss in the cingulate cortex of PSAPP mice (Tg2576 line crossed with PS1-M146L transgenic mice). Using statistical physics methods these authors demonstrated that large and dense fibrillar (thioflavine-S positive) Aβ plaques were responsible for local cell loss. In another double-crossed APP (KM670/671NL and V717I) /PS1 (M146L) model, Schmitz et al. [Schmitz 2004] also showed a reduction of neuron number (-30% in Ammon's Horn, fields CA1-3) but, this time, it was not correlated with the amyloid load (the large amyloid burden was not indicative of enhanced cell loss that may occur in areas distant from plaques). Finally Casas and colleagues [Casas 2004] reported dramatic, macroscopically visible, neuronal loss in CA1-2 (50-60%) in old APP mice bearing additional PS1 M233T/L235P knocked-in mutations (APP/PS1-KI model). This remarkable cell loss was preceded in time by intraneuronal aggregated Aβ accumulation that may be the causative factor.

Inflammation

Chronic inflammation is part of the overall AD neuropathology (for recent reviews, see [Eikelenboom 2002, McGeer 2004]). Cellular and biochemical agents or inducers of inflammation are shown to be in close association with Aβ deposits. Clumps of brain macrophages (activated microglial cells) are observed around plaques of AD brains in

combination with biochemical partners of the inflammatory reaction such as proteins of the complement pathway, cytokines, acute-phase proteins.

Since the first published study [Games 1995], neuroinflammation, shown by gliosis involving astrocytes and microglia, was also reported in transgenic APP mice. Signs of inflammation, based on both cellular and molecular markers, are depicted in different transgenic models, underlining some cross-line constancy (Tg2576 model [Apelt 2001, Benzing 1999]; APP23 model [Bornemann 2001, Sturchler-Pierrat 1997]; TgCRND8 model [Dudal 2004]; YAC APP model [Kulnane 2001]; PSAPP model [Matsuoka 2001]). A great many studies investigating brain inflammation have been carried out using the Tg2576 transgenic line. Interestingly, while reporting similarities of neuroinflammation between species, several reports also emphasize some qualitative/quantitative differences in AD and Tg2576 mice (eg [Mehlhorn 2000, Munch 2003]), suggesting different stages and grading of inflammation in human and animals brains.

BEHAVIOR

Modeling clinical symptoms developed by AD patients in lower mammals might be viewed as a challenge. Memory impairments, associated to early-onset medial temporal lobe pathology, are generally the first outcomes of the disease in humans. With progression of neuropathological lesions in other brain areas, multifaceted clinical manifestations gradually emerge, leading to a severe aphaso-apraxo-agnosic syndrome in the most demented patients.

Considerable efforts have been made to reproduce and identify memory disruptions in APP (or APP/PS1) transgenic mice. Behavioral tests used to evaluate genetically-modified animals are therefore generally aimed at detecting hippocampal (medial temporal-like) dysfunction. The phenotype of these mice does however encompass numerous aspects of the behavioral repertory, not all necessarily hippocampus-dependent. In this sense, several reports indicate basic neurological, non-cognitive, impairments in APP transgenic mice that might interfere with learning abilities in more elaborate cognitive tasks. Characterization of such behavioral abnormalities are hence of particular importance (discussed in [Gerlai 2002]).

Neurological Disorders

APP transgenic mice are occasionally reported to have reduced body weights and enhanced (premature) lethality [Chishti 2001, Kelly 2003, King 2002a, King 1999, Kumar-Singh 2000, Le Cudennec 2003, Moechars 1999] the reasons of which (non favorable background strain? onset of spontaneous seizures? neurodevelopmental abnormalities?) remains somewhat unclear.

Signs of neurological impairments can be described in both single APP and double APP/PS1 transgenic mice from different lines (ie PDAPP, Tg2576, APP23, TgCRND8, PSAPP, APP/PS1 models). Although contested by some (eg [Chapman 1999]), clear neurological symptoms are depicted in several studies and, more importantly, may appear early during ontogenesis. Motor dysfunction and difficulties in coordinating movements are shown by reduced grip strength and altered behavior on a beam or an accelerated rotating

device (rotarod) [Arendash 2001, King 2002a, King 1999, Le Cudennec 2003, Van Dam 2003].

The integrity of sensory functions have not been fully documented in APP transgenic mice; however enhanced acoustic (startle) reflex in TgCRND8 mice [McCool 2003] that may indicate abnormal processing of auditory stimuli has been reported. Similarly, impairments in visually-guided navigation (swimming to a cued location in a spatial environment) could reflect altered motoric function but also compromised visual abilities [King 1999]. Locomotor activity is also abnormal in APP transgenic mice, a number of studies indicating horizontal hyperactivity of these mice [Arendash 2001, Dodart 1999, Holcomb 1999, King 1999, Lalonde 2003, Ognibene 2005]. On the contrary evidence for decreased locomotor activity has been shown in the APP23 model that develops severe cerebral amyloid angiopathy in addition to parenchymal Aβ plaques [Lalonde 2002b, Van Dam 2003].

Anomalous anxiety-related behaviors are occasionally noted in APP transgenic mice either in the form of neophobia or, on the contrary, by hypo-anxiety and reduced inhibition [Dodart 1999, Gerlai 2002, Lalonde 2003, Ognibene 2005]. Finally, decreased thermoregulation and altered wake/sleep patterns have been described by Huitron-Resendiz and colleagues [Huitron-Resendiz 2002] in PDAPP mice.

Cognitive Dysfunctions

Based on the evidence of an amnesic syndrome and early medial temporal lobe pathology in AD patients, behavioral studies searching for cognitive alterations in APP transgenic mice have largely focused on the analysis of mice learning abilities in tasks relying on the integrity of the hippocampus. We will only review here the memory impairments shown in APP mice in three of the most well used tasks for assessing hippocampal function. Additional data concerning behavioral phenotype of APP transgenic mice can be found in recent reviews [Dodart 2002a, Higgins 2003, Kobayashi 2005].

Studies using lesion approach in rats and mice or electrophysiological recordings in freely moving rodents have emphasized a critical role of the hippocampus in the formation and maintenance of spatial (allocentric) maps. From the principle work of Morris et al. [Morris 1982], a standardized task (water maze) is now classically used to assess hippocampal function and dysfunction. In its original version, this test requires the animal to locate and swim towards an invisible platform in a water tank. During learning across several training sessions, it is believed that the rodent forms a cognitive map of the environment in order to guide itself to the escape platform directly, regardless of where it enters the pool. Rodents with damage to the hippocampus are severely impaired in this task. Almost all APP transgenic models have, to date, been screened in the water maze task. The majority of these studies indicate defects in navigation behavior with transgenic mice showing increased response latencies and distance to reach goal location and/or altered memory for remembering the location of the platform when assessed during probe trials. Behavioral deficits, some of which with very early onset [Chishti 2001, Van Dam 2003], have been observed in the PDAPP [Chen 2000], Tg2576 [Hsiao 1996, Westerman 2002], APP23 [Kelly 2003, Lalonde 2002b, Van Dam 2003], TgCRND8 [Chishti 2001], and crossed APP/PS1 [Liu 2003] models. It is important to keep in mind that some reports alternatively failed to demonstrate significant or robust learning and retention deficits in the water maze task [Holcomb 1999, King 2002a,

King 1999] in both APP and APP/PS1 transgenic mice. The reason for such discrepancies remains to be established but might be due to different factors varying between studies such as age of test, gender, behavioral protocol (see also next section for other possible explanations).

A second task that is classically used to evaluate memory function in APP transgenic mice: spatial alternation behavior (assessed in a Y- or T-maze) relies on the natural propensity of rodents to alternate their visits from already-experienced locations to a new location. This behavior, that can either be analyzed spontaneously or conditioned by an explicit reinforced alternation rule, requires intact working memory abilities. Spatial alternation is disrupted following hippocampal lesions and pharmacological manipulations but also relies on extra-hippocampal brain structures such as the frontal cortex [Lalonde 2002a]. Surprisingly spontaneous or reinforced spatial alternation has principally been studied in the Tg2576 model with several reports indicating decreased alternation performance ([Chapman 1999, Corcoran 2002, Holcomb 1998, Hsiao 1996, Lalonde 2003, Middei 2004, Ognibene 2005]; see however [King 1999] for mixed results) with various onset of deficits that, depending on the study, were obtained either at young ages or showed an age-dependent effect. Additional reports illustrated reduced spatial alternation in double APPxPS1 transgenic mice ([Holcomb 1998, Holcomb 1999]; see however [Liu 2002]) but only a very weak disruption of performance in Tg APP23 mice [Lalonde 2002b].

Detecting hippocampal dysfunction has also been demonstrated by testing visual recognition memory. Mice are trained in an object recognition task where they are first familiarized with objects during an acquisition phase. Following a variable delay (from minutes to several hours) mice are replaced in the test arena with both familiar (already-experienced) objects and new objects. The natural tendency of rodents is to explore never-experienced objects (novelty attraction). Good performance in this test depends on intact short-term and intermediate-term visual recognition memory and relies on the hippocampal system and more precisely on hippocampus-interconnected perirhinal and entorhinal cortices. Impaired recognition memory has been demonstrated in both APP and APPxPS1 transgenic mice [Dewachter 2002, Howlett 2004]. Conflicting results have been obtained with PDAPP mice trained in the object recognition task: while Dodart and collaborators [Dodart 2002b, Dodart 1999] showed clear age-dependent deficits, Chen and colleagues [Chen 2000] failed to find any recognition impairments using the same line of mice. While subtle variations in behavioral protocols might account for such differences, other explanations are possible (see next section).

Data obtained from these three behavioral tasks are globally in agreement that memory deficits in APP transgenic mice are linked to a dysfunction of the hippocampus and associated cortical areas. Learning and memory processes rely on different anatomical systems, one of which implicates brain areas of the medial (internal) temporal lobe. This declarative, relational system is severely disrupted in AD patients that show amnestic disorders including disorganization of spatial behaviors and failures of recognition memory. The same system seems also to be compromised in APP transgenic mice. On the other hand AD patients, particularly during the first stages of the disease, show some intact procedural memory abilities involving motor, perceptual or cognitive skills. One may therefore ask whether APP transgenic have similar preserved procedural memory. Unfortunately only a few studies have either directly or indirectly addressed this question. Dodart and collaborators [Dodart 1999] trained PDAPP mice in a simple bar-pressing task (press a lever to get a food reinforcement)

and found only very weak learning deficits, illustrating normal procedural abilities. Two other studies analyzed behavioral strategies (response-stereotyped, "procedural-like" versus spatial, "declarative-like") of APP transgenic mice and have shown interesting results. Huitron-Resendiz et al. [Huitron-Resendiz 2002] trained mice in the Barnes maze (a navigation task where animal have to learn to locate an escape hole from 20 possible locations in a circular arena) and found that wild-type animals were able to progressively develop an efficient spatial search strategy. On the contrary, PDAPP mice showed difficulties in adopting such a spatial strategy and preferred to use a serial search strategy (sampling successive locations with a stereotyped clockwise or anti-clockwise direction). More recently a study from Middei et al. [Middei 2004] also indicated that PDAPP mice trained in a cross-maze preferentially developed a response strategy (always turning the same direction) rather than a spatial strategy compared with control wild-type mice. Both studies suggest that procedural memories and strategies are not only intact in APP transgenic mice but sometimes enhanced, presumably to compensate for deficient spatial declarative capacity.

Possible Pitfalls in Behavioral Studies of APP Transgenic Mice

From the first reports illustrating cognitive impairments in APP transgenic mice [Hsiao 1996], criticism have emerged to question the validity and significance of behavioral studies in AD-like murine models (eg [Routtenberg 1997]). Apart from criticism associated with the validity of the models, these polemic judgments may help understanding 1) the nature of some bias in interpreting behavioral defects as purely cognitive and 2) the origin of inconsistency in results from different studies.

First of all, one may suspect that basic neurological impairments could impede performance in higher-level learning and memory tasks. Arendash and King [Arendash 2002] illustrated correlations between sensorimotor and cognitive measures in mice trained in a battery of tasks. For example basic locomotor activity levels of wild type mice were found to be indicative of subsequent performance in a spatial navigation task (circular maze). Genetically-modified mice with altered sensorimotor phenotypes could therefore be impaired in learning tasks because of non-cognitive impairments. Considering deficits shown in the water maze task (spatial version with immerged platform), some studies indicated, in parallel, that performance of APP transgenic mice is impaired in the sensorimotor control version of the task that simply requires animals to swim to a visible platform [Hsiao 1996, King 2002a, King 1999]. Although some authors [King 1999] claim that such a deficit reflects cognitive impairment (in terms of associative and recognition processes), one must still consider that defects in visual acuity and motor abilities could be the source of the disrupted performance. In the same vein, abnormal thermoregulation function [Huitron-Resendiz 2002] described in PDAPP mice could modify behavioral accuracy in the water maze task in a non specific manner [Rauch 1989].

The lack of a standardized battery of neurological/cognitive tests (see however [Crawley 1999, Crawley 1997]) is undoubtedly a possible cause for recurrent contradictory results in the literature. For example, variations in protocols used to assess object recognition memory in PDAPP mice have been stressed [Kobayashi 2005] and might explain unexpected dissimilarities in the results derived from different research groups working with the same transgenic line but with different training protocols [Chen 2000, Dodart 1999]. Confounding

factors might also be identified as gender, age of testing, training intensity and "personal history" of tested mice. In terms of these two latter points, Dodart [Dodart 2002a] has suggested that variations in duration and strength of acquisition might affect the impact of APP transgenes on water maze performance, possibly explaining discrepant results in the literature (from no deficits to memory impairments; see above), depending on the behavioral protocol used. Also an extended training phase and/or previous testing in a battery of tasks might be viewed as providing some kind of environmental/cognitive enrichment which is known to promote learning abilities and, more importantly, to modulate brain Aβ levels [Lazarov 2005]. Effects of prolonged continuous testing might therefore modify the phenotype of APP transgenic mice and act upon (improve?) their behavioral performances.

An important concern deals with genetic backgrounds and lineages / breeding conditions of tested transgenic mice. The different research groups often maintain independent colonies of transgenic mice that could be affected by genetic drift processes, with consequences of particular importance in the case of mixed genetic backgrounds. For example PDAPP mice have a mixed triple-strained background (C57Bl/6, DBA/2J, Swiss-Webster). Conflicting results obtained by Dodart et al. [Dodart 1999] and Chen et al. [Chen 2000] in the object recognition task might hence be explained by differential genetic drifts in the PDAPP colonies used by the two groups. It is known for example that C57Bl/6 mice have bad recognition performance in comparison to Swiss mice (discussed in [Dodart 2002a]).

Histological Correlates of Behavioral Impairments

There is some general agreement that, in human patients with AD, neurofibrillary lesions and synaptic loss are a better correlate with dementia than Aβ deposits [Berg 1998, DeKosky 1990, Delaere 1991, Nagy 1995, Terry 1991]. This does not mean that cerebral amyloidosis has no impact on the intellectual status but only that in AD subjects, where both tangles and plaques co-exist, the different lesions have graded clinico-pathological outcomes. Neuropathological studies have shown evidence of correlations between amyloid load and dementia, evaluated through clinical rating scales or neuropsychological assessment [Cummings 1996, Naslund 2000, Thomas 2005].

Studies in APP transgenic mice have also addressed the issue of correlations between Aβ and behavioral impairments. Several reports showed the detrimental effects of Aβ accumulation (Aβ load measured from histological sections or biochemical assays) on behavioral performance. Such negative correlations ("the more Aβ, the worst performance") were shown in monogenic APP models (eg [Chen 2000, Dodart 2000]) and double-crossed models (eg [Gordon 2001, Savonenko 2005]) using different behavioral tasks. Also therapeutic approaches showing both decreases of amyloid load and concomitant rescue of the behavioral phenotype (see next section) strongly suggest a link between Aβ accumulation and a disruption of behavior. The fact that declarative and executive functions (relying on plaques-enriched hippocampal and isocortical areas) are impaired in most of transgenic models while motor procedural learning (requiring the integrity of basal ganglia less affected by amyloidosis) is spared, might be considered as additional evidence for a pathogenic role of Aβ lesions.

All these data fit well with the ideal description of an age-related increase in density of Aβ plaques paralleling progression of cognitive impairments. However some behavioral deficits can clearly be obtained at pre-plaques ages (see for instance [Van Dam 2003]), challenging the contention that parenchymal Aβ deposits are the causative factor. Evidence of deficits with early onset in the absence of aggregated deposits has suggested that plaque-independent Aβ assemblies that can not be visualized by classical immunohistochemical approaches (but by biochemical measurements; see however [Kayed 2003, Takahashi 2004]) are responsible for behavioral defects. These structural assemblies might include Aβ in insoluble oligomeric or protofibrillar forms [Liu 2003, Westerman 2002] and also soluble Aβ [Van Dam 2003]. The pathogenic role of non-plaque aggregated assemblies of Aβ peptides is reinforced by the growing literature reporting detrimental effects of intracerebral injections of Aβ peptides (see [Davis 2003, Stephan 2005] for reviews).

To conclude, let us now consider other factors or alterations in brain morphology in APP transgenic mice that may hamper cognitive functions. As an important point Westerman and colleagues [Westerman 2002] demonstrated that the simple overexpression of wild-type hAPP does not lead to behavioral deficits, excluding the possibility of an uncontrolled effect of the transgene. Besides brain Aβ accumulation, APP transgenic mice show additional brain lesions (see above) with putative pathogenicity at the behavioral level. Weiss et al. [Weiss 2002] reported a correlation between hippocampal atrophy and learning performance in PDAPP mice. Synaptic abnormalities, as assessed by synaptophysin immunoreactivity, are also reported to affect behavioral performance [Dodart 2000, King 2002b]. Finally and more speculatively, amyloid angiopathy and other vascular anomalies such as those developed by APP23 transgenic mice [Beckmann 2003] might have deleterious consequences on behavior.

VALUE OF TRANSGENIC MODELS FOR APPLIED RESEARCH

Transgenic mice recapitulate several traits of the Alzheimer's phenotype and consequently are considered to be instrumental for applied research. The efficiency of potential treatments is currently evaluated through post-mortem measures of lesion loads and in-vivo via behavioral testing. The detection of amyloid-related alterations by in-vivo imaging methods can provide new biomarkers that might be helpful for evaluating disease-modifier treatments. Using in-vivo imaging, the effect of therapy can be monitored in the same animal and compared with a reference state before treatment. Such paired designs increase the statistical power of the studies. In this chapter, we will first review what the potential in-vivo imaging markers are that may be used to follow AD pathology in mice. We will then briefly describe recent preclinical drug assays involving transgenic models.

Imaging AD-related Brain Lesions in Transgenic Mice

Modifications Associated to the Amyloid Pathology

Several MR approaches have been developed to evaluate disease progression in mouse models. Transverse (T2) or longitudinal (T1) relaxation times are parameters that can be measured by MRI. They are closely dependent on the biophysical environment of water in the

tissues and are modified by tissue alterations, suggesting they might be modified by the Alzheimer's pathology. A recent transversal study reported a T2 decrease in various brain regions of 16-23 month old APP(Tg2576)/PS1 mice compared to non transgenic littermates [Helpern 2004]. More recently, we reported T2 decrease in the subiculum of APP/PS1 mice as well a T1 decrease in amyloid-rich cortical regions [El Tannir El Tayara 2004]. The origin of these alterations however still requires further assessment. They might be directly related to the amyloid deposits or may be due to secondary events, such as iron accumulation, associated with Aβ deposits [Falangola 2005].

Diffusion modification is another proposed potential marker of AD pathology. Diffusion methods analyze randomized movement of water molecules in tissues [Le Bihan 2001]. Diffusion weighted images and calculations of apparent diffusion coefficient (ADC) provide information on water diffusion in tissues. Studies in APP23 transgenic mice show reduced ADC values in some cortical areas from 25 month old animals [Mueggler 2004]. However, these observations were not reproduced in all brain regions with amyloid load [Mueggler 2004] and failed to be replicated, even in studies using the same strain [Sykova 2005]. Index of diffusion anisotropy is another parameter based on diffusion measurement. It provides information on the integrity of oriented tissues such as fiber tracts. Studies in humans showed a reduction of white matter anisotropy in human AD patients [Hanyu 1999, Rose 2000, Sandson 1999]. Hippocampal alterations of diffusion have also been reported in MCI patients [Kantarci 2001]. Studies in two different strains of transgenic mice have shown a reduction in the water diffusion parallel to axonal tracts ($\lambda_{\parallel}$; a parameter that might be a marker for axonal injury) and/or an increase in water diffusion perpendicular to axonal tracts ($\lambda_{\perp}$; a parameter that might be a marker of myelin integrity [Song 2004, Sun 2005]). However, these results involved animals older than 15 months. This suggests that these alterations are a late surrogate marker of amyloid related pathology. These results are consistent with our data that showed fiber tract atrophies [Delatour In press] and suggest that Aβ or mutated APP overexpression is associated with white matter alterations.

Proton MR spectroscopy has also been used to detect amyloid-related pathology in mice. Studies in human AD patients report a decrease in N-acetylaspartate (NAA) peak [Adalsteinsson 2000] and an increase in the myo-inositol peak [Moats 1994, Valenzuela 2001]. In PS2APP mice, a mouse strain in which amyloid deposits starts at 5/8 months [Richards 2003], a reduction in NAA and Glutamate peaks have been reported, starting at 12 months and reaching significative levels in 20 month old animals. Furthermore in 24 month old animals the NAA index was significantly correlated with the amyloid load [von Kienlin 2005].

Direct Imaging of Amyloid Lesions

Alterations such as those described in the previous paragraphs are late markers of the pathology. Being able to detect primary events associated to the amyloid deposition in mice would permit rapid screening of the effects of new drugs. In terms of application to human patients, these methods will give opportunities for early diagnosis. Up to now, several approaches have been evaluated to attain this goal.

First, methods based on multiphoton microscopy are able to detect amyloid deposition by scanning through a small skull window. In order to be detected with this method, plaques have to be labeled with a specific fluorophore such as Thioflavine S [Christie 2001b] or Thioflavine T derivative such the PIB (Pittsburgh compound B) [Bacskai 2003] that can be

either injected in the brain or in the venous system and detected in association with plaques using low-energy multiphoton excitation. The spatial resolution reached is on the order of one micron and plaques located up to 150µm underneath the cortical surface can be revealed [Christie 2001b]. The use of this method in mice has allowed in-vivo visualization of the turn over of plaques [Christie 2001b] and associated lesions [Tsai 2004] and the effects of drug treatment [Bacskai 2001, Brendza 2005]. It has also been very useful to evaluate new contrast agents that can then be used with other instruments such as PET [Bacskai 2003].

Recent developments of positron emission tomography (PET) radiopharmaceuticals that bind to Aβ have also allowed the detection of amyloid deposits in the brain of AD patients [Klunk 2004, Nordberg 2004, Shoghi-Jadid 2002]. The development of these agents have been largely based on preliminary studies in mouse models of AD [Bacskai 2003]. However, these methods are not well suited to follow-up amyloid pathology in mouse models because they suffers from a low resolution (and eventually a limited access to scanning devices for animal studies). Moreover, for a still unknown reason, the current radiopharmaceuticals do not label rodent amyloid plaques as efficiently as human lesions [Toyama 2005].

MRI is a more widely distributed method with a better spatial resolution and might thus be used to detect amyloid deposition in transgenic mice. The current difficulty with this method is to find what the contrast is that is associated with senile plaques. First results on post-mortem human brain samples provided contradictory results [Benveniste 1999, Dhenain 2002]. However, recent post mortem [Lee 2004] or in-vivo [Jack 2004, Vanhoutte 2005] studies in aged transgenic mice modelling amyloid deposition succeeded in detecting plaques in T2 or T2*-weighted images. The deposits appear as dark spots that are caused by the presence of iron within the amyloid deposits. Unfortunately, because iron accumulation only occurs in aged animals, it is predictable that this method will only be able to detect amyloid deposits in these animals. The difficulties in detecting amyloid by MRI can be partly overcome by using dedicated contrast agents [Dhenain 2004]. To date, most of the approaches use probes made up of amyloid peptides associated with a MR contrast agent (gadolinium or monocrystalline iron oxide nanoparticle (MION)). The chemical can label the amyloid deposits if it crosses the blood brain barrier, which is made possible by associating it with putrescine [Poduslo 2002] or by injecting it with mannitol to permeabilize the hemato-encephalic barrier [Zaim Wadghiri 2003]. This method allows detection of amyloid during in-vivo experiments [Zaim Wadghiri 2003]. More recent MR studies, based on the use of fluor-based contrast agents, described new methods to detect amyloid deposits in living mice [Higuchi 2005].

Near-infrared imaging is another in-vivo imaging technique that has been recently applied to the quantitative evaluation of cerebral amyloidosis in transgenic mice [Hintersteiner 2005, Skoch 2005]. This promising optical method exploits the high transmission of near-infrared light through tissues. Recent development of specific dyes allows assessment of the amyloid load in APP23 mice [Hintersteiner 2005]. This method is particularly interesting because it is cost effective and requires a simple experimental design. This might make it a reference strategy for high-throughput screening of drug candidates.

Usefulness of Tg Models for Preclinical studIes

From recent years different new therapeutic strategies have benefited from the availability of AD's transgenic models. The line of attack was to characterize in-vivo disease modifiers with compelling action on Aβ pathology.

Anti-inflammatory Drugs

As mentioned above AD's neuropathology includes an inflammatory component. From many epidemiologic studies it appears that chronic nonsteroidal anti-inflammatory drugs (NSAIDs) are associated with a reduced risk of developing AD. Preclinical studies using the NSAID ibuprofen (but not only, see [Jantzen 2002]) have been performed in APP transgenic mice [Lim 2000, Yan 2003]. Results from these investigations showed that mice treated with NSAIDs have a decreased amyloid burden. The detailed mechanism of action of NSAIDs on Aβ pathology is yet to be determined but recent in vitro studies indicate that ibuprofen modifies APP processing, specifically decreasing Aβ 42 production [Yan 2003] and inhibits Aβ aggregation [Agdeppa 2003]. The density of different plaque-associated lesions, such as activated microglia, astrocytocis and dystrophic neurites, is also decreased following NSAIDs treatments [Lim 2000]. All these results provide supplementary support to the "anti-inflammatory trail" to wrestle with AD. Nonetheless, to our knowledge, there are no reports indicating what are the effects of NSAIDs on the behavior of APP transgenic mice. Lim and colleagues [Lim 2001] have shown that Tg2576 female mice treated with NSAIDs recover from locomotor defects after treatment but learning and memory functions of the same mice have not been evaluated.

Cholesterol-Lowering Drugs

There are numerous connections between AD and cholesterol homeostasis. Cholesterol is known to play a role in APP processing and Aβ generation. Data from epidemiology show linkage between apolipoprotein E (APOE) genotypes and AD and, more importantly, indicate high risk to develop the disease in people with high cholesterol levels and decreased risk in case of chronic treatments with cholesterol-lowering drugs. Data from APP transgenic mice confirmed that high cholesterol diets potentiate brain amyloidosis [Refolo 2000] and conversely cholesterol-lowering drugs decrease amyloid burden ([Refolo 2001]; see however results from [Park 2003] showing increases of plaque densities in lovastatin-treated Tg2576 mice). Interestingly a recent study has shown that inhibition of the Acyl-coenzyme A: cholesterol acyltransferase (ACAT) both severely reduces brain amyloid load in APP transgenic mice and has beneficial effects on spatial learning performance assessed in the Morris water maze task [Hutter-Paier 2004].

Metal Chelators

The presence of metals (eg iron, copper, zinc) in plaques from AD brains is a known fact and these metal ions could modulate aggregation and toxicity of Aβ. Metal chelators have proven to be efficient in dissolving amyloid plaques in post-mortem samples from AD and APP transgenic mouse brains [Cherny 2000]. Other studies have been conducted to assess the effects of treatments with metal chelators in mouse models in-vivo. Administering clioquinol (an antibacterial agent with zinc/copper chelating properties) or other lipophilic metal

chelators such as DP-109 or XH1 have been shown to reduce amyloid plaque burden and Aβ concentrations in Tg2576 or APP/PS1 mice [Cherny 2001, Dedeoglu 2004, Lee 2004b]. How chelating agents counteract Aβ pathology appears complex [Bush 2003] and may involve degradation of insoluble Aβ deposits to soluble forms of the peptide [Lee 2004b]. The consequences of treatment with metal chelators on the behavior of APP transgenic mice have not been published to our knowledge.

Immunotherapy

In a landmark paper, Dale Schenk from Elan Pharmaceuticals and colleagues [Schenk 1999] reported that treating PDAPP mice with aggregated Aβ 42 induced a clear immune response with serum anti-Aβ 42 antibody titers > 1:10.000. Outstandingly, this reaction was accompanied with spectacular withdrawal of Aβ plaques and associated brain lesions (astrocytosis, microgliosis, neuritic dystrophy) suggesting that treated mice were "immunized against AD". Reductions of amyloid pathology by means of Aβ vaccination (either active or passive, using different routes - see [Billings 2005, Oddo 2004] for effects of direct intracerebral anti-Aβ antibodies injections) have subsequently been reported in a large number of monogenic and double- triple-crossed transgenic mouse models [Gelinas 2004, Oddo 2004]. The mechanisms of Aβ clearance that involve both parenchymal deposits and intracellular aggregates [Billings 2005, Oddo 2004]) are still being examined and may rely on alternative processes such as phagocytis of amyloid complex through microglial activation, inhibition of Aβ toxicity/fibrillogenesis, traping of soluble Aβ in peripheral reservoirs. An interesting observation was recently reported by LaFerla and colleagues [Oddo 2004] in a triple (APP/PS1/Tau) transgenic mouse model that develop both Aβ and tau pathologies: vaccination was efficient in clearing Aβ and "early tau" lesions whereas aggregated tau inclusions remained unaffected. These results strikingly parallel those published from the first vaccinated human cases [Masliah 2005, Nicoll 2003] that showed reduced amyloid burden but intact mature tau pathology (intracytoplasmic tangles and neuropil threads) indicative of high-grade neurofibrillary Braak staging [Masliah 2005].

Vaccination against Aβ does not only lead to attenuation of brain lesions but also has potent effects on learning and memory skills of APP transgenic mice. Immunotherapy therefore protects and rescues spatial learning in different versions of the water maze task [Janus 2000, Kotilinek 2002, Morgan 2000]. Intracerebral injections of anti-Aβ antibodies remarkably have promnesic effects in the water maze task that rely on the hippocampus, a brain area close to the injection site (3rd ventricle) but not in an inhibitory avoidance task that involve the amygdala complex, located ventrally in the brain, far away from the injection site [Billings 2005]. The fact that only the hippocampus (but not the amygdala) showed reduced amyloidosis following vaccination, strengthens the link between Aβ clearance and recovery of function [Billings 2005]. Puzzling data have, nonetheless been reported by Dodart and collaborators [Dodart 2002b] demonstrating that antibody treatments can reverse memory deficits in PDAPP mice without affecting plaque load. Additional observations indicated that Aβ plasma concentrations were dramatically increased in treated mice and that Aβ - antibody complexes were detected in plasma and cerebrospinal fluids. This may suggest enhanced soluble Aβ sequestration following passive immunization. In fact, one may consider that the full process of Aβ generation, polymerization, deposition and regulation may be affected by

vaccination, with beneficial outcomes for behavior [Jensen 2005]. Data from immunized human AD patients have been scarcely unveiled and showed a global slowing down of cognitive decline and dementia progression [Hock 2003] although the recent study of Gilman et al. [Gilman 2005] emphasized a somewhat limited protective effect of immunization when placebo and antibody responder groups were compared.

Other Possible Therapeutic Strategies

While clinical trials for Aβ immunization have been prematurely halted because of important side effects of the treatment in a subset of patients who showed signs of aseptic meningoencephalitis [Hock 2003, Orgogozo 2003], research of safer anti-Aβ immunotherapies is still under development and will require new preclinical studies using mouse transgenic models.

Prevention of cerebral Aβ deposition through inhibition of APP (β- or γ-) secretases is also a promising direction for research that can be evaluated in-vivo in APP transgenic mice, although obstructed for different reasons such as difficulty of pharmacological compounds to cross the blood-brain barrier and potential side-effects of γ-secretase inhibitors affecting Notch activity.

Apart from pharmacological treatments, recent work from Lazarov et al. [Lazarov 2005] highlighted the impact of "behavioral therapies" on neuropathological lesions developed by APP/PS1 transgenic mice (see also [Adlard 2005]). For five months, mice experienced an enriched environment and, in comparison to animals housed in standard conditions, showed highly reduced Aβ burden when sacrificed at 6 months of age. Behavioral effects of enrichment have not yet been fully described in these transgenic mice but will certainly confirm an already known beneficial effect of environmental stimulation and even physical training on cognitive performance (see [Adlard 2005] for preliminary data).

CONCLUSION

Different validity criteria have been proposed to assess the relevance of animal models to human pathologies. These criteria can be appreciated and discussed in the context of transgenic models of AD to summarize the data presented in this paper.

"Face validity" means that phenotypes highlighted in human patients and models should share similarities. The present review has largely centered on this comparative aspect. At the neuropathological level, it appears that some of the lesions developed by APP transgenic mice resemble cerebral alterations in AD patients. These include primary brain Aβ deposits in the parenchyma but also in blood vessels as amyloid angiopathy and secondary brain alterations such as neuritic dystrophy, synaptic and cell loss, inflammatory response. Needless to say there are still several limitations. First, not all lesions are reproduced in genetically-modified mice and this is particularly relevant for neurofibrillary tangles that despite cytoskeletal disorganisation, are absent from the brain of APP transgenic mice. Also neuronal loss and brain atrophy appeared to be different in mice and humans, both quantitatively and qualitatively. Direct comparison of mice behavioral phenotypes and AD symptoms looks at first sight to be hazardous and, at least, requires caution and multilevel screening (including basic neurological evaluation) of the effect of mutated APP transgenes. Numerous data

indicate however there are detrimental effects of Aβ overproduction in learning and memory functions. In this sense, the lack of reliable relationship between Aβ plaque burden and cognitive deficit, together with the evidence of early onset behavioral impairments, strongly suggest a pathogenic role for non-aggregated Aβ assemblies. The growing literature (eg [Cleary 2005]) focusing on the properties and functions of Aβ oligomers support this hypothesis that undoubtedly will help in guiding new therapeutic approaches.

"Predictive validity" requires identity of drug and treatment effects in the model and human pathological conditions (ie pharmacological isomorphism). APP transgenic mice seem, at least partly, to match this criterion and have proven helpful in dissecting the mode of action of different drugs. Also, experiments carried out in genetically modified mice may be very useful for the research and validation of in-vivo disease markers. Implementation of imaging approaches in humans, on the basis of mice studies, is today somewhat premature or technically unachievable, and application of these methods to human patients, allowing early diagnosis and treatment opportunities, will require supplementary research efforts.

"Etiological validity" is defined as an identity between underlying biological mechanisms in both humans and animals modeling the disease. Oversynthesis of brain Aβ deriving from mutated genes associated with familial forms of AD has been effectively reproduced in transgenic mice. However, the whole disease phenotype, including neurofibrillary alterations, severe neuronal loss, brain atrophy, is not successfully mimicked in APP or APP/PS1 mice. This suggests that some pieces of the physiopathogenic puzzle leading to Alzheimer's disease are still missing in these mice models. Recent models using APPxTau transgenic mice are more prone to reproduce all brain lesions of the human pathology (plaques and "tangles"), hence strengthening "face validity". However, the "etiological validity" of these double-triple mutants is reduced as, to date, human neurofibrillary alterations are independent of tau gene mutations.

Finally, the etiological validity of the APP and APP/PS1 transgenic lines appears also limited to genetically-caused AD pathology which only occurs in a restricted subset of patients. Causal factors for sporadic AD have not been yet fully determined and reproduced in animal models. This is obviously a challenge for future research.

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In: Alzheimer's Disease Diagnosis and Treatments ISBN: 978-1-61122-064-3
Editor: Marisa R. Boyd

Chapter 12

RELEVANCE OF COX-2 INHIBITORS IN ALZHEIMER'S DISEASE

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ABSTRACT

Cyclooxygenase 2 (COX-2) is one of the main enzymes involved in inflammation and a major player in prostaglandin synthesis. There exists data that suggest a potential role of COX-2 in Alzheimer's disease (AD) pathogenesis. AD is the most prevalent form of dementia affecting 10% of individuals over the age of 65 and 50% of individuals over 85 years of age and is characterized by the presence of beta-amyloid (Aβ) deposits and neurofibrillary tangles (NFT) comprising of hyperphosphorylated tau. Aβ peptides have been shown to trigger inflammation and to stimulate COX-2 activity in various cell types including neurons, glia (microglia and astrocytes) and cerebrovascular cells. Several epidemiological studies have shown that the use of non-selective COX inhibitors are associated with reduced risk of developing AD. COX-2 inhibitors have also been shown to alter AD pathology and ameliorate some behavioral impairment in transgenic mouse models of AD. Furthermore, in these mouse models, it has been shown that COX-2 inhibitors may influence APP processing. More studies are required to determine whether COX-2 inhibitors have beneficial or detrimental effects on the treatment of AD.

ALZHEIMER'S DISEASE, INFLAMMATION AND COX-2 INHIBITORS

Alzheimer's disease (AD), named after Dr. Alois Alzheimer is a neurodegenerative disorder characterized by intracellular hyperphosphorylated tau and extracellular beta-amyloid (Aβ) peptide deposits [1]. AD is the most prevalent form of dementia in the western world. The number of people afflicted with this disease in the U.S. alone is approximately 4

million and is expected to reach 14 million by the year 2050 [2].The clinical progression of the disease typically includes memory loss followed by physical incapacitation and finally death. The only current definitive diagnosis of AD pathology is the presence of tau tangles and Aβ plaques during post-mortem analysis. These abnormalities are considered the hallmark of AD pathology and Aβ is hypothesized to play a central role in the disease process. Sporadic AD, has an age of onset of 65 or over and the etiology is multi-factorial [3-6]. The pre-disposing risk factors involved in the pathogenesis of sporadic AD include age, diabetes, hypertension, elevated cholesterol levels and head injury [7, 8]. The familial cases of AD are mainly due to genetic mutations in the presenilin genes (PS1 and PS2) and in the amyloid precursor protein (APP) gene which results in the overproduction of Aβ. The apolipoprotein E (APOE) ε4 allele is also reported to be a robust genetic risk factor for AD [9].

In addition to the amyloid plaques and NFTs, extensive Aβ deposition in the cerebrovasculature (cerebral amyloid angiopathy) and white matter lesions have also been observed in AD [10-14]. Furthermore, several converging lines of evidence indicate that both the amyloid plaques and the cerebrovascular deposits of Aβ are sites of inflammatory processes, suggesting that inflammation may also play an important role in the etiology of AD [15-17]. This is further confirmed by the presence of reactive microglia and astrocytes in and around the Aβ deposits, which may contribute to the neurodegeneration observed in AD, by initiating pro-inflammatory cascades leading to the release of cytokines, chemokines and prostaglandins (PGs) [18].

The cyclooxygenase (COX) enzymes COX-1 and COX-2 responsible for the production of PGs from the substrate arachidonic acid are also upregulated in regions of the AD brain undergoing degeneration [19-21]. The constitutively expressed COX-1 enzyme is mainly responsible for housekeeping functions in addition to the production of PGs and thromboxane (TA) in the gastric mucosa [22]. By contrast, the inducible COX-2 enzyme is expressed mostly in the central nervous system (CNS) and inflammatory cells [23, 24].

Non-steroidal anti-inflammatory drugs (NSAIDs) block both COX-1 and COX-2 to differing degrees. NSAIDs are therapeutically used in patients suffering from rheumatoid arthritis, osteoarthritis and various other indications but they commonly produce gastrointestinal (GI) side effects. The GI side effects are attributed mainly to the inhibition of COX-1 enzyme which is essential for normal functioning of the gastric mucosa. Thus, newer NSAIDs have been designed to be selective toward COX-2 and are shown to have reduced GI toxicity [25, 26]. The putative therapeutic benefit of NSAIDs for AD is based on the observations of several major epidemiological studies showing reduced prevalence of AD among patients suffering from arthritis [27, 28]. More recently, it has been shown that some NSAIDs decrease the production of Aβ1-42, the major component of senile plaques in the AD brain. The proposed mechanism for this effect is an allosteric modulation of gamma (γ) -secretase activity, one of the enzymes responsible for the production of Aβ from APP [29]. Other studies have also shown that a subset of NSAIDs lower Aβ1-42 production by possibly affecting the substrate APP or by directly modulating the γ-secretase complex itself to affect amyloid production [30]. Other evidence for the putative benefit of COX-2 inhibitors in AD is also shown by the observation of reduced inflammation and vasoconstriction in transgenic mouse models of AD [31, 32].

However, by decreasing PG production, COX-2 inhibitors may lead to increased prothrombotic activity due to the increased production of thromboxane A2 (TXA2) as revealed by recent studies in rat peritoneal cells [33, 34]. Penglis and colleagues (2000) report a disproportionate ratio of PGE2/ TXA2 due to the differing kinetics of PGE synthase and TXA synthase enzymes [34]. Hence the hypothesis for increased cardiovascular risk in the long-term use of COX-2 inhibitors could be attributed to this imbalanced ratio of the above two mentioned products. Also prolonged use of the COX-2 inhibitors Rofecoxib (Vioxx) and Celecoxib (Celebrex) has been associated with increased cardiovascular risk in two recent cancer trials [35, 36].

Although there is a plethora of research on AD, there is no known cure for this disease todate and the neurodegeneration it causes is without remission [37]. Given that the current treatments, acetylcholinesterase inhibitors and NMDA receptor antagonists, offer symptomatic relief and only slow the progression of the disease to some extent [38], probing therapeutic targets for AD in the COX-2 inflammatory pathway seems like a logical adjunctive approach. However, the cardiovascular side-effects currently associated with selective COX-2 inhibitors need to be addressed first.

CLINICAL IMPLICATIONS OF THE USE OF COX-2 INHIBITORS IN AD PATIENTS

Numerous epidemiological studies have shown that treatments with NSAIDs are associated with reduced risk for AD. A retrospective study conducted at Johns Hopkins Alzheimer's Disease Research Center showed reduced prevalence of dementia among NSAID users [39]. Also, the Rotterdam study showed that protection by NSAIDs was specific for AD and remained significant even after the adjustment of possible confounding factors [40, 41]. Subsequent investigation revealed that this protective effect was present only among long-term users [42]. Similarly, the results from the Baltimore Longitudinal Study of Aging were consistent with the previous studies indicating protection against AD among NSAID users [43]. More recently, the data from the Cache County study also confirmed a reduced occurrence of AD among NSAID users [44]. However, a case-control analysis of the Quebec participants in the Canadian Study of Health and Aging failed to observe any significant difference in the proportion of cases and controls who had received NSAID prescriptions in the 3 years prior to the onset of symptoms of dementia [45].

Reduced GI toxicity for selective COX-2 inhibitors has resulted in the investigation into their putative therapeutic value in AD [46, 47]. However, several clinical trials have failed to show any therapeutic benefit in patients already diagnosed with AD. For instance, a clinical trial conducted by Alzheimer's Disease Cooperative Study using Rofecoxib (a selective COX-2 inhibitor) and naproxen (non-selective COX inhibitor) for AD treatment failed to show efficacy with either drug [48]. Another clinical trial assessing Rofecoxib as a treatment for AD was also unsuccessful [49]. Although there was no benefit of COX-2 inhibitors once the onset of disease had occurred, the possible benefit of these drugs as prophylactic compounds has not yet been ruled out. Unfortunately, the treatment phase of a National Institute of Health funded multi-center prevention trial testing this hypothesis was prematurely halted due to the observation of an increased cardiovascular risk in cancer

patients taking selective COX-2 inhibitors [35, 50]. A recent study aimed at delaying the progression to AD in mild cognitive impairment (MCI) patients, the clinical state prior to the diagnosis of AD found no effect of Rofecoxib on cognition or reduction in the development of AD in MCI patients [51]. These controversial clinical findings could be clarified by performing additional studies *in vitro* and *in vivo* to elucidate the mechanism of action of selective COX-2 inhibitors and thereby modify the deleterious effects associated with them.

COX-2 INHIBITORS AND APP PROCESSING: EVIDENCE FROM *IN VITRO* STUDIES

We have previously shown that inflammatory cascades initiated by amyloid peptides [52] can lead to the production of arachidonic acid via the activation of phospholipases and MAP kinases which are then metabolized by 5-Lipoxygenase and COX-2 enzymes to generate pro-inflammatory eicosanoids. Since several studies have revealed increased activity or overexpression of COX-2 enzyme in AD patients and the subsequent increase in PG production, studies of the effects of COX-2 on APP metabolism are being conducted.

It is suggested that COX-2 inhibitors may be able to alter the production of Aβ peptides in the brain by modulating the γ-secretase activity. Levels of COX-2 enzyme are increased in the AD brain, supporting a neuroinflammatory role for this cascade in the process associated with AD pathology [53-55]. Numerous studies involving administration of COX inhibitors (both NSAIDs and COX-2 selective inhibitors) have been undertaken both *in vitro* (in cultured cells) and *in vivo* (in transgenic AD mice) in order to determine whether COX inhibitors can affect the processing of APP. *In vitro* studies have attempted to elucidate the potential impact of COX-2 inhibitors on the enzymatic processing of APP [56, 57]. Cell lines which over-express mutant forms of APP or the PS-1 enzyme have been used [58-61], to determine the effects of COX inhibitors on the processing of APP. APP is a large transmembrane protein cleaved by three secretase enzymes namely alpha (α)-secretase, beta(β)-secretase and γ-secretase. According to the amyloid cascade hypothesis the two major enzymes, which enable the generation of Aβ from βAPP, are the β and γ secretases [62]. β-secretase cleaves APP at the N-terminus to release sAPPβ (a 100-kD soluble N-terminal fragment) and C99, (a 12-kD C-terminal fragment which remains membrane bound) [63]. Cleavage by α-secretase produces sAPPα (a large soluble N-terminal fragment) and C83, (a 10-kD membrane-bound C-terminal fragment). Both C-terminal fragments, C99 and C83, then become the substrate for one or more γ-secretases that cleave the fragments within their transmembrane domains, leading to the release and secretion of Aβ and the nonpathogenic p3 peptide, respectively [64]. The output measured to determine APP processing is quantification of the production of Aβ peptides, secreted fragments of APP (sAPPα and sAPPβ), and intracellular C-terminal fragments of APP. Many of these studies have suggested a potential link between COX enzymes and γ- secretase mediated (amyloidogenic) processing of APP [65, 66]. In support of this notion, a subset of NSAIDS have been shown to alter γ-secretase mediated cleavage of APP in cultured cells by shifting away from production of Aβ1-42 and towards Aβ1-38 [67]. Other mechanisms regarding the effects of NSAIDs on APP metabolism have also been proposed including the possibility that certain NSAIDs work

by changing the conformation of PS-1, affecting the proximity of APP to PS-1 [68], or that NSAIDs reduce Aβ1-42 generation by inhibition of the Rho (GTP binding protein) pathway [69]. The observation that a subset of NSAIDs can directly modulate γ secretase activity [70-72] may indicate a direct link between COX enzyme activity and the amyloidogenic processing of APP.

EFFECT OF COX-2 INHIBITORS *IN VIVO* IN TRANSGENIC MOUSE MODELS OF AD

Some studies have shown that selective COX-2 inhibitors increase Aβ production [73]. In order to further elucidate if COX-2 inhibitors decrease or increase Aβ production subsequent studies have been conducted *in vivo* in different mouse models of AD. Studies with transgenic mice treated with Ibuprofen for 4 months revealed a reduction in microglial activation and reduced brain Aβ levels suggesting that NSAIDs could affect either APP processing or Aβ clearance [74]. To further elucidate the effect of COX-2 enzyme *in vivo* on APP processing Xiang Z et al (2002) performed studies on mice expressing the 'Swedish' mutation (TgAPPsw)/mutant PS1/COX-2 (mice expressing human COX-2 selectively in neurons). Their studies have revealed potentiation of brain amyloid plaque formation and increased PGE2 levels in mice at 24 months of age suggesting that COX-2 influences APP processing and promotes amyloidosis in the brain [75]. COX-2 inhibitors could therefore be beneficial in treating AD patients either by affecting APP processing or by decreasing amyloid burden by increasing the clearance of Aβ from the brain.

Reports have shown increased levels of COX-2 enzyme in the brain of AD patients. In order to analyze the effect of Aβ on COX-2 activity we treated organotypic rat brain slices with synthetic Aβ peptide and showed that it stimulated the production of PGE2 and TNFα via a COX-2 dependent manner [53]. Studies with TgAPPsw mice which have the mutation that causes early onset AD in humans reveal the presence of abundant Aβ deposits that are visible beginning at 9 months of age. Our studies using organotypic brain slice cultures of TgAPPsw mice (10, 14 and 17 month-old) showed an increased secretion of both PGE2 and TNFα as compared to brain slice cultures of wild type mice. A selective COX-2 inhibitor NS-398 potently inhibited the increased production of PGE2 and partially reduced TNFα production in brain slice cultures from 14 month-old TgAPPsw mice as compared to control cultures. Our results suggested that the increased eicosanoid and cytokine levels observed in these transgenic mouse models are dependent on COX-2 activity and that COX-2 is up-regulated in the brain of these TgAPPsw mice [32].

Products of the COX-2 enzyme are also known to affect long-term potentiation (LTP) in hippocampal neurons and postsynaptic membrane excitability [76] suggesting that a stimulation of COX-2 activity by Aβ may impair neuronal functions and affect learning and memory [77]. Metabolites of the arachidonic acid cascade are important mediators of LTP and neuronal plasticity; the abnormal stimulation of COX-2 activity observed might therefore lead to impaired neuronal function, which has been elucidated by other studies showing that increased COX-2 causes neurotoxicity by increasing the production of pro-inflammatory molecules [78]. Hence COX-2 inhibitors may provide a therapeutic target for AD by

decreasing the production of pro-inflammatory products resulting in reduced glial activation and also improve learning and memory by preventing the deleterious effects of COX-2 on LTP in hippocampal neurons.

EFFECT OF COX-2 INHIBITORS ON BEHAVIORAL CHANGES IN MOUSE MODELS OF AD

In vivo studies suggest that COX-2 enzyme is involved in learning and memory, not only in AD, but also under normal physiological conditions when it is activated or over-expressed. For example, transgenic mouse models overexpressing COX-2 in neurons produce elevated levels of PGs in the brain and display cognitive deficits from 12-20 months of age [79]. Furthermore, in a Sprague Dawley rat model of traumatic brain injury (TBI), the administration of the COX-2 inhibitor Nimesulide resulted in a significant improvement in cognitive function compared to vehicle-treated controls after injury [80]. Using a Barnes Maze, in which the animal has to use spatial cues to escape to a hidden box placed under a circular platform, Cernak et al (2002) reported statistically significant positive effects of COX-2 inhibitors on spatial memory.

In the case of AD, different animal models have been genetically engineered to present AD-like symptoms such as accumulation of Aβ peptide, hyper-phosphorylation of tau, increase in presenilin expression and/or increase in pro-inflammatory cytokines [81, 82]. These models are often used to evaluate the effect of potential therapeutic drugs against AD. For example, Hwang and colleagues (2002) demonstrated a modulation of COX-2 expression by Aβ in the brain of their transgenic mouse model expressing a mutant PS2 (hPS2m) (N141I) [83]. Using the Morris water maze, a standard spatial memory test in which animals locate a submerged platform by using visual cues, they correlated memory dysfunction to elevations of Aβ1-42 which induced COX-2 activation.

Chen and colleagues have shown, in hippocampal brain slices that COX-2 regulates PGE2 signaling in LTP [84]. They observed a negative regulation of LTP by selective COX-2 inhibitors. Such evidence would suggest that treatment of patients or animals with COX-2 inhibitors would result in reduction of synaptic plasticity. It is possible that the positive effects observed with COX-2 inhibitors are due to the reduction of neuroinflammation. On the other hand, when there is no chronic/excessive inflammation, COX-2 inhibitors may be detrimental as they disrupt learning and memory circuits and may even promote the amyloidogenic processing of APP.

FUTURE IMPLICATIONS OF COX-2 INHIBITORS IN THE TREATMENT OF AD

Are COX-2 inhibitors good alternatives to NSAIDs? Selective COX-2 inhibitors were first introduced in 1999 for the management of pain and inflammation primarily in patients with osteoarthritis and rheumatoid arthritis on the premise that they would have similar efficacy as NSAIDs, but a lower risk of GI complications. NSAIDs are among the most widely prescribed drugs for the treatment of pain and inflammation. NSAIDs inhibit both

COX enzymes and can cause severe electrolyte disturbances and renal complications such as hyponatremia, hyperkalemia, edema, hypertension, acute and chronic tubulointerstitial nephritis, papillary necrosis and glomerular lesions. Hence the reasoning was that selective COX-2 inhibitors would be a better alternative to NSAIDs due to reduced side-effects associated with the inhibition of COX-1 enzyme.

Chronic inflammation as evidenced by astrogliosis and microgliosis is believed to be a major factor in the pathogenesis of AD. Therefore, it was thought that NSAIDs, more specifically COX-2 inhibitors, could be beneficial in the treatment of AD [85]. This was supported by evidences from *in vitro* and *in vivo* studies using transgenic mouse models of AD showing that selective COX-2 inhibitors are capable of reducing the elevated production of pro-inflammatory cytokines and eicosanoids in transgenic mouse models of AD [31, 32]. Also other studies in transgenic mouse models of AD showed reduction in amyloid burden and dystrophic neurite formation along with decreased inflammation after treatment with Ibuprofen [86].

However, currently, there is intense debate on the potential use of NSAIDs, more specifically selective COX-2 inhibitors in AD due to the recent findings showing increased cardiovascular risk in patients taking Celecoxib and Rofecoxib. From a scientific standpoint the increased cardiovascular risk associated with COX-2 inhibitors is attributed mainly to the fact that COX-2 catalyzes the conversion of arachidonic acid to eicosanoids that play an important role in maintaining cardiovascular homeostasis [87]. TXA2 which is derived mainly from COX-1 activity via thromboxane synthase, causes irreversible platelet aggregation, vasoconstriction and smooth muscle proliferation, while PGI2 synthesized by COX-2 counteracts the effects of TXA2. As a result of COX-2 inhibition TXA2 levels are increased, thereby possibly elevating the risk of coronary heart disease [87-89].

Recent clinical trials contradict the hypothesis that selective COX-2 inhibitors could be beneficial in AD patients, since they found no effect in delaying MCI or AD [51]. Kukar et al (2005) show that treatment with the specific COX-2 inhibitor Celecoxib increases Aβ42 levels in mouse models of AD [73]. Due to contradicting results from various clinical trials and studies using animal models of AD, more research is needed to investigate the efficacy of COX-2 inhibitors in AD. Other studies suggest that COX-2 inhibitors have a negative effect by killing neurons instead of protecting them due to increased production of Aβ1-42 [90]. Hence there is an ongoing debate as to the efficacy of COX-2 inhibitors in the treatment of AD.

Although there have been positive results with COX-2 inhibitors *in vitro* and *in vivo* in animal mouse models, the recent cardiovascular risks associated with these drugs have caused a setback in the clinical trials and warranted further evaluation on the safety of these drugs. Some researchers at Johns Hopkins have another line of thought for the cardiovascular complications associated with COX-2 inhibitors. They suggest that not all metabolites produced by the activation of COX-2 enzyme are deleterious and that certain metabolites may actually be protective. PGs are involved in a wide variety of bodily activities including relaxation and contraction of muscles and blood vessels, control of blood pressure and inflammation. Recent studies have found that PGD2 (PG most produced in the brain) is either protective or harmful depending on where it docks on the neuronal cell surface [91].

Because COX-2 inhibitors have tremendous potential for the prevention and/or treatment of cancer and AD, it is extremely important that thoughtful consideration of risks versus

benefits be given to current as well as proposed future uses of these drugs in the treatment of AD. It has been speculated that particular genetic traits may make some individuals more susceptible to side effects of COX-2 inhibitors than others and that these factors must be taken into account in future research. But as of now, current research regarding the effects of COX-2 inhibitors indicates that they may be useful as a prophylactic treatment for AD. Selective COX-2 inhibitors may not provide any long-term benefit and may actually be harmful since they raise Aβ levels. However, given all these scenarios, AD pateints need to evaluate the benefits of these COX-2 inhibitors against the potential cardiovascular risk factors asociated with them. Future research should be targeted at developing COX-2 inhibitors with fewer side effects whilst still retaining their therapeutic efficacy in order to find a cure for this 'disease of the mind'.

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In: Alzheimer's Disease Diagnosis and Treatments ISBN: 978-1-61122-064-3
Editor: Marisa R. Boyd

Chapter 13

COPPER STUDIES IN ALZHEIMER'S DISEASE

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ABSTRACT

Abnormalities of brain metal homeostasis in Alzheimer's disease (AD) could contribute to set up chemical conditions where β-amyloid (Aβ) toxicity and deposition are promoted. Recent studies, some also *in vivo*, have shown the possible implication of copper in AD pathogenesis. In particular, evidence collected in the last five years showed that abnormalities in copper distribution deriving from blood stream variations, or as a consequence of aging, correlate with functional or anatomical deficits in AD. Serum copper increases specifically in AD and its assessment may help to non-invasively discriminate AD from normalcy and vascular dementia. Moreover, changes in distribution of the serum copper components, consisting of an increase of a copper fraction not related to ceruloplasmin, seem to be characteristic of AD and possibly implicated in the pathogenesis of the disease.

INTRODUCTION

Alzheimer's disease (AD) is an irreversible, progressive neurodegenerative disorder, characterized by gradual cognitive deficits associated with abnormal behavior, personality changes, ultimately leading to dementia. These deficiencies are related to loss of neurons and presence of dystrophic neuritis and synapses. AD advances by stages, from early, mild

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forgetfulness to severe dementia. The earliest symptoms often include loss of recent memory, faulty judgment, and change in personality. AD patients will progressively lose all reasoning abilities and become dependent on other people. On average, AD patients live 8 to 10 years after diagnosis.

Redox transition metals and oxyradicals are reactive species implicated in a number of human diseases such as cataract, arthritis, renal and liver failure, heart and lung diseases, diabetes, and ischemia-reperfusion syndromes [1]. In the last seven years considerable evidence has been accumulated relative to the role played by iron, copper and oxidative species in the neurodegeneration of AD [2,3]. There is compelling evidence that beta amyloid (Aβ) deposition in AD involves oxidative stress and anomalous metal–Aβ protein interaction. New studies have implicated redox active metals such as copper, iron, and zinc as key mediating factors in these processes. Iron and copper are highly concentrated within senile plaques (SPs) and neurofibrillary tangles (NFTs), the histopathologic hallmarks of AD [4-6]. Both metals can catalyze Fenton's reactions generating a flux of reactive oxygen species that can damage functional and structural macromolecules [6].

METALLOCHEMISTRY IN AD

According to the hypotheses that oxidative stress and metal imbalance are potential factors leading to AD, metals seem to play an important role in the aggregation and toxicity of amyloid. Although previous studies have attempted to quantify cerebral copper levels in AD had produced highly variable results, recent studies found a 2-fold increase of copper levels in the CSF [7], serum [8,9], and amyloid plaque rim [4], along with an increase in the levels of brain and CSF ceruloplasmin, the main copper-binding /transporting ferroxidase protein [10].

The metallochemistry of AD has gradually developed in the mid '90s, with the observation that the amyloid precursor protein (APP) possesses selective zinc and copper binding sequences. These sites appear to mediate redox activity and cause precipitation of Aß under mildly acidic condition even at very low concentrations [11,12]. Such events might therefore be also occurring in the brain affected by AD. In addition, Aß possesses selective high and low-affinity metal-binding sites, binding equimolar amounts of copper and zinc. In conditions of acidosis, copper completely displaces zinc from Aß. This metal-induced precipitation of Aß is completely reversed by chelation [12] as observed in post mortem AD brain samples.

Apart from metal dependent aggregation, it is metals such as copper and iron that confer the Aß peptide its redox activity: Aß in fact reduces the metal ions, producing hydrogen peroxide by transferring electrons to O_2 [13,14]. This reduction reaction seems to mediate Aß-induced oxidative stress and toxicity. Hydrogen peroxide is in fact a prooxidant molecule, triggering Fenton's like reactions and generating hydroxyl radicals.

There is now convincing evidence that Aβ does not always spontaneously aggregate, rather it does so as an age-dependent reaction. A current hypothesis suggests that, in AD, stochastic neurochemical events, such as the oxidation of Aβ or a rise in copper or iron, may convert a small portion of Aβ to a rouge form with redox reactivity [7]. The copper ions found "in situ" in plaques and tangles are redox competent [5]. Aβ is a normal component of

healthy CSF [15] and the peptide is ubiquitously expressed in the cerebral cortex. Therefore, additional, possibly age-dependent, neurochemical reactions other than simple production of Aβ, must contribute to amyloid formation and deposition in AD, also accounting for the fact that amyloid deposits are focal (related to synapses and the cerebrovascular lamina media) and not uniform in their distribution [7]. The abnormal combination of Aβ with copper or iron confers redox properties to Aβ-metal complexes and this could induce the precipitation of the protein into metal-enriched masses (plaques), as well as the production of hydrogen peroxide, which may, in turn, mediate the conspicuous oxidative damage observed in the AD brain [7].

IN VIVO STUDIES IN AD

In AD an abnormal brain homeostasis of metals, and copper in particular, possibly due to variations in circulating levels, or as a consequence of aging, could contribute to set up chemical conditions where toxicity and deposition of Aβ are promoted. Recent studies have shown the possible implication of copper in the pathogenesis of AD also *in vivo*. Even though [16,17] found no differences in serum copper levels between AD and controls, new studies of [8,9] – one of the authors of the present chapter – have shown an increase in serum copper in AD. In addition, there is evidence that copper measurements may help to non-invasively discriminate AD from normalcy [9] and vascular dementia [18]. The potential implications of a better understanding of the molecular bases and the role of oxidative stress and redox metal in the pathogenesis of AD are far reaching and this knowledge would certainly be key in the prevention, diagnosis and eventually the treatment of this form of dementia.

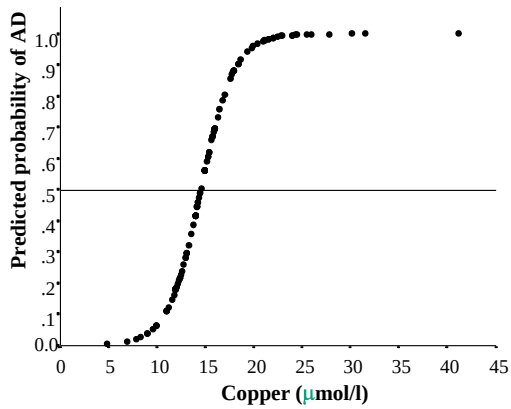

Figure 1A. Model predicted probability to belong to the AD group according to serum copper levels (μmol/l). With permission by Squitti et al., Neurol 2002; 59(8): 1153-61.

Studies by our group in the last 5 years strongly support the evidence of the implication of copper in the pathogenesis of AD *in vivo*. We investigated copper, iron, total hydro- and lipoperoxides, transferrin and ceruloplasmin levels, and the antioxidant capacity (Total Radical Trapping Antioxidant capacity or TRAP) in the serum of patients affected by AD, vascular dementia (VAD) and normal subjects [9,18]. In addition we attempted to determine whether biological variables of serum oxidative stress correlated with functional or

anatomical deficits in the AD brain [9]. Our first results indicated that copper levels were higher in AD patients compared to normal elderly controls [9]. We found that copper, unlike other various peripheral markers of oxidative stress and trace metals, could discriminate between AD patients and healthy individuals in a high percentage of cases (95%). An increase of 1 μmol/l in serum copper, accounted for more than 80% of the risk to belong to the AD group (Figure 1A), while TRAP increments of 0.1 mmol/l decreased the risk by 37%. A serum copper level of 16 μmol/l (1.02 mg/l) separated effectively AD cases from controls with a specificity of 95%, and an approximate sensitivity of 60% (Figure 1B,C) [9].

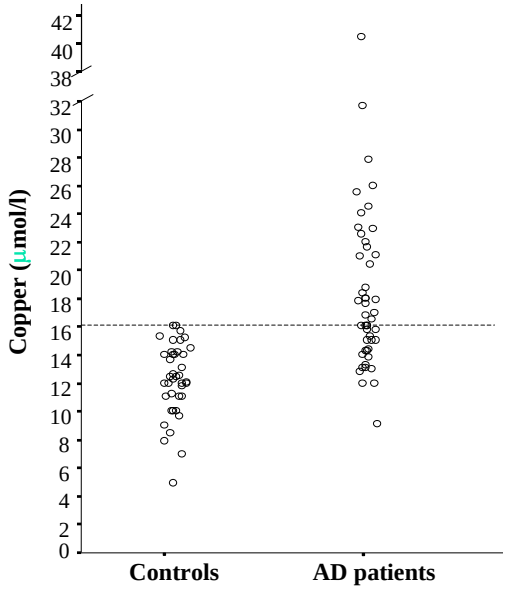

Figure 1B. Serum copper levels (μmol/l) in individual controls and AD patients. The dotted line indicates the proposed cut-off based on ROC curves (16 μmol/l). Controls were below the cut-off. With permission by Squitti et al., Neurol 2002; 59(8): 1153-61.

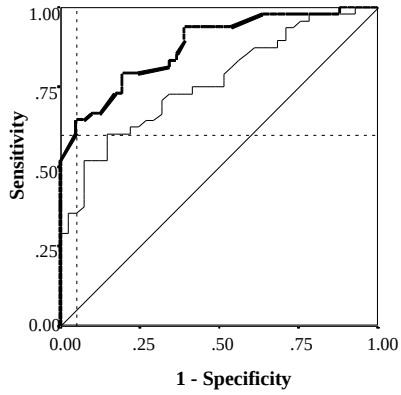

Figure 1C. ROC curves showing specificity and sensitivity of serum total peroxides (regular line) and copper (bold line) as peripheral markers of AD. For copper a cut-off of 16 μmol/l best discriminated AD patients from controls. With permission by Squitti et al., Neurol 2002; 59(8): 1153-61.

The data presented are in agreement with previous reports of altered peripheral copper metabolism in AD [8,19,20], and compound previous evidence of central copper homeostasis perturbation, as also evidenced by increased amounts of copper and iron in the SPs and in the CSF of AD patients [4,21], even though at least one recent study has failed to shown similar results [17].

Impairment of copper plasma levels, as suggested by our results, could determine abnormal brain copper concentrations in AD, these concentrations being dependent on circulating copper [22].

To assess whether copper changes in the serum of AD patients is related to abnormalities specific of this disease, we analyzed the correlation between copper content and neuropsychological performance, as well as cerebrovascular or atrophic burden, as estimated by brain MRI and ultrasonography of the cerebral blood vessels.

Elevated copper levels in particular, as well as low TRAP capacity, were correlated with typical neuropsychological deficits found in AD patients (Table 1). TRAP capacity reflects antioxidant vitamin status (mainly vitamin E and C) and presence of compounds with indirect antioxidant effects (vitamin B and folate) [23,19,24]. Its correlation with poor cognitive performance supports recent evidence for a major role of inadequate group B vitamin levels in AD (B6, B12 and folate in particular), as well as a probable effect of vitamin E on disease progression [25-27].

In our clinical studies, we also found a positive correlation between isolated medial temporal lobe atrophy, estimated by visual inspection of brain MRI, and serum oxidative-trace metals values and copper levels (Spearman rho=0.308, p=0.033) and TRAP (Spearman rho= -0.379, p=0.009).

Table 1. Correlation between outcomes of neuropsychological examination and peroxides, copper and TRAP level.

Neuropsychological test	Peroxides (n=54)	Copper (n=54)	TRAP (n=54)
RVLT Immediate Recall	r=-0.231; p=0.093	r=-0.413; p=0.002	r=0.285; p=0.039
RVLT Delayed Recall	r=-0.303; p=0.026	r=-0.391; p=0.003	r=0.304; p=0.027
Immediate Visual Memory	r=-0.158; p=0.254	r=-0.246; p=0.073	r=0.303; p=0.028
Copy Drawing	r=-0.242; p=0.078	r=-0.376; p=0.005	r=0.242; p=0.081
Copy Drawing with Landmarks	r=-0.257; p=0.061	r=-0.427; p=0.001	r=0.315; p=0.022
Raven's Progressive Matrices	r=-0.289; p=0.036	r=-0.439; p=0.001	r=0.354; p=0.01
Sentences Construction	r=-0.031; p=0.825	r=-0.204; p=0.139	r=0.129; p=0.357
Verbal Fluency	r=-0.106; p=0.446	r=-0.354; p=0.009	r=0.357; p=0.009
Digit span	r=-0.262; p=0.056	r=-0.285; p=0.037	r=0.233; p=0.094
Corsi Test	r=-0.173; p=0.21	r=-0.288; p=0.035	r=0.255; p=0.065
MMSE	r=-0.227; p=0.089	r=-0.331; p=0.012	r=0.252; p=0.061

r= Pearson Correlation coefficient. Significant at the 0.005 level (2-tailed, after Bonferroni's adjustment). With permission by Squitti et al., Neurol 2002; 59(8): 1153-61.

The correlation between elevated copper and TRAP decrement with volume loss in the medial temporal lobe suggests that these biological variables could be related more specifically to the neurodegenerative process typical of AD, possibly through copper-

mediated toxicity [11,5,28,14,29]. Moreover, as other pathologic conditions alter plasma copper content [30] and oxidative status [1], we studied the usefulness of this putative marker for AD regardless of the presence or absence of additional pathological conditions that might increase serum copper. On one hand we observed that AD patients without additional medical conditions had increases in copper levels, again suggesting that copper is a peripheral marker specific for the functional and anatomical deficits of this disease. On the other hand, serum copper levels in elderly controls with comorbidities were still below the cut-off that identifies AD, and still overall lower than in AD patients, making copper a valid discriminating factor even in the presence of comorbidities [9].

We extended our research in the metallochemistry of AD by testing copper levels in VAD patients, in order to study the specificity of the copper abnormalities for AD [18]. Serum copper levels paralleled cognitive deficits of AD but were not perturbed in the serum of VAD patients. Setting a cut-off of 16 μmol/l, serum copper levels discriminated AD from VAD, with a specificity of 85% and a sensitivity of 60% (12) (Figure 2A, B). Data from this investigation support the notion that copper may be a rather specific marker for AD, and not a non-specific correlate of brain damage and dementia.

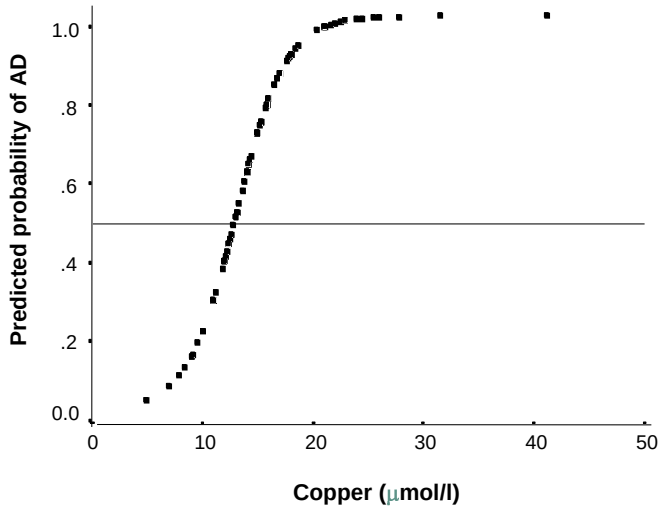

Figure 2A. Model predicted probability to belong to the AD group according to serum copper levels (μmol/l).

A study on the reliability of copper in discriminating AD from Parkinson disease is currently in progress. Preliminary results seem to reveal that copper is not elevated in Parkinson's patients, confirming that copper-mediated pathogenetic mechanisms are probably specific to AD and not related to aspecific neurodegenerative mechanisms.

A clinical study of a pair of 73 year old female monozygotic twins discordant for AD, who had had very similar habits and lifestyle, permitted also to make additional considerations about the role of copper and oxidative dysfunction [31]. The twin case was considered a good opportunity to study particularly environmental and life-style factors that could affect individual antioxidant efficiency and oxidative stress markers in AD, such as smoking, pregnancies and other sexual hormonal influences and so on. We found that differences in copper levels corresponded to a different clinical picture, in agreement with our

previous reports (Table 2) [9,19]. The patient who had greater serum levels of copper and oxidative stress indicators, whom we called Twin A, had overall worse scores on all cognitive testing and met criteria for a diagnosis of AD. Her twin sister, or Twin B, whose serum copper levels were much lower, yet slightly increased compared to normal, remained free of dementia four years after her sister's death and despite a stroke who had brought her to our attention [31]. Only a longitudinal follow up of the surviving twin will eventually determine whether she will also develop dementia, however, all current evidence suggests that her minimal cognitive deficits, far from meeting criteria for a diagnosis of dementia, are purely on a vascular basis. The twin case supports again the view that cerebrovascular dysfunction has little impact on serum copper variations [18,31].

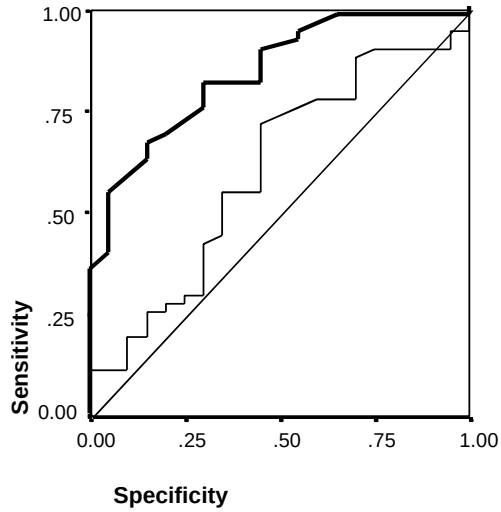

Figure 2B. ROC curve showing specificity and sensitivity of serum total peroxides (thin line) and copper (thick line) as peripheral markers. A cut-off of 16 μmol/l best discriminated AD from VAD patients.

In more recent studies we studied copper-enzymes that might be perturbed in AD, that is Copper, Zinc superoxide dismutase (Cu, Zn SOD) [32] and ceruloplasmin [33]. AD patients have higher Cu, Zn SOD activity in comparison with controls, confirming previous reports [24,34].

The increase of Cu, Zn SOD in AD is consistent with the increased serum copper levels of AD patients [8,9,18,19], thus confirming a perturbation of copper homeostasis. Copper modulates the expression of Cu, Zn SOD, as demonstrated in models *in vivo* of copper depletion [35], resulting in decreased levels of the enzyme, or in copper overload in human cells [36]. It must be emphasized that the variations of both Cu, Zn SOD and copper levels observed in AD are detected in the peripheral blood, suggesting that these changes are probably indicative of a systemic perturbation of copper homeostasis. Moreover, an abnormal level of Cu, Zn SOD was evident in 74% of patients already at 18-24 months after the documented onset of cognitive disturbances, an argument for the use of this assay as a potential tool for early diagnosis, if our findings are confirmed by others.

Table 2. Comparison of trace metals and oxidative stress species assessed at Jun 2000 in both twins.

Biological variables of trace metals and oxidative stress	Twin A absolute value	Twin B z-score	Twin B absolute value	Twin B z-score	*Normal reference range
† Copper (μmol/l)	22.9	6.3	16	2.1	9.1-15.8
‡ Copper (mg/l)	2.47	6.3	1.45	1.5	0.7- 1.55
Total peroxides (U CARR)	543	6.8	376	2.5	205-350
Homocystein (μM)	20.3	3.06	19.8	3.00	<10
TRAP (mmol/l)	1.38	0.4	1.4	0.5	1.1-1.6
Iron (μg/dl)	46	-1.2	70	-0.3	30-126
Transferrin (g/l)	2.76	0.1	2.78	0.2	1.9-3.5

* Normal range as established on our normal elderly population (mean ±2 SD). (See ref. [7,8] for details).

† Copper assay according to the Abe method.[16] With permission by Squitti et al., Arch Neurol 2004; 61: 738-43.

Dysfunction of redox status and trace metals homeostasis could be related to an inflammatory response. Significant changes in copper absorption, transport, metabolism or excretion, do occur in inflammation, where plasma copper levels rise, along with levels of the acute phase copper-protein ceruloplasmin, the main carrier of fasting serum copper [37]. Ceruloplasmin is in fact increased due to an augmented rate of its hepatic synthesis and secretion [37,30]. Much evidence supports the presence, in AD, of an inflammatory component leading to brain tissue damage, possibly through activated glia [38]. In addition there is yet somewhat controversial evidence of a concomitant inflammatory response in the general circulation of AD patients [39,40].

In order to assess the role of peripheral markers of redox trace metals in a putative inflammatory response in AD, we started studying levels the biological variables of trace metals and oxidative stress in relation to peripheral markers of inflammation, including ceruloplasmin. In addition, we attempt to define changes most specific to AD patients, comparing the results of the same assays in patients with vascular dementia (VAD).

To address the disequilibrium between copper and transferrin or ceruloplasmin we calculate the copper:transferrin and copper:ceruloplasmin ratios. We found that the copper:transferrin ratio was higher in AD compared to both normal elderly controls and VAD patients ($p<0.001$; Figure 3A).

These data suggest an "excess" of serum copper with respect to metal transporting proteins that is specific for AD patients. This initial evidence prompted us to investigate in details the relationship between copper and its main transporting protein in serum, that is ceruloplasmin.

Comparisons of copper:ceruloplasmin ratios, confirmed higher values in AD patients ($p=0.028$ vs. controls, $p=0.015$ vs VAD patients; Figure 3B).

In order to explain the "physiological" relationship between ceruloplasmin and copper, we applied both lowess non-parametric and polynomial regression models to data obtained from the control group. About 56% of copper variability could be explained by ceruloplasmin. We calculated that, in healthy subjects, for each 10 points of ceruloplasmin levels increase, copper is expected to increase by 3.28 points (95% confidence interval = 2.15 - 4.41). On the basis of this "physiological" relationship, we computed two copper measures:

the first, copper "explained by ceruloplasmin", corresponds to the theoretically expected level of copper, given a ceruloplasmin level; the second, copper "not explained by ceruloplasmin", corresponds to the residual copper with respect to the regression line determined in controls (Figure 4).

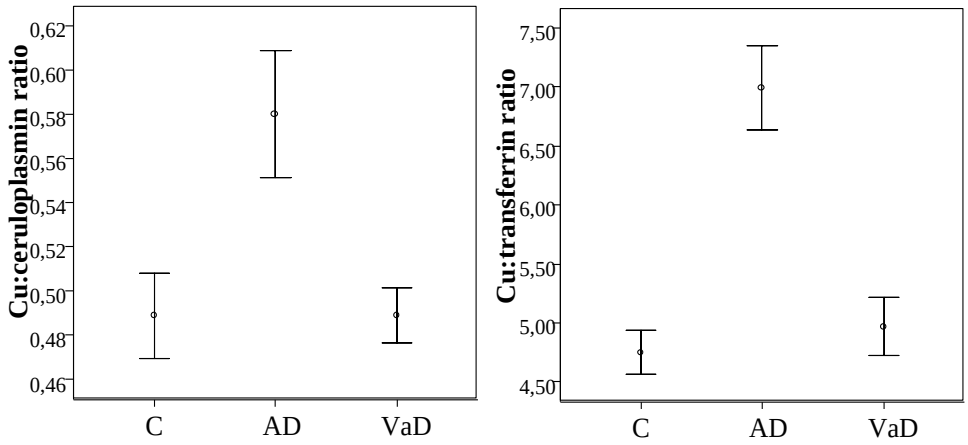

Figure 3. Mean values (± 1 standard error of the mean) of copper:ceruloplasmin ratio (A) and copper:transferrin ratios (B). With permission by Squitti et al., Neurol 2005; 22(6): 1040-6.

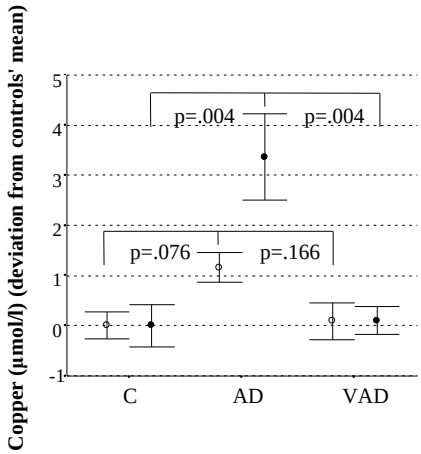

Figure 4. Mean values (± 1 standard error of the mean) of copper "explained by ceruloplasmin" (open circle ○) and copper "not explained by ceruloplasmin" (filled circle ●) in the three groups of subjects. Data are expressed in terms of deviations from controls' means. Eta-squared resulted equal to 0.06 (p=.056) for copper "explained by ceruloplasmin" and 0.14 (p=.001) for copper "not explained by ceruloplasmin". Post-hoc Tukey's comparisons vs. AD are reported. Post-hoc Tukey's comparisons vs. AD are reported. With permission by Squitti et al., Neurol 2005; 22(6): 1040-6.

The results of this study show that the portion of serum copper unexplained by ceruloplasmin, as calculated based on the ratio between ceruloplasmin and copper levels in healthy controls is higher in AD patients. It can also discriminate AD from normalcy and VAD better than copper explained by ceruloplasmin (Figure 4). Ceruloplasmin, an α-2 globulin with ferroxidase and copper transport functions is a marker of both plasma copper

status and inflammation [30,41]. Also another study analyzing indices of copper metabolism in AD found alterations in the relationship between copper and ceruloplasmin suggesting that the ceruloplasmin-copper relationship, rather than absolute serum copper levels, probably represents the key in interpreting *in vivo* copper studies on AD [42,43]. These results, in addition, make less relevant the discrepancy among studies that did [8,9], or did not find differences in serum copper levels in AD, since the relationship between copper and ceruloplasmin was not addressed [16,17]. Normally, over 90% of human serum copper is considered to be tightly bound to ceruloplasmin [44], even though some authors [37] suggested a value closer to 60% for ceruloplasmin-bound copper, similar to what determined in our study. The rest of the copper would be distributed among transcuprein (12%), albumin (12%), aminoacids (e.g. histidine), and small molecular weight complexes (0.5-5%), named "exchangeable component". Studies on transgenic mouse models show that copper can bind and interact with APP (or Aβ), which has been proposed to function as a copper/zinc metalloprotein that in AD is part of a failing metal homeostatic mechanism [45,46]. In particular, copper-binding APP has been hypothesized to represent a means for removing excess copper from brain tissue [45-47]. The excess of serum copper we estimated in AD could be explained by an efflux from cortical cells, as also proposed by some authors [48], which would also explain why the rise observed is mainly due to copper unbound to ceruloplasmin, rather than the bound fraction biosynthesized, in fact, by the liver. This hypothesis is coherent with the APP-/- knockout mouse model, where absence of APP, proposed to balance cell copper concentration, is considered causative of 40% of the observed increase in brain copper. Alternatively our results could be ascribed to a failure of copper incorporation into APP in the liver [45]. In the APP-/- mouse model copper increases by 80% in the liver and 40% in the brain, while apparently no serum variations are found [45]. This situation closely resembles Wilson's disease, a degenerative condition where copper metabolism abnormalities are key to the pathogenesis, even though serum copper levels, in this condition, are within the normal range. In this disease, in fact, the micronutrients-associated fraction, that is the exchangeable serum copper component, is extremely elevated [22]. In Wilson's disease copper is bound to small molecules that can easily reach organ tissues and cross the blood brain barrier, which normally functions as an effective mechanism to control redox metals brain tissue levels [49,7]. In studies on the distribution of copper among serum components in cancer patients, variations in the four serum fractions were found and some patients with very high level of low-molecular weight copper were also described [37]. The increase in ceruloplasmin unexplained copper calculated in our AD patients is far from the unbound copper estimated in Wilson's disease, yet it approaches the levels described in cancer patients. Even at these levels copper could be toxic, partaking in Aβ-mediated toxicity of AD, as it can easily cross the blood brain barrier [7]. In addition, our findings are coherent with the proposed activity of APP in balancing copper concentrations [45,46,48]. A recent report showed that the ingestion of low concentrations of copper (2 μM) added to drinking water markedly impairs biometals homeostasis and increases brain parenchymal Aβ in rabbits fed cholesterol-supplemented diets [50]. The specific increase in total serum copper in our AD patients was in the micromolar range, yet some authors have suggested that even increases in the nanomolar range could have an impact on AD pathogenesis [46]. The authors found no variation of ceruloplasmin in rabbit blood, suggesting that

the copper ingested could supply the fraction unbound to ceruloplasmin, similarly to what our results seem to suggest.

Though we could not look, *in vivo*, for direct evidence of a toxic effect or a change in brain Aβ burden, we developed a statistical approach to assess the potential implication of copper in the pathogenesis of AD. Our aim was to estimate the specificity of the biological indices of copper metabolism and oxidative stress in discriminating AD patients from healthy and demented controls, and correlating them to the most relevant clinical characteristics of the disease. Discriminant analysis was applied to assess the potential implication of indices of copper metabolism in AD. In particular, we developed a model to identify which biological (copper, ceruloplasmin, peroxides and TRAP) and demographic variables (age and sex) could discriminate among AD, VAD and controls. This procedure automatically identified two functions as linear combinations of the biological variables. Function 1, was obtained combining three biological variables high in the AD group, that is copper, peroxides and ceruloplasmin. These clearly separated AD patients from healthy controls (Figure 5, Function 1). The second linear combination (Function 2) provided by the discriminant procedure was due almost exclusively to the TRAP contribution to the statistical model. This function distinguished VAD from controls patients, being TRAP lower in the VAD group. According to this model, when AD patients are compared to controls the biological variables of trace metals and oxidative stress can correctly classify 80% of subjects. When the model is applied to AD and VAD populations and the MRI indices are included in the canonic function, they can discriminate 90% of patients. Indeed, a correlation between Aβ burden in cerebrospinal fluid and copper in the serum is also suggested by preliminary evidence from our laboratory (manuscript in preparation).

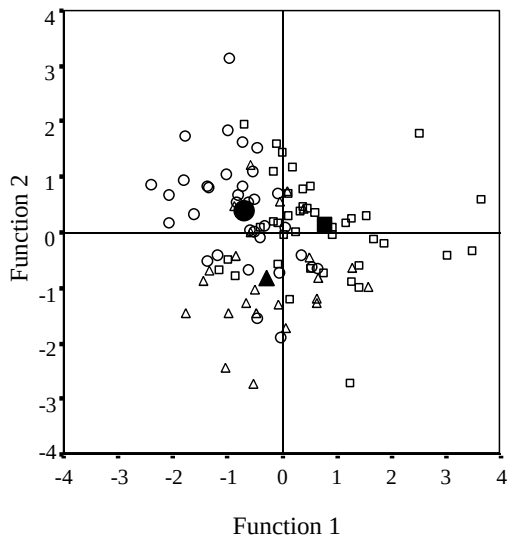

Figure 5. Discriminant analysis applied to differentiate Alzheimer's disease patients (open squares □), Vascular dementia patients (open triangles △) and control subjects (open circles ○) on the basis of biological (copper, ceruloplasmin, peroxides and TRAP) and demographic variables (age and sex). Group centroids: (filled large square ■) Alzheimer's disease patients; (filled large triangle ▲) Vascular dementia patients; (filled large circle ●) controls. Function 1 is a linear combination of copper, peroxides and ceruloplasmin levels; Function 2 is almost exclusively accounted for by TRAP levels. With permission by Squitti et al., Neurol 2005; 22(6): 1040-6.

Much evidence suggests a role of inflammatory processes in AD pathogenesis, as well as a concomitant peripheral inflammatory response [51]. No inflammatory processes were determined to be specific to AD in our patient sample, since the markers of inflammation, namely the erythrocyte sedimentation rate (ERS), albumin, electrophoretic α1,α2, γ and Interleukin 1β (IL-1β) and tumor necrosis factor α (TNF α) analysed did not differ among the three groups. Moreover, no correlation was present between cognitive testing and these physiological indices of peripheral inflammation.

The concomitant increase in our AD patients of the copper fraction explained by ceruloplasmin, a marker of inflammation, suggests that at least in part our findings could relate to general inflammatory mechanisms, even though no additional significant differences in other peripheral markers of inflammation were present.

In AD, therefore, the changes in Cu,Zn superoxide dismutase and ceruloplasmin, along with the specific copper elevation, strongly support the hypothesis of copper abnormalities. The bulk of the evidence reported from *in vivo* studies fits well with the proposed model of a major role of biometals in the pathogenesis of AD [7]. Though our results are strongly in favor of a copper-mediated tissue damage hypothesis [7]. Data on copper chemistry in AD are not univocal [16,17]. In addition recent experiments carried out on transgenic mouse models of AD have given results suggestive of a beneficial effect of copper in the prevention of amyloid formation [52,53]. Further investigation taking into consideration regional variation of diet, life style or genetic makeup as well as early and presymptomatic cases are needed.

Table 3. Neuropsychological evaluation of AD patients from the D-penicillamine and placebo groups at study entry (t0) and at the end of the observation period (t1)

	D-penicillamine group		placebo group		Time	Treatment	Time X Treatment
	baseline (t0)	end therapy (t1)	baseline (t0)	end therapy (t1)	df=1,16	df=1,16	df=1,16
Rey's Immediate Recall	21.6 (8.7)	18.8 (10.1)	24.9 (5.8)	25.3 (7.1)	F=1.316; p=0.268	F=1.814; p=0.197	F=2.510; p=0.133
Rey's Delayed Recall	2.8 (1.5)	2.9 (1.1)	4.0 (1.1)	3.8 (0.7)	F=0.000; p=1.000	F=4.414; p=0.052	F=0.302; p=0.590
Immediate Visual Memory	15.1 (4.5)	12.6 (7.0)	16.5 (3.3)	16.2 (2.9)	F=2.298; p=0.149	F=1.541; p=0.232	F=1.621; p=0.221
Copy Drawing	4.5 (3.1)	3.4 (2.7)	7.4 (3.3)	6.9 (3.5)	F=4.695; p=0.046	F=4.889; p=0.042	F=0.862; p=0.367
Copy Drawing with Landmarks	29.6 (23.7)	22.6 (21.2)	51.3 (28.2)	45.4 (26.3)	F=8.044; p=0.012	F=3.700; p=0.072	F=0.060; p=0.811
Raven's Progressive Matrices '47	16.4 (5.1)	13.8 (4.3)	22.8 (5.2)	21.8 (8.0)	F=3.419; p=0.083	F=7.831; p=0.013	F=0.654; p=0.430
Sentences Construction	11.4 (7.9)	8.7 (5.3)	13.4 (5.0)	13.6 (5.5)	F=0.579; p=0.458	F=2.198; p=0.158	F=0.808; p=0.382
Verbal Fluency	17.0 (5.2)	15.4 (8.9)	21.0 (5.4)	22.3 (6.5)	F=0.005; p=0.943	F=3.909; p=0.066	F=0.902; p=0.356
MMSE	16.8 (3.5)	15.1 (4.3)	18.3 (4.8)	17.9 (4.2)	F=2.136; p=0.163	F=1.362; p=0.260	F=0.716; p=0.410
Geriatric Depression Scale	10.3 (6.3)	9.8 (6.0)	8.4 (2.9)	9.3 (5.5)	F=0.043; p=0.838	F=0.238; p=0.632	F=0.812; p=0.381
NeuroPsychiatry Inventory	9.1 (3.3)	9 (6.5)	9.5 (3.2)	8.8 (6.0)	F=0.579; p=0.458	F=1.362; p=0.260	F=0.302; p=0.590
Gottfries Brane Steen	36.3 (8.2)	39.8 (14.1)	39.5 (14.7)	37.3 (10.7)	F=0.005; p=0.943	F=1.541; p=0.232	F=0.716; p=0.413

Values are mean (SD). With permission by Squitti et al., Eur J Clin Invest 2002;32:51-59.

The bulk of the evidence collected on our first studies has prompted us to carry out a pilot pharmacological study challenging the potential therapeutic effect of copper cheletation on AD progression, by administrating D-penicillamine, the most widely used agent in Wilson's disease. The study was carried out in with a double blind placebo controlled design. Both clinical and biochemical data from the patients enrolled were collected. Both serum and urine copper and red blood cells Cu,Zn SOD were measured [19,32]. While serum copper remained stable, its urine levels were drastically elevated by therapy, indicating that large amounts of copper were indeed being removed from the tissues. The activity of Cu,Zn SOD was drastically reduced, below control levels, revealing that this enzyme could be used as a good indicator of general depletion of the bioavaible copper. We noted a significant reduction in peroxide levels in AD patients by decreasing the bio-available copper with D-penicillamine, although it is uncertain whether clinical improvement or slowing of progression could result from this therapy (Table 3) [19].

In fact, even though we could reach the positive result of decreasing the bioavaible copper and total peroxides content in patients *in vivo*, the clinical relevance of our data could not be fully assessed, because the 24 week observation period of the study was unfortunately insufficient to detect differences in cognitive decline compared to the placebo control group. Indeed, Crapper McLachlan and colleagues [54] have shown a positive effect of chelation therapy in AD by treating patients for 24 months. More recently, Ritchie et al. [55], reported that copper chelation with clioquinol significantly slowed the rate of cognitive decline in a subset of patients with AD, as compared to untreated control subjects In our study with D-pennicillamine, surprisingly, in spite of the abundant literature existing in the field, the follow up period of 24 weeks was not sufficient to detect any cognitive decline in the placebo group and a comparison with treated patients was therefore impossible (Table 3). It must be noted however that in that pilot study we had also observed an important antioxidant change in the serum of both treated and placebo patients, possibly because both groups were taking vitamin B6 to prevent the expected D-penicillamine induced deficit of this vitamin (Figure 6). It was therefore impossible to say whether the lack of an expected 24 week cognitive decline in the AD patients from the placebo group was related to enhanced antioxiodant mechanisms (Table 3). Indeed, TRAP capacity reflects antioxidant vitamin status (mainly of vitamins E and C) as well as the effect of compounds with indirect antioxidant activity (vitamin B group and folic acid in particular; [1,24,23]. The correlation of the global serum antioxidant activity with poor cognitive performance and medial temporal atrophy supports the recent evidence for a major role of inadequate group B vitamin levels in AD (B6, B12 and folate in particular), as well as a probable effect of vitamin E on disease progression [23,25-27,56-58]. Moreover, vitamin B6 catalyzes the synthesis of glutamate, the principal aminoacid mediating neurotransmission in the brain, its uptake being necessary to restore neurotrasmitter reservoirs. In our studies we found [59,60] that TRAP as expressed by lower TRAP values, was inversely correlated with the allele ε4 of the apolipoprotein E, an established genetic risk factor for AD, both in patients and in normal elderly controls. Our findings of a decreased antioxidant activity in AD patients and in ε4 carriers in general, support the notion that presence of ε4 impairs some of the protective mechanisms against oxidative damage [60]. ApoE is related to lipid membrane integrity and oxidative injury is increased in ε4 carriers [59,60]. Total peroxides and TRAP disruption were noted in either AD and VAD, suggesting a significant oxidative stress in both dementias in comparison with normalcy [18]. Despite contrasting reports about specific pro-

or anti-oxidant agents, general agreement exists on a perturbation of oxidative balance in dementia. One can therefore assume that oxidative abnormalities accompany brain tissue degeneration, and that susceptibility to dementia is increased in individuals who have defective antioxidant machinery. Some authors have described a characteristic antioxidant blood profile in AD and VAD patients, that allows a discrimination between these two diseases [61]. Our results confirm this hypothesis, and in particular AD and VAD can be discriminated on the basis of copper levels. Copper homeostasis, therefore, seems specifically disrupted in AD, supporting the hypothesis of a selective copper-mediated toxicity as part of the pathogenic process of this disease [28,7].

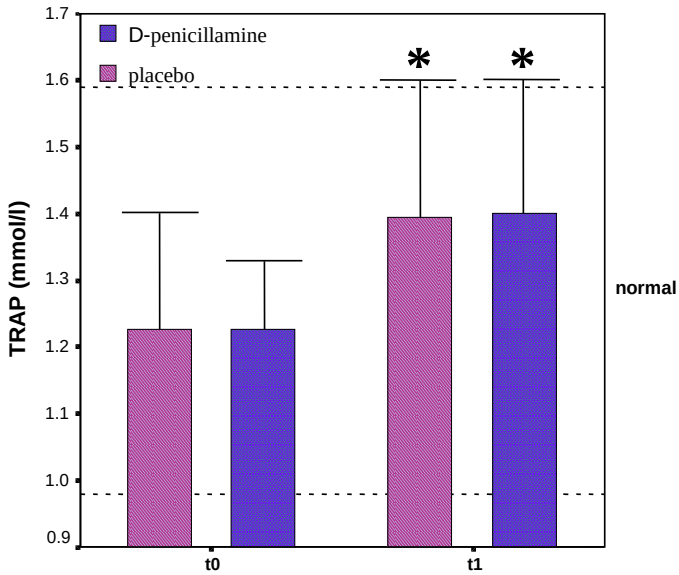

Figure 6. TRAP in the serum of patients with Alzheimer's disease participating in the D-penicillamine trial at baseline (t0) and at the end of the study (t1): No difference between groups was evident following chronic treatment. In both groups total antioxidant capacity increased after the observation period (t-test, *$p<0.05$). Data are means SD. With permission by Squitti et al., Eur J Clin Invest 2002;32:51-59.

CONCLUSION

In conclusion our recent results seem to support, even from a clinical point of view, the model proposed for copper pathogenesis in AD [7]. In particular the accuracy of the clinical characterization and the positive correlations between the biological variables suggestive of copper and antioxidant metabolism dysfunction in AD, appear to represent a valid methodological approach for confirming *in vivo* and on human subjects a conceptual hypothesis of AD pathogenesis. We propose, therefore, that, along with other, yet non-mutually exclusive hypothesis of AD neurodegeneration, two mechanisms involving abnormalities in copper metabolism may be operating: an inflammatory, ceruloplasmin-related component with excessive absorption of copper, resulting in serum level elevations; and a direct toxic mechanism related to a non ceruloplasmin-bound copper fraction that, entering the exchangeable plasma copper pool, easily crosses the blood brain barrier.

ACKNOWLEDGMENTS

Work was supported by: Grants of the Italian Ministero della Salute, PF/DML/UO4/2002 (Ricerca Finalizzata "Stress ossidativo nei parkinsonismi occupazionali da metalli di transizione"), and by a grant from the AFaR Foundation - Osp. Fatebenefratelli, Rome, Italy.

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In: Alzheimer's Disease Diagnosis and Treatments ISBN: 978-1-61122-064-3
Editor: Marisa R. Boyd

Chapter 14

THEORETICAL COMPARISON OF COPPER CHELATORS AS ANTI-ALZHEIMER AND ANTI-PRION AGENTS

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Neurodegenerative diseases, such as Alzheimer's disease (AD) and prion diseases (PDs), are among the most serious threats to human health[1,2]. Although the pathogenetic mechanisms of these diseases are not very clear, it is widely accepted that transition metal ions (*e.g.*, copper ions) and reactive oxidative species (ROS) are implicated in the pathogenesis of AD and PDs[3-5]. As a result, there is growing interest in using metal chelators and antioxidants to combat both diseases. Some metal chelators have showed promising preventive effects on AD and PDs. For instance, desferrioxamine, clioquinol and D-(-)-penicillamine are effective to prevent AD *in vitro* and/or *in vivo*[6-8] and D-(-)-penicillamine can delay the onset of PD in mice[9]. As to antioxidants' effects, although convincing clinical evidence is still lacking, some modest therapeutic effects on AD and PDs have been observed for antioxidant combinations[10-12].

Considering the preliminary success of metal chelators in treating AD and PDs and the fact that some superoxide dismutase (SOD) mimics are metal chelates, we proposed a new strategy to combat these diseases. That is, using SOD-mimetic ligands to chelate copper ions, then the chelates will hold radical-scavenging potential, which may lead to better clinical effects than pure metal chelators. It is interesting to note that this strategy is supported by recent *in vitro* experimental findings that copper chelators whose copper complexes have high SOD-like activity are potential anti-prion drug candidates[13]. To evaluate the potential of existing copper chelators as anti-Alzheimer and anti-prion drug candidates, we attempted to compare the copper-binding ability and SOD-like activity of various chelators and derived

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chelates by theoretical calculations. The results may help screen new anti-Alzheimer and anti-prion drugs.

Our previous study revealed that the copper-binding ability of chelators can be measured by binding energy (BE) and the SOD-like activity of chelates can be characterized by electron affinity (EA)[14]. According to the definitions of BE and EA, BE = $TE_s - TE_c$, in which, TE_s is the sum of total electronic energies for ligand and metal ion, TE_c is the total electronic energy of chelate; EA = $TE_{Cu(I)} - TE_{Cu(II)}$, in which, $TE_{Cu(I)}$ is the total electronic energy of Cu(I)-chelate, $TE_{Cu(II)}$ is the total electronic energy of Cu(II)-chelate. The higher the BE is, the stronger the copper-binding ability; the lower the EA is, the higher the SOD-like activity[14].

All of the molecules were calculated at full optimization level in gas phase by density functional theory[15,16] with B3LYP functional[17-19]. During the calculations, standard double zeta basis set was used for all light elements, while for metals, non-relativistic effective core potential (ECP) was employed. The valence basis set used in connection with the ECP is essentially of double zeta quality (the LANL2DZ basis set)[20]. This method has been justified by a series of previous studies[14,21]. As the molecules are rather large, the B3LYP/LANL2DZ method failed to give zero point vibrational energy and thermal correction to energy. However, according to the previous studies[14,21-23], the physicochemical parameters derived from total electronic energy are applicable in a relative sense. All of the calculations were performed with Gaussian 98 package of programs[24].

Figure 1 illustrates some known metal chelators and SOD mimics. Their BEs and EAs are listed in table 1. Clioquinol, (5-chloro-7-iodo-8-hydroxy-quinoline, figure 1), a hydrophobic moderate metal chelator, has exhibited promising treatment effect in a Phase II clinical trial of moderately severe AD patients[8,25]. It can be seen from table 1 that the BE of clioquinol-copper(II) was significantly higher than those of the SOD mimics, *e.g.*, (N,N'-ethylene bis-(2-acetylpyridine iminato) copper(II), APEN; N,N'-propylene bis-(2-acetylpyridine iminato) copper(II), APPN; N,N'–butylene bis-(2-acetylpyridine iminato) copper(II), APTN, figure 1)[14,21,26]. Nevertheless, the EA of clioquinol-copper(II) was also much higher than those of the SOD mimics, which implies that the clioquinol-copper(II) complex is very inert in scavenging superoxide radical. Similar properties are revealed for curcumin-copper(II) and 8-hydroxyquinoline-copper(II) complexes, which also show high BEs and EAs simultaneously[13,27-29]. In comparison, according to the experimental determination and theoretical calculation results (table 1), the superoxide-scavenging potentials of 2,2'-biquinoline-copper(II), neocuproline-copper(II), bathocuproine-copper(II) and nicotine-copper(II) complexes are comparable with those of SOD mimics[13,30], whereas their copper(II)-chelating abilities are rather low comparing with that of clioquinol-copper(II) complex. Thus, it seems it is a great challenge to find molecules that hold good copper-binding ability as chelators and high SOD-like activity as chelates.

Recently Li *et al* designed two novel copper(II)-chelator complexes, *i.e.*, copper(II)-1-(benzimidazole-2-ylmethyl)-1,4,7-triazacyclononane (1-BYT) and copper(II)-1,4-bis(benzimidazole-2-ylmethyl)-1,4,7-triazacyclonone (1,4-BYT), which have high SOD-like activity and good thermodynamic stability[31]. The theoretical calculations indicated that 1-BYT and 1,4-BYT are well balanced between chelating copper ions and mimicking SOD (when binding copper(II)). Their BEs are higher than that of clioquinol (table 1), suggestive of strong copper(II)-binding ability of both ligands. In addition, their EAs are also comparable with those of SOD mimics, in good agreement with their high superoxide-

scavenging activity (table 1)[31]. Therefore, 1-BYT and 1,4-BYT are likely to provide appropriate starting points to fulfill our new anti-Alzheimer and anti-prion strategy. We are attempting to verify their effects by experiments.

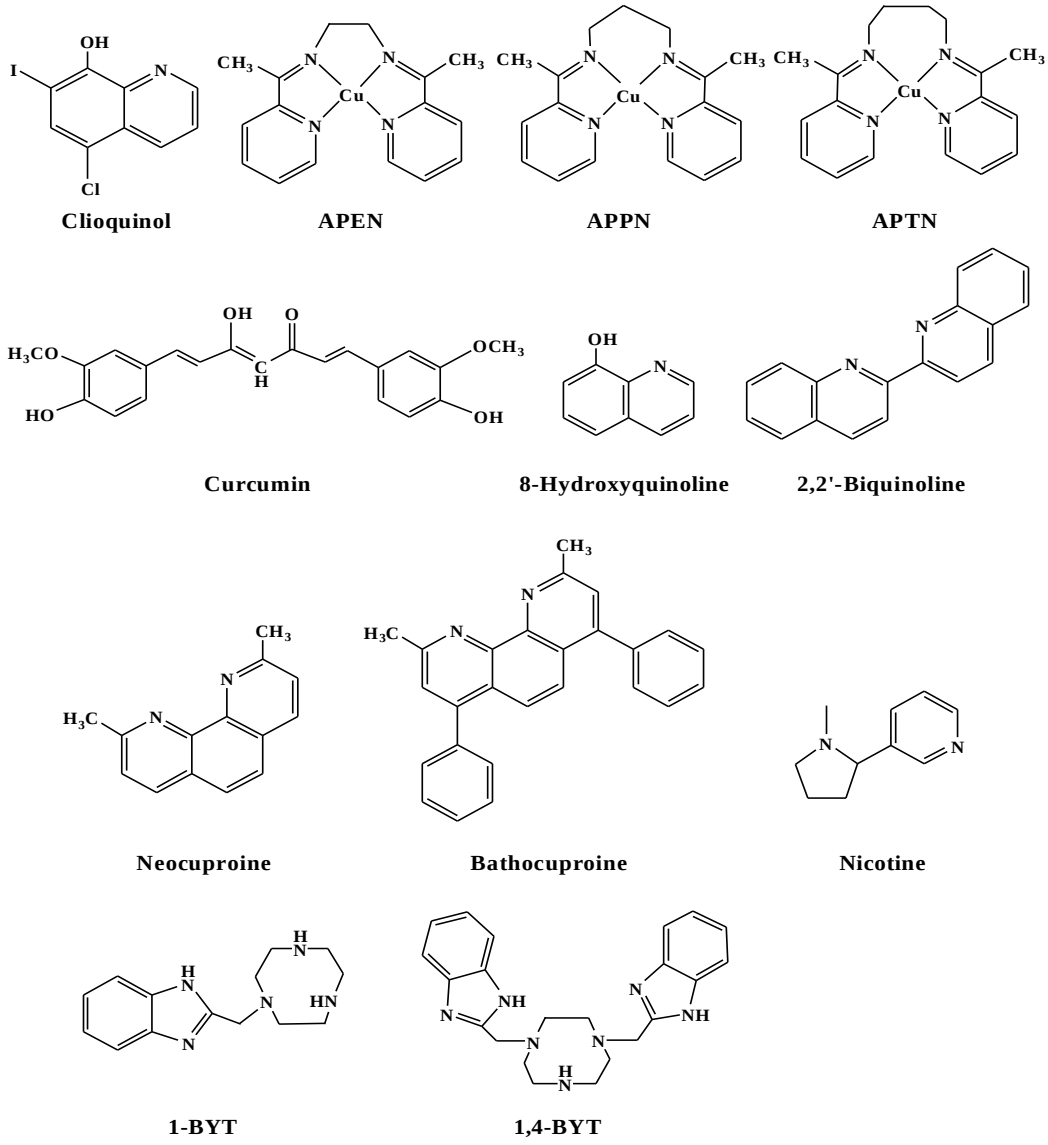

Figure 1. Molecular structures of some metal chelators and SOD mimics.

Table 1. Theoretical binding energies (BEs), electron affinities (EAs) and experimental SOD-like activities (IC_{50}) of copper(II)-chelates

	BE (kcal/mol)	EA (kcal/mol)	IC_{50} (μM)
clioquinol-copper(II)	685.26[a]	-64.06[a]	140[b]
N,N'-ethylene bis-(2-acetylpyridine iminato)-copper(II)	461.14[a]	-187.37[a]	11.04[c]
N,N'-propylene bis-(2-acetylpyridine iminato)-copper(II)	460.69[a]	-190.58[a]	2.33[c]
N,N'-butylene bis-(2-acetylpyridine iminato)-copper(II)	465.90[a]	-192.90[a]	0.56[c]
curcumin-copper(II) (1:1)	694.64[d]	-73.61[d]	
curcumin-copper(II) (2:1)	688.39[e]	-40.67[e]	
8-hydroxyquinoline-copper(II)	706.18	-35.03	263[b]
2,2'-biquinoline-copper(II)	456.96	-202.96	3[b]
neocuproine-copper(II)	450.48	-207.16	50[b]
bathocuproine-copper(II)	470.32	-192.61	32[b]
nicotine-copper(II) (1:1)	407.67[f]	-225.56[f]	
nicotine-copper(II) (2:1)	435.14[f]	-207.04[f]	
1-(benzimidazole-2-ylmethyl)-1,4,7-triazacyclononane-copper(II)	798.46[a]	-164.79[a]	0.90[g]
1,4-bis(benzimidazole-2-ylmethyl)-1,4,7-triazacyclonone-copper(II)	709.08[a]	-184.50[a]	0.76[g]

[a] data from ref. 21
[b] data from ref. 13
[c] data from ref. 26
[d] data from ref. 27
[e] data from ref. 29
[f] data from ref. 30
[g] data from ref. 31.

ACKNOWLEDGMENTS

This study was supported in part by the National Basic Research Program of China (2003CB114400) and the National Natural Science Foundation of China (30570383 and 30700113).

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In: Alzheimer's Disease Diagnosis and Treatments ISBN: 978-1-61122-064-3
Editor: Marisa R. Boyd

Chapter 15

TOWARD A MORE RATIONAL APPROACH TO THE TREATMENT OF PATIENTS WITH DEMENTIA WITH PSYCHOSIS AND BEHAVIORAL DISTURBANCE

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Psychosis and behavioral problems are very common in patients with dementia and the burden this causes caregivers cannot be overstated. Behavioral problems in dementia are the leading reason that families place dementia patients in facility settings, yet facilities themselves are often overwhelmed by such behaviors. No less important, patients suffer when they feel agitated, psychotic or combative and the humane treatment of dementia patients includes treating their symptoms for quality of life.

Currently, there are no FDA approved treatments for dementia with psychosis or behavioral disturbance. Atypical antipsychotics have been prescribed for these behaviors. They had been considered to have a better side effect profile compared with typical antipsychotics, with lower rates of adverse effects such tardive dyskinesia, extrapyramidal symptoms and orthostasis. However, recent concerns including increased risk of cerebrovascular adverse events and death have resulted in an FDA warning, bringing into question their use in the demented population.

However, the research examining efficacy and safety of treatment of such patients has been fraught with difficulty. The main problem is that dementia with psychosis and behavioral disturbance is a heterogeneous group of patients, not a single disorder. Treating dementia patients with behavioral problems as if they have a single diagnosis that can all be treated by a single type of medicine is a mistake. Unfortunately, most studies examining treatment of behavioral disturbance in dementia have been designed in this way.

There are many steps to evaluate such behavioral problems in dementia before the prescription of an atypical antipsychotic should be given. Our group considers the four contributors to behavioral disturbance in dementia: (1) *Environmental Factors*; (2) *Medical*

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factors; (3) *Psychiatric Co-morbidity/depression*; and (4) *Behaviors Due to the Dementia itself.*

Environmental factors need to be assessed that may be contributing to the behaviors. In our experience, many behaviors can be addressed by education of family/caregivers and developing specific behavioral plans. Environmental factors can only be determined by a thorough history. Once Environmental factors are ruled out, *Medical Factors*, such as urinary tract infections or changes in medication, which commonly cause behavioral disturbance must be considered. Even chronic behavioral problems can be caused by chronically undiagnosed or untreated medical illness. Once the medical illness is properly treated, the behavioral disturbance improves, thus avoiding the need for atypical antipsychotics. Finally, patients need to be carefully examined for *Psychiatric Co-Morbidity*, most commonly depression which typically causes behavioral disturbance in dementia patients. A diagnosis of depression in patients with dementia is difficult due to their cognitive deficits, and language impairment. Depression in dementia commonly presents as agitation, irritability and aggression. These features are so prominent that any depressive features are overlooked. Treatment with antidepressants is crucial in any case of agitation in dementia that could possibly be depression, and this will often eliminate the need for atypical antipsychotics.

Finally, and only when these three factors (Environmental, Medical or Psychiatric co-morbidity/depression) have been carefully ruled out, should it be concluded that the behavioral problem is *Due to the Dementia itself*. Only at this point should other psychiatric medications be considered. However, there are medications besides atypical antipsychotics used to treat such symptoms. If a patient is not psychotic, buspirone or valproate acid for example may be used, depending on the behaviors. If a patient is psychotic or has not responded to other medications, then antipsychotics are appropriate.

A recent widely-publicized article in the New England Journal by Schneider et al., as part of the CATIE-AD study group, (NEJM 2006:355:1525-1538) "Effectiveness of Atypical Antipsychotic Drugs in Patients with Alzheimer's Disease" demonstrates some of the difficulty and hazards in evaluation of treatment of psychosis and behavioral disturbance in dementia patients. The abstract concluded that "adverse effects offset advantages in the efficacy of atypical antipsychotics for the treatment of psychosis, aggression, or agitation" (p1525).

Let's now look a bit closer at this research, and the problems it demonstrates. First, examination of the first three factors of behavioral disturbance described above are very briefly mentioned and dispensed with as a matter of course. No details are given about any structured way that environmental factors or medical factors are ruled out. What kind of thorough history was taken about the type of agitation to ensure environmental factors were not contributing? Did all patients have blood work and urine samples to rule out infection or UTI? Was the medication list thoroughly examined for possible simplification and consideration for contribution to the behavioral problems? Notably, depression was not an exclusion factor in this study – patients were excluded if they "were going to receive treatment with ...antidepressant medication" but not if they were otherwise depressed. Amazingly, a majority of the patients were depressed by NPI measures (61%) and yet they were enrolled in an antipsychotic trial anyway! Patients with depression should have been excluded from the trial and received appropriate antidepressant therapy since their agitation may have been depression-related.

Until researchers understand that dementia patients have a multitude of reasons for being behaviorally disturbed and can methodically examine carefully for each factor and then actually treat each factor, little progress will be made in finding effective and safe treatments for these behavioral problems. One wonders what the results would have been if the 61 % of depressed patients in the above study had been given antidepressants, and the remaining 39% the antipsychotics? As it stands, readers of the article may fail to realize the importance of antidepressant therapy in these patients.

Another unfortunate outcome of the above study is it will dissuade physicians from using atypical antipsychotic drugs when appropriate. Fear of litigation due to product liability may influence treatment decisions to a greater degree than medical evidence. Overemphasis on the negative potential side effects and neglecting to mention the possible outcomes of no treatment, influences physicians or patients to avoid certain treatments that may be effective.

In our experience, families of patients with dementia want their patients to be safe, but are also interested in having their loved ones be as happy and comfortable as possible. Being paranoid, and having hallucinations are usually painful symptoms from which families do not want their loved ones to suffer. In addition, being irritable, angry and agitated, especially to the point of combativeness, is equally not a pleasant or comfortable way to feel. No one should spend their last months or years feeling irritable and angry, just as no one would want patients to have untreated physical pain.

These can be difficult times for clinicians trying to treat patients with dementia with behavioral problems. With no approved FDA treatments and problems as noted in the current research about available treatments, clinicians may feel in a bind what to do. Future studies need to more carefully examine this population not as a homogenous group, but a group of people exhibiting behaviors that have different causes. Only then will we move forward in understanding the appropriate treatments to really help our patients and their families.

In: Alzheimer's Disease Diagnosis and Treatments ISBN: 978-1-61122-064-3
Editor: Marisa R. Boyd

Chapter 16

AMYLOID CLEARING IMMUNOTHERAPY FOR ALZHEIMER'S DISEASE AND THE RISK OF CEREBRAL AMYLOID ANGIOPATHY

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ABSTRACT

Immunization strategies which aid in the clearance of beta-amyloid (Aβ) plaques have raised new hopes for the treatment of Alzheimer's disease (AD). Two particularly promising passive immunization therapies currently being investigated include intravenous immunoglobulins (IVIG) containing Aβ antibodies and specifically developed monoclonal antibodies for Aβ. These Aβ antibodies may reduce amyloid accumulation in the brain by binding to the amyloid peptide and drawing it in through the blood-brain barrier for subsequent removal from the capillaries. However, as this strategy aims at removing extracellular amyloid through cerebral vessels, a redistribution of amyloid pathology may manifest as increased cerebral amyloid angiopathy (CAA). CAA occurs when Aβ becomes embedded in the walls of cerebral vessels associated with weakening of the vessel walls. Antibody mediated Aβ clearance from the parenchyma could significantly increase the Aβ burden in the vessel lumen and wall, therefore increasing the risk of vessel rupture and hemorrhage. This chapter will review the current literature on Aβ immunotherapy for AD and explore the mechanisms as well as possible risks of amyloid clearance treatment, particularly cerebral amyloid angiopathy.

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INTRODUCTION

As our average life expectancy increases with modern advances, Alzheimer's disease (AD) is becoming more prevalent. The incidence of AD approximately doubles every 5 years after age 65 and is rapidly approaching near 50% of persons over age 85 [1]. It would be unfortunate to extend the length of life without corresponding extension of meaningful cognitive function.

AD is a neurodegenerative disorder and the most common cause of dementia. Since its original description by Alois Alzheimer in 1906, this clinical syndrome of progressive cognitive decline has been correlated with the pathological findings of neurofibrillary tangles and beta-amyloid (Aβ) plaques. Novel treatment strategies, which specifically target Aβ plaques, are currently being investigated. One strategy is to reduce the production of Aβ by inhibiting the enzyme, beta-secretase, which cleaves this pathological peptide [2]; however, development of these specific enzyme-inhibitors remains particularly challenging [3]. Another approach, which appears to be more feasible at this time, is to develop methods of enhancing Aβ clearance. One such technique generating much excitement in the AD research community is immunotherapy, however we must explore the potential risks of this novel treatment before becoming prematurely zealous. Cerebral amyloid angiopathy (CAA) is a third component of Alzheimer's pathology that may potentially be exacerbated by immunotherapy.

IMMUNOTHERAPY

Evidence is mounting that immunotherapy may be an effective therapy for AD by fostering the clearance of Aβ plaques. First, in 1996-1997 Solomon et al. [4,5] demonstrated that Aβ monoclonal antibodies both prevented and disassembled Aβ fibrils *in vitro*. This notion of immunotherapy for AD was then further spurred in 1999 by Schenk et al. [6] who found that Aβ vaccination diminished Aβ plaques in mice. Morgan et al. then took this an additional step in 2000 and showed that mice vaccinated against Aβ maintained superior memory function with age.

After a promising phase I human trial, the 2001 phase II trial of active immunization against Aβ42 also yielded evidence for immunotherapy as a potentially successful treatment for AD: the group of subjects who successfully developed Aβ antibodies showed not only a significantly slower rate of cognitive decline over one year compared to the placebo group, but also improvement in memory scores which positively correlated with antibody titer [7]. Unfortunately, 6% of the 298 participants who were immunized developed an aseptic meningoencephalitis and the trial was discontinued [8].

A leading theory regarding the etiology of this postvaccination meningoencephalitis is T-cell mediated inflammation triggered by the Aβ42 molecule used for active immunization [8]. One strategy to avoid this adverse inflammation is to administer the Aβ antibodies passively such as via intravenous immunoglobulins (IVIG). Commercial IVIG has been shown to contain antibodies to Aβ [9,10].

Dodel et al. [11] investigated IVIG for AD and found that Aβ levels in the CSF decreased by 30% whereas serum Aβ levels increased, thus supporting amyloid clearance theories.

Dodel and colleagues also found that these patients demonstrated mean cognitive improvement (mean improvement of 3.7 points on the ADAS-cog) after 6 months of treatment.

Instead of exposing a patient to a plethora of antibodies as in IVIG, another method of passive immunization for AD currently being investigated uses monoclonal antibodies for Aβ. These monoclonal antibodies can be specifically developed to bind to various epitope regions of the Aβ peptide, thus instilling differing characteristics which may alter both efficacy and adverse properties. A phase II investigation of the Aβ monoclonal antibody AAB-001 is currently underway in patients with mild to moderate AD.

MECHANISMS OF AB CLEARANCE BY IMMUNOTHERAPY

The molecular mechanism of Aβ clearance by IVIG was studied *in vitro* by Istrin and colleagues in 2006. Similar to earlier investigations of immunotherapy, they found that the antibodies in IVIG disassembled the Aβ fibrils and enhanced their clearance. APOE and APOJ have been proposed to bind to Aβ for bi-directional transport across the BBB [12].

Furthermore, Instin et al reported that IVIG enhanced microglial migration and lead to the phagocytosis of Aβ. This proposed mechanism involving Aβ antibodies crossing the BBB to bind Aβ plaques and subsequently activate microglia was also supported by Bard et al. [13] who conducted *ex vivo* assays demonstrating that these antibodies can cross intact BBB. The microglia are also thought to eventually deposit the Aβ into the vascular lumen [14].

DeMattos et al. [15] offered another mechanism of Aβ clearance by passive immunization. They proposed that Aβ antibodies do not cross the BBB, but instead stay in the peripheral blood and lower free serum Aβ. This is thought to cause a net efflux of Aβ from the brain parenchyma into the vessels; thus this is referred to as the "sink" hypothesis.

A neuropathological examination of three AD patients who were immunized against Aβ in the halted phase II active immunization trial (whose deaths were not attributed to the immunization) revealed remarkable clearance of cortical Aβ [16]. Interestingly, the pathologists also observed phagocytosed Aβ within microglia. However, all three cases were notable for CAA. Another pathological investigation of a fourth immunized patient from the same active immunization trail (whose cause of death was "failure to thrive") also demonstrated a relative absence of Aβ plaques but the presence of CAA [17].

CEREBRAL AMYLOID ANGIOPATHY

Up to 20% of all non-traumatic hemorrhagic infarcts in the elderly are due to CAA, and some degree of CAA is present at autopsy in nearly all AD patients [14, 18]. Nakata-Kudo et al. [19] performed gradient echo MRI on 50 AD patients and found microbleeds in 16.7% of the AD patients (without cerebrovascular disease) compared to no microbleeds in the control group. Approximately 20% of AD cases have "severe" CAA, in which circumferential amyloid deposits are present in many cerebral vessels. Olichney and colleagues reported that not only do AD cases with severe CAA have an increased risk of cerebral hemorrhage [20], but they also have a greater than two-fold increase in the prevalence of microinfarctions and

other ischemic lesions [20,21]. Interestingly, the severe CAA cases did not have increased severity of dementia, compared to AD cases with milder CAA [21]. Perhaps this is related to CAA being primarily composed of the Aβ40 peptide [22], whereas the primary peptide in parenchymal plaques is the less-soluble Aβ42 peptide.

Greenberg and colleagues [23] reported that 47% of patients who presented after a cerebral hemorrhage thought to be due to CAA showed MRI evidence of additional microhemorrhages over a 17 month follow-up. Greenberg and colleagues [24] have also suggested that these recurrent microhemorrhages may contribute to cognitive decline in AD. It is also very conceivable that CAA could contribute to decline in AD via ischemic mechanisms (e.g. granular cortical atrophy due to multifocal microscopic infarctions).

CAA occurs when Aβ becomes embedded in the walls of parenchymal arterioles and capillaries or leptomeningeal vessels resulting in weakening of the vessel walls [25]. This predisposes the cerebral vessels to hemorrhage.

Rensink and colleagues [25] reviewed four theories for the deposition of Aβ in the vessel wall, stated briefly below:

1. Transfer of Aβ from the blood into the vessel wall occurs due to disruption of the BBB (possible causes of impair BBB include head trauma, hypertension, arteriosclerosis, stroke and Aβ itself);
2. Production of Aβ is produced intrinsically by smooth muscle cells of the vessels;
3. Neuronally produced Aβ is drained in the interstitial fluid along peri-arterial spaces [26] and then internalized into smooth muscle cells via APOE (this pathway may be protective means of Aβ clearance, which becomes pathologically saturated in AD thus leading to CAA);
4. Vascular changes associated with aging such as thickening of the basement membrane may contribute to Aβ deposition in the vessels.

CAA ASSOCIATED WITH IMMUNOTHERAPY

As immunotherapy for AD aims at clearing extracellular amyloid through cerebral vessels, a redistribution of the amyloid pathology could manifest itself in the form of even higher rates of CAA. Antibody mediated Aβ clearance from the parenchyma could significantly increase the Aβ burden in the vessel lumen and wall, therefore increasing the risk of vessel rupture and hemorrhaging.

As noted above, neuropathology of AD patients from the phase II Aβ42 active immunization trial showed reduced Aβ42 plaques but persistent CAA [16,17]. In a different subject actively immunized against Aβ42 in this same trail, Ferrer and colleagues [27] observed similar findings of Aβ42 plaques reduction and additionally found evidence of multiple microhemorrhages due to CAA.

Although the main Aβ species in CAA is Aβ1-40, Attems et al. [28] noted that both Aβ40 and Aβ42 contribute to CAA in the leptomeningeal and cortical arterial vessels. They further suggested that capillary CAA is mainly characterized by Aβ42 deposition. This may explain why clearance of Aβ42 from the cortex may cause a redistribution phenomenon of saturated vessel Aβ load, i.e., CAA.

Pfeifer et al. [29] further investigated this possible relationship between immunotherapy and CAA. In a mouse model of AD (APP23 transgenic mice), they studied the effects of passive immunization against Aβ (at the N terminus) and found that the immunized mice had a greater than two-fold increase in CAA-associated hemorrhages. They found an inverse correlation of significantly decrease in Aβ42 plaques in the cortex and increased amyloid in the vessel walls with associated hemorrhages. It was noted that Aβ40 showed no significant change.

Other researches have also found that antibodies directed against N terminus of Aβ were associated with CAA microhemorrhages, however Racke et al. [30] demostrated that antibodies directed against central domain of Aβ were not associated with CAA microhemorrhages. Asami and colleagues [31] investigated a C terminus Aβ antibody and suggested that this may render Aβ42 more soluble while inhibiting the deposition of both Aβ42 as well as Aβ40. Theoretically, this may also be a means of reducing risks associated with immunotherapy such as exacerbation of CAA

STRATEGIES TO MINIMIZE CAA RISK WITH IMMUNOTHERAPY

While immunotherapy seems to be effective in clearing Aβ plaques and even improving cognitive function, it also seems to carry an increased risk of CAA. So how do we proceed from here? First, we need to attempt to identify those at highest risk for hemorrhage. Secondly, we need to continue our efforts to find safer methods of administrating immunotherapy, such as developing new, more specific Aβ antibodies.

After a review of the literature, the following is a list of probable or potential risk factors for CAA: advanced, severity/stage of AD [32], presence of vascular disease [33], prior hemorrhage (clinical or subclinical identified with gradient echo MRI), APOE2 [16] and APOE4, especially ε4 homozygotes [34]. We should perhaps have a higher threshold to pursue immunotherapy in this group and, of course, this group should be thoroughly educated about the potential risks of TIA and stroke (ischemic or hemorrhagic) before immunotherapy is elected or implemented.

The following is a list of possible protective strategies: more specific monoclonal N-terminus antibodies [35], central (instead of N terminus) domain anti-body [30], C terminus antibodies [31], different adjuvants [32], altering pulse rate possibly with exercise [36], and the development of novel medications to specifically reduce CAA and/or the associated risk of hemorrhage. Greenberg and colleagues [37] have conducted a phase II study of a low molecule weight candidate agent, tramiprosate, which may reduce the risk of hemorrhage for cerebral amyloid angiopathy.

CONCLUSION

This is a very exciting time for the development of anti-amyloid therapies, such as immunotherapy, for AD. These have the potential to not just ameliorate symptoms, but could actual reverse the underlying disease. Thanks to significant recent advances in neuroimaging, we now have available relatively non-invasive methods to track amyloid deposition

throughout the brain with *in vivo* amyloid imaging methods such as PET scanning with Pittsburgh compound B (PIB) [38]. What remains unproven is that removing Aβ plaques and reducing the extracellular brain amyloid will, in fact, translate to robust clinical improvement or reversal in disease. Neuropathological studies have shown that the density of pre-synaptic terminals correlates more strongly with dementia severity than does amyloid burden [39], or even the extent of neurofibrillary tangles. Some recent basic research suggests that intracellular amyloid metabolism and trafficking may be more critical to AD pathogenesis than is extracellular amyloid .

To summarize, immunotherapy has provided much new hope for the treatment of AD. The preliminary clinical data look encouraging. However, with the reduction of Aβ plaques there appears to be an elevation of CAA with the associated potential risks of cerebral hemorrhage or infarct. We have reviewed possible mechanisms for Aβ clearance with immunotherapy as well as the mechanisms of cerebral amyloid angiopathy. Possible strategies to minimize the risk of CAA in AD patients being evaluated for immunotherapy were offered.

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In: Alzheimer's Disease Diagnosis and Treatments
Editor: Marisa R. Boyd
ISBN: 978-1-61122-064-3

Chapter 17

USE OF ANTIDEPRESSANTS IN OLDER PEOPLE WITH MENTAL ILLNESS; A SYSTEMATIC STUDY OF TOLERABILITY AND USE IN DIFFERENT DIAGNOSTIC GROUPS

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ABSTRACT

Aims: The objective of the study was to provide observational clinical data on psychotropic drugs used in older people with mental illness.

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Method: This was an observational, single-centre, one-week prevalence study of psychiatric symptoms, disorders and psychotropic/antidepressant drug use in older people with mental illness cared for by the South West people Yorkshire Mental Health NHS Trust (Wakefield Locality), UK. The clinical assessment included completion of the Psychosis Evaluation Tool for Common use by Caregivers.

Results: A total of 593/660 older patients with mental illness (mean±SD age, 76±8.1 years) were assessed). 44.5% had dementia (excluding vascular dementia) and 33.7% had a mood disorder. Of the total, 20.4% did not receive CNS active medication and 46.2% of patients were prescribed an antidepressant. Antidepressants were commonly prescribed where the primary diagnosis was not depression including vascular dementia (31%), dementia (26.1%), schizophrenia and related disorders (26.2%) and anxiety disorders (51.5%). SSRIs were the most commonly prescribed drugs (63.2%) followed by TCAs (22.4%), venlafaxine (9%), mirtazapine (3.2%), reboxetine (1.8%) and phenelzine (0.36%). The single most commonly prescribed drug was paroxetine (n=77) which accounted for 27.7% of all prescriptions. Medications were well tolerated but some patients prescribed a TCA received relatively small doses. Patients with non-vascular dementia received a significantly lower dose of paroxetine compared with other diagnostic groups ($F=3.14$, $p<0.02$) though this was still within the recommended/therapeutic range.

Conclusions: Antidepressants are commonly used in older people with mental illness including dementia, schizophrenia and anxiety disorders as well as for patients with a primary diagnosis of depression. Antidepressants are generally well tolerated and patients were broadly satisfied with their medication. The evidence for the use of low dose TCAs in older people remains controversial and further work is needed in this area.

Declaration of interest: None

Keywords: psychotropics, antidepressants, older people, mental illness.

Depression in older people is similar to major depression at other times of life. However ageing and other factors may alter the presentation of depression in later life. In particular older people are less likely to complain of sadness compared with younger patients, they are more likely to complain of physical symptoms (NIH, 1992), memory complaints and anxiety symptoms (Baldwin et al., 2002). In addition, depression in patients with dementia is a common cause of behavioural disturbance (Dwyer and Byrne, 2000). Depression is one of the leading causes of disability, leads to a greater risk of hospitalisation as well as prolonging hospitalisation and is the single most important predictor of suicide. It also reduces compliance with medical treatments, reduces the patient's quality of life and is an independent predictor of mortality (Baldwin et al., 2002).

Depression in older people is two to three times more prevalent than dementia (Katona, 1994) and is the most common mental health problem amongst older adults. In community samples the prevalence of mild depression has been estimated to be 11% (Alexopoulous, 1992) rising to 22- 33% in residential and nursing homes (Ames et al., 1988) and 45% in hospitalised elderly patients with physical illness (Koenig et al., 1988). There is also some evidence of increased prevalence in the very old (Stek et al., 2006).

The aetiology of depression in older people is complex. Genetic susceptibility is less important compared with younger patients. Female gender, a previous history of depression and loss of spouse increases susceptibility. Reductions in levels of noradrenaline and serotonin, decreases in brain weight and the greater prevalence of deep white matter and

subcortical grey matter lesions all play a part. Hypertension, vascular risk factors, impaired function, being chronically ill and being a carer are also important risk factors as are life events such as bereavement, separation, acute physical illness and moving into residential care. A number of drugs can also cause or aggravate depression including beta-blockers, methyldopa, calcium channel blockers, digoxin and steroids (NIH, 1992; Jonas and Mussolino, 2000; Ariyo et al., 2000; Penninx et al., 2000; Baldwin et al., 2002). For these reasons, antidepressants are usually only part of the solution for the treatment of depression in older people.

In a recent Canadian study (Beck et al., 2005) the prevalence of psychotropic drug use in the general population was 7.2% and selective serotonin reuptake inhibitors and venlafaxine accounted for 25.2% of all psychotropic drug usage. Although there is now a reasonably good body of evidence for the efficacy of antidepressants in older people (Wilson et al., 2001; Oslin et al., 2003; Guaiang et al., 2004; Sheikh et al., 2004; Nelson, 2005) very little information has been published on the use of antidepressants in this age group. The aim of the present study was to provide a better understanding of psychotropic drug use and particularly antidepressant use in older people with mental illness as well as exploring tolerability and prescribing issues in different diagnostic groups.

METHOD

Study Design

This was an observational, single-centre, one week prevalence study of psychiatric symptoms, disorders and psychotropic drug use carried out in the Wakefield Locality, South West Yorkshire Mental Health NHS Trust, UK over 12 months in 2003/2004. The service consisted of two acute wards, one day-hospital, outpatient clinics for three consultant teams, three Community Units for the Elderly, and two Community Mental Health Teams. The study was approved by the Wakefield Research Ethics Committee.

Patient Selection

All consenting patients under the care of psychiatric services for older people in the Wakefield Locality (total population over 65 years approximately 55,000) were included in the study. Patients identified from Trust records were contacted by a Research Nurse to ask if they would like to take part in the study. All patients and caregivers received an information sheet before taking part in the study and gave written consent.

Assessments

The Research Nurse undertook a detailed clinical assessment, which included demographic details, clinical information, diagnosis and treatment response (classified as first episode, stable-dissatisfied, stable-satisfied, treatment resistant, and uncontrolled),

medication, symptoms and side-effects. These were part of a computer-based package, the Psychosis Evaluation Tool for Common use by Caregivers (PECC), developed from the work of Lindstrom et al. (1997). The PECC was specifically designed to be used by a wide variety of health care workers including nurses. The reliability and validity has been described in both younger and older people (de Hert et al., 1999). Prior to undertaking the study the Research Nurse attended a three-day training course organised by the PECC development team in Belgium.

The assessment also included an interview with the caregiver, discussions with medical and nursing staff and a review of medical notes including GP records. This specifically included a review of patients' current physical health and laboratory and other investigations. Patients were assessed in a variety of settings including the two acute wards, OP clinics, the three Community Units for the Elderly and in their own homes. The assessment took approximately one hour to complete and after the assessment a copy was made available to the appropriate clinical team. Diagnosis was based on DSM-IVR criteria (APA, 1994). Some patients attended several parts of the service e.g. day hospital and OP clinic but they were only included once.

Symptoms and side-effects were based on the previous seven days and a standardised protocol was used for *defining* and *scoring* individual symptoms and side-effects. Symptoms were recorded on a seven-point scale (1=absent, 7=extreme burden, all areas of functioning are disturbed, supervision necessary) and included positive (e.g. delusions and hallucinations) and negative symptoms (e.g. motor retardation, blunted affect, poor rapport and passive social withdrawal) as well as depressive, cognitive and excitatory symptoms. Side-effects were measured on a four-point scale (1=absent; 4=severe, obvious influence on functioning, intervention necessary) and included extrapyramidal side-effects (EPS), anticholinergic, hormonal, dizziness, daytime somnolence, drowsiness, sexual dysfunction, insomnia, weight gain and orthostatic hypotension.

Statistical Analysis

Statistical analyses were carried out using SAS/STAT software (version 8.12). Comparisons of continuous variable used ANOVA, and pair-wise comparisons (Chi squared test - χ^2, Cochran-Mantel-Haenzel test) for categorical variables were performed with adjustment for multiple comparisons employing the Tukey-Kramer's method.

RESULTS

Patient Characteristics

Of a total of 660 older patients, 593 (89.8%) patients took part in the study. 293 patients (approximately 50%) had a diagnosis of dementia with 4.9% of the total population having vascular dementia (VaD). Of the remaining patients 200 (33.7%) had an affective disorder and 65 (11%) schizophrenia or a related disorder. In addition, the majority of patients had had their mental illness for a relatively short period (table 1).

Table 1. Frequency distribution of main diagnoses and time in years since the main diagnosis was made

Diagnosis	Main diagnosis n (%) patients	Time in years since main diagnosis Mean ±SD years (range)
Vascular dementia	29 (4.9)	0.4±0.8 (0-4.0)
Non vascular dementia	264 (44.5)	0.5±1.2 (0-8.9)
Affective disorders	200 (33.7)	0.4±0.9 (0-7.3)
Schizophrenia, schizotypal and delusional disorders	65 (11.0)	1.7±5.0 (0-28.0)
Anxiety disorders	33 (5.6)	0.3±0.3 (0-0.9)
Unknown	2 (0.3)	1.2±1.3 (0.3-2.1)
Total	593 (100)	0.6±2.0 (0-28)

Age of the patients ranged from 44 to 97 years, the mean age±SD was 76±8.1years, and 44% were aged 71 to 80 years. There was a statistical difference in the age of the patients between the diagnostic groups ($F=8.37$, $p<0.001$). More specifically, patients with VaD and non vascular dementia were older than patients with affective disorders ($p=0.035$, $p<0.001$, respectively) and were older than those with schizophrenia and related disorders ($p<0.0005$).

Sixty-nine percent (n=409) of patients were female and there were more females (≥67%) in each diagnostic category (χ^2, $p=0.001$), with the exception of VaD dementia (males n=19, 65.5%; females n=10, 34.5%). There were no differences in the level of education, occupational status or marital status between the diagnostic groups. Treatment response was rated as "stable-satisfied" for the majority of patients (n=537, 90.6%) with 7 patients (1.2%) rated as "stable-dissatisfied." Only 2 patients (0.3%) were rated at "treatment resistant." The time in years since patients were first diagnosed with their principal mental disorder ranged from 0 to 28 years. This was numerically greater for patients with schizophrenia and related disorders but there were no statistically significant differences between the diagnostic groups ($p=0.97$ - table 1).

Psychoactive Drugs

Of the 593 patients, 121 (20.4%) did not receive a psychoactive drug. A total of 304 (51.3%) patients were taking an antipsychotic, 274 (46.2%) an antidepressant, 130 (21.9%) an hypnotic, 42 (7.1%) an anxiolytic, 29 (4.7%) an anticonvulsant and 29 (4.9%) anticholinergic drugs.

Intake of Antidepressants

In total 46.2% of patients were prescribed an antidepressant and these were more likely to be prescribed to patients with depression (81%) compared with other diagnoses (VaD 31%, dementia 26.1%, schizophrenia and related disorders 26.2% and anxiety disorders 51.5%) ($\chi 2=155.5$, $p<0.001$). SSRIs were the most commonly prescribed drugs (63.2%) followed by TCAs (22.4%), venlafaxine (9%), mirtazapine (3.2%), reboxetine (1.8%) and phenelzine (0.36%). The single most commonly prescribed drug was paroxetine (n=77) and this

accounted for 27.7% of all prescriptions. All antidepressants were prescribed in therapeutic doses although some patients prescribed a TCA received subtherapeutic doses. The mean daily doses for the most commonly prescribed TCAs were amitriptyline (mean 62.8 mg, range 50-150 mg), doxepin (mean 100 mg, range 50-150 mg), imipramine (mean 66.7 mg, range 25-150 mg and lofepramine (mean 131.3 mg, range 140-210 mg). In addition use of sertraline ($F=1.34$, $p<0.27$) and fluoxetine ($F=0.71$, $p<0.55$) did not differ significantly between the diagnostic groups. However, patients with dementia received a significantly lower dose of paroxetine compared with other diagnostic groups ($F=3.14$, $p<0.02$) though this was still within the recommended/therapeutic range.

Evaluation of Symptoms

There were significant differences between the different diagnosis groups for the mean scores of cognitive ($F=56.7$, $p<0.001$), depressive ($F=44.4$, $p<0.001$), negative ($F=8.5$, $p<0.001$), and positive ($F=27.9$, $p<0.001$) symptoms (table 2). Not unexpectedly, patients with dementia had more problems with cognitive function, those with affective disorders had greater depressive symptoms, and negative and positive symptoms were greatest in patients with schizophrenia and related disorders. Excitatory symptoms (e.g. hyperactivity, agitation, poor impulse control and hostility) were not significantly different between the diagnostic groups ($F=2.1$, $p= 0.08$) (table 2).

Table 2. Symptom scores (1=absent, 7=extreme burden, all areas of functioning are disturbed, supervision necessary) Mean score ±SD

Diagnosis	Cognitive Depressive Excitatory Negative Positive
Vascular dementia (n=29)	1.277 1.121 1.078 1.129 1.043 ±0.208 ±0.207 ±0.178 ±0.456 ±0.150
Non vascular dementia (n=264)	1.354 1.150 1.054 1.046 1.106 ±0.200 ±0.251 ±0.160 ±0.189 ±0.208
Affective disorders (n=200)	1.077 1.649 1.032 1.181 1.078 ±0.167 ±0.552 ±0.183 ±0.399 ±0.194
Schizophrenia, schizotypal and delusional disorders (n=65)	1.173 1.349 1.108 1.254 1.450 ±0.353 ±0.526 ±0.334 ±0.450 ±0.559
Anxiety disorders (n=33)	1.053 1.583 1.039 1.045 1.061 ±0.104 ±0.499 ±0.129 ±0.159 ±0.166
Total (n=593)	1.219 1.363 1.052 1.118 1.129 ±0.245 ±0.480 ±0.194 ±0.329 ±0.285

Evaluation of Side-Effects

Medication was generally well tolerated but anticholinergic side-effects (range 1-3) and drowsiness (1-2.4) were significantly higher in patients with an affective disorder compared with other diagnoses ($F=2.9$, $p=0.02$ and $F=7.8$, $p<0.001$ respectively).

DISCUSSION

The principal objective of this study was to obtain a better understanding of the use and tolerability of psychotropic drugs in older people and this paper makes particular reference to antidepressants. The study was undertaken just before the CSM guidance was issued on venlafaxine (CSM, 2004). Antidepressants drugs are commonly used in older people with mental illness including dementia, schizophrenia and related disorders and anxiety disorders as well as for depression.

It is interesting that there were no differences in the level of education, occupational status or marital status between the diagnostic groups. The time in years since patients were first diagnosed with their main mental disorder was relatively short and ranged from 0 to 28 years. It is likely that since most patients developed their illness later in life this did not have a significant impact on their education and life choices such as occupation and marriage.

In addition over 90% of patients reported feeling "satisfied" with their treatment. Seven percent reported feeling "dissatisfied" and 2% were classified as treatment resistant. The definition of treatment resistant depression (TRD) was not clearly defined in this study. Overall this is probably an underestimate of the true prevalence of TRD. However, the main focus of this study was drug use and tolerability rather than efficacy.

This study confirms that antidepressants are commonly prescribed to older people with mental illness. 46.2% of patients were prescribed an antidepressant and whilst the largest proportion was for patients with depression, 26.1% of patients with dementia, 26.2% with schizophrenia and related disorders and 51.5% of patients with an anxiety disorder received an antidepressant. A wide range of antidepressants were prescribed and of those prescribed an antidepressant six patients (2.2%) were prescribed two. The most commonly prescribed antidepressant was paroxetine which was prescribed at lower doses in older patients with dementia compared with other diagnoses. In addition, doses of TCAs tended to be lower compared with other antidepressants. However, in a relatively recent Cochrane review Furukawa et al. (2004) concluded that low dose TCAs can be justified though the debate on this issue has not concluded. Antidepressants were generally well tolerated but patients with depression reported significantly more drowsiness and anticholinergic side-effects compared with other diagnostic groups. Clinicians need clear advice on the use of antidepressant in older people. This advice should be based on good quality efficacy, tolerability and safety data from randomised-controlled studies and more research is needed in this area.

ACKNOWLEDGMENTS

This was an Investigator Initiated Project funded by an unconditional educational grant from Janssen-Cilag (UK).

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In: Alzheimer's Disease Diagnosis and Treatments ISBN: 978-1-61122-064-3
Editor: Marisa R. Boyd

Chapter 18

CYSTATIN C ROLE IN ALZHEIMER DISEASE: FROM NEURODEGENERATION TO NEUROREGENERATION

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ABSTRACT

In the brain cystatin C is synthesized by the choroid plexus and leptomeningeal cells, and it is localized in glial cells and in neurons. Its physiological high concentration in the cerebrospinal fluid (CSF) of the central nervous system and its proliferative effect on neural rat stem cells strongly suggest that cystatin C could exert a trophic function in the brain. Acute and chronic neurodegenerative processes induce an increase of cystatin C expression levels, mainly in activated glial cells. In brains from Alzheimer disease (AD) patients neuronal concentration of cystatin C protein is increased and its association to beta-amyloid peptide (A-beta) was revealed. A direct interaction of cystatin C and A-beta, resulting in an inhibition of amyloid formation, was demonstrated. An involvement of cystatin C in the pathogenesis of AD was further suggested by genetic studies in which the allelic haplotype B in cystatin C gene (CST3), determining an Ala25Thr substitution in the signal peptide, was associated with risk to develop late-onset AD. The B/B haplotype is specifically associated to highly reduced levels of extracellular cystatin C. In this view, the molecular correlate of the genetic risk conferred by cystatin C B variant could be the reduction in cystatin C secretion, which may result in A-beta formation and deposition. Alternatively, a reduced secretion of this protein could cause an impairment in neuroregeneration in response to brain damage.

Keywords: Alzheimer disease, cystatin C, CST3, amyloid-β peptide, amyloidosis

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INTRODUCTION

Alzheimer disease (AD) is a progressive degenerative disease clinically characterized by loss of memory, cognitive function, and personality changes. Neuropathologically, AD has been defined by the occurrence in brain regions serving memory and cognition of the following types of lesions: neurofibrillary tangles, amyloid plaques and cerebral amyloid angiopathy. Neurofibrillary tangles are masses of paired, helically wound protein filaments (PHF) lying in the cytoplasm of neuronal cell bodies and neuritic processes [1,2].

The other major lesion is the amyloid plaque. These are extracellular deposits of insoluble, 8/10-nm amyloid fibrils that are polymers of the amyloid-β peptide (A-beta) [3,4] intimately surrounded by dystrophic dendrites and axons as well as by activated microglia and reactive astrocytes. A-beta peptide is also found to form cerebral amyloid angiopathy (CAA), a cerebrovascular amyloid deposition [5].

Here, we discuss the role of cystatin C in the pathogenesis of AD. In detail, functions of cystatin C in the brain, the role of cystatin C in neurodegenerative events affecting the central nervous system, the interaction of cystatin C with A-beta and the more recently reported genetic data supporting a role of cystatin C protein as a risk factor for AD, will be discussed.

The Physiological and Pathological Role of Cystatin C in the Brain

In most of the investigated human body fluids, cystatin C is established to be the predominant cysteine protease inhibitor.

In the brain cystatin C is synthesized by the choroid plexus and leptomeningeal cells, by astrocytes and neuronal progenitor cells [6-9]. Its physiological high concentration in the cerebrospinal fluid (CSF) strongly suggest that it could exert a protective function in the brain [10].

In accordance to this hypothesis, it has been demonstrated that a glycosylated form of cystatin C is an autocrine/paracrine cofactor of basic fibroblast growth factor necessary for the proliferation of neural stem cells, and thus plays a central role in supporting neurogenesis *in vivo* and *in vitro* [8]. Moreover, Palmer and colleagues demonstrated that the propagation of neural progenitor cells from human post-mortem tissues was greatly improved by using conditioned medium from rat stem cells producing the glycosylated form of cystatin C [11]. This important role of cystatin C in the control of cell proliferation and survival is supported by the evidence that tumor growth is reduced in the cystatin C knock-out mouse model [12].

Taken together, these observations suggest that in the brain cystatin C might be involved in neuroregeneration or in the protection of neurons following brain injury.

Involvement of cystatin C in degenerative processes in the central nervous system has been largely described, following both acute and chronic injuries. Different brain injuries, including ischemia, axotomy, surgery and epilepsy, induce an increase of cystatin C expression levels, both in activated glial cells and in neurons [13-18]. Cystatin C has been demonstrated to be neuroprotective during brain ischemia: brain damage after focal ischemia is increased in cystatin C knock-out mice [19]. Moreover, administration of cystatin C can suppress neurodegeneration induced by lesion of nigrostriatal pathway, mimicking Parkinson degeneration [18].

Concerning AD, neuronal concentration of cystatin C protein is increased in activated glia cells in the brain of patients [9,20]. In AD brain cystatin C protein is accumulated within intracellular vesicles in the most susceptible neurons [20].

Cystatin C is an amyloidogenic protein: its dimerization is accelerated by the pathogenic L68Q mutation that causes hereditary cerebral hemorrhage with amyloidosis-Icelandic type (HCHWA-I), an autosomal dominant disorder characterized by repeated cerebral hemorrhages caused by massive amyloidosis of the brain vasculature [21-24]. The mutant protein lacks the first 10 amino terminal residues, it aggregates more readily than the wild type protein, and it is deposited predominantly in small blood vessels throughout the brain [22,24]. The E693Q mutation of the A-beta precursor protein (APP) causes the Dutch type of the hereditary cerebral hemorrhage with amyloidosis disorder (HCHWA-D) [25-27].

Co-deposition of A-beta and cystatin C occurs in vascular amyloid deposits of patients suffering from either HCHWA-I or HCHWA-D. Because of the different etiology, the deposition of these two proteins differs: The major protein component of amyloid in the Icelandic form is the mutant form of cystatin C, whereas A-beta deposition in patients carrying the Dutch APP mutation contains additional cystatin C [28,5,29]. Consistent with this co-deposition of A-beta and cystatin C in the vascular amyloid structures, the clinical phenotypes of the distinct diseases are similar.

Cystatin C deposits within A-beta plaques are known in aged canine brains [30], as well as in several amyloid deposits in the brains of AD patients [31,32].

A separate line of investigation demonstrated that cystatin C levels are increased in activated astrocytes throughout the brain of the transgenic mice expressing the Swedish APP mutation; moreover analysis of the amyloid plaques demonstrated the deposition of cystatin C layers onto the amyloid plaque cores [33]. These data strongly suggest an early role of cystatin C in amyloid plaque growth by apposition of cystatin C to preexisting plaque cores, followed by the apposition of A-beta amyloid.

Cystain C Role in Alzheimer Disease: Molecular Aspects

As previously detailed, in patients with AD cystatin C has been shown to be co-deposited with A-beta in amyloid plaques as well as in brain arteriolar walls [30-33]. These neuropathological observations suggest a functional link between cystatin C and A-beta.

Data reported in literature support a role of cystatin C in the processing of the A-beta precursor protein (APP). APP is a single transmembrane domain protein that is alternatively cleaved to generate a large soluble extracellular fragment, having a trophic function, or A-beta, the amyloid generating peptide. Cathepsin S, which is strongly inhibited by cystatin C, is known to cleave APP into derivatives containing A-beta *in vitro*: treatment of cells with E64, a synthetic cystatin C based inhibitor of lysosomal cysteine proteases, significantly reduces A-beta secretion induced by cathepsin S transfection [34,35].

Recently Sastre and coworkers demonstrated a colocalization of the two proteins, both intracellularly and at the cell surface [36]. Moreover a direct interaction of cystatin C with APP has been demonstrated: cystatin C binds to the full length protein as well as to its secreted forms (A-beta peptide and sAPPβ): the binding of cystatin C to A-beta results in an inhibition of in vitro amyloid fibrils formation [36].

Cystain C Role in Alzheimer Disease: Genetic Aspects

Alzheimer disease is the most common form of dementia in the elderly and it is believed to be genetically complex. To date, three genetic loci have been identified that contribute to early-onset autosomal dominant AD: presenilin 1 (*PSEN1* [MIM 104311]), presenilin 2 (*PSEN2* [MIM 633044]), and *APP* [MIM 104760]). However, only apolipoprotein E (*APOE* [MIM 107741]) has been well-established as contributing to late-onset AD [37,38]. Daw et al. [39] found evidence for multiple genetic loci contributing to the age at onset of AD with effect sizes similar to or larger than the effect of the *APOE* locus, suggesting a role of additional genetic-risk factors influencing the age at onset of AD. However, clear evidence of additional loci that contribute to risk or age at onset has remained elusive, although substantial linkage evidence exists for regions on chromosomes 9 [40], 10 [41-43], and 12 [40].

The gene coding for cystatin C (*CST3* [MIM 604312]), maps on chromosome 20p11.2; this gene contains three exons, and two KspI polymorphisms are known in the 5' untranslated sequence, combined with an additional KspI polymorphism that results in a threonine for alanine substitution at the penultimate position of the signal peptide [44,45] (Figure 1).

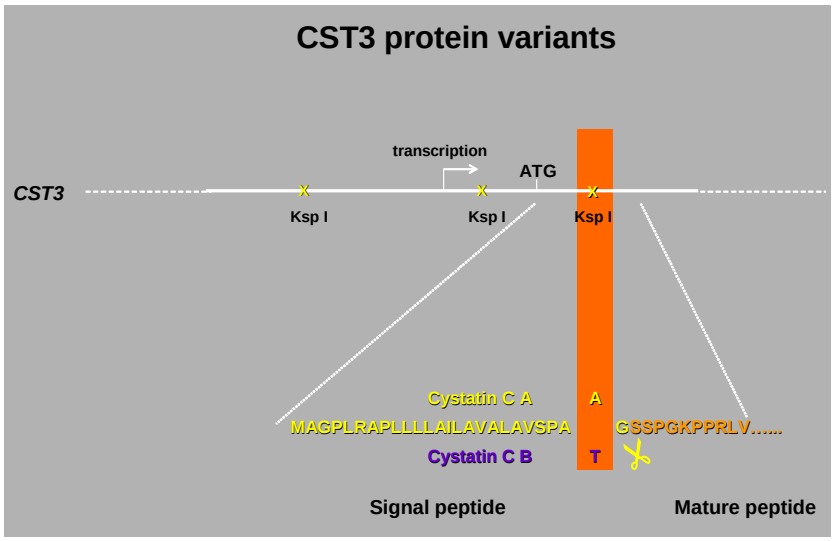

Figure 1.

Evidence of genetic association between late-onset AD and these *CST3* polymorphisms has been described in four case-control studies [46-49]; these studies showed that the strength of this association increased with increasing age. No association with *CST3* was found in a Japanese sample [50]. In the largest study, Finckh and collaborators demonstrated an excess of the *CST3* B/B genotype in AD patients compared to control subjects in two independent populations. *CST3* B/B was present in 4.7 to 9.1 % of AD patients – as compared to 10 to 14% APOE e4/e4 in this study. In addition, *CST3* B/B significantly reduced the average disease-free survival by 4 years. Taken together, these data indicate that *CST3* B/B is a risk factor for late-onset AD (Table 1).

In support to these data, Olson and collaborators [51] obtained strong evidence for linkage to chromosome 20p: *CST3* is the major candidate gene, as its locus is <15 cM proximal to this peak location. Moreover, it has been reported evidence for strong epistasis between the 20p region and the region of the APP locus, thus supporting the hypothesis that these two proteins interact affecting susceptibility to AD.

Table 1. CST3 case-control studies.

Population	References	AD cases (n)	Normal controls	Results
Spain	Beyer, 2001 (48)	159	155	Positive (B haplotype)
USA	Crawford, 2000 (46)	309	134	Positive (A haplotype)
Germany	Finckh, 2000 (47)	110	150	Positive (B haplotype)
USAandEurope	Finckh, 2000 (47)	407	240	Positive (B haplotype)
USA	Cathcart, 2005 (49)	179	141	Positive (B haplotype)
Japan	Maruyama 2001 (50)	179	228	Negative
Germany	Dodel, 2002 (58)	287	181	Negative
USA	Goddard, 2004 (59)	130	112	Negative
Netherlands	Roks, 2001 (60)	101	117	Negative
Taiwan	Lin, 2003 (61)	124	115	Negative

Cystatin C Role in Alzheimer Disease: from Neurodegeneration to Neuroregeneration

Summing the neuropathological, genetic and experimental evidences here presented, it can be stated that cystatin C may be involved in different neurodegenerative events occurring in AD brain.

Genetic data, demonstrating a role of cystatin C gene as a risk factor for late-onset AD, strongly support the hypothesis that cystatin C may be involved in the onset of AD.

Studies in which the cDNA for cystatin C, with or without the nucleotides encoding the leader sequence, was fused to cDNA for Enhanced Green Fluorescent Protein (EGFP) demonstrated that the leader sequence targets the precystatin C fusion protein to the Golgi apparatus and the secretory pathway [52,53]: The findings thereby established that the leader sequence functions as a signal sequence.

The Ala25Thr variation alters the hydrophobicity profile of the signal sequence, and it reduces its ratio of predicted alpha-helix to beta-sheet contents by approximately 42%. This variation was associated with changes in secretory processing of cystatin C: fibroblasts homozygous B/B displayed a reduced secretion of cystatin C, due to a less efficient cleavage of the signal peptide [54,55] (Figure 2). As reported for L68Q variant [56,57], the reduced cystatin C levels detected in fibroblasts derived from *CST3*B/B carrying subjects might be

reflected in an altered metabolism of cystatin C in the central nervous system. Thus, in the brain, the association of the *CST*3B/B genotype with AD might be due to the defective intracellular processing of cystatin C. Similarly to what observed in HCHWA-I patients, the accumulation of the unprocessed protein might lead to its intracellular self-aggregation, which may have a toxic effect on neurons.

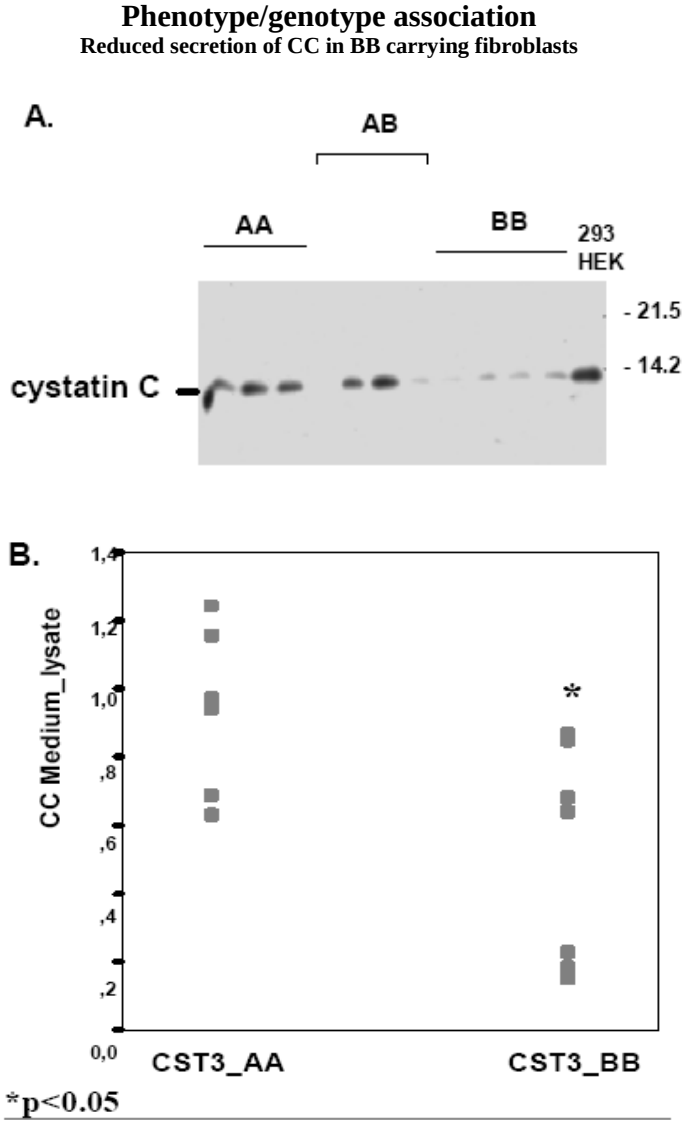

Figure 2. A. Western blot of cystatin C secreted into the culture media. Primary skin fibroblasts from 11 human subjects with CST3 A/A, CST A/B and CST3B/B and as a control, a human embryonic kidney (293) cell line. B. Level of secreted cystatin C. A statistical difference is demonstrated in B/B subjects, as compared to A/A.

In addition, we can hypothesize that the molecular correlate of the genetic risk conferred by cystatin C B variant could be associated with the reduction in cystatin C extracellular levels. Reduced level of cystatin C in B/B subjects may promote A-beta formation and

deposition. Thus, we may speculate that the impaired production of Cystatin C in *CST3* B/B carrying subjects may predispose them to be more susceptible to neurodegeneration.

Several observations suggest that cystatin C could exert a protective role on neurons. Cystatin C is an essential cofactor of FGF2 for the proliferation of rat brain derived stem cells [8]; cystatin C may be involved in the proliferation of adult neuronal stem cells in the human brain, as already demonstrated for rat cystatin C on cells derived from post-mortem human brains [11]. The impaired secretion of cystatin C observed in *CST*3 B/B subjects may result in a defective proliferation of stem cells in the brain. Since AD is characterised by continuous loss of neurons not replaced, a failure in neural stem cells replacement may contribute to progression and pathogenesis of this disease.

In conclusion, in the brain the reduced level of cystatin C may represent the molecular factor responsible for of the increased risk of AD. Understanding the contribution of cystatin C in the pathogenesis of AD might highlight new therapeutic prospective, such as the opportunity of combating neurodegeneration by preventing cellular damage and helping a patient's own stem cells and repair mechanisms work more effectively.

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In: Alzheimer's Disease Diagnosis and Treatments ISBN: 978-1-61122-064-3
Editor: Marisa R. Boyd

Chapter 19

A THEORETICAL EVALUATION ON ACETYLCHOLINESTERASE-INHIBITORY POTENTIAL OF QUERCETIN

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One century has passed since the discovery of Alzheimer's disease (AD), however, there has been no effective therapeutics to the disease. Since multiple factors are involved in the pathogenesis of AD, finding multipotent agents that can hit the multiple targets implicated in the disease is attracting more and more attention[1-3]. Recently, accumulating evidence indicated that quercetin (figure 1), a flavonoid abundant in fruits and vegetables, is a multipotent anti-AD agent. It can block Aβ- or τ-aggregation with IC_{50}s of < 1 μM[4] and inhibit monoamine oxidases A and B (MAO A and MAO B) with IC_{50}s of 0.01 μM and 10.89 μM, respectively[5,6]. Besides, quercetin is an efficient inhibitor for butyrylcholinesterase (BChE, a recently recognized potential target for treating AD[7]) with an IC_{50} of 1 μM[8]. Of course, quercetin is also an excellent antioxidant, both as reactive oxygen species (ROS) scavenger and transition metal chelator[9,10]. As quercetin is highly bioavailable and can pass through the blood-brain barrier (BBB)[11,12], it is highly possible to be responsible for the benefits of fruit and vegetable juices to AD.[13] However, considering the fact that the current strategy in the fight against AD depends largely on inhibiting acetylcholinesterase (AChE), it is of interest to explore the AChE- inhibitory potential of quercetin.

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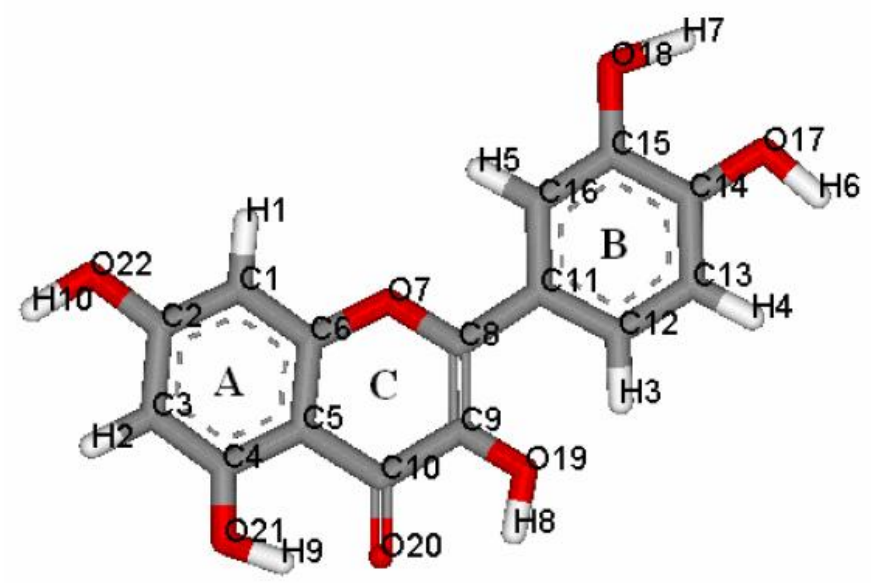


Figure 1. The molecular structure of quercetin.

Sívori *et al.* has indicated that quercetin is a weak inhibitor for insect AChE[14]. To evaluate the human AChE inhibitory activity of quercetin, we cooperated with the National Center for Drug Screening of China to do some preliminary experimental evaluations. The result showed that quercetin has no inhibitory effect on human AChE even at 25 μM[15]. However, recently Jung and Park reported that quercetin is a potential AChE inhibitor with an IC_{50} of 19.8 μM[16], which stimulated our interest to examine the AChE inhibitory potential of quercetin by theoretical methods.

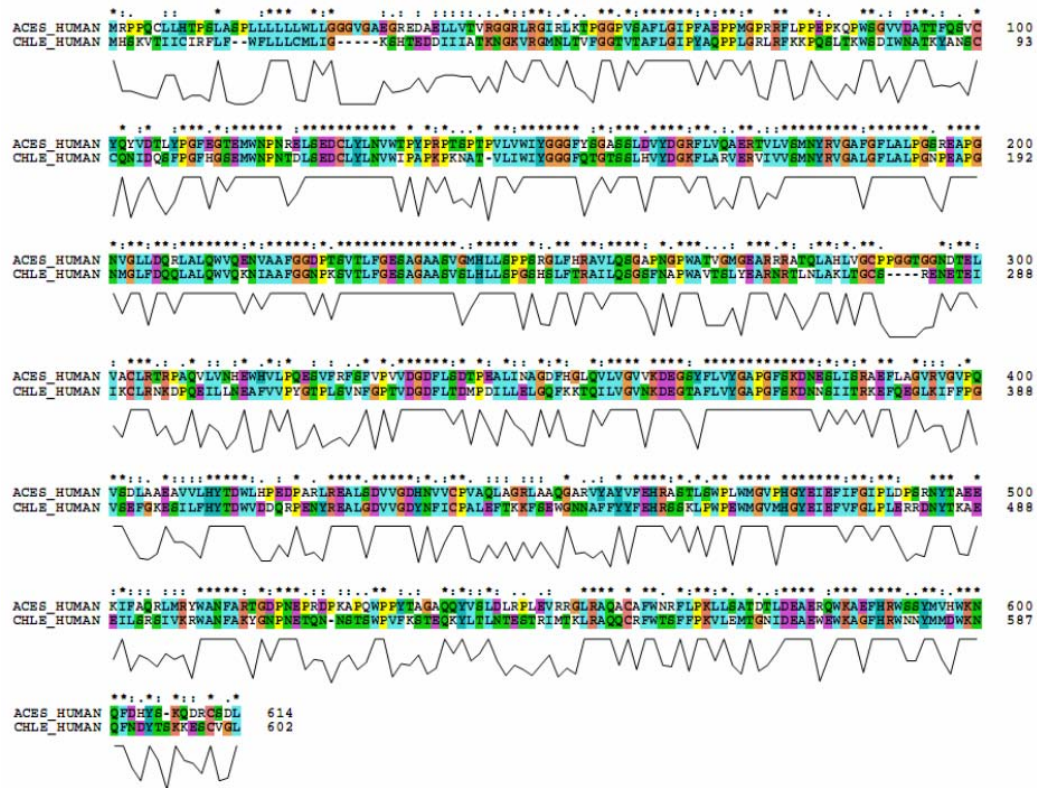
The structure coordinates for human AChE and BChE were taken from the Protein Data Bank (PDB entries: 1B41 and 1P0I, respectively)[17,18], which have been successfully used in previous virtual drug screening efforts[15,19]. The 3D structure of quercetin was first constructed using standard geometric parameters of SYBYL 6.92[20], then was optimized using 200 steps of steepest descent, followed by conjugate gradient minimization to a root mean square (RMS) energy gradient of 0.001 kcal/(mol·Å^2). Tripos force field and Gasteiger-Hückel charges were employed throughout this study. FlexX[21] embedded in SYBYL 6.92[20] was employed to conduct docking experiments, which is a fast, flexible docking method that uses an incremental construction algorithm to place ligands into an active site[22]. The active sites for the proteins were selected on the basis of experimentally reported key residues which play key roles in their catalytic activities. 30 conformations of quercetin were selected to dock with targets. Standard parameters of FlexX, as implemented in SYBYL 6.92[20], were used to estimate the binding affinity characterized by FlexX[21]. The structure alignment between human AChE and BChE was performed by using the Combinatorial Extension (CE) algorithm, which compares pairs of protein polypeptide chains[23].

Through docking quercetin with human AChE and BChE, it was found that the FlexX score for quercetin-BChE couple (-26.3 kcal/mol) is much lower than that for quercetin-AChE counterpart (-16.1 kcal/mol), indicating that quercetin is a more efficient inhibitor to BChE than to AChE. Since human AChE and BChE are similar in sequence (with the identity of 52.7%) and structure (with the RMSD of 1.3 Å) (figure 2), it is reasonable to infer that the

different activities of quercetin to inhibit human AChE and BChE arise from the differences in the binding modes of quercetin with both enzymes.

The analysis on the binding modes of quercetin to human AChE and BChE indicates that the binding is governed by hydrophobic interactions and hydrogen bonds. As illustrated in figure 3, the hydrophobic interactions are formed between Phe297 of AChE and ring A of quercetin and between Phe329 of BChE and ring A of the flavonoid. As to the hydrogen bonds, four exist between quercetin and AChE, while eight exist between quercetin and BChE (figure 3 and table 1). Thus, the hydrogen bond advantage in BChE-quercetin binding is likely to be responsible for the stronger inhibitory effects of quercetin on BChE than on AChE.

In brief, through examining the binding modes of quercetin to human AChE and BChE, we can conclude that although the AChE-inhibitory effect of quercetin can not be completely excluded, it must be much weaker than the effect on BChE. Quercetin's higher inhibitory effect on BChE is likely to result from the more hydrogen bonds formed in BChE-quercetin couple than in AChE-quercetin counterpart.



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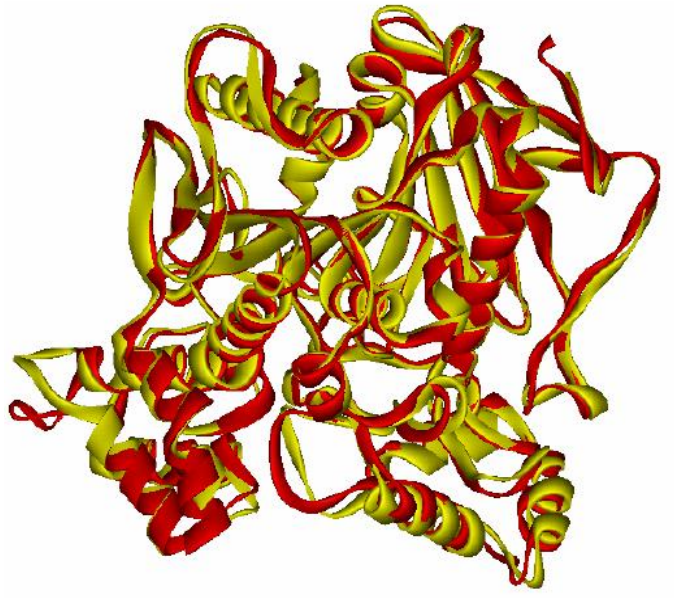
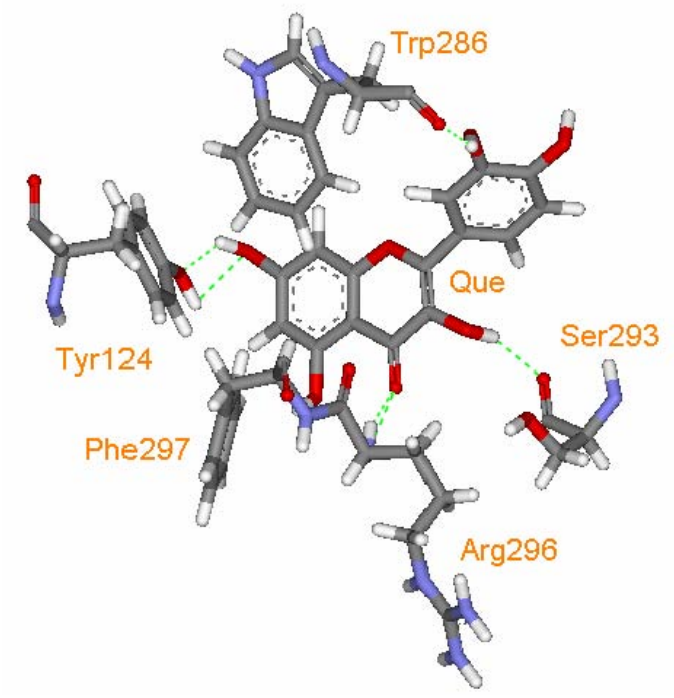


Figure 2. (a) Sequence alignment of human AChE (PDB entry: 1B41) and human BChE (PDB entry: 1P0I) (Sequence identity = 52.7%). Symbols above the alignment indicate sequence conservation: (*) 100% conserved identities; (:) highly conserved identities. (b) Structure alignment of human AChE (in red, PDB entry: 1B41) and human BChE (in yellow, PDB entry: 1P0I) (RMSD = 1.3 Å).



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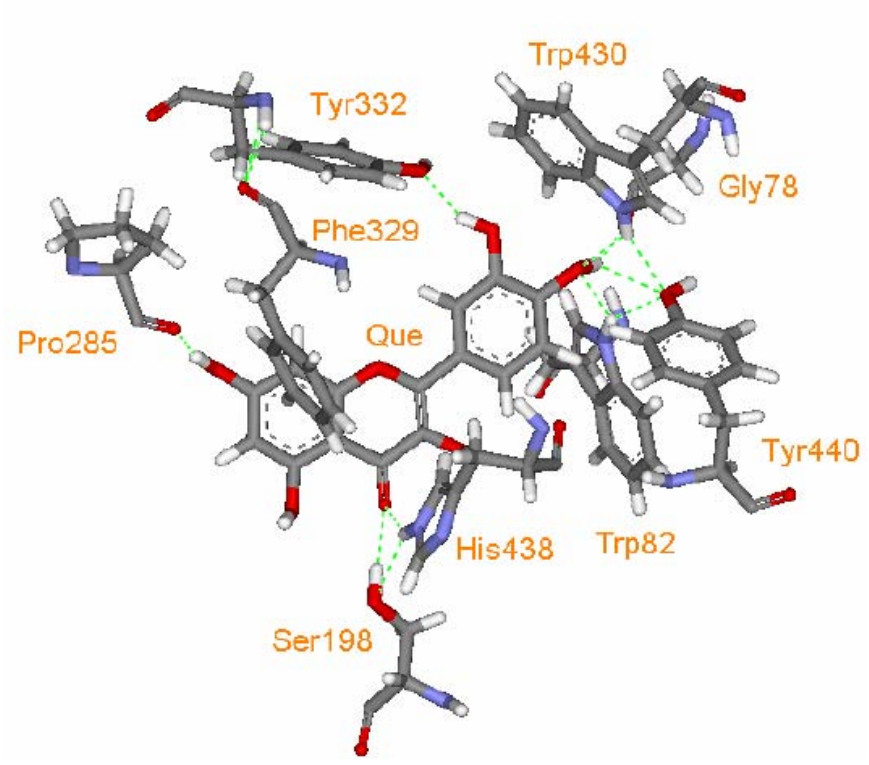

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Figure 3. Close-up views of binding modes of quercetin to human AChE (a, PDB entry: 1B41) and human BChE (b, PDB entry: 1P0I). The hydrogen bonds are marked in green dotted lines.

Table 1 Hydrogen bonds formed between quercetin (Que) and human AChE (PDB entry: 1B41) and human BChE (PDB entry: 1P0I)

PDB entry	H-bond donor	H-bond acceptor	Distance	Angle
1B41	Arg296:H	Que:O20	1.67	158.95
	Que:H7	Trp286:O	1.73	150.45
	Que:H8	Ser293:O	2.06	156.27
	Que:H10	Tyr124:OH	1.60	130.83
1P0I	Trp430:HE1	Que:O17	2.21	140.18
	Trp82:HE1	Que:O17	2.18	148.06
	Ser198:HG	Que:O20	2.09	172.42
	His438:HE2	Que:O20	1.91	141.12
	Que:H6	Tyr440:O	1.93	156.21
	Que:H6	Gly78:O	1.93	164.01
	Que:H7	Tyr332:OH	2.07	150.03
	Que:H10	Pro285:O	1.74	166.82

ACKNOWLEDGMENTS

This study was supported in part by the National Basic Research Program of China (2003CB114400) and the National Natural Science Foundation of China (30570383 and 30700113).

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In: Alzheimer's Disease Diagnosis and Treatments ISBN: 978-1-61122-064-3
Editor: Marisa R. Boyd

Chapter 20

THERAPY WITH DRUG PRODUCT AZD-103 MAY EASE ALZHEIMER'S DISEASE

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Alzheimer's disease (AD) is a group of disorders involving the areas of the brain that control thought, memory, and language. AD is the most common form of dementia among the elderly. Almost four million Americans and eight million more worldwide suffer from AD; after the age of 65, the incidence of the disease doubles every five years and, by the age of 85, it affects nearly half of the population. Currently approved Alzheimer's therapies primarily treat the disease symptoms but do not reverse or slow down the disease progression. [1] The increasing awareness of the diverse factors involved in the onset of AD has outlined new paths of research for prevention and pharmacological treatments. A pivot clinical trial using Abeta1-42 immunization (AN1792) on AD patients showed a possible therapeutic effect, in line with previous experiments using animal models; [2-4] however, the trial was interrupted because of meningoencephalitis probably due to the activation of T-cells and microglia, in 6% of participants. Although no significant amelioration of cognitive dysfunction was observed, CSF tau decreased in anti-AN1792 antibody responder patients. [5] A MRI study on AD patients with immunotherapy demonstrated decreased volume of neuronal tissue including hippocampus, which is unrelated to worsening cognitive dysfunction; this shows a possible amyloid removal by immunotherapy. [6] Another approach to observe the decrease of Abeta-associated amyloidogenesis is the inhibition of Abeta aggregation and its clearance.

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In this commentary, the Authors express their opinion regarding the *Questio* of AZD-103 (scyllo-cyclohexanehexol) and AD concomitantly with the publication of the paper by McLaurin J et al. [4] The findings in the *Nature Medicine* publication show that oral treatment of AZD-103 (scyllo-cyclohexanehexol) reduces accumulation of amyloid beta and amyloid beta plaques in the brain, and it also reduces, or eliminates, learning deficits in an AD transgenic mouse model. Transition Therapeutics Inc. (Canada) is pursuing the clinical drug development of AZD-103 in an expedited manner and it has also announced that dosing with AZD-103 has commenced in Phase I clinical trial. The Phase I trial is a single blind, randomized, placebo controlled study in which healthy volunteers will receive placebo or increasing acute doses of AZD-103. The primary aim of the trial is to evaluate AZD-103 safety, tolerability, and pharmacokinetics.

In our humble opinion, the paper by McLaurin and colleagues might be very significant since AZD-103 has many of the properties sought in a disease-modifying drug for AD, as subsequently confirmed. [7] It is a small, orally bioavailable compound with enantiomeric specificity. It addresses a well-documented target, the soluble assemblies of secreted Abeta that have been widely validated as interfering with the hippocampal synaptic function in a variety of AD animal models. In addition, the compound is soluble, it can be readily administered orally, and penetrates into the brain in quantities sufficient to prevent cognitive deficits produced by levels of Abeta similar to those that occur in human CSF. Although side effects unrelated to its mechanism of action cannot be excluded and initial *in vivo* toxicity data appear benign, the findings provide evidence that AZD-103, neutralizing the neurotoxic activity of soluble Abeta oligomers, becomes a promising therapeutic option for AD.

COMPETING INTERESTS

The author(s) declare that they have no competing interests.

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In: Alzheimer's Disease Diagnosis and Treatments ISBN: 978-1-61122-064-3
Editor: Marisa R. Boyd

Chapter 21

NSAIDS IN ANIMAL MODELS OF ALZHEIMER'S DISEASE

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ABSTRACT

Brain inflammation is an underlying factor in the pathogenesis of Alzheimer's disease (AD) and epidemiological studies indicate that sustained use of non-steroidal anti-inflammatory drugs (NSAIDs) reduces the risk of AD and may delay its onset or slow its progression. Nevertheless, recent clinical trials have shown that NSAIDs do not alter the progression of AD. Neuroinflammation occurs in vulnerable regions of the AD brain where highly insoluble β-amyloid (Aβ) peptide deposits and neurofibrillary tangles, as well as damaged neurons and neurites, provide stimuli for inflammation. To elucidate the complex role of inflammation in neurodegenerative processes and the efficacy of NSAIDs in AD we developed an animal model of neuroinflammation/neurodegeneration *in vivo*. An "artificial plaque" was formed by injecting aggregated ß-amyloid peptide (Aβ(1-40) or Aβ(1-42)) into the nucleus basalis magnocellularis (NBM) of rats. We investigated several aspects of the neuroinflammatory reaction around the "artificial plaque" such as microglia and astrocyte activation, production of proinflammatory compounds, activation of cyclooxigenase-2 (COX-2), p38 Mitogen Activated Protein Kinase (p38MAPK) and induction of inducible Nitric Oxide Synthase (iNOS). Finally, degeneration of cortically projecting cholinergic neurons was also evaluated by means of immunohistochemistry and microdialysis. We examined whether the attenuation of brain inflammatory reaction by NSAIDs and NO-donors may protect neurons against

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neurodegeneration. The data reported in this review show that in *in vivo* model of brain inflammation and neurodegeneration, the administration of NSAIDs and NO-donors prevent not only the inflammatory reaction, but also the cholinergic hypofunction. Our data may help elucidating the role of neuroinflammation in the pathogenesis of AD and the ability of anti-inflammatory agents to reduce the risk of developing AD and to slow its progression.

Keywords: NSAIDs, Alzheimer's Disease, β-amyloid, brain inflammation, acetylcholine.

INTRODUCTION

Alzheimer's disease (AD) is a neurodegenerative disease characterized clinically by progressive and severe memory loss that begins early in the disease. Other cognitive (disorientation, confusion and problems with reasoning) as well as behavioural (agitation, anxiety, delusions, depression and insomnia) disturbances appear as the disease progresses and impair daily living (Terry and Katzman, 1983). Elucidating the pathogenetic mechanisms leading to AD is a major goal for neuroscientists with the aim to find efficacious disease-modifying agents, but identification of the steps most amenable to intervention has been a difficult task to achieve. Nevertheless, in the last several years substantial consensus has developed that certain cellular and biochemical changes, which start years or even decades before clinical symptoms, are prominent neuropathological hallmarks of the AD brain and an outline of the disease cascade has emerged. The key neuropathological features of AD are amyloid plaques with associated dystrophic neurites, and neurons containing paired helical filaments in neurofibrillary tangles.

Neuritic (senile) plaques contain extracellular deposits of the 40– and 42–amino acid β-amyloid peptides (Aβ(1-40) and Aβ(1-42)) surrounded by dystrophic neurites (axons and dendrites), activated microglia, and reactive astrocytes. A large proportion of the β-amyloid protein in these neuritic plaques is in the form of insoluble amyloid fibrils, but these are intermingled with a poorly defined array of the peptide in nonfibrillar form. Amyloid immunohistochemistry has also revealed, in brain of AD patients, deposits that lack the dystrophic neurites and altered glia characteristic of neuritic plaques, referred to as "diffuse" plaques. The diffuse plaques are composed of the 42-residue form of the peptide, which is far more prone to aggregation than the slightly shorter and less hydrophobic 40-residue form (β-amyloid protein 40) (Iwatsubo et al., 1994). In the AD brain, β-amyloid plaques do not occur simply in these two extreme forms (neuritic and diffuse) but plaques actually exist in a morphological continuum, in which mixtures of nonfibrillar and fibrillar forms of the peptide can be associated with varying degrees of surrounding neuritic and glial alteration. Neuritic plaque number does not itself correlate with the severity of dementia, although a clinical correlation between elevated levels of Aβ peptide in the brain and cognitive decline has been reported (Naslund et al., 2000). The current neuropathological data suggest that plaques are closely associated with a locally induced chronic inflammatory process.

A recurring concern in the study of AD is that Aβ plaques can be found at autopsy in individuals who had few or no cognitive symptoms during life. However, it is important to note that almost all plaques in aged normal brain tissue are of the diffuse type —that is, they lack associated neuritic and glial cytopathology—and they are accompanied by very few or

no neocortical tangles. On this basis, it has been postulated that diffuse plaques are "pre-clinical" lesions not yet associated with microscopically visible injury to neurons and their processes.

The other classic lesions observed in large numbers in the AD brain are the neurofibrillary tangles. Tangles are intraneuronal masses of paired, helically wound filaments (paired helical filaments) (Goedert and Spillantini, 2000). The subunit protein of the paired helical filaments is the microtubule-associated protein, tau (Kondo et al., 1988;Lee et al., 1991). Biochemical studies revealed that the tau proteins present in paired helical filaments are hyperphosphorylated, insoluble forms of this normally highly soluble protein (Goedert and Spillantini, 2000). Paired helical filaments are not limited to the tangles found in the neuronal cell bodies, but also occur in smaller bundles in many of the dystrophic neurites present around the amyloid plaques.

β-Amyloid peptides are derived by sequential cleavage from the β-amyloid precursor protein (APP) (Selkoe, 1998;Selkoe, 2001). APP is a ubiquitous single transmembrane glycoprotein with a long N-terminal extracellular region and a short C-terminal cytoplasmic tail. (Selkoe, 2001;Price and Sisodia, 1998;Wisniewski et al., 1997;Kang et al., 1987). Nine APP isoforms are produced from a single APP gene by alternative mRNA splicing and by post-translational modifications, such as addition of sugar or phosphate groups to the protein, and encode proteins ranging from 365 to 770 amino acids. Alternatively spliced forms of APP containing 751 or 770 amino acids are widely expressed in cells throughout the body and also occur in neurons. However, neurons express much higher levels of a 695–amino acid splice form. Mature APP is processed proteolytically by distinct α-secretase or β-secretase pathways. The α-secretase activity cleaves the Aβ domain within Lys16 and Leu17 residues to prevent formation of full-length Aβ peptide. This pathway yields a soluble N-terminal APPα and a 10-kDa C-terminal APP fragment that can be further processed by γ-secretase to generate Aβ17–40 or Aβ17–42. The α-secretase cleavage occurs mostly at the cell surface, although it can be mediated to some extent during the secretory intracellular trafficking of APP (Selkoe, 2001;Clippingdale et al., 2001). The β-secretase pathway, which results in the formation of intact Aβ peptide, is mediated by the sequential actions of β-secretase (β-APP cleaving enzyme [BACE]) and γ-secretase enzymes.

Although several evidences are strongly in favour of the hypothesis that increased β-amyloid protein accumulation is an early and necessary event of AD, considerable debate remains as to whether this can explain the full Alzheimer phenotype. The β-amyloid hypothesis predicts that gradual elevation of β-amyloid levels in brain parenchyma, and perhaps inside neurons (Skovronsky et al., 1998;Walsh et al., 2000), may lead to the oligomerization of the peptide and eventually to its fibrillization into an insoluble form and deposition as diffuse plaques, associated with local microglia activation, astrocytosis, and cytokine and acute phase protein release (Akiyama et al., 2000). Whether β-amyloid peptide oligomers trigger synaptic dysfunction through this intermediate inflammatory process or produce direct synaptotoxic effects by subtly disrupting receptors, channel proteins, and other macromolecules on the plasma membrane (Walsh et al., 2002;Snyder et al., 2005) may prove difficult to sort out because these changes may develop almost simultaneously *in vivo* at the very early stages of the disease.

Nevertheless, increasing evidence suggests that an inflammatory reaction accompanies the Aβ deposition in pathologically vulnerable regions of the brain (Akiyama et al., 2000;Akiyama et al., 2000). The accumulation of reactive microglia in the neuritic plaques

may contribute to the neurodegenerative process through the excessive generation of pro-inflammatory cytokines, reactive oxygen species, nitric oxide, excitatory amino acids, all of which may damage proteins and other macromolecules in neurons (Gonzalez-Scarano and Baltuch, 1999;Behl et al., 1994;Harris et al., 1995;Markesbery, 1999).

Studies in APP transgenic mice (Games et al., 1995;Hsiao et al., 1996;Calhoun et al., 1998;Bondolfi et al., 2002) and in nontransgenic adult animals injected intracerebrally with Aβ (Giovannelli et al., 1995;Harkany et al., 1995;Itoh et al., 1996;Geula et al., 1998) reinforce the notion that overexpression of Aβ peptide, or injection of aggregated Aβ, induces subcellular alterations, and neuronal loss in specific brain regions. Furthermore, it has been suggested that overexpression or injection of Aβ peptide may potentiate the formation of neurofibrillary tangles in tau transgenic mice (Gotz et al., 2001;Lewis et al., 2001), posing a positive correlation between the β-amyloid hypothesis and tau deposition, the two pathogenic hallmarks of AD. Although these results suggest a role for Aβ peptides in the neurodegenerative process, both the role of Aβ in the normal brain and the mechanisms by which it causes neuronal loss and tau abnormalities in AD remain matter of investigation.

In AD, as well as in APP transgenic mouse, activated microglia cells are distributed both diffusely, throughout the cerebral cortex and the hippocampus, and focally concentrated in and around Aβ plaques where they deeply interdigitate neuritic plaques (Griffin et al., 1995;Griffin et al., 1989;Itagaki et al., 1989;Mackenzie and Munoz, 1998;Frautschy et al., 1998;Stalder et al., 1999). Furthermore, activated astrocytes are also integral and prominent components of neuritic plaques (Griffin et al., 1995;Griffin et al., 1989;Mrak et al., 1996). Plaque-associated activated microglia, classified into morphological subtypes representing progressive stages of activation and defined as primed, enlarged, and phagocytic (Sheng et al., 1997), has been suggested to play a relevant role in the transformation of nonfibrillar Aβ into amyloid fibrils. Thus, activated microglial may contribute to the transformation of supposedly non pathogenetic diffuse amyloid deposits into neuritic plaques typical of AD (Cotman et al., 1996;Griffin et al., 1995;Mackenzie et al., 1995;Sasaki et al., 1997;Mrak and Griffin, 2005). Moreover, Wisniewski and colleagues provided ultrastructural evidence that microglia participate in the deposition of amyloid fibrils within the plaques (Wisniewski et al., 1989). Exposure of microglia to Aβ causes its activation leading to an increase in cell surface expression of major histocompatibility complex II (MHC II) along with increased secretion of the pro-inflammatory cytokines which give rise to the so-called "cytokine cycle", a cycle of self propagating, inflammatory events that drives neurodegeneration (Griffin et al., 1998). This positive feedback loop may cause further dysregulation of the β-amyloid precursor protein and local production of complement proteins and acute-phase proteins (Eikelenboom et al., 1998). Furthermore, activated microglia release the excitotoxins glutamate (Piani et al., 1992) and quinolinic acid (Espey et al., 1997) which may further contribute to the development of inflammatory and neurodegenerative processes. However, one opposing view is that microglia may also play a role in plaque evolution by phagocyting and/or degrading deposited Aβ, in line with the view that the amyloid burden in AD brain results from a dynamic balance between amyloid deposition and removal (Hyman et al., 1993). In fact, different laboratories have shown that microglia, both *in vivo* and in culture, phagocyte exogenous fibrillar Aβ (Paresce et al., 1997;Shaffer et al., 1995).

It is more difficult to define the astrocyte role in the inflammatory process associated with AD. It is known that reactive astrocytes cluster at sites of Aβ deposits (Dickson, 1997). However, the position of astrocytes in the plaques differs from that of microglia. Astrocyte

somata form a corona at the perimeter of the neuritic halo that, in turn, may surround a dense core of Aβ deposit. Processes from the astrocytes cover and interdigitate the neurite layer (Mrak et al., 1996) in a manner reminiscent of glial scarring. Astrocytes have been shown to secrete many pro-inflammatory molecules, such as interleukins, prostaglandins, leukotrienes, thromboxanes, coagulation factors, complement factors, proteases, and protease inhibitors, similar to and overlapping with that of the microglia (Tuppo and Arias, 2005), which may take part in the neurodegenerative events typical of AD. However, it has been recently reported that astrocytes may play a crucial role in the degradation of Aβ and it has been proposed that astrocyte defects that lead to reduced Aβ clearance may be implicated in the pathogenesis of AD (Wyss-Coray et al., 2003).

How and when inflammation arises in the course of AD has not yet been fully understood, and for some researchers the pathophysiologic significance of AD inflammation itself still needs to be clarified. Inflammatory mechanisms are highly interactive and almost never occur in isolation from each other.

Degenerating neurons in the brain of individuals with AD are located predominantly within regions that project to or from areas displaying high densities of plaques and tangles. The most severe loss of neurons has been observed in the hippocampus, entorhinal cortex, amygdala, neocortex, dorsal raphe and locus coeruleus (Braak H and Braak E, 1994;Geula C and Mesulam MM, 1994;DeKosky et al., 1996;Ladner and Lee, 1998), but mainly in subcortical areas such as basal forebrain (Bartus et al., 1982;Mesulam et al., 1983). Indeed, the first transmitter abnormality to be documented in AD brain tissue was the loss of enzymes that synthesize and degrade acetylcholine (Davies and Maloney, 1976;Perry et al., 1977). Accordingly, cholinergic neurons in the septum and basal forebrain were found to decline in both size and number in AD (Whitehouse et al., 1982). These findings led to the development of a "cholinergic hypothesis" of AD (Bartus et al., 1982). This hypothesis posits the degeneration of the cholinergic neurons in the basal forebrain and the loss of cholinergic transmission in the cerebral cortex and other areas as the principal cause of cognitive dysfunction in patients with AD (Ladner and Lee, 1998;Francis et al., 1999;Davies and Maloney, 1976;Blusztajn and Berse, 2000;Perry et al., 1978;Bartus et al., 1982). This hypothesis is supported by evidence that drugs that potentiate central cholinergic function (such as the cholinesterase inhibitors donepezil, rivastigmine and galantamine) have some value as a symptomatic treatment during early stages of the disease (Ladner and Lee, 1998;Trinh et al., 2003).

In the last ten years, epidemiological evidence indicated that NSAIDs may reduce the risk of developing AD (Etminan et al., 2003;Hoozemans et al., 2003;Breitner, 1996;McGeer et al., 1990;in, V et al., 2002;McGeer et al., 1996;Pasinetti, 2002;Rich et al., 1995) and may delay its onset or slow its progression (Akiyama et al., 2000), further indicating that inflammation is closely related to the clinical manifestation of the disease (McGeer and McGeer, 1999). Indeed, since patients with rheumatoid arthritis and osteoarthritis are treated with NSAIDs for long periods of their life, epidemiological studies have looked into the association of these diseases and AD. An inverse relationship between the incidence of AD in arthritis patients treated with NSAIDs (Zandi and Breitner, 2001) was observed. Moreover, a prospective population-based study also showed a significant reduction in the risk of AD in subjects who had taken NSAIDs for a cumulative period of 24 months or more (in,'t V et al., 2001). Post-mortem studies have also shown the ability of NSAIDs to reduce the inflammation that is consistently seen in AD brain tissue (Mackenzie, 2001). Therefore, based on the compelling

evidence that inflammatory processes are involved in the pathogenesis of AD, research has looked into the use of anti-inflammatory drugs as a treatment option for patients with AD. The NSAIDs, a family of drugs that include the salicylates, propionic acid, acetic acid, fenamate, oxicam, and the cyclooxygenase-2 (COX-2) inhibitor classes are among the most widely used drugs worldwide owing to their anti-inflammatory, antipyretic and analgesic properties. They function by inhibiting the COX enzyme that catalyses the conversion of arachidonic acid to several eicosanoids. Eicosanoids play major regulatory roles in cell function including immune and inflammatory functions. COX is known to exist as two isoenzymes, COX-1 and COX-2, both of which occur in the brain. With the exception of COX-2 inhibitors, which selectively inhibit the COX-2 enzyme, all classes of NSAIDs inhibit both COX-1 and COX-2 enzymes.

The hypothesis was put forward that NSAIDs might reduce AD risk by inhibiting COX-2 in the brain. In many experimental models of AD, inflammation contributes to neuronal damage, and anti-inflammatory treatments have been shown to offer some neuroprotection. It has been shown that COX-2 mRNA and protein are considerably up-regulated in affected areas of AD brain (Pasinetti and Aisen, 1998;Ho et al., 1999;Yasojima et al., 1999a), with COX-2 immunoreactivity noted mainly in pyramidal neurons in the cerebral cortex (Pasinetti and Aisen, 1998) and the hippocampal formation (Ho et al., 1999), where it may be associated to some neuropathological aspects of the disease, such as potentiation of Aβ (Ho et al., 1999) and glutamate (Kelley et al., 1999) neurotoxicity. COX-2 up-regulation is also found in transgenic mouse models of AD (Hwang et al., 2002;Xiang et al., 2002). Inflammation occurring in the brain of mice with a transgene for amyloid is reduced by ibuprofen (Lim et al., 2000), and, in the rat brain, the microglia response to an excitotoxin injection or Aβ infusion is reduced by nimesulide (Scali et al., 2000) and indomethacin (Netland et al., 1998). Primary neuron cultures (Ho et al., 1999) from transgenic mice overexpressing human (h)COX-2 are more susceptible to excitotoxicity (Kelley et al., 1999), and neuronal death mediated by either synthetic aggregated Aβ (Ho et al., 1999) or *N*-methyl-D-aspartate is prevented by COX-2 inhibitors (Hewett et al., 2000). The injection of the excitotoxin quisqualic acid results in neuronal death and has been used to induce cholinergic hypofunction, mimicking that occurring in AD (Casamenti et al., 1998;Scali et al., 2000). In this regard, evidences indicate that excitotoxicity contributes to inflammation that leads to the neurodegeneration in AD brain (Olney et al., 1997). However, it is not known whether the inflammation-related events in this pathological cascade are always detrimental or whether some elements of this sequence of events could even be protective. As already pointed out, inflammation may at first start as a beneficial host defense response to the Aβ deposition (Akiyama et al., 2000) and only at later times becomes detrimental. Indeed, several studies in transgenic mice encoding the familial AD mutations have shown that immunization with Aβ peptide reduces deposition of cerebral fibrillar Aβ deposits followed by beneficial behavioural effects (Morgan et al., 2000;Schenk, 2002). Antibodies against Aβ may stimulate the removal of Aβ by microglial cells, therefore triggering the positive, beneficial inflammatory response. The hypothesis of treatment with anti-inflammatory drugs is based on reduction of the inflammatory reaction *in toto*, whereas immunization leads to stimulation of the inflammatory response that may be beneficial for Aβ removal.

Despite the encouraging epidemiological and experimental data, the therapeutic results from several recently published trial reports on AD patients treated with NSAIDs have been

thus far disappointing (Aisen et al., 2003). Indomethacin, diclofenac, celecoxib, prednisone and hydroxychloroquine are the anti-inflammatory drugs that have been used in attempts to slow the disease progression in AD (Aisen et al., 2000;Rogers et al., 1993;Sainetti SM et al., 2000;Scharf et al., 1999;van Gool et al., 2001) with no clinical results. An important difference which may explain the disappointing results obtained in clinical trials is the different time-frame of intervention with anti-inflammatory drugs between epidemiological or experimental studies and clinical trials. The underlying pathology might be too advanced in patients with established diagnosis of AD for an antiinflammatory treatment to alter the course of the disease. It is feasible that inhibition of the inflammatory process even at the very beginning of clinical symptoms of dementia is too delayed to block the detrimental effects of the inflammatory process. Indeed, it has been demonstrated (Lim et al., 2000) that in a transgenic mouse strain, model of AD, ibuprofen can significantly delay some forms of AD pathology, including amyloid deposition, when administered at an early stage of brain pathology.

Therefore, intervention with antiinflammatory drugs probably ought to start at the early stages of the pathogenesis, before any clinical symptom is evident. Studies on primary prevention were started under the sponsorship of National Institute of Aging, the Alzheimer Disease Anti-inflammatory Prevention Trial (ADAPT). The effects of the selective COX-2 inhibitors celecoxib and naproxen on the incidence of AD were compared in a population with increased risk of AD, defined as presence of a first relative with dementia. Unfortunately, in Dec 2004 the Food and Drug Administration announced the premature suspension of the ADAPT trial. The trial was stopped after an average follow-up of 3 years because of an apparent increase in cardiovascular and cerebrovascular events in the naproxen arm compared to placebo, but not in the celecoxib arm. This trial was the first placebo-controlled clinical trial to indicate that naproxen was associated with excessive adverse cardiovascular events and contradicted multiple epidemiologic studies and randomized trials that have suggested a cardioprotective effect of naproxen (although inferior than that of aspirin). Similar results were obtained in different clinical trials with celecoxib, rofecoxib, valdecoxib (Konstantinopoulos and Lehmann, 2005) which were then suspended.

To elucidate the complex role of inflammation in the neurodegenerative process and the efficacy of NSAIDs in AD in the last few years we developed a model of brain neuroinflammation and neurodegeneration by injecting into the NBM of adult rats Aβ(1-42) or Aβ(1-40) peptide, aggregated *in vitro* before injection. The aggregated peptide forms a congophilic deposit with characteristics typical of an “artificial plaque”, surrounded by an inflammatory reaction with microglia and astrocytes activation, inducible nitric oxide synthase (iNOS) induction, COX-2 activation as well as activation of the p38 Mitogen Activated Protein Kinase (p38MAPK) pathway, neuronal degeneration and cholinergic hypofunction. On this animal model of neuroinflammation we examined whether the attenuation of brain inflammatory reaction by different classes of NSAIDs may protect neurons against neurodegeneration. To this aim, the effects of NSAIDs with different levels of selectivity for COX-2 (FitzGerald and Patrono, 2001;Chan et al., 1999;Warner and Mitchell, 2004)**,** were investigated in the rat brain *in vivo*, as well as NO derivatives of flurbiprofen derivatives (Del Soldato et al., 1999;Burgaud et al., 2002).

The NO-NSAID compounds are generated by adding a nitroxybutyl moiety to the parent NSAID (aspirin, flurbiprofen, naproxen, ketoprofen, etc.) via a short-chain ester linkage (Del Soldato et al., 1999;Burgaud et al., 2002), and may offer an interesting alternative to the

existing NSAIDs (Prosperi et al., 2001). These compounds exhibit markedly reduced gastrointestinal toxicity (Wallace et al., 1994), while retaining the anti-inflammatory and antipyretic activity of the parent NSAID. Indeed, experimental studies indicate that NO-NSAIDs are more effective than conventional NSAIDs in reducing inflammation (Williams et al., 2001). The rationale for their development was based on the hypothesis that NO and nitrogen oxide compounds (e.g. NO_2^-, NO_3^-) released from these derivatives might exert beneficial effects on the gastric mucosa by enhancing its defensive ability and preventing pathogenic events that occur subsequently to the suppression of prostaglandin biosynthesis, i.e. reduced gastric mucosal blood flow and leukocyte-endothelial cell adherence (MacNaughton et al., 1989;Kitagawa et al., 1990;Santucci et al., 1995;Loscalzo, 2001). Thus, the NO released by these compounds may counteract the detrimental effects of NSAIDs on COX inhibition.

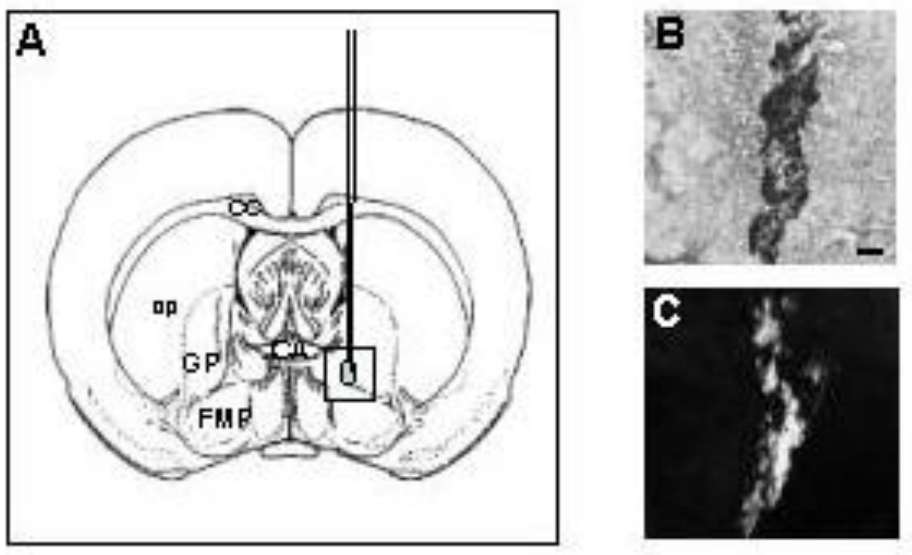

Figure 1.(A) Diagram of a coronal brain slice of rat showing the injection site in the NBM in correspondence with the tip of the needle. CA: Commissura Anterior; CC: Corpus Callosum; cp: Nucleus caudatus putamen; GP: Globus Pallidus; FMP: Fasciculus Medialis Prosencephali. The Aβ(1-42) peptide (Sigma Chemical Co, Milan, Italy) was dissolved in distilled water at the concentration of 5 μg/μl, and the solution was incubated at 37 °C for one week (Aβ(1-40)) or 3 days (Aβ(1-42)), before use. One μl of the solution (containing 5 μg of the peptide) was injected into the right NBM under sodium pentobarbital (45 mg/kg i.p.) anesthesia at the stereotaxic coordinates: AP = - 0.2 mm; L = - 2.8 mm from the bregma; H = - 7.0 mm from the dura (Paxinos and Watson, 1982). The injection lasted 3 min and the syringe was left in place for 5 min after completing the infusion. Control rats were injected with saline solution (1 μl) using the same procedure. (B,C) The presence of a deposit of Aβ(1-42) at the injection site was verified by means of immunohistochemistry using an antibody against the peptide and Congo Red staining in two consecutive brain slices. In (B), Aβ(1-42) was stained using a specific primary antibody followed by DAB staining and light microscopy. The dark staining is indicative of the presence of an Aβ(1-42) deposit. Panel (**C**) shows a polarized light microphotograph of a coronal slice of an Aβ(1-42)-treated animal stained with Congo Red. Note the typical birefringent (white) material indicative of the presence of a fibrillary deposit of Aβ(1-42). The figure shows that the deposit was densely packed, resembling an "artificial plaque" (see also Fig. 2). Scale bar = 75 μm.

INFLAMMATORY REACTION INDUCED BY Aβ(1-42) INJECTION INTO THE NBM

The characteristics of the deposit resulting from Aβ(1-42) or Aβ(1-40) intracerebral injection and of the ensuing inflammatory reaction have been extensively investigated (Casamenti et al., 1998;Giovannini et al., 2002;Giovannelli et al., 1998) (Fig. 1). As previously reported, the injection of Aβ(1-42) into the NBM resulted in a Congo red-positive deposit consisting of aggregated, fibrillary material exhibiting a typical birefringence when observed under polarized light (Fig. 1C). The deposit was densely packed, resembling an "artificial plaque", with infiltration of activated microglial cells and other markers of inflammation The deposit formed by Aβ(1-40) was aggregated in a fibrillar form up to 4 months after surgery, whereas at 6 months no trace of birefringency was visible at the injection site, indicating a loss in the fibrillar organization (Giovannelli et al., 1998;Giovannelli et al., 1995). Scrambled Aβ peptides do not form a Congo Red positive deposit but only induce a non-specific tissue reaction, not different from that observed in saline-injected animals, both in terms of inflammatory reaction and of cholinergic hypofunction (Giovannelli et al., 1995).

IL-1β Production

IL-1β production around the β-amyloid deposit is one of the primary events leading to a self-amplifying inflammatory cascade that may be the cause of subsequent neurodegeneration (Griffin et al., 1998). IL-1β injection in the NBM induces glial activation and iNOS enzyme in the area of the injection site (Casamenti et al., 1999). Aβ(1-42), injection induced a three-fold increase in IL-1β formation ($p < 0.05$, Student's t test vs saline-treated animals) 24 h after injection (Fig. 2A).

COX-2 Immunoreactivity

In coronal brain sections taken at the level of the injected area, COX-2 immunoreactivity (IR) was visible neither in the NBM of naive rats (Breder et al., 1995) nor in the NBM of saline-injected animals, but it was present in neurons of the cortex, hippocampus and amygdala with no noticeable differences among the groups. Injection of Aβ(1-42) induced COX-2 IR in cells surrounding the deposit (Fig. 2B). Seven days after injection of Aβ(1-42) the number of COX-2 positive cells was well above that found in saline-injected animals. The immunopositive cells showed very dark staining both in their bodies and long arborizations, and from their shape they appear to be microglial cells, as also observed after double immunostaining in a model of brain inflammation performed injecting quisqualic acid in the NBM (Scali et al., 2000).

These results demonstrate that a local inflammatory reaction, characterized by early production of the proinflammatory cytokine IL-1β and sustained expression of COX-2, is triggered by the artificial deposit of Aβ(1-42).

To further explore the intracellular events underlying the inflammatory cascade, we studied glial reaction, activation of the MAPK pathways, induction of iNOS and neurodegeneration in this brain inflammation model.

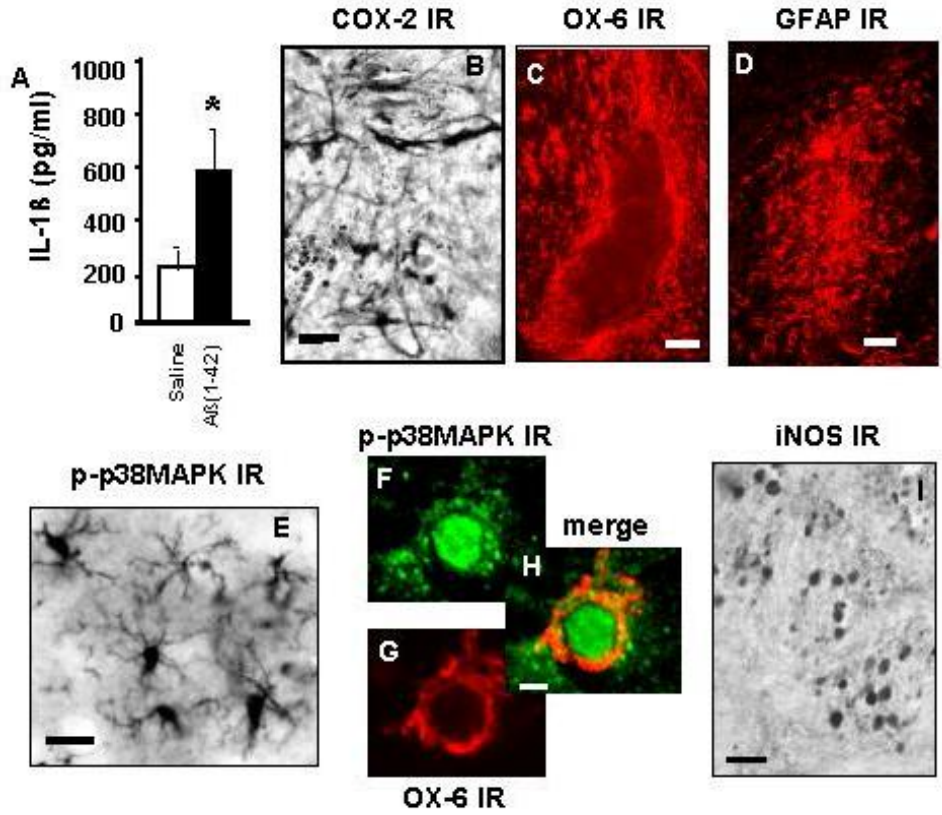

Figure 2. (A) IL-1β formation was detected by means of a rat specific ELISA kit. IL-1β is expressed as pg/ml of homogenate solution. Open bar: saline-injected animals (n=4); black bar, Aβ(1-42)-injected animals (n=4). * $P < 0.02$, Student's t test. (B) COX-2 IR was visualized using a polyclonal antibody and DAB staining; scale bar: 20 µm. (C) Activated microglial cells were revealed by their immunoreactivity for MHC II, visualized using the monoclonal antibody OX-6 and a Texas Red-conjugated secondary antibody followed by immunofluorescence microscopy. Scale bar = 50 µm. (D) Astrocytes were demonstrated by their immunoreactivity for GFAP, visualized using a specific monoclonal antibody and a Texas Red-conjugated secondary antibody followed by immunofluorescence microscopy. Scale bar = 50 µm. (E) activation of p38MAPK was visualized using a specific antibody for its phosphorylated form and DAB staining; scale bar: 25 µm. (F,G,H) Double-labeled confocal microscopy images obtained from slices labelled using antibodies specific for activated phospho-p38MAPK and a Fluorescein conjugated secondary antibody (F) and activated microglia and a Texas Red-conjugated secondary antibody (G) followed by laser confocal microscopy. The images were obtained from one single z-scan (1.7 µm) acquired 22 µm deep into the slice through the cell body and nucleus. Scale bar = 3 µm. The digitally combined image (H) shows that phospho- p38MAPK colocalizes in activated microglial cells and translocates to the nuclear region. (I) iNOS IR was visualized using a polyclonal antibody and DAB staining; scale bar: 20 µm. For further experimental details refer to publications (Giovannini et al., 2002;Giovannelli et al., 1995;Scali et al., 2000).

Microglia and Astrocyte Activation

Seven and 21 days after Aβ(1-42) injection into the NBM, the parenchyma surrounding the deposit was characterized by an intense glial reaction, including microglia and astrocytes (Fig. 2C and D). Many MHC II immunopositive microglial cells, surrounding and infiltrating the deposit, were visualized by the specific antibody OX-6. The cells were numerous, hypertrophic and showed phenotypes ranging from densely arborized to a bushy appearance with swollen cell bodies and intensely stained short processes, to round-shaped microglial cells (Giovannini et al., 2002). Conversely, minimal glia activation was observed after saline injection into the NBM. Quantitative analysis, performed by measuring the immunopositive area using an image analyzer, revealed significant increase in OX-6 IR around the Aβ(1-42) or Aβ(1-40) at 7 and 21 days after injection, as compared to saline controls (Giovannelli et al., 1998;Giovannelli et al., 1995;Giovannini et al., 2002) (Table 1).

Table 1. NSAIDs prevent the inflammatory reaction evoked by Aß(1-42) deposit into the NBM of rats

	(% increase vs saline-treated animals)						
	Aß(1-42)	Aß(1-42)+ rofecoxib	Aß(1-42)	Aß(1-42) + flurbiprofen	Aß(1-42) + HCT	Aß(1-42) + NCX	Aß(1-42)+ naproxen
	7 DAYS		**21 DAYS**				
Activated microglia (OX-6 IR)	343 (4)	135* (5)	368 (5)	184# (4)	129# (5)	196# (5)	160# (5)
Astrocytes (GFAP IR)	145 (6)	89* (5)	217 (5)	159# (4)	147# (5)	162# (5)	ND
	7 DAYS		**7 DAYS**				
i-NOS IR	245 (5)	135* (3)	+++	+	+	+	ND
Phospho-p38 MAPK IR	243 (6)	125* (4)	+++	+	+	+	ND

Immunoreactivity was measured using Scion Image and expressed as area in pixels above background. Values in the table are percentages of respective saline-treated animals. Rofecoxib: 3 mg/kg p.o. for 7 days. Flurbiprofen 15 mg/kg, NCX-2216 (15 mg/kg) HCT-1026 (15 mg/kg) naproxen (15 mg/kg) p.o. for 7 or 21 days. Statistical analysis: * at least $p < 0.05$ (Student's t test) and # at least $p < 0.05$ (one-way ANOVA and Neuman-Keuls multiple comparison test) vs respective Aß(1-42)-treated groups.

+++ high increase of i-NOS IR and phospho-p38MAPK IR vs saline treated rats; + the effect was reversed by treatment with NSAIDs. ND: not detected.

For more detailed methods and statistical analysis see (Giovannini et al., 2002;Scali et al., 2000;Prosperi et al., 2004).

The astrocyte reaction was visualized by means of the immunoreactivity for glial fibrillar acidic protein (GFAP), a specific marker of astrocytes (Fig. 2D). β-amyloid deposit induced massive infiltration of astrocytes around the NBM, as well as the transformation of astrocytes from resting to activated state, highlighted by phenotypic changes characterized by cell hypertrophy and long, thick branching. Quantitative analysis, performed by measuring the immunopositive area using an image analyzer, revealed significant increase in GFAP IR

around the Aβ(1-42) or Aβ(1-40) at 7 and 21 days after injection, as compared to saline controls (Table 1) (Giovannelli et al., 1998;Giovannelli et al., 1995;Giovannini et al., 2002).

In saline-injected animals, only a small number of microglial cells and astrocytes with enlarged cell bodies, resembling an early stage of activation, were seen along the needle track in the injected NBM.

Activation of the p38MAPK Pathway

To date, three major protein kinase pathways have been demonstrated to be responsive to IL-1 stimulation (for rev. see (Rothwell and Luheshi, 2000)). These include the p38MAPK, Extracellular Regulated Kinase$_{1,2}$ (ERK$_{1,2}$) and Stress Activated Protein Kinase/JunKinase (SAPK/JNK) pathways. Therefore, we studied by immunohistochemistry the activation of the MAPK pathways by means of specific antibodies for the phosphorylated forms of p38MAPK, ERK$_{1,2}$ and SAPK/JNK 7 days after the injection of Aβ(1-42) to evaluate if the inflammatory reaction triggers and/or involves the activation of the MAPK cascade around the injected area.

Seven days after injection, the parenchyma surrounding the deposit was densely infiltrated with cells that showed very strong immunoreactivity for phospho-p38MAPK but not for phospho-ERK$_{1,2}$ and SAPK/JNK (Giovannini et al., 2002). Immunopositive cells were not only more densely stained but also more numerous in Aβ(1-42)-treated animals than in the saline-treated animals (Fig. 1H). The effect was statistically significant, as shown by the quantitative analysis reported in Table 1. Phospho-p38MAPK-positive cells in Aβ(1-42)-treated animals had a bushy appearance with swollen cell bodies and intensely stained processes, representing successive stages of activation, enlarged, and phagocytic, while those present in saline-injected rats were smaller and more fusiform in shape. In brain of rats treated with Aβ(1-42) the phospho-p38MAPK IR was mostly localized within activated microglia cells, as demonstrated by double-label confocal laser microscopy with the activated microglia-specific antibody OX-6 (Fig. 2F-H). Phospho-p38MAPK IR was never detected within GFAP-positive astrocytes. In the double stained microglial cells, the phospho-p38MAPK showed typical nuclear localization, although staining was still present in the cytoplasm where it colocalized with MHC II. The nuclear translocation of the activated form of p38MAPK is indicative of its involvement in phosphorylating transcription factors such as AP-1 which appears to be one of the critical regulators of genes, including cytokines, growth factors, inducible enzymes and cell adhesion molecules associated with inflammatory diseases (for rev. see (Lewis and Manning, 1999)). On the other hand, its cytoplasmic localization seems to indicate that downstream effectors other than transcription factors might be the target of p38MAPK activation. In this respect it has been demonstrated that p38MAPK increases the expression of COX-2 by stabilizing its mRNA (Lasa et al., 2000;Faour et al., 2001).

iNOS Immunoreactivity and Nitrite Production

In recent years, it has been suggested that microglia-produced NO and reactive nitrogen intermediates mediate neuronal cell death in neurodegenerative disorders (McCann, 1997).

Cytokine-stimulated human astrocytes have also been shown to damage neurons via NO-mediated mechanisms (Chao et al., 1996). In activated glial cells, NO is synthesized by iNOS, which has been demonstrated to be rapidly expressed upon stimulation (Casamenti et al., 1999;Hu et al., 1999). iNOS IR was markedly induced around the injection site by Aβ(1-42) (Fig. 2I and Table 1). iNOS-positive cells had a round shape and in a double-labeling experiment they were mostly identified with activated microglial cells (Casamenti et al., 1999). No iNOS-positive cells were observed in other brain regions at any time after Aβ injection.

Cholinergic Hypofunction

We evaluated the possible neurodegenerative effects brought about by this model of brain inflammation by studying the morphology and the functionality of the cholinergic neurons of the NBM, an area amongst the mostly affected in AD (Whitehouse et al., 1982), where the cell bodies of cortically-projecting cholinergic neurons are localized (Mesulam et al., 1983).

The cholinergic neurons were visualized by means of immunohistochemistry for the enzyme choline acetyltransferase (ChAT). ChAT-immunoreactivity is localized in intensely labeled magnocellular neurons of oval or triangular shape located at the border between the internal capsule and the globus pallidus. The possible neurodegenerative effects in this model of brain inflammation was evaluated by counting the number of the cholinergic neurons projecting from the NBM to the cortex and assessing their function. Quantitative analysis of ChAT IR showed that the Aβ(1-42) or deposit decreased significantly the number of ChAT positive neurons in the NBM 7 and 21 days after injection (-32 and -50%, respectively vs. uninjected contralateral side) (Table 2). Preliminary data show that ChAT IR was still significantly decreased 30 days after injection of Aβ(1-42), and up to 4 months after injection of the Aβ(1-40) peptide (– 33% vs. uninjected contralateral side), indicating that the loss of ChAT positive neurons persists long after injection. At 6 months after Aβ(1-40) injection, concomitantly with the loss of fibril conformation, a complete recovery of ChAT positive neurons in the NBM occurred (+ 15% vs. uninjected contralateral side) (Casamenti et al., 1998). Therefore, the cholinergic hypofunction temporally paralleled the presence of the deposit in fibrillary form (Giovannelli et al., 1998).

Table 2. NSAIDs prevent the loss of cholinergic neurons evoked by Aß(1-42) injection into the NBM of rats

	(% variation vs saline-treated animals)						
	7 DAYS		21 DAYS				
	Aß(1-42)	Aß(1-42)+ rofecoxib	Aß(1-42)	Aß(1-42)+ flurbiprofen	Aß(1-42)+ HCT	Aß(1-42)+ NCX	Aß(1-42)+ naproxen
ChAT IR (NBM neurons	- 41 (5)	+ 12* (5)	- 45 (5)	- 33 (5)	- 25# (5)	- 30 (5)	- 11# (5)

ChAT IR was evaluated counting the positive neurons throughout the length of the injected NBM.

Rofecoxib: 3 mg/kg p.o. for 7 days. Flurbiprofen 15 mg/kg, NCX-2216 (15 mg/kg) HCT-1026 (15 mg/kg) naproxen (15 mg/kg) p.o. for 7 or 21 days. Statistical analysis: * at least $p < 0.05$

(Student's t test) and # at least $p < 0.05$ (one-way ANOVA and Neuman-Keuls multiple comparison test) vs respective Aß(1-42)-treated groups.

For more detailed methods and statistical analysis see (Giovannini et al., 2002;Scali et al., 2000;Prosperi et al., 2004).

The activity of the cortically-projecting cholinergic neurons was evaluated by *in vivo* microdialysis, positioning a transversal membrane in the parietal cortex ipsilateral to the injected NBM. Basal and K^+-stimulated ACh release was evaluated in saline- and Aβ(1-42)- and Aβ(1-40)-treated animals, at different times after injection. Both basal and 100 mM K^+ stimulated ACh release were significantly reduced in Aβ(1-42) and Aβ(1-40) treated animals up to 2 months after surgery (Table 3). For further readings, refer to (Giovannelli et al., 1998;Giovannelli et al., 1995;Giovannini et al., 2002).

Table 3. NSAIDs prevent the cholinergic hypofunction evoked by Aß(1-42) injection into the NBM of rats

	Saline	Aß(1-42)	Aß(1-42)+ rofecoxib
	7 DAYS		
ACh release (K^+ stimulated)	+ 167 ± 48 (5)	+57 ± 12.8* (6)	+110 ± 23.8 (5)

ACh release from the parietal cortex ipsilateral to the Aβ(1-42) injection side was measured by microdialysis followed HPLC. Samples were collected at 20 min intervals at a flow rate of 3 µl/min. Four baseline samples were collected to evaluate baseline release of ACh. After this time, a challenge with high K^+ (100 mM for 20 min) was delivered, and two more samples were collected. Basal ACh release in saline treated animals was 15.7 ± 2 fmol/µl. Values are expressed as percent variation vs baseline ACh release. Rofecoxib: 3 mg/kg p.o. for 7 days.

Statistical analysis: * at least $p < 0.05$ vs saline (Student's t test). For more detailed methods and statistical analysis see (Giovannini et al., 2002;Scali et al., 2000;Prosperi et al., 2004)

EFFECT OF DIFFERENT NSAIDS ON THE INFLAMMATORY REACTION AND NEURONAL DEGENERATION

Drugs

Given the importance of brain inflammation in the pathogenesis of AD and the potential use of sustained administration of NSAIDs in AD, the above findings prompted us to study the effects of different classes of NSAIDs on the inflammatory reaction in our *in vivo* model.

We chose the selective inhibitor of COX-2, rofecoxib (FitzGerald and Patrono, 2001) which has been demonstrated to selectively inhibit COX-2 derived PGE_2 synthesis with an IC_{50} value of 0.53 ± 0.02 µM (Chan et al., 1999). It has a COX-1/COX-2 ratio IC_{50} of 36 (whole blood) and shows no effect on COX-1 in clinical trials (Chan et al., 1999). Rofecoxib was administered orally at the dose of 3 mg/kg (dissolved in 0.5% methocel) for 7 days once daily starting 1 h before the injection of Aβ(1-42). Unfortunately, rofecoxib (Vioxx) was withdrawn from the market after compelling evidence of increased cardiovascular risk in a randomized, double blind, placebo-controlled clinical trial to assess its role (25 mg/kg daily vs placebo) in adenomatous polyposis prevention (APPROVe trial) (Bresalier et al., 2005).

Furthermore, we used flurbiprofen and its NO-NSAID derivatives HCT-1026 and NCX-2216, which may offer an interesting alternative to the existing NSAIDs. Prosperi (Prosperi et al., 2001) demonstrated in the rat that, after oral administration of NO-flurbiprofen (HCT-1026), a significant increase in extracellular nitrite levels can be detected in the cortex, and its subchronic administration markedly reduces the brain inflammatory reaction brought about by intracerebral injection of quisqualic acid. According to Wenk (Wenk et al., 2000) NO-flurbiprofen reduces the activation of microglia cells and prevents damage to cholinergic neurons of the basal forebrain caused by continuous infusion of lipopolysaccharide (LPS) into the fourth ventricular space. In transgenic Tg2576 mice, the NO-flurbiprofen derivative NCX-2216 was found to be more active than ibuprofen and celecoxib in clearing Aβ deposits from the brain (Jantzen et al., 2002). Flurbiprofen, HCT-1026 (15 mg/kg), and NCX-2216 (15 mg/kg) were administered orally to rats. To study the anti-inflammatory effects the animals were treated once a day for 7 or 21 days.

Some experiments were also performed using naproxen at the dose of 15 mg/kg.

Treatment with NSAIDs significantly blocked the inflammatory reaction evoked by the artificial plaque, both at 7 and 21 days after injection of Aβ(1-42). Seven days of treatment with rofecoxib significantly reduced the microglia and the astrocyte reaction around the Aβ(1-42) injection site, as shown by the quantitative analysis in Table 1. Furthermore, treatment with rofecoxib prevented the increase of iNOS IR induced by the Aβ(1-42) deposit, as shown by the quantitative analysis in Table 1. The effect of administration of rofecoxib was also evaluated on p38MAPK phosphorylation evoked by Aβ(1-42). Unexpectedly, we found that rofecoxib completely prevented the increase in phospho-p38MAPK-positive signal around the injection site (Table 1).

Flurbiprofen, NCX 2216 and naproxen at 21 days after injection were equipotent in reducing microglia activation, while HCT-1026 virtually abolished it in this model. In addition, flurbiprofen, HCT 1026 and NCX-2216 significantly attenuated the astrocyte reaction induced by the Aβ(1-42) injection. Oral administration of either flurbiprofen (15 mg/kg) or its NO derivatives (15 mg/kg each) for 7 days prevented the increase in iNOS-immunopositive cells around the deposit, as shown in Table 1. No significant differences between the effects of the three drugs were observed. Oral administration of 15 mg/kg of NCX-2216, HCT-1026, and flurbiprofen for 7 days prevented the increase in p38MAPK phosphorylation around the deposit. No significant differences between the effects of the three drugs were observed.

The effect of the drugs was also evaluated on the cholinergic hypofunction caused by Aβ(1-42) deposit (Table 2) at 7 and 21 days after injection. Interestingly, 7 days of treatment with 3 mg/kg of rofecoxib completely prevented the loss in ChAT IR (Table 2). Quantitative analysis, carried out 21 days after Aβ(1-42) injection, showed that the deposit induced a significant decrease in the number of ChAT-positive neurons in the NBM (-45 % vs. saline treated animals). This decrease was significantly attenuated by the administration of HCT-1026 (15 mg/kg) and naproxen (15 mg/kg). Only a tendency towards recovery in the number of ChAT-positive neurons was observed in rats treated with NCX-2216 (15 mg/kg) and flurbiprofen (15 mg/kg). Therefore, it seems that the inhibition of the inflammatory reaction by pre-treatment with NSAIDs led to neuroprotection, as revealed by a number of ChAT positive neurons.

Interestingly, 7 days of treatment with 3 mg/kg rofecoxib also significantly attenuated the decrease in K^+-stimulated ACh release induced by the deposit of Aβ(1-42) (Table 3).

CONCLUSION

These finding reported in this chapter are relevant for further understanding the molecular mechanisms through which Aβ plaques induce brain inflammation and neuronal degeneration in AD. Indeed these data show that injecting pre-aggregated Aβ(1-42) peptide into the rat NBM produces an "artificial plaque" showing several features of the plaques found in AD brain, characterized by an inflammatory reaction and by a decrease in the number of cholinergic neurons around the deposit and hypofunction of the cortical cholinergic system. Treatment with different NSAIDs prevents both the inflammatory reaction and the decrease in the number of ChAT-positive neurons and cholinergic hypofunction. The mechanism through which the Aβ(1-42) peptide deposition damages the surrounding cholinergic neurons has not yet been elucidated. However, the finding that NSAIDs treatment prevents the damage supports a role for proinflammatory products, including prostaglandins, NO and cytokines. In particular, despite the growing evidence from several laboratories (Pasinetti and Aisen, 1998;Ho et al., 1999;Xiang et al., 2002;Bazan et al., 2002;Yasojima et al., 1999b) implicating COXs in the pathophysiology of AD and in models of AD, their precise role in the clinical progression of AD is little understood (Pasinetti and Pompl, 2002). Thus, further understanding of the role of COX activity (specifically COX-derived PG) in mechanisms leading to Aβ generation is critical for the future development of antiinflammatory therapy for AD. Findings showing that COXs may promote Aβ generation via a PGE_2-mediated pathway (Qin et al., 2003) and the current evidence suggesting that certain NSAIDs may also directly influence γ-secretase activities (Weggen et al., 2003;Weggen et al., 2001) support the hypothesis that certain NSAIDs may bear therapeutic relevance to antiamyloidogenic strategies. Indeed, select nonsteroidal antiinflammatory drugs are capable of lowering amyloid levels both *in vitro* and *in vivo* (Eriksen et al., 2003), altering the γ-secretase activity without significantly modifying other APP processing pathways (Weggen et al., 2003;Sagi et al., 2003). By contrast, it has recently been demonstrated that a number of compounds, among which some COX-2 selective NSAIDs as well as other compounds devoid of COXs inhibiting properties, increase amyloid peptide levels both *in vitro* and *in vivo* targeting the γ-secretase complex and increasing γ-secretase–catalyzed production of Aβ(1-42) (Kukar et al., 2005).

Different mechanisms of action other than inhibition of COXs activity have also been reported to explain the antiinflammatory effects of a subset of NSAIDs, suggesting that multiple and different actions may participate in their pharmacological activities. It has been reported that aspirin, mefenamic acid, indomethacin and ketoprofen prevent NOC18-induced neuronal damage by directly and dose-dependently scavenging nitric oxide radicals in neuronal cells (Asanuma et al., 2001), and ibuprofen reduces caspase activity per plaque in Tg2576 mice (Lim et al., 2001) and prevents quinolinic acid- and cyanide-induced lipid peroxidation and superoxide radical generation, respectively, in rat brain homogenates (Lambat et al., 2000). Furthermore, ibuprofen, indomethacin and sulindac decrease by 80% the highly amyloidogenic peptide Aβ(1-42) in cultured cells. This effect is not seen with all NSAIDs and seems not to be mediated by inhibition of COX activity (Weggen et al., 2001).

Other laboratories have shown that these effects are mediated through inhibition of transcription factors directly or indirectly via alterations of the activity of intracellular kinases such as the MAPK pathway(s) (for rev. see (Tegeder et al., 2001)). For instance, NSAIDs suppress T-cell activation by selectively inhibiting p38MAPK activation (Paccani et al.,

2002). In our experiments, the prevention of the inflammatory reaction, and of cholinergic cell loss and hypofunction by NSAIDs were concomitant with the inhibition of p38MAPK phosphorylation. These findings are of clinical significance as an intense phospho-p38MAPK IR in neuritic plaques, neuropil threads, and neurofibrillary tangle-bearing neurons have been detected in AD brain (Hensley et al., 1999), thus playing a role in the progression of the neuropathology.

Given that p38MAPK is both upstream and downstream of proinflammatory agents (cytokines, PGE_2, NO), it appears that conditions exist in the AD brain for a self-propagating cycle of autocrine and/or paracrine stimulation initiated by activated microglia cells (Hull et al., 2002). The finding that several NSAIDs used in our experiments inhibit p38MAPK activation emphasizes the role of COXs activation and PGE2 production in the cycle. It is worth mentioning that other putative anti-inflammatory drugs, the cytokine-suppressive anti-inflammatory drugs (CSAIDs) (Lee et al., 1994), are currently being developed and their use for the treatment of AD is suggested. The basis for the anti-inflammatory activity of these compounds resides in their ability to inhibit a subset of the p38MAPKs, and the consequent activation of AP-1, as well as cytokine induction (Lee et al., 1994), thereby blocking the "cytokine cycle" responsible for the initiation of inflammation (Griffin et al., 1998). All of the above indicates that blocking one step in this vicious cycle may be enough to interrupt the cycle itself, posing further basis for the therapeutic use of anti-inflammatory drugs in AD.

Therefore, the disappointing trial outcome with anti-inflammatory treatment in AD patients together with the unfortunate premature suspension of the clinical trials due to increased severe cardiovascular adverse effects have cast a dark shade on the use of these compounds. Nonetheless, scientific interest in addressing the role of inflammatory processes in the pathogenesis of AD remains. Different classes of NSAIDs can therefore be used as pharmacological tools to further understand the pathogenetic mechanisms leading to AD as well as a pharmacological basis for developing alternative agents with different targets, devoid of the severe side effects of the present drugs and useful in the treatment of AD patients. The ability of some NSAIDs to inhibit both COX activity and γ-secretase complex should be exploited to design new molecules endowed with both activities which might be clinically more efficacious than the presently available compounds.

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