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The Role of Aβ-Peptide on Spatial Memory, EEG, Auditory Evoked Potentials And Nicotinic Cholinergic Receptors in A/P Transgenic Mice

Doctoral dissertation

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ABSTRACT

Accumulation of amyloid β-peptide (Aβ) in the brain and its deposition into plaques is currently thought to be one of the key pathological features in Alzheimer's disease (AD), and lead to neuronal dysfunction or neuronal death and cognitive impairment. However, the mechanisms how the Aβ exerts its effects neurobiologically remain elusive. Transgenic mice co-expressing mutated human amyloid precursor protein (APP695swe) and presenilin 1 (PS1-A264E) protein and developing AD-like amyloid pathology with age provide a useful tool for studying the neurobiological effects of Aβ. Using these mice as a model, this study was designed to assess the role of Aβ in several clinical features of AD, including sex difference in the disease progression, correlation of Aβ levels with memory impairment, neurophysiological changes, and reduction in the number of nicotinic cholinergic receptors (nAChRs).

Methods. Immunohistology and enzyme linked immunosorbent assay (ELISA) were used to detect the Aβ accumulation and its deposition into amyloid plaques. Radio immunoassay (RIA) was used to determine changes in binding of α4β2 and α7 nAChR subtypes. Recording of electroencephalogram (EEG) and auditory event related potentials (ERPs) were used to observe progressive effects of Aβ accumulation on brain function. Water maze, T-maze and plus maze tasks were used in behavioral testing of possible cognitive impairment.

Results. A/P mice developed age-dependent AD-like amyloid pathology. Aβ40 and Aβ42 levels, number of Aβ plaques and Aβ burden robustly increased with age (from 3 weeks to 17 months). Furthermore, Aβ42 level, number of Aβ plaques and Aβ burden were significantly correlated with each other at the age of 12 months. Female A/P mice had higher Aβ40 and Aβ42 levels, number of Aβ plaques and Aβ burden than the males, and also the progression of amyloid pathology with age was faster in the females. No Aβ plaques were found in APP and PS1 single transgenic mice up to 12 months of age. The A/P mice were impaired in the acquisition and retention of water maze spatial navigation task at the age of 12 months, but not at the age of 4 months, and the impairment in spatial memory retention correlated with the total amount of Aβ42 in the hippocampus. Several alternations in the EEG and auditory ERPs were observed between the A/P mice and their controls. However, despite robust increase in the Aβ levels between 7 and 13 months of age and the appearance of amyloid plaques, the genotype differences remained similar in magnitude. All EEG changes and increased latency of auditory ERP were similar in A/P and APP single transgenic mice. In contrast, impaired auditory gating was observed only in A/P mice. Binding to α4β2 and α7 nAChRs was similar in A/P and control mice at all age groups between 3 weeks to 17 months.

Taken together, these results indicate that the rate of Aβ accumulation with age may be faster in females than in males. Furthermore, age-dependent accumulation of Aβ-peptide in the hippocampus is associated with impaired spatial memory in A/P mice. Electrophysiological changes in A/P mice were independent of Aβ accumulation with the exception of auditory gating, which was observed in double mutant but not single mutant mice. Aβ accumulation did not affect binding of α4β2 and α7 nAChRs. These findings suggest that some but not all clinical features of AD are likely to be associated with accumulation of Aβ-peptide in the brain.

This book is dedicated to Xiaohui and Fangfang

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My deepest thanks are given to my parents, my brother and sister for their love and support during these years.

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ABBREVIATIONS

Aβ	amyloid β peptide
Aβ40	amyloid β peptide 1-40 (40 amino-acids)
Aβ42	amyloid β peptide 1-42 (42 amino-acids)
AChE	acetylcholinesterase
AChI	acetylcholinesterase inhibitor
AD	Alzheimer's disease
ANOVA	analysis of variance
ApoE	apolipoprotein E
A/P	amyloid precursor protein and presenilin 1 double transgenic mice
APP	amyloid precursor protein
α-BTX	α-bungarotoxin
CERAD	the Consortium to Establish a Registry for Alzheimer's Disease
ChAT	choline acetyltransferase
EEG	electroencephalography
ELISA	enzyme linked immunosorbent assay
EPs	evoked potentials
ERPs	event-related potentials
FAD	familial Alzheimer's disease
MEG	magnetoencephalography
MMN	mismatch negativity
MMNm	magnetic mismatch negativity
nAChRs	nicotinic acetylcholine receptors
NFT	neurofibrillary tangle
NT	nontransgenic mouse
P50m	magnetic P50
PHF	paired helical filament
SPL	sound pressure level
PS1	presenilin1
PS2	presenilin2

LIST OF ORIGINAL PULICATIONS

This thesis is based on the following original publications that are referred to in the text by the Roman numerals **I-V**

I   **Jun Wang,** Heikki Tanila, Inga Kadish, Thomas van Groen. Gender Differences in the Deposition of Amyloidβ in APPswe and PS1 Double Transgenic Mice. **Neurobiology of Disease** Submitted. 2002

II   Jukka Puoliväli, **Jun Wang**, Taneli Heikkinen, Matti Heikkilä, Tero Tapiola, Thomas van Groen, Heikki Tanila. Hippocampal Aβ42 levels correlate with spatial memory deficit in APP and PS1 double transgenic mice. **Neurobiology of Disease** 9;339-347 2002.

III   **Jun Wang**, Sami Ikonen, Kestutis Gurevicius, Thomas van Groen, Heikki Tanila. Alteration of cortical EEG in mice carrying mutated human APPswe transgene. **Brain Research** 943; 181-190 2002.

IV   **Jun Wang**, Sami Ikonen, Kestutis Gurevicius, Thomas van Groen, Heikki Tanila. Altered auditory evoked potentials in mice carrying mutated human amyloid precursor protein and presenilin-1 transgenes. **Neuroscience** 116:511-517 2002.

V   Amelia Marutle, Christina Unger, Ewa Hellström-Lindahl, **Jun Wang**, Jukka Puoliväli, Heikki Tanila, Agneta Nordberg, Xiao Zhang. Elevated levels of Aβ1-40 and Aβ1-42 do not alter the binding sites of nicotinic receptor subtypes in the brain of APPswe and PS1 double transgenic mice. **Neuroscience Letters** 328; 269-272. 2002.

CONTENTS

1. INTRODUCTION

Alzheimer's disease (AD) is the most common cause of dementia in western countries, accounting for up to 70 % of all dementia cases. Prevalence and incidence of AD increase with age. In Finland, the number of dementia patients is estimated to rise to 95000 by the year 2030 and at same time, the proportion of individuals over 65 years of age is estimated to increase to 24.8% by the year 2030 (Sulkava et al. 1986), which will cause heavy burden on society and cause public health problems. Based on epidemiological, genetic, and clinical studies, AD is etiologically heterogeneous, multifactorial disease, in which both genetic and environmental factors play important roles in the pathogenesis (Kivipelto et al. 2001, Tyas et al. 2001, Fratigloini and Rocca, 2001, Hiltunen et al. 2001). The major recent contribution in understanding the disease mechanism(s) is the discovery of genetic mutations that underlie certain familial forms of AD. More than 100 rare and highly penetrate mutations have been described in three genes: amyloid precursor protein (APP), presenilin-1 (PS1) and presenilin-2 (PS2) (Hardy 1997, Tandon et al. 2000, Tanzi and Bertram 2001). However, more than 90% of AD cases are sporadic cases.

Mutations in APP, PS-1 and PS-2 genes result in the overproduction of β-amyloid (Aβ) from its precursor protein, APP, and formation of amyloid plaques (Hardy 1997). These mutant gene products cause dysfunction and death of vulnerable population of nerve cells, with the resulting clinical syndrome of progressive dementia. In order to understand the roles of these mutant genes on the pathobiology of AD, many laboratories have generated gene-targeted mice, such as APP knock-out (Zheng et al. 1995) and PS1 knock-out (Shen et al. 1997) mice, as well as and transgenic mice carrying certain familial forms of AD mutations, such as APP single transgenic (Hsiao et al. 1996) or APP and PS1 double transgenic mice (Borchelt et al. 1997). These genetically modified mice mimic some features of pathophysiological processes of AD and can be used as useful tools to study AD.

Currently, the relationship between amyloid pathology and impaired cognitive functions is one of hot issues in AD research. For example, at an early stage AD pathology is largely restricted to the hippocampus and medial temporal cortical structures (Hyman et al. 1984), which play a pivotal role in declarative memory (Eichenbaum 2000). Similarly, transgenic mice lines carrying mutations of human APP and PS1 genes develop progressive, age-related Aβ neuropathology with amyloid plaques and elevated levels of Aβ in the brain, especially in the hippocampus and neocortex, and also cognitive impairment in different tests of learning and memory (Hsiao et al.

1996). Several studies have attempted to correlate the level of hippocampal amyloid pathology with impaired cognitive performance in transgenic mice, but the data has remained only suggestive. Whereas some studies report correlation between the degree of spatial memory impairment and the amyloid plaque load (Chen et al. 2000, Gordon et al. 2001), other studies have found similar impairment in mice that do not develop plaques despite elevated levels of Aß peptide (Nalbantoglu et al. 1997) or no significant age-related spatial memory impairment at all (King et al. 1999). Furthermore, the mouse line in which the correlation between amyloid plaque load and impaired spatial memory was demonstrated, have marked hippocampal atrophy already at an early age (Dodart et al. 2000), thus, making the interpretation of correlation analyses difficult.

Sex-difference in prevalence and rate of progression is another interesting question in the pathogenesis of AD. Especially after the age of 85 years, a high incidence rate of AD in women has been reported in some studies (Fratiglioni et al. 1997). The basis for this sex difference is still under debate. One plausible explanation for this sex-difference in AD is that estrogen deficiency associated with menopause may contribute to the development of AD (Monk and Brodaty 2000). This notion is supported by many studies showing decrease in the risk of AD among women taking estrogen replacement therapy (Henderson et al. 1994). On the other hand, some data from epidemiological investigations suggest that this difference may be simply ascribed to greater longevity of women than the men (Molsa et al. 1982). However, a recent post-mortem study from our department demonstrated heavier Aβ load and higher levels of abnormal tau protein in female than male brains of diagnosed Alzheimer patients (Alafuzoff et al. in press). Also one recent experimental study (Callahan et al. 2001) reported that in aged transgenic mice with APPswe mutation females exhibit greater amount of amyloid plaques in their brain than males. As no other study has addressed the possible sex difference in the degree of amyloid pathology in transgenic mice, we wanted to see if females have more severe amyloid pathology also in another transgenic mouse line.

The clinical diagnosis of AD in its early stage is very important, because any therapy - whether symptomatic or aiming at slowing disease progression - is predicted to have its best efficacy during early stages of AD. Thus new methods for more reliable diagnosis and follow-up of the disease pathology are warranted. Recording of spontaneous activity with electroencephalography (EEG) or event-related potentials (ERPs), which are time-locked responses in EEG to presented stimuli, are non-invasive diagnostic methods that are especially suitable for follow-up studies. Several abnormalities in EEG or ERPs have been reported in AD patients, but the

underlying pathology has remained unclear, because AD affects several neurotransmitter systems that are potentially related to the abnormal EEG and ERPs (Rice et al. 1990, Soininen et al. 1991, Pekkonen et al. 2001). Among those neurotranmitters, the role of cholinergic system on EEG and ERPs has been widely investigated (Riekkinen et al. 1990, Riekkinen et al. 1991a, Soininen et al. 1992, Jääskeläinen et al. 1999, Pekkonen et al. 2001). Slowing of occipital alpha of EEG (and corresponding shift to theta) correlates inversely with the frontal ChAT activity in AD patients (Soininen et al. 1992), and mid-latency ERP components (P50 or P1) that are abnormal in AD patients (Buchwald et al. 1989, Green et al. 1992, Jessen et al. 2001) show cholinergic modulation (Jääskeläinen et al. 1999, Pekkonen et al. 2001). However, the contribution of Aβ accumulation to electrophysiological changes in AD patients is totally unknown. So far no studies have looked at possible EEG and ERP changes in transgenic mice carrying certain mutations related to familial forms of AD. Using these transgenic mice with AD-like amyloid pathology we directly address the question whether EEG and ERP changes are related to Aβ accumulation or rather arise from other degenerative chances such as cholinergic deficit that is largely missing in our transgenic mice.

Furthermore, transgenic mice with AD-like amyloid pathology also provide a useful tool to study the relationship between the Aβ and observed chances in AD at the receptor level. There is a significant loss of nicotinic acetylcholine receptors (nAChRs) in the cerebral cortex and hippocampus in AD patients, which may consistently contribute to the brain dysfunction observed in AD (Nordberg 2001). However, it is still controversial whether muscarinic cholinergic receptors are also lost in AD (Quirion et al. 1989, Rinne et al. 1989, Aubert et al. 1992). Of particular relevance to our studies on the role of Aβ in auditory gating in A/P mice are potential changes in the number or binding of α7 nAChRs, which are closely involved in the control of auditory gating in the hippocampus (Luntz-Leybman et al. 1992). On the one hand, Aβ binds selectively with picomolar affinity to α7 nAChR (Wang et al. 2000a, 2000b), directly modulates nAChRs (Pettit et al. 2001), and blocks the α7 nAChR mediated responses (Liu et al. 2001). On the other hand, nicotine and nicotinic agonists have effects against Aβ toxicity (Zamani et al. 1997). Those data are based on *in vitro* studies however, and less is known about the relationship between Aβ and nAChRs *in vivo*. One earlier study reported increased numbers of α4β2 and α7 nAChRs in the brains of APPswe transgenic mice (Bednar et al. 2002), which is contrary to the findings in AD patients. Therefore we wanted to study whether these findings are can be reproduced in a different mouse line effects of Aβ accumulation.

To conclude, AD is etiologically heterogeneous, multifactorial disease, whereas the A/P transgenic mice provide an excellent research tool to study the specific contribution of amyloid pathology to several clinical features of AD. Namely, except for Aβ deposition these mice do not have the common pathological features of AD, such as cholinergic deficits, formation of neurofibrillary tangles or presence of Lewy bodies. The present series of experiments addressed the role of amyloid pathology in gender difference in the disease prevalence, the biological effects of Aβ-peptide on spatial memory, EEG and ERP changes, and the number of nAChRs. The findings suggest that Aβ accumulation may be attributed to some but not all clinical features of AD-like amyloid pathology observed in Alzheimer patients. First, female A/P mice show higher production of Aβ than males. Second, the age-dependent spatial memory impairment in A/P mice correlates with their hippocampal Aβ levels. On the other hand, the accumulation of Aβ-peptide correlates poorly with observed EEG or ERP alterations (except for auditory gating) and no effect of Aβ-peptide accumulation was found on specific ligand binding to major neuronal nAChRs.

2. REVIEW OF LITERATURE

2.1. ALZHEIMER'S DISEASE

2.1.1. Epidemiology

Dementia is one of the major public health problems, and the increasing number of patients with dementia will impose a major financial burden on the health care systems. More than half of the patients with dementia have Alzheimer's disease (AD). The proportion of elderly people is growing in the western countries, and the prevalence and incidence of AD have been shown to increase exponentially with age. The prevalence for AD in Europe is 0.3% for ages of 60-69 years, 3.2% for ages 70-79 years, and 10.8% for ages of 80-90 years (Rocca et al. 1991). The incidence rate of mild AD in Europe for individuals 65-69 years old is 2.5, for 75-79 years old 10.7, and for 85-89 years old 46.1 per 1,000 person year (Jorm and Jolley 1998).

Based on epidemiological, genetic, and clinical studies, AD is etiologically heterogeneous, multifactorial disease. Both genetic and environmental factors play important roles in the pathogenesis of AD. More and more mutations in the genes and environmental risk or protective factors have been found that contribute to the pathogenesis of AD (Kivipelto et al. 2001, Tyas et al. 2001, Fratigloini and Rocca 2001, Hiltunen et al. 2001). Clinically, AD can be divided into familial and sporadic cases based on known hereditability, and can also be divided into early-onset (onset before age of 65) and late-onset (onset after age of 65), with early-onset more often seen in familial cases and late-onset more often in sporadic cases. The major recent contribution in understanding the disease mechanism(s) is the discovery of genetic mutations that underlie certain familial forms of AD.

2.1.2. Clinical course

AD is a progressive neurodegenerative disease with certain characteristic clinical and pathological features. The clinical symptoms of AD develop insidiously and progressively, including profound memory loss, decline in ability to perform daily tasks, impairment of judgement, disorientation, personality change, difficulty in learning, and finally loss of language. In its early stage, the typical feature is memory loss, uncharacteristic forgetfulness, and misplacement of items. As disease progresses, patients show memory loss, forgetfulness increases, and some patients

become apathetic or show irritability, agitation, paranoid ideas, sleep disorders, or incontinence. In the very advanced stage of the disease, the patients cannot walk or talk and become totally incapable of caring for themselves (McKhann et al. 1984, Hyman et al. 1989). In longitudinal assessment, many patients with AD show progressive loss of recent memory followed by disorders of language (aphasia), motor skills (apraxia), or visual perception (agnosia) (McKhann et al. 1984). However, clinical variations are common, including differences in age of onset, rate of progression, patterns of neuropsychological deficits, and occurrence of neuropsychiatric symptoms.

The median duration of AD from onset to death is usually 9 to 10 years (Heyman et al. 1996), although a wide variability is seen. Increased age and greater dementia severity adversely affect survival (Heyman et al. 1997). The cause of death in AD include infectious disease, such as pneumonia and sepsis, and other common causes of mortality in the elderly, such as cardiovascular disease and stroke (Friedland 1993, Beard et al. 1996).

2.1.3. Neuropathology

In AD brain, the most profound neuropathological changes are amyloid plaques, neurofibrillary tangles (NFTs) and neuronal loss (Braak and Braak 1991). Other changes include synaptic alterations, Lewy bodies, Hirano bodies, amyloid angiopathy, granulovacuolar degeneration and inflammation (Esiri et al. 1997, Kalaria and Ballard 1999, Farkas and Luiten 2001). However, the relative importance of each of these pathological changes remains still obscure, and many of these changes can also be found in normal aging brain without clinical signs of dementia, or in other neurodegenerative diseases.

Plaques The amyloid-β-peptide (Aβ) deposits extracellularly forming amyloid plaques in AD brain. There are two major types of amyloid plaques in AD brain: neuritic plaques and diffuse plaques. Neuritic plaques contain dense bundles of amyloid fibrils and are surrounded by dystrophic neurites, astrocytes, and microglia (Morris 1995). Diffuse plaques contain nonstructured amyloid and are not surrounded by dystrophic neurites. Neuritic plaques may develop from diffuse plaques. The plaques deposition show a typical anatomical specificity, so that in the early phase of the disease the medial temporal lobe structures, entorhinal cortex and the hippocampus are mainly affected (Braak and Braak 1991), and associative temporal and parietal cortical areas are affected in the later phase. The number of plaques has been shown to be significantly increased in the initial stages of the disease (Haroutunian et al. 1998, Naslund et al. 2000, Morris and Price 2001), which

supports the hypothesis that Aβ deposition may be an initial pathogenic event in the development of AD. However, whether the Aβ plaques are associated with the severity of dementia has been a matter of controversy. Some studies have not shown a clear correlation between the number of neuritic plaques and the severity of dementia in AD (Terry et al. 1991, Arriagada et al. 1992), whereas some recent studies have supported a correlation between Aβ plaques and cognitive impairment (Cummings and Cotman 1995, Kanne et al. 1998, Naslund et al. 2000). Moreover, some cognitively preserved aged individuals have such density of neuritic plaques in brain that the diagnosis of AD would be made if the individuals exhibited any clinical signs of dementia (Katzman et al. 1988, Price and Morris 1999).

Amyloid plaques are formed of insoluble Aβ peptides that are cleaved from amyloid precursor protein (APP) (Small and McLean 1999). Aβ peptides occur principally in two lengths, 40 or 42 amino acids, the latter being more susceptible to aggregate. In AD, the proportion of Aβ42 is increased, and the mutations causing familial forms of AD enhance the production of Aβ42. Interestingly, the levels of Aβ 42 seem to be central to the pathogenesis of AD and correlate with the cognitive impairment in AD (Younkin 1995).

Neurofibrillary tangles (NFTs)   NFTs are found intracellularly in the AD brain. They consist of 8-20 nm wide paired helical filaments (PHF), and to a lesser extent of straight filaments. The main component of PHFs is the abnormal phosphorylated microtubule associated protein tau (Lee et al. 1991). NFTs affect primarily large neurons in which they form abnormally fiber masses in the cytoplasm. It has been suggested that NFTs are related to the progression of AD (Braak and Braak 1995), and a correlation between the number of NFTs and cognitive decline in AD has been reported in several studies (Wilcock and Esiri 1982, McKee et al. 1991, Arriagada et al. 1992, Samuel et al. 1994, Cummings et al. 1996). During the preclinical phase of the disease, NFTs appear in the entorhinal region spreading then to the hippocampus at the early phase with mild dementia, and finally to the neocortex during the later stages of AD (Braak and Braak 1995). In addition to AD, NFTs can also be found in individuals without dementia (Price and Morris 1999), and in a variety of degenerative disorders, such as frontotemporal dementias, Down's syndrome, corticobasal degeneration, and progressive supranuclear palsy (Buee et al. 2000).
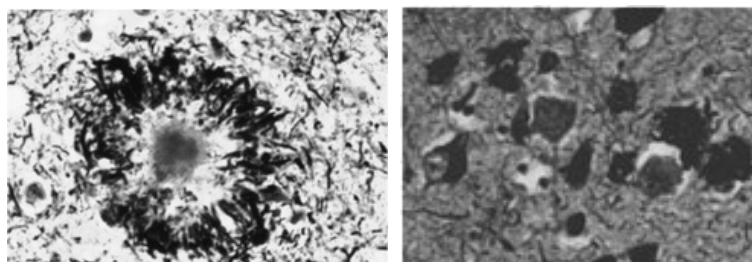
Figure 1. The neuropathology of Alzheimer's disease. Adapted from Sisodia S, 2002. **Left:** High-power photomicrograph of an amyloid plaque. The central core is composed of Aβ fibrils and is surrounded by a halo of dystrophic nerve terminals that are filled with deposits of hyperphosphorylated tau. **Right:** Photomicrograph of silver-stained (black) neurofibrillary tangles in the cell bodies and processes of cerebral cortical pyramidal neurons.

Neuronal loss The number of cholinergic neurons is markedly decreased in all basal forebrain cholinergic cell groups and the loss of cortical cholinergic markers, and the degeneration of basal forebrain cholinergic neurons are the most consistent and severe biochemical deficits in AD brain. Biochemical measurements of acetylcholine synthesizing enzyme, choline acetyl transferase (ChAT) - and acetylcholine hydrolyzing enzyme, acetyl choline esterase (AChE)-activities, ChAT immunohistochemistry and AChE histochemistry have demonstrated severe but regionally variable loss of cholinergic activity in AD brain (Davies and Maloney 1976, Reinikainen et al. 1990, Geula and Mesulam 1996), which correspondingly correlates with memory loss and cognitive impairments. The loss of cholinergic innervation is greatest in cortical structures within the temporal lobe (Geula and Mesulam 1996). In the nucleus basalis of Meynert (NB), neuronal loss ranging from 30 to 95% has been reported (Whitehouse et al. 1982, Rinne et al. 1987, Geula and Mesulam 1999). However, cholinergic activity in cortical areas is also affected in normal ageing (Sparks et al. 1992, Smith et al. 1999), and cholinergic deficits occur in a number of neurodegenerative diseases other than AD, such as dementia with Lewy bodies, Parkinson's disease, Down's syndrome, and head injury (Arendt et al. 1983, Perry et al. 1985, Kish et al. 1990, Murdoch et al. 1998).

2.1.4. Aetiology

Both genetic and environmental factors play important roles in the pathogenesis of AD. Known genetic and environmental risk factors are summarized below.

Amyloid precursor protein (APP) APP is a type I transmembrane protein, encoded by a gene on chromosome 21, which has a long extracellular or luminal N-terminal domain and a short intracellular C-terminal domain containing these Aβ region (Hardy 1997), which undergoes a series of endoproteolytic events referred to as α-, β-, and γ-secretase cleavage. It is now known that Aβ is

a normal product of APP processing, which is cleavaged by β- and γ-secretases to produce Aβ1-40 and Aβ1-42 peptide (Estus et al. 1992, Golde et al. 1992, Haass et al. 1992). Currently, a total of sixteen different APP mutations have been described in AD families and all of them are located close to or within the domain coding the Aβ peptide (Alzheimer's Disease Mutation Database http://molgen-www.uia.ac.be/ADMutations). The evidence for a pathogenetic role for APP comes from genetic studies of early onset FAD (Hardy 1997), because several mutations in APP gene increase the production of both Aβ1-40 and Aβ1-42 peptides, which enhance the deposition into neuritic plaques (Hardy 1997), The clinical, neuropathological, and genetic characteristic of APP-linked FAD with different APP mutations have several shared features, including onset before the age of 60 (mean age of onset between 43-55 years), autosomal dominant fully penetrant inheritance, and also clinical and pathological phenotype indistinguishable from individuals with sporadic AD. Although the exact biological function of APP is currently unknown, more and more research supports the "amyloid cascade hypothesis" that is, the accumulation of Aβ plays a key role in the pathogenesis of AD (Hardy 1997, Sisodia and St George-Hyslop 2002).

Presenilins (PS1 and PS2) PS1 and PS2 proteins are encoded by genes on chromosomes 14 and 1, respectively. They are highly homologous 43- to 50 kDa proteins (Levy-Lahad et al. 1995, Sherrington et al. 1995) predicted to contain eight transmembrane helices (Doan et al. 1996). Thus far, 124 missense mutations have been identified (Alzheimer's Disease Mutation Database http://molgen-www.uia.ac.be/ADMutations) in PS1, and they account for up to 18-50% of early-onset cases of FAD (Cruts and Van Broeckhoven 1998). The common pathogenic feature for all of these PS1 mutations is that they are accompanied by the increased release of the amyloidogenic Aβ1-42 peptide from the APP, which in turn leads to the abnormal accumulation of Aβ1-42 into the amyloid plaques in AD brain (Scheuner et al. 1996, Citron et al. 1997). PS2 displays 67% homology with PS1. To date, 8 AD causing missense mutations have been found in PS2 gene. Also PS2 mutations favor the overproduction of Aβ42 peptide both *in vitro* and *in vivo* (Levy-Lahad et al. 1995, Mann et al. 1997, Marambaud et al. 1998). These results indicate similar mechanisms for both PS1 and PS2 mutations in the pathogenesis of AD

2.1.5.   Risk factors

Age Age is the single most important and established risk factor for AD (Fratiglioni and Rocca 2001). The prevalence of dementia doubles approximately every 5 years between the age 65

and 95 years (Zhang et al. 1990, Aronson et al. 1991, Fratiglioni et al. 1991, Fukunishi et al. 1991, Rocca et al. 1991, Ueda et al. 1992).

Family history of AD   A history of AD in the first-degree relative has been shown by case-control studies to increase the risk of developing dementia by approximately threefold, and about 30% of AD cases have a positive family history (van Duijn et al. 1991). In a number of families in whom AD is inherited as an autosomal dominant pattern, mutations have been inherited in APP, PS1 and PS2 genes (Tanzi and Bertram 2001). In contrast, in late-onset AD, only genetic factor associated with the disease so far is the apolipoprotein ε4 allele (Mahley and Rall 2000). As its presence does not necessarily result in AD, it has been considered more as a risk factor than genetic cause of the disease. However, mutations in APP, PS1 and PS2 and the susceptibility gene ApoE cannot entirely account for the familial aggregation of AD, suggesting that additional susceptibility genes exist (Hiltunen et al. 2001, Tanzi and Bertram 2001). Furthermore, since the majority of AD cases do not have any family history, environmental factors must contribute to the development of the disease.

Apolipoprotein E (ApoE)   ApoE is a polymorphic protein, which exists in three common isoforms (E2, E3, and E4), encoded by three alleles (ε2, ε3, and ε4) of a single gene on chromosome 19q 13.2 locus (Emi et al. 1988). ApoE has an important function as a regulator of lipid metabolism during development, and is also involved in the growth and regeneration of injured neurons (Poirier 1994). The association between the ε4 allele and AD has been confirmed in a number of studies both in sporadic and late-onset familial cases of AD (Farrer et al. 1997). To date, only ε4 allele has been established as a significant genetic risk factor for AD in the general population (Mahley and Rall 2000). The risk for developing AD associated with ε4 allele increases in a dose-related manner, as well as the age of onset. Increase in the number of ε4 alleles is also known to correlate with increased number of senile plaques, NFTs, and more severe cholinergic deficits in the sporadic AD (Schmechel et al. 1993, Soininen et al. 1995a, Soininen et al. 1995b, Beffert and Poirier 1996, Pirttila et al. 1997). ApoE bounds to Aβ plaques (Namba et al. 1991), and ε4 allele promotes β-amyloid aggregation (Ma et al. 1994, Sanan et al. 1994, Wisniewski et al. 1994), whereas ε3 inhibits the aggregation of Aβ (Evans et al. 1995), ε4 allele is also linked to increased density of NTFs in brain (Nagy et al. 1995, Ohm et al. 1995, Polvikoski et al. 1995). On the other hand, ε3 allele has been shown to bind avidly to microtuble-associated proteins (Huang et al. 1994) and to promote their polymerisation, whereas ε4 allele is claimed to depolymerize

microtubules (Nathan et al. 1995). In contrast, AD patients carrying the ε2 allele have a decreased amyloid burden (Polvikoski et al. 1995). It has also been suggested that the ε4 allele increases vascular Aβ deposition (Zarow et al. 1999) and amyloid accumulation after brain injury (Nicoll et al. 1995). In addition to its effect on increasing the risk and lowering the age of onset, and on neuropathology, more recent data suggested that ε4 allele has a significant contribution to memory loss in the elderly by enhancing the severity of hippocampal atrophy, being associated with more severe memory impairment and impaired learning ability in AD (Soininen and Riekkinen 1996). Although ε4 allele is an established risk factor for AD, some studies have not been able to replicate these findings (Blennow et al. 1996, Morris et al. 1996).

Education   Lack of education is a risk factor for dementia, probably for both AD and vascular dementia. Mortimer (1988) predicted that low education would be a positive risk factor for dementia in the very elderly. Zhang et al (1990) confirmed that prediction in Shanghai survey of dementia. An uneducated person older than 75 years is at about twice the risk of dementia compared with one who has completed at least eight grades of school (Snowdon et al. 1989, Katzman 1993).

Gender   Women seem to be at a greater risk for dementia, and in most epidemiological studies, women are at a greater risk for AD when compared to men, especially after the age of 85 years (Fratiglioni et al. 1997). The basis underlying this gender difference is still not known, but implicates an effect of hormones, particularly post-menopausal reduction in estrogens, on the development of AD. The leading hypothesis for this gender difference in AD is that estrogen deficiency associated with menopause (Monk and Brodaty 2000) may contribute to the development of AD. This notion is supported by many positive results from epidemiological investigations and clinical trials, that lower serum estrogen values are found in women with AD than in age-matched controls (Fillit et al. 1986, Henderson et al. 2000), and that estrogen replacement therapy (ERT) may decrease the risk or delay the onset of AD in women (Henderson et al. 1994, Paganini-Hill and Henderson 1996, Harwood et al. 1999, Waring et al. 1999). However, some epidemiological investigations suggest that the sex differences partially correlate with the greater longevity among women or with the survival of women with AD longer when compared to men (Molsa et al. 1982). However, recent *in vivo* and *in vitro* studies in rodents have shown that ovariectomy increases Aβ levels in brain, and that this effect is partly reversed with either low-dose or high-dose 17β-estradiol treatment (Petanceska et al. 2000). Also Callahan et al. (2001) reported that in aged APPswe transgenic mice, females exhibit a greater amyloid burden in the brain than

males, implicating a key role of estrogen on the pathogenesis of AD. Moreover, in addition to the direct effect on Aβ, estrogen also has some other indirect effects that potentially affect Aβ generation: estrogen has trophic and protective effects on neurons (Luine 1985), and has also some antioxidant properties to decrease neuronal damage caused by oxidative stress (Behl et al. 1995).

Some studies have also found an interaction between gender and ApoE, with women having a higher ApoE4-associated risk of AD than men (Duara et al. 1996). Since ε4 allele has been suggested to promote aggregation of amyloid (Heinonen et al. 1995), and to enhance NFTs formation, the carriers of ε4 allele may have more severe neurodegeneration in AD (Martinoli et al. 1995). A more recent study confirmed the association between ε4 allele and AD-related NFTs formation and senile plaques, and showed that this association is differentially modified by age and gender (Ghebremedhin et al. 2001). However, there is still a debate if the ApoE genotype independently, or in combination with other risk factors, such as estrogen, causes the gender difference in AD (Kushwaha et al. 1991, Srivastava et al. 1997b).

Head Injury   Head injury has been reported as a risk factor for AD (Chandra et al. 1989, Gedye et al. 1989, Mortimer et al. 1991). Indeed, repeated head injuries have been reported to lead to the accumulation of NFTs and amyloid plaques (Roberts et al. 1990), but the connection between these changes and the injury is still controversial (van Duijn et al. 1992).

High Blood Pressure   Until recently, hypertension has rarely been considered as a risk factor for AD. However, more recent population-based studies showed that high midlife systolic blood pressure is an independent risk factor for late-life AD (Kivipelto et al. 2001, 2002b, Skoog et al. 1996, Launer et al. 2000).

High Serum Cholesterol Level   Hypercholesterolemia, just like hypertension, is a known risk factor for atherosclerosis and coronary artery disease. Recent studies have also shown that hypercholesterolemia in midlife is associated with AD later in life (Hofman et al. 1997, Sparks 1997, Kivipelto et al. 2001, 2002a), suggesting that cholesterol may be involved in the pathogenesis of AD.

Anti-inflammtory Drug   Use of nonsteroidal anti-inflammtory drugs (NSAIDs) has been reported to reduce the risk of developing AD (Andersen et al. 1995, Stewart et al. 1997). It suggests that inflammatory processes resulting in microglial activation play an important role in the pathogenesis of AD. Furthermore, anti-inflammatory drugs might also modulate Aβ production.

Interestingly, anti-inflammatory drugs such as indomethasine, ibuprofein and sulindac sulphide reduced the amount of Aβ42 in *in vitro* cell culture models, and this property seemed to be independent of their cyclo-oxygenase (COX) inhibition (Weggen et al. 2001).

Smoking   Smoking was found to be a protective factor for AD in early case-control studies (van Duijn and Hofman 1991), but it has recently been reported to increase the risk of AD (Kukull 2001).

2.1.6. Clinical diagnosis and treatment

Clinical diagnostic criteria   There are several guidelines for the clinical diagnosis of AD: the National Institute of Neurological and Communicative Disorders and Stroke – Alzheimer's Disease and Related Disorders Association Work Group (NINCDS-ADRDA) criteria (McKhann et al. 1984), the Diagnostic and Statistical Manual of Mental Disorders, 4th edition (DSM-IV) (American Psychiatric Association 1994) and the International classification of diseases, 10th revision (ICD-10). The NINCDS-ADRDA criteria have been most widely used in research settings because they are well validated, provide high diagnostic accuracy and allow comparison between studies (Blacker et al. 1994, Gearing et al. 1995). The NINCDS-ADRDA criteria divide AD into three categories with increasing reliability of the diagnosis (possible AD, probable AD and definite AD). The patient has probable AD when dementia is characterized by gradual onset and progression, when deficits are documented by examination and testing in two or more cognitive areas, and when other disorders that could cause dementia are absent. The onset should be between the ages from 40 to 90 years, and no disturbances of consciousness should be present. The probable AD diagnosis is strengthened by a positive history of dementia, normal findings in routine cerebrospinal fluid analysis, atrophy in brain imaging, impairments in activities of daily life and a change in behaviour. Histopathological AD changes in autopsy and biopsy of a probable AD patient confirm the diagnosis of definite AD. Possible AD is diagnosed when the patient has another potentially dementing disorder that is not considered to be the primary cause of dementia, or when the patient has variations in the clinical presentation of dementia. The accuracy of the clinical diagnosis of AD using NINCDS-ADRDA criteria is over 80% (Blacker et al. 1994, Galasko et al. 1994, Gearing et al. 1995, Lopez et al. 1999). The sensitivity has been better than the specificity, and the follow-up of patients improves the diagnostic accuracy.

Neuropsychology   Neuropsychological deficits in AD include changes in a variety of cognitive functions, such as episodic memory, semantic memory, language, executive abilities, attention, and visuospatial and visuoperceptual processes. The Neuropsychological tests include the assessments of memory functions (Wechsler Logical Memory Test, Wechsler 1945), verbal functions (Wechsler Adult Intelligence Scale, Wechsler 1981), visuospatial functions, praxis, and executive functions. Neuropsychological tests can be influenced by education (Doraiswamy et al. 1995), practice (Galasko et al. 1993), and sociocultural or ethnic factors (Manly et al. 1998).

Neuropathology   The presence of both amyloid plaques and NFTs in normal aging and in other dementing disorders complicates the neuropathological diagnosis of AD. The widely accepted criteria for neuropathological diagnosis of AD have been established by the Neuropathology Task Force of the Consortium to Establish a Registry for Alzheimer's Disease (CERAD) (Mirra et al. 1991). The analysis includes macroscopic examination of brain, spinal cord, meninges and cerebral blood vessels and semiquantitative microscopic analysis of neuritic plaques by conventional staining in different brain regions. The frequency of plaques is combined with the age of the patients to obtain an age related plaque score (0, A, B, C) that is then integrated with the clinical information regarding the absence or presence or dementia. The CERAD-criteria define the presence of possible, probable and definite AD. Neurofibrillary lesions are not taken into account in the CERAD-criteria.

Neurophysiology   The neurophysiological tests are usually used for research purposes or for an additional tool to dementia diagnosis, and can include spontaneous EEG or event-related potential (ERPs) measurements.

EEG   EEG has been used for clinical diagnosis of neurological diseases since 1920s (Berger, 1929). The electrophysiological study of dementia started with Obrist and Henry (1958) who found that diffuse EEG slowing was related to intellectual deterioration. In AD patients, the EEG slowing is characterized by: 1) decrease of alpha and beta activities, 2) slowing of the dominant occipital rhythm and the mean overall occipital frequency, and 3) increase of theta and delta power (Soininen et al. 1982, Coben et al. 1983, Penttila et al. 1985, Brenner et al. 1986).

Abnormalities in EEG have been found to correlate with the severity of AD including different patterns of cognitive decline (Helkala et al. 1991, Claus et al. 1999, Huang et al. 2000, Kowalski et al. 2001) and extrapyramidal symptoms (Wetter et al. 2001, Levy et al. 2002),

suggesting that the abnormalities of EEG are more marked with more severe dementia. In addition, One study also showed that the decrease in both beta and alpha power in the parieto-occipital region are good predictors of mortality (Claus et al. 1998). On the other hand, the EEG findings may vary a lot between individuals, and therefore compromise its applicability as a diagnostic tool, especially in detecting cases with mild pathology from age-matched controls (Coben et al. 1985, Penttila et al. 1985). Abnormal EEG slowing in AD has been most consistently related to the cholinergic deficit (Bondareff et al. 1982, Riekkinen et al. 1991a, Soininen et al. 1992). However, controversies still exist as to the neurochemical underpinnings of EEG alterations in AD patients because a variety of neurotransmitter systems are affected (McCormick 1989, Rice et al. 1990, Soininen et al. 1991). Furthermore, little is known about the contribution of amyloid pathology on EEG changes in AD.

EPs Evoked potentials, first conceived by Dawson (1954), have been used for decades to investigate brain dynamics and also neurological dysfunction. These have been called either "evoked potentials" - emphasizing their passive character as an electrophysiological response to exogenous physical stimuli of auditory, somatosensory or visual modality - or "event-related-potentials" (ERPs) - emphasizing the endogenous processing of stimuli. This review uses the broader term ERP without making any special implications about their exogenous or endogenous nature.

To date, most ERP studies in AD patients have utilized long-latency potentials such as P300 (or P3), which is a positive deflection in EEG with a latency around 300ms after stimulus presentation. It is recorded in the context of so called oddball paradigm, in which the subject has to indicate, e.g. by pressing a button, the detection of infrequent deviant target stimuli, which are embedded in a sequence of frequent standard tones, either visual or auditory (Pfefferbaum et al. 1990). The P300 component is known to index discrimination and evaluation of stimulus, and it has been considered to be a measure of neural activity underlying attentional processes and immediate memory. P300 has been used to measure cognitive function (Polich and Herbst 2000). Many reports have shown that delayed latency of P300 wave in patients with various dementias (Brown et al. 1982, Polich et al. 1986, Polich et al. 1990, O'Donnell et al. 1992), especially with AD, discriminates the patients from normal subjects (Goodin et al. 1978, Polich et al. 1990, Holt et al. 1995). Unfortunately, because of individual variation, P300 based discrimination works at the group level, but is not reliable at the individual level.

Another frequently recorded ERP potential in the context of oddball tasks is the mismatch negativity (MMN) occurring about 100-200 ms after the stimulus onset. The MMN is elicited by physically deviant auditory stimuli occurring among frequent ("standard") stimuli and reflects memory-based comparison process of incoming stimulus to existing stimulus trace (Näätänen 1992, Näätänen and Alho 1995). With MMN, it is possible to study auditory sensory memory also. To elicit MMN for the deviant tone, memory trace of the proceeding standard tone must exist in the auditory system. When the stimulus interval is increased, MMN to the deviant tones will attenuate and finally disappear, indicating that memory trace has decayed. EEG studies using different stimulus intervals have found that memory trace exists up to 10s in the auditory system, while aging is accompanied with a faster decay of the memory trace. MMN can even be elicited by stimuli that fall outside the focus of attention, and hence MMN is considered to be an automatic, pre-attentive response to stimuli deviance. MMN has been widely used in studies of early development, aging, frontal-lobe lesions, Alzheimer and Parkinson's disease, perceptual capabilities, and learning (Näätänen and Alho 1995). AD is characterized by deficits in cognition, and attention is one of the first cognitive functions to deteriorate in AD (Parasuraman and Nestor 1993). Attention deficit may be coincident with or follow the memory impairment in AD. There are some studies about the MMN in AD patients. Pekkonen et al (1994) found that MMN to frequency deviance did not differ between AD patiens and age-matched controls with 1 s interstimulus intervals (ISI), but after prolonging the ISI to 3 s hardly any MMN was elicited in the AD patients, while the controls still demonstrated a clear MMN. The attenuated MMN with longer ISIs in AD patiens is likely to be caused by faster decay of the memory trace. In this regard, it is noteworthy that MMN to frequency change was reduced in normal ageing, when a 4.5 s ISI was used, but not with 1.5 s ISI (Pekkonen 1996). In addition, scopolamine has been shown to reduce MMNm (magnetic MMN corresponding to electic MMN) to amplitude in response to a change in frequency, but not in duration (Pekkonen 2001). Yokoyama et al. (1995) measured MMN to frequency change in AD patients, patiens with vascular dementia and age-matched healty controls. The MMN amplitude did not differ between the groups, but MMN latency was prolonged in the AD group. On the other hand, Gaeta et al (1999) reported that patients with mild to moderate AD showed no MMN changes compared with age-matched controls.

Lately, also other components of ERPs, such as P50 (Boutros et al. 1995b, Jessen et al. 2001), N100 (Boutros et al. 1995a, Yokoyama 1995, Soininen et al. 1995c), P200 (Yokoyama 1995, Swanwick et al. 1996, Tachibana et al. 1996) and P400 (Ford et al. 1996, Tachibana et al. 1996) have been studied, and delayed latency, increased amplitude and depressed habituation in AD

patients have been observed. Taken together, these findings suggest that ERPs may be useful, at least at the group level, indices to evaluate cognitive function (Polich et al. 2000), to discriminate dementias from depression-associated pseudo-dementia (Brown et al. 1982), to distinguish between subcortical (e.g. Parkinson's disease) and cortical (e.g. AD) dementias (Rosenberg et al. 1985, Goodin and Aminoff 1986), and to study psychiatric disorders (Bruder et al. 1995, Boutros et al. 1997), if appropriate task conditions are employed. The obvious advantage of ERPs as an additional diagnostic tool is that they are free of risk for the patient and equipments are relatively inexpensive. On the other hand, large inter-subject variability is a common problem with these measures and substantially reduces the sensitivity and specificity of the method, especially in detecting early changes in the course of AD.

Sensory processing and sensory gating are the basic neurobiological function by which the brain controls responsiveness to sensory stimuli. Normal human subjects have diminished evoked responses to repeated auditory stimuli (Davis et al. 1966), such as P50 and N100. Laboratory rodents exhibit gating of auditory evoked responses to the N40 component similar to that seen to P50 component in normal human subjects (Knight et al. 1985, Adler et al. 1986). Similarity between the habituating mid-latency ERP components in rodents and humans offers a useful model of sensory gating to delineate the neuronal mechanisms that underlie this brain function (Adler et al. 1986, Adler et al. 1988). However, it is worth noticing when interpreting the results of sensory gating from animal models that some differences exist between the rodent and human responses (Bickford-Wimer et al. 1990). First, due to the difference in brain size, the latency of the mid-latency resonse differs between the species, such that the N35 in mice corresponds to N40 in rats and P50 in humans. Second, the human scalp ERPs is dominated by N100 and P300, whereas the largest amplitude in mouse ERP is that of N35. Third, the generators of ERPs have a different location. The human electric P50 is assumed to consist of several subcomponents, some originating from the auditory cortex, others being subcortical (Ninomiya et al. 1997), whereas the magnetic P50 is supposed to originate mainly in the auditory cortex (Mäkelä et al. 1994). In contrast the rodent N40 (N35) is reported to be generated in both the hippocampus and auditory cortex (Bickford-Wimer et al. 1990).

Cholinergic symptomatic treatment The symptomatic treatment of AD is currently based on the cholinergic hypothesis, which states that many of the cognitive, functional and behavioural symptoms derive from the reduction in brain acetylcholine activity secondary to the loss of cholinergic neurons in the nucleus basalis of Meynert and other cholinergic nuclei projecting to the

hippocampus and mesial cortical regions (Cummings and Masterman 1998, Geula 1998). These symptomatic AD drugs target the cholinergic neurotransmission at several levels (Figure 2). Manipulation at presynaptic levels have had only limited success, although attempts have been made to augment ACh synthesis with precursor loading using choline, lecithin (Etienne et al. 1981, Calvani et al. 1992), as well as ACh-releasing compounds such as linopirdine (Rockwood et al. 1997). Similarly, drugs selectively stimulating muscarinic and nicotinic receptors at the pre- and post-synaptically have not been able to demonstrate converging evidence of efficacy (Gauthier 2001). Currently, only acetylcholinesterase inhibitors (AChIs), which prolong the action of ACh at the postsynaptic receptors by preventing its hydrolysis, are at clinical use for AD (Gauthier 2001). AChIs show some common features: measurable short-term (6 months) improvement in cognition and global functions, the measurable benefit on activities of daily living (ADL), and improvement of some neuropsychiatric symptoms. Age, gender, and apoE genotype do not seem to determine the response to therapy, nor does the disease stage within the mild to moderate stage (Gauthier 2001). Tacrine was the first AChIs that was approved for clinical use. Because of liver-toxicity, it has practically been replaced by second generation AChIs of which three are currently in clinical use. Donezepil and rivastigmine are pure AChIs, whereas galantamine has intrinsic nicotinic activity as well (Pontecorvo 1998).
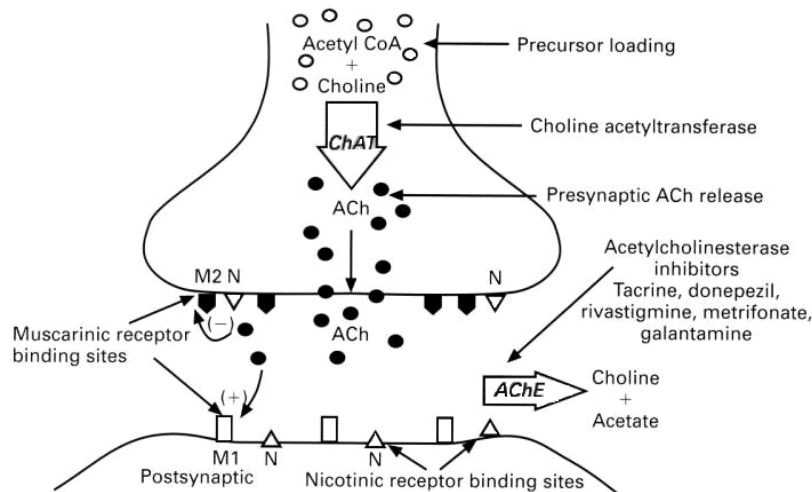
Figure 2. A schematic drawing of the cholinergic synapse and the possible sites for pharmacological interventions. Adapted from Feldman H and Grundman M, 2001.

2.2. β–AMYLOID PEPTIDE

The amyloid fibrils present in senile plaques and cerebral vessel walls are composed of 39-43 amino acid long Aβ peptide with molecular weight of around 4kDa. Aβ peptide is a cleavage product of a larger amyloid precursor protein (APP).

2.2.1. APP processing

APP gene contains 18 exons, and represents a family of at least eight different transmembrane isoforms, which arise by alternative splicing of exons 7, 8 and 15. The APP isoforms expressed mainly by neurons (APP isoforms 695, 714 and 770) contain exon 15, are more amyloidogenic and release much more $A\beta40-42$ peptides than the non-neuronal (APP isoforms 677, 693, 733 and 752) APP isoforms (Hartmann et al. 1996, Sandbrink et al. 1996).
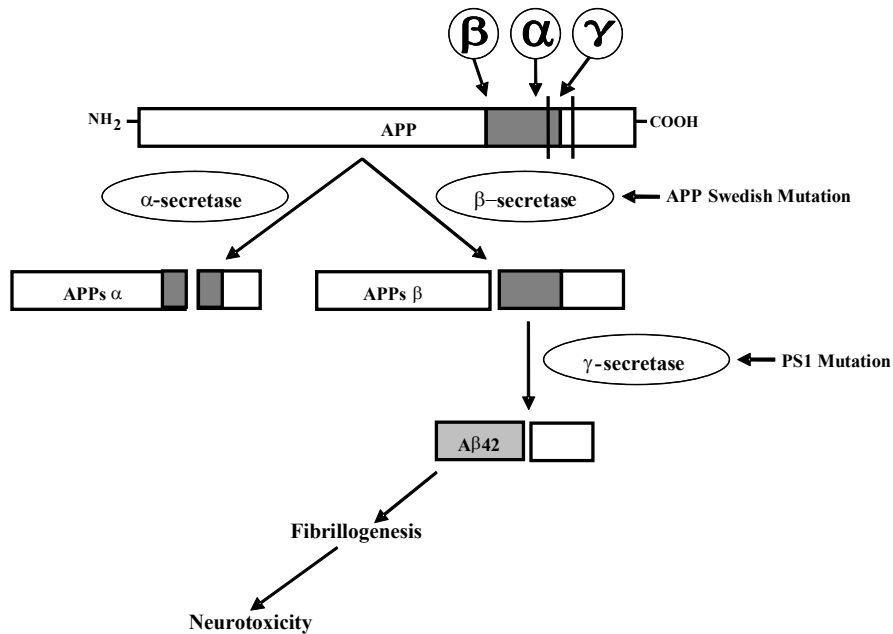
Figure 3. A schematic figure depicting the endoproteolytic events and cleavage products that are generated during the processing of APP. The interaction sites of APPswe and PS1 mutation have also been marked.

APP is processed by at least two pathways in all cells (Hardy 1997). The cleavage site of α-pathway (called the α-secretase process) is within the $A\beta40-42$ domain between residues 687 and 688 of the APP. Thus, after cleavage of another enzyme, γ-secretase, it does not yield $A\beta40-42$ peptides. The cleavage site of the β-pathway (called β-secretase process) is between residues 671 and 672 of the APP, and it yields $A\beta40-42$ after γ-secretase cleavage. The γ-pathway (called γ-secretase process) involves a cleavage in the vicinity of residues 712 of the C-terminus. In the β-pathway, the cleavage site of γ-secretase is of key importance. A short $A\beta40$ peptide results from a cleavage site at residue 712-713, and if the cleavage site is situated at the residue 714, long $A\beta42$ peptide is produced. Thus, the γ-pathway is fundamental to the production of the longer $A\beta42$ peptide (Sisodia and St George-Hyslop 2002).

2.2.2. Aβ40 and Aβ42

It is now clear that Aβ peptide is a normal product of cells (Haass et al. 1992, Shoji et al. 1992, Lorenzo and Yankner 1994), and around 90% of secreted Aβ is Aβ40 which is fairly innocuous, soluble form of the peptide. The remaining secreted Aβ peptides are Aβ42(43) (some antibodies used do not differentiate between Aβ40 and Aβ42). Many studies indicate that the aggregation of Aβ42 may be the critical event in the pathogenesis of AD (Younkin 1995, Borchelt et al. 1996). First, Aβ42 contains two more hydrophobic amino acid residues when compared to Aβ40. Thus, it aggregates, forms oligomers more rapidly and is more fibrillogenic than Aβ40 (Burdick et al. 1992, Jarrett et al. 1993, Jarrett and Lansbury 1993). Second, several FAD-linked mutations in APP alter the processing of APP in cultured cells, leading to increased levels of Aβ42 in culture medium (Citron et al. 1992, Cai et al. 1993, Suzuki et al. 1994). Third, the initial extracellular Aβ deposits are composed mainly of non-filamentous Aβ42 (Roher et al. 1993, Iwatsubo et al. 1994, Kuo et al. 1996). Fourth, immunocytochemical and biochemical studies have documented early and selective deposition of Aβ42 in brains of AD (Iwatsubo et al. 1994, Gravina et al. 1995). Aβ peptides are generally released into the extracellular fluid, but recent studies have shown that Aβ42 also accumulates inside the cells (Hartmann et al. 1997, Tienari et al. 1997, Wild-Bode et al. 1997, Gouras et al. 2000). In the mature plaques, the neuritic plaques contain both aggregated Aβ40 and Aβ42 (Iwatsubo et al. 1994).

2.2.3.   Amyloid cascade hypothesis of AD

The original amyloid cascade hypothesis proposed that amyloid plaque depositions or partially aggregated soluble Aβ trigger a neurotoxic cascade, thereby causing neurodegeneration and finally pathology of AD (Selkoe 1996, Hardy 1997). This hypothesis is based on *in vitro* studies showing that Aβ is toxic to neurons (Yankner 1989, Yankner et al. 1990, Pike et al. 1991, Howlett et al. 1995), and on the measurements of increased release of Aβ by cells carrying FAD mutant genes (Selkoe 1996, Hardy 1997). The basic framework of the amyloid cascade hypothesis has been stated as: "Aβ precipitates to form amyloid, which in turn, causes neurofibrillary tangles and cell death" (Hardy and Higgins 1992).

Missense mutations in APP, PS1 and PS2
⬇
Altered proteolysis of APP
⬇
Increased production of Aβ1-42 and /or total Aβ
⬇
Progressive aggregation and insolubilization of Aβ1-42 in brain interstitial fluid
⬇
Deposition of aggregated Aβ1-42 as diffuse plaques
⬇
Aggregation of Aβ1-40 onto diffuse Aβ1-42 plaques
⬇
"Inflammatory" response:
- microglial activation and cytokine release
- astrocytosis and acute phase protein release
⬇
Progressive neuritic injury within amyloid plaques and elsewhere in the neuropil
⬇
Disruption of neuronal metabolic and ionic homeostasis; Oxidative injury
⬇
Altered kinase/phosphatase activities ➡ Hyperphosphorylated tau ➡ PHF formation
⬇
Widespread neuronal/neuritic dysfunction and cell death in hippocampus and cerebral cortex with progressive neurotransmitter deficits
⬇
DEMENTIA

Figure 4. A hypothetical amyloid cascade in the pathogenesis of familial forms of AD. Simplified from Selkoe 1999.

2.2.4. Aβ *In vitro* neurotoxicity
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Most of our information about the pathobiological activities of Aβ derives from its observed neurotoxicity in cell culture (Yankner 1989, Pike et al. 1991, Howlett et al. 1995), especially in its aggregated but not monomeric form (Pike et al. 1993, Lorenzo and Yankner 1994). This toxicity is enhanced if the Aβ peptides are "aged" (incubated from hours to days), which increases amyloid fibril formation (Pike et al. 1991). The mechanism of neurotoxicity is unclear (Small and Mclean. 1999). Some studies suggest that Aβ can disrupt calcium homeostasis (Mattson et al. 1992, Mattson et al. 1993), cause free radical generation (Hensley et al. 1994) and various forms of peroxidative injury (Behl et al. 1994), and activate microglia (Combs et al. 2001) which may release some unidentified neurotoxic agent(s).

Although the process of "aging" increases the number of amyloid fibrils formed from Aβ, this is not evidence *per se* that fibrils are the major toxic form of Aβ. Recent *in vivo* studies suggest that the levels of soluble oligomeric species of Aβ are also increased by the process of aging and

potentially underlie the neurotoxicity. Aβ is probably secreted as a monomer and subsequently aggregates into soluble oligomers or fibrils (Podlisny et al. 1998). There is evidence for the existence of low-molecular-weight Aβ oligomers in the brain. Kuo et al. (Kuo et al. 1996) have isolated water soluble Aβ oligomers from normal and AD brains, and in this study the interesting finding was that not only was the level of soluble Aβ greater in the AD brain when compared with controls, but as with some familial mutations, the proportion of soluble Aβ42/43 was significantly increased over soluble Aβ1–40 species in AD patients. Similar results have been obtained by Funato et al. (1998). Roher et al. (1996) suggest that the water soluble dimeric species are neurotoxic, whereas in a recent study, Lambert et al. (1998) found that small, low-molecular-weight oligomers of Aβ42 are several orders of magnitude more potent neurotoxins than high molecular-weight fibrillar species of Aβ40. Walsh et al. (2002) showed that naturally secreted oligomers of Aβ potently inhibit hippocampal long-term potentiation. These studies have implications for our view of amyloid toxicity. If oligomeric soluble forms of Aβ have a pathogenic role, then it is possible that amyloid plaques are not the only or major toxic form of Aβ in the brain.

2.2.5. Correlation between the number of plaques and cognition

There has been considerable interest over the last decade about the relationship between progressive cognitive decline and underlying neuropathology associated with AD. Despite considerable effort, the relative contributions of plaques, as well as some other markers of pathology to cognitive impairment remain unclear and controversial. Although some reports (Blessed et al. 1968, He et al. 1993, Dickson et al. 1995) showed significant correlations of plaque number with dementia, numerous studies have found only weak or no correlation between neocortical plaque densities and clinical severity measures (Wilcock and Esiri 1982, Neary et al. 1986, McKee et al. 1991, Terry et al. 1991, Arriagada et al. 1992, Berg et al. 1993, McKeel et al. 1993). The poor correlation between the number of amyloid plaques and cognitive impairment seems to present a serious challenge to the "amyloid cascade hypothesis". However, the problem of poor correlation between amyloid plaques and clinical features probably arises from several aspects (Cummings et al. 1996). First, few studies have included a full representative range of individuals from normal to mildly and severely demented, employed sensitive and specific detection system and controlled the time intervals between death and last neuropsychological evaluation, and between death and tissue fixation. Second, other neuropathological markers, such as the tangle formation may also contribute to dementia severity (McKee et al. 1991). Third, recent data

suggested that toxic forms of Aβ are not only aggregated fibrils (Yankner et al. 1990, Pike et al. 1993, Lorenzo and Yankner 1994), but relative soluble Aβ oligomers (Roher et al. 1996) as well.

2.2.6.   Transgenic models

A major recent advancement in understanding the disease mechanism(s), especially the correlation between cognitive decline and Aβ accumulation, was the generation of transgenic animals carrying mutated genes which are involved in certain familial forms of AD. (Games et al. 1995, Hsiao et al. 1996, Borchelt et al. 1997). These transgenic mice not only develop age-related Aβ accumulation which results in plaque formation in brain, but also are impaired in various learning and memory tasks (Hsiao et al. 1996, King et al. 1999, Chen et al. 2000, Dodart et al. 2000, Gordon et al. 2001). Mainly, there are two major transgenic mice types that mimic AD-like amyloid pathology. One type is mice carrying APP single mutated genes based on different promoters. Games et al. (1995) used PDGF β-promotor to drive the expression of human APP minigene that encoded the FAD-linked APP (V717F) mutation in an outbred strain (so-called PDAPP line). In this mouse, the levels of Aβ42 are slightly higher than those of Aβ40 (Suzuki et al. 1994). The brains of these mice showed diffuse Aβ depositions and plaques with tau-negative dystrophic neurites around Aβ cores (Masliah et al. 1996). Hsiao et al. (1996) used hamster PrP promoter that overexpressed human APP-695 with two points (K595N, M596L, based on 752 APP positions) mutations, the so-called APPswe mutation. In this so-called Tg2576 line, the brain levels of Aβ40 stay higher than those of Aβ42 at least up to 13 months of age (Hsiao et al. 1996). Immunohistology revealed dystrophic neurites and Aβ deposition in the amygdala, hippocampus and cortex. Similar result was found when mouse PrP promoter was used in human APPswe transgenic mice, but this mouse developed Aβ plaques as late as about 18~20 months of age (Borchelt et al. 1997). Another major type of transgenic mice mimicking AD-like amyloid pathology is one carrying APP and PS1 double transgenes. Borchelt et al. (1997) found that transgenic mice coexpressing human PS1-A246E and APP695swe had numerous amyloid depositions in the brain, many of which were associated with dystrophic neurites and reactive astrocytes. Interestingly, parallel analyses of brains from age-matched mice that expressed APP695swe alone or human PS1-A246E alone were free of Aβ deposition (Borchelt et al. 1997), convincingly suggesting that human PS1-A246E acts synergistically with APP695swe to accelerate the rate of Aβ deposition, most likely by increasing the relative amount of the more amyloidogenic Aβ42 peptide (Borchelt et al. 1997). In conclusion, these transgenic models are excellent model

systems in studying the pathological process and potential therapeutics in AD (Price et al. 1995, Borchelt et al. 1997).

2.2.7. Correlation between Aβ accumulation and cognitive impairment in transgenic mice

Although transgenic mice develop AD-like amyloid pathology and are impaired in cognitive tasks (Hsiao et al. 1996, Chen et al. 2000, Gordon et al. 2001), it is still not clear whether these cognitive impairments are age-dependent, or whether there is a correlation between Aβ deposition and cognitive impairment. Hsiao et al. (1996) reported an age-related increase in the total levels of Aβ and number of amyloid plaques, as well as an age-associated impairment in water maze learning in APPswe mice. However, they did not find a correlation between hippocampal pathology and the behavioral deficit at an individual mouse level. Furthermore, a later study with the same mouse line could not confirm the age-related water maze impairment (King et al. 1999). Nalbantoglu et al. (1997) reported extracellular Aβ deposition and impaired water maze learning in transgenic mice expressing the carboxy terminus of APP, but they included only one age group in the study. Recently, Chen et al. (2000) reported an age-related impairment in PDAPP mice in a modified version of the water maze task and also a correlation between hippocampal Aβ load and impaired water maze learning. However, the interpretation of their finding is complicated by marked hippocampal atrophy that occur already at an early age in this mouse line (Dodart et al. 2000), which is likely to account for their age-independent impairment of performance in the traditional water maze. More recently, Gordon et al. (2001) reported that the double transgenic APPswe+PS1(M146L) mice at the age of 15-17 months are cognitively impaired in the radial arm version of the water maze task (Gordon et al. 2001), and that this impairment is inversely related to the amount of Aβ deposited in the frontal cortex and hippocampus. However, in an earlier study by the same group, the same mice were found to be impaired in the rate of acquisition but not in the retention of the traditional water maze (Arendash et al. 2001). In addition, because of extensive amyloid depositions in all cortical areas and the striatum in these mice, the contribution of hippocampal pathology to the behavioral impairment is difficult to address (Gordon et al. 2001).

In conclusion, more and more evidence support the hypothesis that Aβ accumulation is the underlying cause of familial and sporadic AD. However, more work is needed to define the precise nature of the toxic form of Aβ.

2.3. NICOTINIC ACh RECEPTORS (nAChRs)

Nicotinic cholinergic receptors (nAChRs) belong to the superfamily of ligand-gated ion channels that includes glutamatergic N-methyl-D-aspartate (NMDA) and α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA), γ-aminobutyric acid A (GABA$_A$), glycine, and serotonin 3 (5-HT$_3$) receptors (Albuquerque et al. 1997, Jones et al. 1999, Dani et al. 2000). The nAChRs subtypes can be separated into three general functional classes that are consistent with their evolutionary development, and their pharmacological and physiological properties: muscle subunits (α1, β1, δ, ε, γ), standard neuronal subunits (α2-α6 and β2-β4) that form nAChRs in different αβ combinations, and subunits (α7-α9) capable of forming homomeric nAChRs that are inhibited by α-bungarotoxin (McGehee et al. 1995, Colquhoun and Patrick 1997). Of the receptor of the third class, only α7 (but not α8 or α9) is widely distributed in the mammalian central nervous system. In the neocortex the predominant subtype is α4β2. The hippocampus, on the other hand, is rich in α7 subunits (Wonnacott 1997). The nAChRs are distributed at presynaptic, postsynaptic and somatic sites (Wonnacott 1997). In the hippocampus, presynaptic activation of nAChRs is thought to dominate over the postsynaptic activation, which enhances neurotransmitters release at excitatory, inhibitory and modulatory synapses (Vidal 1996, Wonnacott 1997, Fu et al. 1999, Radcliffe et al. 1999).

2.3.1. Cholinergic hypothesis of AD

Cholinergic neuronal loss in the basal forebrain was recognized as a hallmark of dementia in 1980s (Bartus et al. 1982, Coyle et al. 1983). The cholinergic hypothesis of AD was first described by Bartus et al (1982) and Coyle et al (1983), and stated as follows. First, cholinergic neurons in the basal forebrain are severely affected in the course of the disease, detectable histopathologically as a loss of neurons and neurochemically as a loss of marker enzymes for acetylcholine synthesis and degradation. Second, the resulting cerebral cholinergic deficit leads to memory loss and other cognitive and non-cognitive symptoms, which are characteristic for the illness (Frolich 2002). The hypothesis is supported by several lines of evidence. First, the loss of cortical cholinergic markers and degeneration of basal cholinergic neurons are the most consistent and severe biochemical deficits in AD (Geula and Mesulam 1996, 1999). Second, pharmacological studies have demonstrated that drugs blocking central cholinergic activity can produce a dementia-like syndrome, with concomitant memory loss (Davis et al. 1985, Blokland 1995, Ebert and Kirch 1998), and similarly, cognitive impairments were reversed by AChE inhibitors in animals with a medial septum lesion, which results into cholinergic deafferentation of the hippocampus (Riekkinen et al. 1991b, Ikonen and Riekkinen 1999). Third, treatment of AD patients with AChE inhibitors (e.g. tacrine,

donepezil, rivastagmine, galanthamine) improves cognitive function and decrease neuropsychiatric problems (Francis et al. 1999, Perry et al. 1999, Gauthier 2001). However, although cholinergic hypothesis has the basis of numerous pathophysiological studies, and also forms the basis of current drug treatment of AD, the relevance of these changes for the pathophysiological disease process in AD is not totally resolved. Also the relationship between cholinergic deficits and other pathology in AD, such as amyloid plaques, and NFTs still needs to be elucidated.

2.3.2. Nicotinic ACh receptors (nAChRs) in AD

A loss of nicotinic binding sites, especially in the cortex and hippocampus has been reported in patients with AD (Nordberg et al. 1988, Sugaya et al. 1990, Rinne et al. 1991, Nordberg 1993, Newhouse et al. 1997, Shimohama and Kihara 2001). Martin-Ruiz et al. (1999) studied AD brain tissues after autopsy and found a significant reduction in α4 receptors subunit but not α7 or α3 ones as compared with age-matched samples. Some other reports have documented a great loss of α7 subunits in the midtemporal gyrus (but not in the frontal cortex and hippocampus) in AD brain (Davies and Feisullin 1981, Sugaya et al. 1990, Warpman and Nordberg 1995). Also a significant reduction in α3 and α4 subunit binding has been reported in FAD brain with APPswe mutation (Marutle et al. 1999). Moreover, more recent studies documented significant reduction in α3 and α4 subunits in the temporal cortex, and α7 subunit in the hippocampus in postmortem AD brains (Marutle et al. 1999, Guan et al. 2000). Burghaus et al. (2000) also reported that both α4 and α7 nAChRs were significantly decreased in postmortem AD brains. These studies support the notion that decrease in nAChRs may contribute to the pathogenesis or symptoms of AD.

The role of nAChRs in cognitive function and development is well documented (Levin 1992, Granon et al. 1995, Meyer et al. 1998). The potential therapeutic benefit of nAChRs stimulation in AD is based on the fact that nicotine improves memory in animals, healthy subjects and AD patients (Newhouse et al. 1997, Levin and Rezvani 2000, Newhouse and Kelton 2000). Some studies have demonstrated that nicotine administration via injection or skin patches has been shown to improve attention (Jones et al. 1992, White and Levin 1999), learning (White and Levin 1999), and memory (Newhouse et al. 1988, Wilson et al. 1995, Parks et al. 1996) in patients with AD.

2.3.3. Interaction between Aβ and nAChRs

A very interesting issue is whether there is a relationship between the Aβ accumulation and cholinergic neuronal loss. Recently, several laboratories have documented an interaction between Aβ and the cholinergic neurotransmission (Cheung et al. 1993, Monteggia et al. 1994, Itoh et al. 1996, Kihara et al. 1997, Kim et al. 1997, Zamani et al. 1997, Kihara et al. 1998). The interaction between Aβ and nAChRs includes two aspects. On the one hand, Aβ binds selectively and with picomolar affinity to α7 nAChRs (Wang et al. 2000a, 2000b), directly modulates nAChRs (Pettit et al. 2001), blocks the response of α7 nAChRs (Liu et al. 2001), or activates mitogen-activated protein kinase (MAPK) cascade via hippocampal α7 nAChRs (Dineley et al. 2001). On the other hand, nicotine and nAChRs act against Aβ toxicity (Zamani et al. 1997) by inhibiting Aβ aggregation (Zeng et al. 2001), upregulating Bcl-2 and Bcl-x proteins (Shimohama et al. 2001), or inducing production of soluble APP (Kim et al. 1997). The neuroprotective effect of nicotine may be receptor subtype specific. Accordingly, specific nicotinic receptor agonists for α4β2 or α7 subunit have shown a dose-dependent neuroprotective effect (Kihara et al. 1998, Meyer et al. 1998). Interestingly, the muscarinic system appears not to be involved in neuroprotection, since the application of muscarine itself did not offer any protection against Aβ toxicity (Kihara et al. 1997).

The relationship between Aβ accumulation and nAChRs has a broad biological importance. For example, the molecular mechanism(s) of how Aβ peptide forms insoluble fibrils is largely unknown, but nicotine probably is involved in this process by binding to small-sized soluble Aβ-sheets to inhibit Aβ aggregation (Zeng et al. 2001), implicating a potential therapeutic approach for treatment of AD.

2.3.4. The role of α7nAChR in auditory gating

An important characteristic of hippocampal pyramidal cells is their rapid habituation, or sensory gating of a response to repeated sensory stimulation. One method to demonstrate such sensory gating is to record auditory evoked responses using a paired stimulation or conditioning-testing paradigm (Bickford-Wimer et al. 1990). Pharmacological studies have provided evidence that cholinergic system serves a critical role in auditory sensory processing (Luntz-Leybman et al. 1992). More specifically, evidence supports the involvement of the α7 nAChRs in the mechanisms of habituation (Luntz-Leybman et al. 1992). Bickford and Wear (1995) reported that nicotine administration to rats with fimbria-fornix lesion (which deprived the hippocampus of its ACh innervation) was able to reinstate the normal suppression of the second auditory evoked response to

a paired stimulus, although the fimbria-fornix lesion is not specific for nicotinic cholinergic system. Furthermore, a more recent study showed that after fimbria-fornix lesion in rats, 3-(2,4-dimethoxybenzylidene) anabaseine (DMXB-A), an agonist of $\alpha 7$ nAChRs, was able to restore the auditory gating, indicating that activation of the $\alpha 7$ nAChRs alone is sufficient to restore the sensory gating after fimbria-fornix lesion (Adams et al. 2000). Also Stevens et al. (1998) reported that in the inbred DBA mouse strain, with defective sensory gating and reduction in the number of hippocampal α-bungarotoxin-sensitive nicotinic receptors (containing $\alpha 7$ subunit), GTS-21, a selective $\alpha 7$ nAChRs agonist, was able to produce almost normal sensory gating.

3. AIMS OF THE STUDY

The exact role of β-amyloid accumulation in the disease progression is one of the central questions in AD research. Based on the amyloid cascade hypothesis of AD, this study was designed to assess the role of Aβ-peptide accumulation in memory deficit, changes in EEG and evoked potentials and its interaction with nicotinic cholinergic receptors in A/P transgenic mice.

The specific aims of present study were:

1) To evaluate whether amyloid pathology is more severe in female than male transgenic mice.

2) To determine whether Aβ accumulation underlies the spatial memory impairment in A/P mice. More specifically, we addressed the following questions:
Is the spatial memory impairment age-dependent?
Is the spatial memory specific to tasks that are dependent on the hippocampus?
What is the correlation between plaque count and memory impairment?

3) To test whether Aβ accumulation in A/P mice is related to EEG and ERP alterations, and to further assess whether those alterations are genotype-dependent and correlate with the number of plaques in the brain.

4) To assess whether accumulation of Aβ is related to changes in the number of nAChRs subtypes.

4. MATERIALS AND METHODS

4.1. ANIMALS

Transgenic mice expressing either human PS1 protein harboring familial AD-linked A246E mutation or chimeric mouse/human APP695 harboring a human Aβ domain and mutations (K595N, M596L, 752 based) linked to Swedish familial AD pedigrees (APPswe) (Borchelt et al., 1997) were back-crossed to C57BL/6J strain for 6 generations and then mated to generate double transgenic mice coexpressing both transgenes (A/P mice). Male A/P double transgenic mice and nontransgenic littermates (control mice) were used for this study. The housing conditions (National Animal Center, Kuopio, Finland) were controlled (temperature +21°C, light from 7:00 A.M to 7:00 P.M; humidity 50-60%), and food and water were freely available. The experiments were conducted according to the Council of Europe (Directive 86/609) and Finnish guidelines, and approved by the State Provincial Office of Eastern Finland.

4.2. BEHAVIOURAL TESTS

4.2.1. Water maze

Before the actual water maze testing took place, the mice were given two days of pretraining in a 1 m x 14 cm x 25 cm (length x width x height) alley with a black rubber-coated platform (14 x 14 cm), located 1 cm below the water surface. This gave them experience in climbing onto the platform from the water. The mice were allowed to swim until they found the platform or for a maximum of 20 s, after which they were placed on the platform for 10 s. This was repeated four times on both pre-training days.

We used a black plastic circular pool, diameter 120 cm, and a black painted stainless steel square platform; 14 x 14 cm, 1.0 cm below the water surface. The pool was divided into three annuli of equal surface area, and the escape platform was in the middle annulus. The starting locations, which were labelled North, South, East and West, were located on the pool rim. The timing of the latency to find the submerged platform was started and ended by the experimenter. A computer connected to an image analyzer (HVS Image, Hampton, UK) monitored the swim pattern. Mice were placed in the water with their nose pointing towards the wall at one of the starting points in a random manner. If the mouse failed to find the platform, it was placed there by the

experimenter. Mice were allowed to stay on the platform for 15 s. A 10 min recovery period was allowed between the training trials. The temperature of the water was kept constant throughout the experiment ($20 \pm 0.5^{\circ}$C).

The training schedule consisted of 7 consecutive days of testing. During the first 5 days of testing, the mice were trained with a hidden platform for five 60 s trials per day. The platform location was kept constant during this period of training. On the sixth day, the platform was removed and the mice were allowed to swim for 50s. After the probe trial, the mice were trained two days (days 6 and 7) with a visible platform for five 60 s trials per day to control for possible visual impairment. During visible platform training, the platform location was changed after each trial. During the platform training trials, the swimming length to find the platform was measured. During the spatial probe trial, the time that the mouse spent in the vicinity (within a radius of 12 cm from the former platform center) of previous platform position was measured.
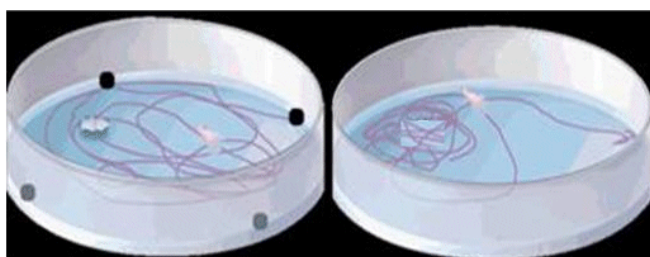
Figure 5. A schematic drawing of water maze task. **Left:** The swim path of the mouse during one of the early trials with the hidden platform. The four starting locations are indicated by black dots. **Right:** The probe trial with the platform removed. The mouse swims most of the time near the former platform location.

4.2.2. T-maze

After the water maze task the animals were tested in the T-maze. The T-maze consisted of a stem (38 cm x 7 cm) and two arms (35 cm x 7 cm). A sliding door separated the first 11 cm of the stem as the starting compartment, and a door at the each arm separated the arm from the stem 8.5 cm from the intersection. The walls were painted black and were 14 cm high to encourage the use of an egocentric response strategy. The source of illumination was an incandescent light located above the stem of the maze. The mice were first familiarized to the T-maze for two days by letting them freely explore it for 10 min until they repeatedly ate the rewards (rice cereals) at both arms of the maze. After two days of pretraining, testing was conducted as follows. On the initial trial of the first testing day both arms of the maze were rewarded to test the spontaneous turning preference of the animal. After this trial, the arm that the mouse had not spontaneously selected was rewarded for 15 consecutive trials. A 1-min inter-trial interval was introduced between the trials, during which time the animal was confined in the starting compartment. At the beginning of each trial, the animal

was given 5 s to leave the starting compartment. If this did not happen, the mouse was encouraged to start by gently pushing it with a paintbrush. The rewarded arm remained the same for all four days of testing. The number of correct choices was recorded. Our pilot studies indicated that fimbria-fornix lesion does not affect performance in this task, so we used it as a control spatial task, which should be independent of hippocampal functioning.

| Trial 0 | Trial 1 (C) | Trial 2 (E) | Trial 3 (C) |

Figure 6. A schematic drawing of the T-maze task with examples of correct and erroneous choices (E=error, C=correct choice.

4.3. ELECTROPHYSIOLOGICAL RECORDINGS

4.3.1. Experimental design

This electrophysiological study consisted of two experiments. In Experiment 1, we followed A/P double transgenic and non-transgenic (NT) control mice from 7 to 11 months of age by recording the cortical and hippocampal EEG and auditory ERPs once a month. At the end of the study, the mice were sacrificed, and samples were taken for histological and biochemical analysis. In Experiment 2, cortical and hippocampal EEG and auditory ERPs were recorded three times in two weeks at the age of 8 months in four groups of mice: NT, APP single mutant, PS1 single mutant and A/P double mutant mice. The single mutant mice were sacrificed at the age of 12 months, and samples were taken for histological and biochemical analysis. A separate group of 8-month-old A/P mice was used to evaluate age-related progression of amyloid pathology.

4.3.2. Surgery of electrodes implantation

Hippocampal electrodes (two stainless steel wires, 100 μm in diameter, tip separation 500 μm) were implanted in the left hemisphere at AP: -2.2 mm (from bregma), ML: 1.5 mm (from the midline) and DV: -1.5 mm (from the dura). The longer electrode was aimed at the hippocampal fissure and the shorter at the stratum oriens of CA1. One stainless steel screw was implanted above the posterior cortex at AP: -5.0 mm (from bregma), ML: -1.3 mm (from the midline). One stainless steel screw above the frontal cortex served as a ground electrode. The electrodes were fixed to the skull with dental cement and two anchor screws (Figure 7). The mice were deeply anaesthetized

with a mixture of pentobarbital and chloral hydrate (each 40 mg/kg i.p), and received 0.1 mg/kg of buprenorphine s.c. (Temgesic; Reckitt & Colman) for post-operative analgesia. The mice were allowed to recover for two weeks before the start of the first experiments.
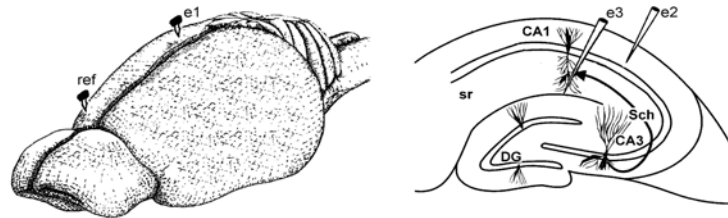
Figure 7. A schematic drawing of location of electrodes

4.3.3. EEG recording

All EEG recordings took place during the daytime. The hippocampal EEG was recorded between the long and short hippocampal electrodes and the cortical EEG between the posterior and frontal cortical screw electrodes. The signal from the recording electrodes was first passed through a source follower mounted on distal end of the recording cable to lower the electrode impedance, amplified 1000-5000 times, and bandpass filtered between 0.1-100 Hz. In addition, a 50 Hz notch filter was applied. The amplified signal was digitized at 2000 kHz with a Data Translation A/D board and was recorded directly onto a PC hard drive by Experimenter's Workbench software (DataWave Technologies, CO, USA). The EEG data was collected in 4-s segments during alert immobility. The data sampling was continuously monitored for movement artifacts, and the recording of each segment was manually verified by the experimenter, blind to the genotype of the animals. All segments with the mouse moving or the amplified EEG amplitude crossing the maximum of $\pm$ 1.25 V were discarded. Eight artifact free segments of 4s were analyzed with Fast Fourier Transformation over 1-40 Hz and averaged to yield power spectra. For cortical EEG, we further analyzed the power for selected frequency bands based on observed peaks on the wide frequency spectrums as follows: maximum theta at 4-6 Hz, beta 14-23 (27) Hz and gamma 28 – 40 Hz (Figure 8A). For hippocampal EEG, dominated by robust theta peak (Figure 8B), we analyzed the maximum power between 5-11 Hz, the frequency at maximum theta power, and the total power over 1-40 Hz. To compare the frequency spectra between A/P mice and their nontransgenic controls (NT), we calculated a difference score for each 1 Hz bin as follows:

$$d\_Power = \frac{Power\ (A/P) - Power\ (NT)}{Power\ (NT)} \times 100\ \%.$$

A. Cortex
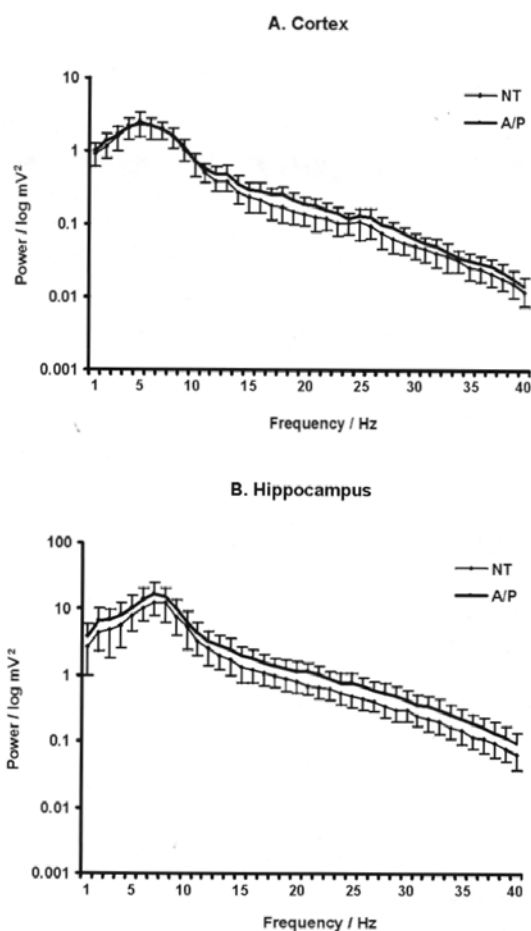
B. Hippocampus

Figure 8. The average cortical EEG power spectrum during rest (A) and hippocampal power spectrum during movement (B).

4.3.4. Auditory ERP recording

All the ERP recordings were performed in daytime during alert immobility. Cortical ERPs were recorded between the posterior and frontal cortical screw electrodes and the hippocampal ERPs were recorded between the long and short intrahippocampal electrodes. The frontal screw also served as the ground electrode. A dual-channel JFET on the headstage acted as a source follower. The auditory stimuli in the gating experiment consisted of 3.3kHz, 80dB (SPL) tones of 10 ms duration generated by a pietzo buzzer. Tones were presented in pairs with an inter-tone interval of 500 ms and the inter-trial interval as 10s. In Experiment 2, only cortical responses were recorded. In this experiment, we also looked at earlier subcortical responses with single tones of 2 ms duration presented with an inter-trial interval of 2s. The signal from the recording electrode was first passed through a source follower mounted on distal end of the recording cable to lower the electrode impedance, the signal was amplified 1000-4000 times and filtered 0.1–1000 Hz. The amplified signal was digitised at 2000 kHz by a Data Translation A/D board. Thirty artifact free auditory ERP

were averaged and recorded directly into a PC hard drive by Experimenter's WorkBench (DataWave Technologies) computer program.

The ERP data were analysed off-line using the Common Processing utility of the DataWave program. The cortical N35 response amplitude was calculated as the difference between the positive deflection at 10-30 ms after the stimulus onset and the peak negative response at 30-40 ms. This method has been consistency used in earlier studies of the N40 wave in rats because it yields more reliable results that comparison to baseline (Bickford-Wimer et al, 1990). However, the waveform in the hippocampal recordings varied between animals (likely due to small differences in electrode locations) so we preferred to measure the hippocampal N35 from the pre-stimulus baseline (Fig. 9). Using the previousBecause the exact shape of the hippocampal response depended on the electrode location and varied between individuals, the N35 latency was not calculated for the hippocampal response. The N8 was identified in some animals as a sharp negative peak between 6-9 ms in the cortical response (Figure 9). Only the latency of N8 was determined. Habituation is the progressive decrease in a response with repetition of the stimulus (Näätänen 1992). The habituation (%) of the N35 response (auditory gating) was calculated as follows:

$$\text{Habituation} = \frac{V_{cond} - V_{test}}{V_{cond}} \times 100\%$$

(V_{cond} = response amplitude to the conditioning stimulus; V_{test} = response amplitude to the test stimulus).

Habituation was only calculated for those conditioning responses that exceeded the mean amplitude of the test response in the control group. The data of the two groups in Experiment 1 were compared using analysis of variance (ANOVA) with repeated measures and the data in Experiment 2 using oneway ANOVA followed by Least Significant Difference test post-hoc.
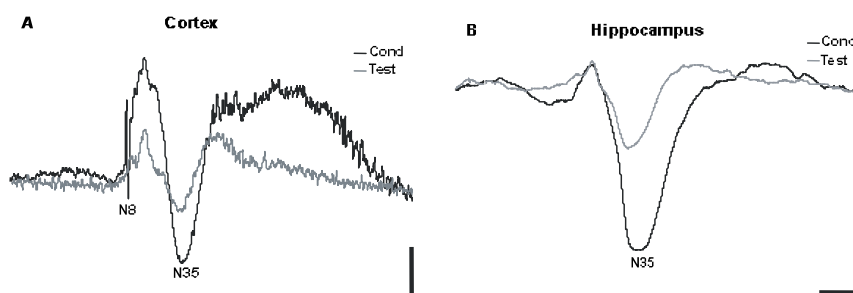
Figure 9. Examples of averaged auditory ERPs recorded from a nontransgenic control mouse. Cortical (A) and hippocampal (B) responses to the conditioning stimulus (black line) and to the test stimulus (grey line). N8 and N35 components are indicated by text.

4.4. BIOCHEMICAL DETECTIONS OF Aβ LEVELS

To analyse total Aβ, the hippocampus from one hemibrain was homogenized in guanidine buffer (5.0 M guanidine-HCl/50 mM Tris-HCl, pH 8.0) in proportion to their weight. The samples and Aβ-peptides used as standards were prepared to contain 0.5 M guanidine-0.5% BSA-1 mM AEBSF in the final composition. The levels of Aβ40 and Aβ42 were quantified using the Signal Select™ Beta Amyloid ELISA Kits (BioSource International Inc.) according to the manufacturer's protocol. The total Aβ40 and Aβ42 levels were standardized to brain tissue weight and expressed as ng (Aβ)/g (brain tissue).

To analyse soluble Aβ42, cortical brain samples were weighed and homogenised in phosphate buffered saline (PBS), pH 7.2, containing a mixture of protease inhibitors (Complete™, Boehringer Mannheim, Germany). Brain homogenates were centrifuged at 218 000 x g for 2 h at 4°C, and the supernatants were collected and stored at −70°C until the analysis. The levels of soluble Aβ42 in the supernatants were quantified using the Innotest β-amyloid(1-42) High Sensitivity Test –ELISA kit (Innogenetics, Belgium) according to the manufacturer's protocol. The soluble Aβ42 levels were standardized to brain tissue weight and expressed as ng (Aβ)/g (brain tissue).

4.5. HISTOLOGICAL DETECTION OF AMYLOID PLAQUES AND AMYLOID BURDEN

The other hemibrain was immersed in 4 % paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) and then transferred to a 30 % sucrose solution for cryoprotection. The hemibrains were cut in coronal sections (four 1 in 6 series of 30 μm) using a freezing, sliding microtome. The first series was stained for cresyl violet, the second series was stained for AChE (Van Groen and Wyss 1995) the third series was stained for Aβ (Mouse anti-human Aβ1-16; W0-2, a generous gift. K. Beyreuther), and the fourth series was silver-stained using the Garvey method (Garvey et al. 1991). The sections were processed for Aβ immunohistochemistry using a standard protocol following a 30 of pre-treatment with a Nacitrate solution at 85°C. In short, the sections were incubated in primary antibody solution (at 1:5000) for 18 h on a shaker table at room temperature (20°C) in the dark, the sections were rinsed and transferred to the solution containing the secondary antibody (Goat anti-mouse*biotin at 1:400; Sigma). After four hours the sections were rinsed and transferred to a solution containing ExtrAvidin® (at 1:1000; part of the mouse ExtrAvidin® Peroxidase

Staining Kit), and following rinsing the sections were incubated with Ni-enhanced DAB. The stained sections were mounted on slides and coverslipped. The appropriate sections (i.e., hippocampal formation) were digitized using a Nikon Coolpix 990 camera, and the images were converted to grey scale using the Paint Shop Pro 7 software. In three sections through the hippocampus the area covered by Aβ (amyloid burden) was measured using the ScionImage (NIH) program, with the Aβ load being reported as stained area fraction. For counting the number of amyloid plaques, all Garvey-stained sections containing the hippocampal formation were used. The plaque number is reported as the total number of plaques counted in this series.

4.6. NICOTINIC RECEPTOR BINDING ASSAYS

A/P and NT controls mice were sacrificed by decapitation at the age of 3 weeks, and 2, 5.5, 8, 12 and 17 months. The dissection of mouse brain was done as follows. The scalp was opened and the site of bregma was marked on the brain surface with a sharp-edged surgical blade. The skull bones were removed with blunt tip surgical scissors. Thereafter, a horizontal cut was made at bregma and 1.5 mm in front of it. The caudate putamen was sampled using a small diameter plastic straw to punch the round nucleus in this coronal section. The hippocampus was carefully dissected out using scissor with blunt tips. Other parts of brain tissue were sampled by cutting them sharply according to the lines depicted below (Figure 10).

HL=Hippocampus Left Part
HR=Hippocampus Right Part
DCL=Dorsal Cortex Left Part
DCR=Dorsal Cortex Right Part
VCL=Ventral Cortex Left Part
VCR=Ventral Cortex Right Part
CE=Cerebellum
OB=OlfactoryBulb
FC=FrontalCortex
MC=Frontal Cortex
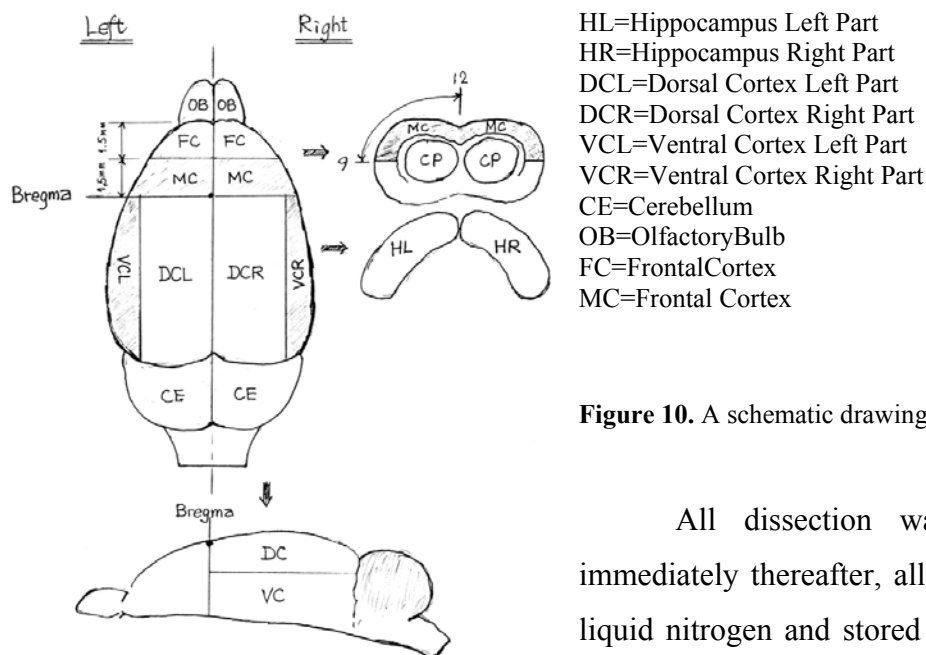
Figure 10. A schematic drawing of dissection of mouse brain

All dissection was done on ice, and immediately thereafter, all samples were frozen in liquid nitrogen and stored at −70°C until use. The dorsal cortex and the hippocampus were used in the nicotinic receptor binding assays. The same brain regions from 2 individual mice were pooled together, yielding 6 to 8 samples per experimental

group. The receptor binding assays were performed at the Karolinka Institute at Huddinge, Stockholm by prof. A. Nordberg's group.

The $\alpha4\beta2$ and $\alpha7$ nAChR binding sites on the membrane preparations of the brain samples were assayed with [^{3}H]cytisine (2 nM, 35.2 Ci/mmol, NEN) and [^{125}I]α-bungarotoxin ([^{125}I]α-BTX, 2 nM, 152 Ci/mmol, NEN), respectively. The non-specific binding was defined by 0.1 mM (-)-nicotine for [^{3}H]cytisine and 1μM cold α-BTX for [^{125}I]α-BTX, respectively (Bednar et al. 1998, Guan et al. 2001).

Brain tissues were homogenised in binding buffer, and the homogenates were centrifuged at 10,000 rpm for 15 min at 4°C. The resulting pellets were resuspended in binding buffer and centrifuged again. The final pellets were suspended in binding buffer ready for receptor binding assay.

[3H]Epibatidine (52 Ci/mmol, DuPont, NEN) binding assay was performed as previously described (Miao et al. 1998). Briefly, the membrane preparations were incubated with [3H]epibatidine in 50 mM Tris-HCl buffer (pH 7.4) at 25°C for 3 hr. The samples were then filtered through Whatman GF/C glass filters presoaked with 0.3% polyethyleneimine solution for 3–4hr and washed three times with assay buffer. Nonspecific binding was determined in the presence of 0.1mM (-)-nicotine. The samples were counted with a scintillation counter.

For [125I]α-bungarotoxin (α-BTX) binding assay, the membrane preparations of brain tissue were preincubated in the binding buffer (10 mM Na-phosphate buffer, pH 7.4, containing 50mM NaCl and 0.1% BSA) with (for non-specific binding) or without (for total binding) 1μM (final concentration) of cold α-BTX at 37°C for 30 min. Then, [125I] α-BTX was added into the binding mixtures with the final concentration of 2nM and the incubation was continued for 30 min. The final binding volume was 200μl. The assay was terminated by addition of 1ml cold binding buffer, followed by centrifugation at 11,000 rpm and 4°C for 5 min. The pellets were washed with the binding buffer by centrifugation at the same condition as above two more times. The radioactivity enwrapped in the pellets was counted with a γ-counter.

Displacement binding assays of either [3H]epibatidine or [125I]α-BTX by different concentrations of Aβ42 (Sigma) and Aβ40 were performed by using the same experimental

protocols as above. It has been recently reported that thoroughly washed membranes must be used in order to obtain significant displacement of [3H]epibatidine and [125I]α-BTX binding by Aβ fragments (Wang et al. 2000a, 2000b). Thus, the homogenates of brain tissues were prepared by centrifugation in binding buffer at 11,000 rpm and 4°C for 5 times, each 15 min, immediately before the binding assays were performed. All of the Aβ fragments were pre-aged by incubation at 37°C for 24 hr before the binding assays.

5.   RESULTS

5.1. PROGRESSION OF Aβ PATHOLOGY WITH AGE

5.1.1.   The rate of Aβ40-42 accumulation in A/P double transgenic mice from 3 weeks to 17 months

In the parietal cortex and hippocampus of A/P mice, soluble Aβ40 was detectable already at 3 weeks of age, while no soluble Aβ42 was detectable yet at 8 months of age. Insoluble Aβ40 and Aβ42 levels were low in the parietal cortex of A/P mice between 3 weeks and 8 months, but a significant increase in insoluble Aβ40 and Aβ42 levels was observed between 8 and 17 months. In the hippocampus, the increase in insoluble Aβ42 levels between 8 and 17 months of age was more robust than in insoluble Aβ40 levels, thus indicating a favorable accumulation of Aβ42 in these mice.

Figure 11 summarizes the time course Aβ accumulation in A/P mice between 3 weeks and 17 months based on combined data from several studies. The levels of Aβ40 and Aβ42 and their ratios are plotted separately.
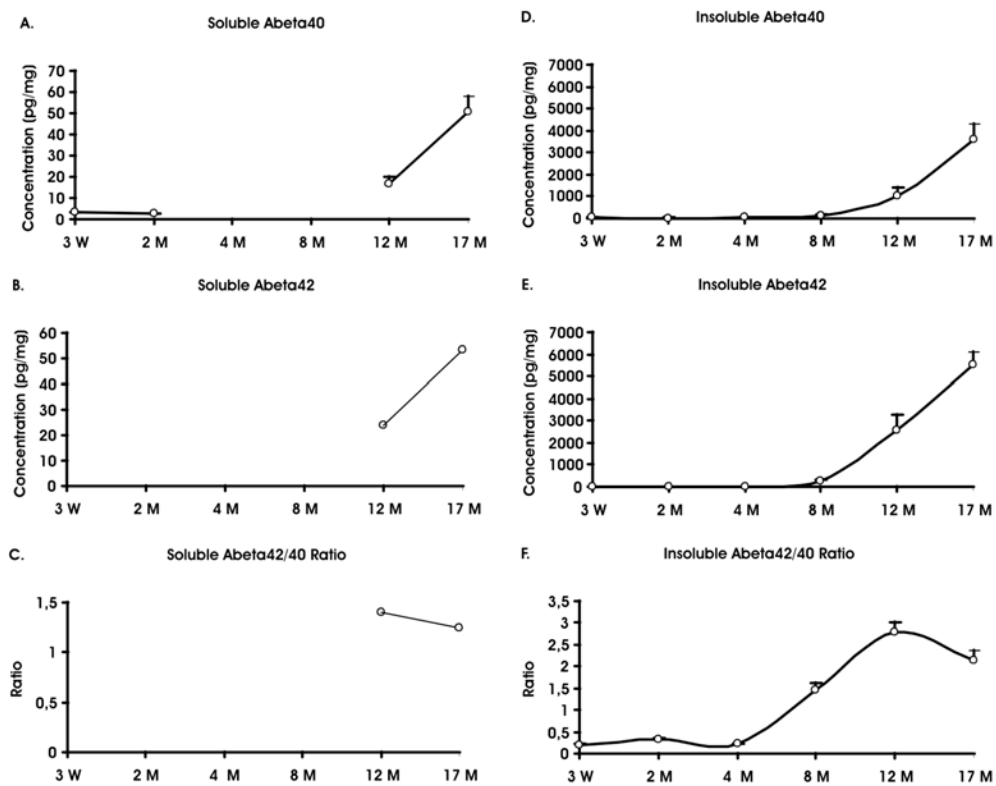
Figure 11. A summary of hippocampal Aβ40 and Aβ42 levels and their ratio at different ages in male A/P mice. The data was collected from the following sources: 3 weeks and 2 months (publication **V**), 4 months (publication **II**), 8

months (Liu et al. 2002c). The data for 12 and 17 months was generated by pooling individual mice from two separate studies (publications **I** and **V**).

5.1.2. Aβ levels in APP and PS1 single and double transgenic mice

Only the sensitive ELISA used for soluble Aβ42 was able to detect Aβ42 in 13-month-old APP single mutant mice. In addition, the APP single mutant mice had moderately elevated levels of insoluble Aβ40. However, all Aβ measures were about 50-fold higher in A/P mice than in APP single mutant mice. Similar pattern was found for cortical and hippocampal Aβ levels.

Table 1 Summary of Aβ40 and Aβ42 levels in 13-month-old A/P, APP and PS1 mice (Data from the EEG experiment, publication **III**) (ng/g , mean±SEM)

	Mouse	Soluble Aβ 42	Insoluble Aβ 40	Insoluble Aβ 42
Cortex	A/P	67.96±15.84	1136.47±298.12	1760.85±343.43
	APP	1.25±0.23	24.04±2.84	-
	PS1	-	-	-
	NT	-	-	-
Hippocampus	A/P	47.42±25.18	445.78±163.46	1162.15±363.91
	APP	2.03±0.24	30.50±6.00	
	PS1	-	-	-
	NT	-	-	-

No amyloid plaques could be detected in PS1 and APP single mutant mice. The A/P mice had some depositions in the hippocampus and cortex at 13 months of age, but not at 8 months of age. Both APP and A/P mice showed positive immunostaining for human APP. (see Figure 4. Aβ pathology in APP and PS1 single/double transgenic mice, publication **III**)

5.1.3. The correlations between biochemical and histopathological measures of Aβ

Using silver staining and W0-2 immunostaining for Aβ we detected amyloid plaques at the age of 12 but not at 4 months. Table 2 summarizes the correlations between histopathological and biochemical results.

Table 2 Correlations between biochemical and histopathological measures of amyloid pathology in 12-month-old male A/P mice (Data from the behavioural experiment, publication **II**)

Variable	Total Aβ40	Total Aβ42	SolubleAβ42	Amyloid burden	Amyloid plaques
Total Aβ40		$r = 0.97$ **$P < 0.001$**	$r = 0.49$ **$P = 0.047$**	$r = 0.52$ **$P = 0.046$**	$r = 0.55$ **$P = 0.02$**
Total Aβ42	$r = 0.97$ **$P < 0.001$**		$r = 0.42$ $P = 0.096$	$r = 0.46$ $P = 0.08$	$r = 0.57$ **$P = 0.02$**
Soluble Aβ42	$r = 0.49$ **$P = 0.047$**	$r = 0.42$ $P = 0.096$		$r = 0.27$ $P = 0.33$	$r = 0.29$ $P = 0.27$
Amyloid burden	$r = 0.52$ **$P = 0.046$**	$r = 0.46$ $P = 0.08$	$r = 0.27$ $P = 0.33$		$r = 0.50$ $P = 0.06$
Amyloid plaques	$r = 0.55$ **$P = 0.02$**	$r = 0.57$ **$P = 0.02$**	$r = 0.29$ $P = 0.27$	$r = 0.50$ $P = 0.06$	

In the table, the number and surface area of plaques (amyloid burden %) correlated with the biochemically measured levels of either Aβ40 or Aβ42 (ng/g). In contrast, the levels of soluble Aβ42 correlated only weakly with the plaque measures (Pearson correlation was used for statistics).

5.1.4. The degree of Aβ pathology differs between female and male A/P mice

Aβ ELISAs The Aβ40 and Aβ42 level were measured in all transgenic mice, i.e., at both ages and in both sexes. For both Aβ40 and Aβ42 levels, the ANOVA revealed a highly significant effect of age (both Aβ40 and Aβ42) and gender. The levels increased with age were higher in females. In addition, the age by gender interaction was significant in Aβ40 and Aβ42, so that the increase with age was faster in females. A significant effect of age and gender was also found for the Aβ42/40 ratio. The ratio decreased with age and was lower in females. No age by gender interaction was found for the Aβ42/40 ratio, however (Table 3).

Aβ histology The ANOVA revealed a highly significant effect of age and gender in the amyloid burden. It dramatically increased with age and was higher in females. The age by gender interaction was nonsignificant (Table 3).

The W0-2 antibody proved to be suitable for staining individual plaques in 12-month-old mice, but was too sensitive for the 17-month-old mice. Therefore, the plaque counts in this age group were based on silver staining, and correspondingly a separate analysis (student *t*-test) was performed for each age group. Among the 12-month-old mice, the plaque count was higher in females than in males. The gender difference was even more pronounced among the 17-month-old mice (Table 3).

Table 3 Summary of the ANOVA for age and gender effects on hippocampal Aβ accumulation in A/P mice (Data from the study on gender differences, publication **I**)

	Age		Gender		Age × Gender	
	F	*P*	*F*	*P*	*F*	*P*
Total Aβ 40	(1,57)=15.28	< 0.001	(1,57)=19.59	< 0.001	(1,57)=4.86	< 0.05
Total Aβ 42	(1,57)=15.31	< 0.001	(1,57)=41.13	< 0.001	(1,57)=5.58	< 0.05
Aβ 42/40 ratio	(1,57)=4.09	< 0.05	(1,57)=4.93	< 0.05	(1,57)=0.25	> 0.05
Aβ Burden	(1,34)=299.82	< 0.001	(1,34)=12.47	=0.001	(1,34)=1.83	> 0.05
Aβ Plaque Number*	12 months *t* = 2.28, *P*< 0.05 ; 17 months *t* =6.19, *P* < 0.001;					

* *t*-test for significance between female and male mice at 12 and 17 months

Table 4 Measurement of hippocampal Aβ accumulation in female and male A/P mice at 12 and 17 months (Data from the study on gender differences, publication **I**) (Aβ concentration= ng/g , Aβ burden=%, mean±SEM)

	12 months		17 months	
	Female	Male	Female	Male
Total Aβ 40	4081.08± 1375.79	1432.79± 512.28	11374.19± 1173.66	3465.53± 868.70
Total Aβ 42	8503.62± 2250.95	3564.16± 993.97	16151.90± 974.90	5455.33± 655.62
Aβ 42/40 ratio	2.25± 0.35	3.04± 0.22	1.80± 0.23	2.30± 0.29
Aβ Burden	1.71± 0.55	0.56± 0.99	11.52± 0.82	8.95± 0.66
Aβ Plaque Number	267.56± 81.28	91.73± 22.70	153.33± 19.37*	38.50± 7.20*

* Amyloid plaque count using Garvey's silver staining materials

Correlations between Aβ42 level, amyloid burden, and amyloid plaque number in male and female mice at the age of 12 months   There was a significant correlation between the Aβ42 levels and amyloid burden, and between amyloid plaque number and amyloid burden in 12-month-old female mice. No significant correlation was observed between Aβ42 levels and amyloid burden, but the correlation between amyloid plaque number and amyloid burden was significant in 12-month-old male mice.

Correlations between the amyloid burden and amyloid plaque number in male and female mice at the age of 17 months   There was a significant correlation between the amyloid plaque number and amyloid burden in 17-month-old female mice. Similarly, a significant correlation was found between amyloid plaque number and amyloid burden in 17-month-old male mice.

5.2. AGE-DEPENDENT MEMORY IMPAIRMENT

5.2.1. Age-dependent impairment in the water maze

Hidden platform   The data from both age groups (4 and 12 months) revealed that during the initial acquisition stage the animals improved their performance as measured in decrease of escape distance over the training days. There was a significant age by genotype interaction, but no overall age or genotype effect on escape distance. The data showed that at the age of 4 months there were no differences on escape distance between the genotypes, and the both genotypes improved their performance similarly over the training days. However, at the age of 12 months the control mice improved their performance faster than A/P mice, and in general the transgenic mice had also longer escape distances than control mice.

The probe trial data from both age groups (4 and 12 months) showed that there were no differences between the genotypes in the time spent in the vicinity of previous platform position. However, an overall effect of age and an age by genotype interaction was found. The data showed that at 4 months of age there were no differences in the probe trial performance between the genotypes, but at the age of 12 months the A/P mice spent less time in the vicinity of previous platform position than the control mice (Figure 12). There were no differences in swimming speed between the control and A/P mice during the initial acquisition stage. However, the older mice swam more slowly than the younger mice.

Visible platform The data from both age groups (4 and 12 months) revealed that during visible platform training the control and transgenic mice improved their performance as measured in the decrease of escape distance over the training days. There was a significant difference between the genotypes, such that transgenic mice had longer escape distances than control mice. However, no significant age by genotype interaction or age effect was found on escape distance. Although the escape distance to find the visible platform was longer in transgenic mice, the average platform finding percentage during visible platform training was only slightly decreased in transgenic mice (85 %) compared to control mice (96 %). There was no overall effect of genotype or genotype by age interaction on swimming speed during the visible platform training.
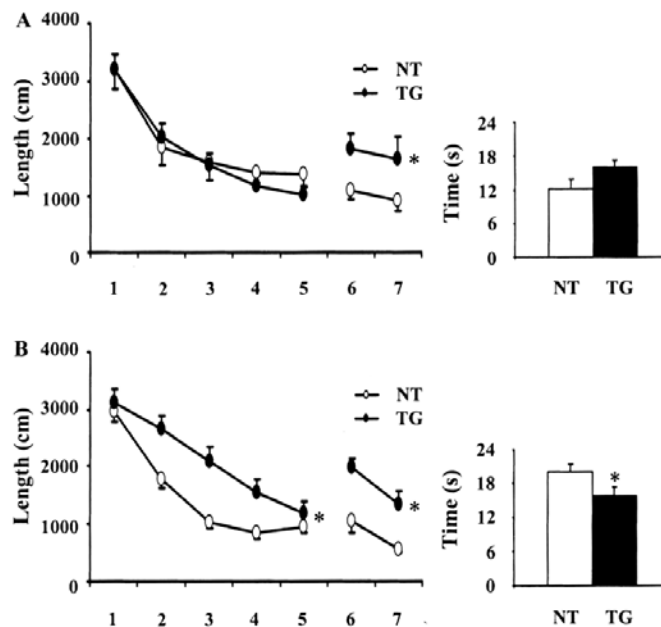
Figure 12. Performance in the water maze task by (A) 4-month-old and (B) 12-month-old male A/P mice (TG) and their nontransgenic controls (NT). The averaged path length on five daily trials are shown for each groups. Days 1-5 show learning to find a hidden platform and days 6-7 subsequent test with visible platform. The column to the right shows the mice performance during the probe trial. The columns indicate the time spent in the vicinity of former platform position (Data from publication II).

5.2.2.   No difference between the genotypes in position discrimination in the T-maze

The analysis of pooled data from both age groups (4 and 12 months) in T-maze revealed that during the initial learning stage the control and transgenic mice improved their performance as measured in the increase of correct choices over the training days. There were no overall effects of genotype and age or an age by genotype interaction on the number of correct choices (Figure 13).
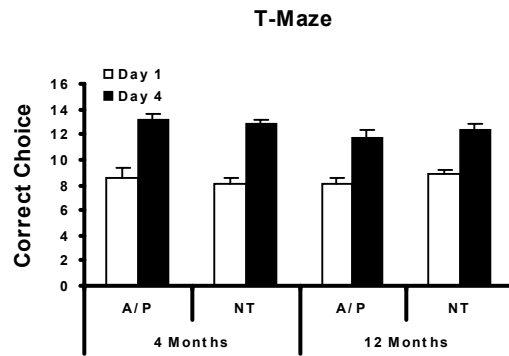
Figure 13. Performance in the T-maze task by (A) 4-month-old and (B) 12-month-old male A/P mice (TG) and their nontransgenic controls (NT). The open columns show the number of correct choices during day 1 and the filled columns during the last, fourth day of task acquisition. Unpublished figure.

5.2.3. Poor spatial nagivation correlates with the hippocampal total Aβ levels

Since the A/P mice differed cognitively from control mice in their water maze performance, we evaluated whether the different measures of Aβ pathology (hippocampal total Aβ40 and Aβ42 levels, cortical soluble Aβ42 levels, number of amyloid plaques, total Aβ burden) correlated with search bias in the hidden platform version and length in the visible platform version of the water maze. The main finding was that only the hippocampal total Aβ42 correlated with spatial search bias.

Table 5 Correlation of performance in the hidden and visible platform version of the water maze with hippocampal total Aβ42 in 12-month-old A/P mice (Data from behavioural experiment, publication **II**)

	Total Aβ40	Total Aβ42	Soluble Aβ42	Amyloid burden	Amyloid plaques
Retention score	$r = -0.41$ $P = 0.11$	$r = -0.49$ ***P = 0.048***	$r = -0.38$ $P = 0.14$	$r = -0.30$ $P = 0.25$	$r = -0.12$ $P = 0.65$
Visible platform	$r = -0.06$ $P = 0.83$	$r = -0.09$ $P = 0.72$	$r = 0.03$ $P = 0.90$	$r = -0.28$ $P = 0.31$	$r = -0.13$ $P = 0.62$

* Measurement of hippocampal total Aβ42 level (concentration=ng/g) was correlated with water maze probe trial performance (retention score, time=seconds) (Pearson correlation: $r = -0.49$, ***P = 0.048***)

5.3. EEG AND ERP CHANGES ARE AGE-INDEPENDENT

5.3.1. No correlations of Aβ accumulation with EEG and ERP changes in A/P mice

EEG The cortical EEG in the mouse during alert immobility was dominated by a broad peak at the theta range with a maximum around 5 Hz. Additional smaller peaks were observed around 12 Hz and 25 Hz.

As illustrated in Figure 2, publication **III,** the A/P mice tended to have lower power than NT mice around the theta peak but increased power over two wide frequency bands ranging from 12 to 23 Hz and 28 Hz to 40 Hz independent of their age.

To quantify these group differences we compared the maximum power of the theta (5 Hz) and the power in the beta band between the two additional peaks (14-23 Hz) and in the gamma band after the third peak (28 – 40 Hz) across all age points using repeated measures ANOVA. The analysis for the maximum theta power yielded a significant effect of the genotype and age, but the genotype by age interaction was nonsignificant. For the beta band, the ANOVA revealed a highly significant genotype effect. The beta power decreased with age in both genotypes. Similarly, the gamma activity differed between the genotypes and decreased with age, but the genotype by age interaction only approached significance.

The hippocampal EEG during alert immobility was dominated by one sharp theta peak with the maximum at 6.6 ± 0.1 (mean$\pm$SEM) Hz in the NT mice and 6.7 ± 0.1 in the A/P mice. This difference was nonsignificant. The frequency of the theta peak decreased dramatically with age, from 7.2 ± 0.2 Hz in 7-month-old to 6.2 ± 0.2 Hz in 13-month-old NT mice. No age by genotype interaction was found, however. The ANOVA yielded no genotype effect in the maximum theta power nor a genotype by age interaction. In contrast, the total power over 1-40 Hz was significantly higher in A/P mice. The total power also decreased with age in both genotypes.

ERP In the cortical recording, habituation to repeated auditory stimuli (auditory gating) decreased steadily with age both in A/P and NT mice. Auditory gating was weaker in A/P mice than in NT mice, but this difference did not increase with age (see Figure 2A, publication **IV**) Also the cortical N35 latency to the first (conditioning) stimulus tended to be longer in A/P than in NT mice independent of age (see Figure 2C, publication **IV**). In addition, the hippocampal recording revealed weaker auditory gating in A/P mice independent of age (see Figure 2B, publication **IV**).

5.3.2.   The role of APP transgene in EEG and ERP alterations

EEG We further analyzed the effect of genotype on the cortical EEG by including also groups of single APP and PS1 mutant mice. The EEG spectrum in the 8-month-old NT mice was essentially similar to that in Experiment 1, except that the third small peak appeared at a higher frequency (around 28 Hz) and an additional peak was found with a peak around 38 Hz. Therefore we analyzed the beta band from 14 to 27 Hz in this experiment. The power at the theta peak did not differ between the genotypes, but the genotype difference in the beta band was still evident. In the post-hoc comparison, the APP mice differed significantly from the NT and PS1 mice, while the A/P mice were close to APP mice (see Figure 5A, publication **III**). The genotype effect was also found for the gamma band (28 - 40 Hz). In the post-hoc comparison, both A/P and APP mice differed from the NT mice (see Figure 5B, publication **III**), but did not differ from each other.

ERP The degree of auditory gating differed among the genotypes. It was weaker in A/P mice compared to PS1 mice and APP single mutant mice (see Figure 3A, publication **IV**). The cortical N35 latencies also differed between the genotypes, but so that both A/P and APP mice had longer latencies than did NT and PS1 mice (see Figure 3B, publication **IV**). In contrast, the latencies to the subcortical (most likely inferior collicular) N8 peak did not differ between the genotypes.

5.4. NO CHANGES IN nAChRs

5.4.1. No changes in the number of nAChRs in A/P mice

No significant differences in $[^{3}H]$cytisine binding sites ($\alpha 4\beta 2$ nAChRs) were observed between A/P double transgenic mice and NT controls in the parietal cortex at any age studied (see Figure 1A, publication **V**). $[^{125}I]\alpha$-BTX binding was measured both in the parietal cortex and hippocampus of A/P mice and NT controls. Similar to $[^{3}H]$cytisine binding, no significant differences in $[^{125}I]\alpha$-BTX binding sites ($\alpha 7$ nAChRs) were found between the two groups at any age studied (see Figure 1B, C, publication **V**).

5.4.2. No correlation between Aβ accumulation and nAChRs

The nAChRs as measured by $[^{3}H]$cytisine and $[^{125}I]\alpha$-BTX binding in the brains of A/P mice did not correlate with the levels of Aβ peptides at any age.

6. DISCUSSION

6.1. MICE CARRYING A/P MUTATIONS DEVELOP AGE-DEPENDENT AD-LIKE AMYLOID PATHOLOGY

6.1.1. Age-dependent accumulation of Aβ

Our main finding was a dramatic age-dependent accumulation of Aβ in the parietal cortex and hippocampus in A/P mice. Soluble Aβ40 was detectable already at 3 weeks of age and robustly increased between 12-17 months. No soluble Aβ42 was detectable until 12 months, reaching highest levels by 17 months of age. The insoluble Aβ40 and Aβ42 levels were low in A/P mice between 3 weeks and 8 months of age, increased rapidly by 12 months of age and continued to increase - although at slower rate - up to 17 months. Interestingly, the rapid increase in the insoluble Aβ levels coincides with the appearance of first amyloid plaques, which takes place at around 9 months of age in this transgenic mouse line (Borchelt et al. 1997). Also the increase in insoluble Aβ42 levels were more robust than in insoluble Aβ40 levels, thus indicating a favourable accumulation of Aβ42 in these transgenic mice. This is in agreement with earlier studies in APP+PS1 doubly transgenic mice reporting a marked increase in biochemically measured Aβ42 levels preceding visible Aβ deposition (Borchelt et al. 1997, Holcomb et al. 1998). A new interesting finding in the present set of studies was the age-dependency of the Aβ42/40 ratio. The ratio stayed quite constant from 3 weeks to 8 months, dramatically increased from 8 months to 12 months, and again decreased from 12 months to 17 months, while the total amount of Aβ (Aβ1-40 + Aβ1-42) kept increasing as the animals aged. The age-related changes in the Aβ levels and Aβ42/40 ratio are schematically described below in Figure 14.
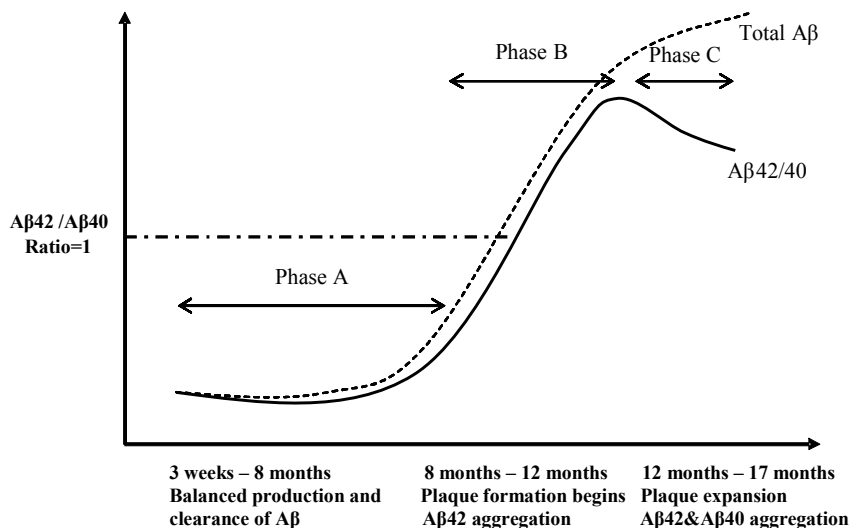
Figure 14. Here we propose a hypothetical model that divides the process of plaque formation into three separate phases in this transgenic mouse line. During phase A (from 3 weeks to 8 months), the Aβ42/40 ratio is lower than 1, because the production of Aβ40 is faster than that of Aβ42. The production and clearance of Aβ are balanced, and no gross accumulation takes place. During phase B (from 8 months to 12 months), the Aβ42/40 ratio rapidly reaches 1 and rises above it. As Aβ42 is far less soluble than Aβ40, it starts to aggregate first after reaching a critical concentration. The Aβ42 fibrils act as seeds to promote further aggregation leading in formation of plaques (Vitek et al. 1994). During phase C (from 12 months to 17 months), although total levels of Aβ still increase and new plaques are constantly formed, the rate of amyloid accumulation clearly slows down. Two factors are likely to contribute to this slowing. First, as the animals age, the turnover rate of Aβ, as of many other proteins, gets slower. Second, several lines of evidence suggest that the formation of amyloid plaques activates the immune response in the brain with activated microglia "eating" some of the deposited Aβ (Wilcock et al. 2001). This phase is also characterized by a decrease in Aβ42/40 ratio, as the more rapidly produced Aβ40 also reaches its critical concentration for aggregation.

6.1.2. Sex difference in Aβ accumulation in A/P mice

The findings from the present study are in agreement with the notion that gender as a variable plays an important role in the pathogenesis of AD, i.e., sex-related differences are present in Aβ levels and in amyloid deposits in the hippocampus. Firstly, the Aβ40 levels and the Aβ42 levels were significantly increased in female mice compared to male mice, at both 12 and 17 months of age. Secondly, at both ages the amyloid burden was substantially higher in female mice compared to male mice.

Although numerous studies have shown the gender difference in the incidence, clinical course and neuropathological changes of AD (Molsa et al. 1982, Fillit et al. 1986, Henderson et al. 1994, Fratiglioni et al. 1997, Asthana et al. 1999, Henderson et al. 2000, Honjo et al. 2001, Alafuzoff et al. in press), the mechanisms that underlie the gender difference are still unclear. The disparity in estrogen level between females and males, together with the dramatic change in estrogen level with menopause has been hypothesized to cause these gender differences. This hypothesis is supported, on the one hand, by epidemiological studies showing a reduced risk of AD among women taking estrogen replacement therapy (Fillit et al. 1986, Asthana et al. 1999, Henderson et al. 2000, Honjo et al. 2001). On the other hand, results from *in vitro* and *in vivo* studies provide direct evidence of biological effects of estrogen on the metabolism of Aβ. Several studies have reported that estrogen down-regulates Aβ generation from APP metabolism in cultured cells (Chang et al. 1997, Xu et al. 1998, Greenfield et al. 2002), and that overiectomy increases the

production of Aβ in animal models (Zheng et al. 2002, Petanceska et al. 2000), implicating a relationship between decline of estrogen and increase of Aβ generation. Except the direct effect on Aβ, estrogen also has some other indirect effects that potentially affect Aβ generation. Estrogen has trophic and protective effects on neurons (Luine 1985, Keefe et al. 1994, Lustig 1994, McEwen and Woolley 1994), and can also act as an antioxidant to decrease neuronal damage caused by oxidative stress (Behl et al. 1995, Gridley et al. 1997, Skoog and Gustafson 1999).

However, contrastingly, some more recent studies have suggested that the gender does not a significantly influence the AD prevalence (Ganguli et al. 2000, Ruitenberg et al. 2001). Also results from some studies suggest that estrogen supplementation is not beneficial for slowing the progression of Alzheimer's disease (Broe et al. 1990, Mortel and Meyer 1995). Furthermore, although Petanceska et al. (Petanceska et al. 2000) have shown that in very young, normal guinea pigs ovariectomy causes a 50% increase in the levels of Aβ, which suggest a relation between a decline in estrogen levels and an increase the generation of Aβ, preliminary data from our laboratory indicate that ovariectomy at 3 months of age in the A/P mice does not affect the amount of deposited β-amyloid at 6-12 months of age.

Another interesting finding in our present study was the lower Aβ42/40 ratio in females compared to males. This observation suggests that the gender difference in Aβ accumulation is more likely due to higher production of Aβ40 in females rather than their increased tendency for amyloid accumulation. This is consistent with earlier findings in ovariectomized (Zheng et al. 2002) and aged APPswe (Tg2576) (Callahan et al. 2001) trangenic mice. Although ovariectomized and aged Tg2576 mice showed a significant increase in soluble and insoluble Aβ40 but not Aβ42, high dose of estradiol reduced production of both Aβ40 and Aβ42. However, these studies should be compared to ours with caution, because combination of APPswe mutation with PS1 mutation selectively increases the Aβ42 to Aβ40 ratio in the double mutant mice (Borchelt et al. 1997, Holcomb et al. 1998).

The striking gender difference in the rate of amyloid accumulation and the less obvious but still significant difference in the Aβ42 to Aβ40 ratio in our APPswe + PS1(A246E) mice is an infrequent finding in transgenic models with AD-like amyloid pathology. So far only one study has reported a difference in the extent of Aβ accumulation between female and male transgenic mice carrying an APP mutation (Callahan et al. 2001). However, when comparing our results with

Callahan's study, it should be noticed that although these two mouse lines showed sex-difference in amyloid accumulation, in APPswe(Tg2576) mice the levels of Aβ40 were higher than Aβ42 levels at 15 months of age, contrary to our mice that showed higher Aβ42 levels. Furthermore, Callahan et al. (2001) showed that 15-months-old female mice had significantly higher levels of Aβ40 than male mice of the same age, whereas no difference was found in the Aβ42 level between female and male mice. In conclusion, these transgenic mouse lines with faster Aβ accumulation in females may be of particular interest and provide a tool to study mechanisms that underlie the sex difference in the incidence, clinical course and neuropathological changes in AD patients.

6.1.3. Amyloid pathology in APP and PS1 single transgenic mice

In our third study (publication **III**) using both double and single transgenic mice for APP and PS1, we found that only the sensitive ELISA used for soluble Aβ1-42 was able to detect Aβ1-42 in the cortex and hippocampus in the APP single transgenic mice at age of 13 months. In addition, the APP single transgenic mice had moderately elevated levels of insoluble Aβ1-40 in the cortex and hippocampus at age of 13 months. However, all Aβ measures were about 50-fold higher in A/P mice than in APP single mutant mice.

In our first study (publication **I**), histological silver staining and Aβ W0-2 immunostaining revealed that at the age of 12 months, the brains of A/P mice had significant amyloid deposits. The amyloid burden significantly correlated with total Aβ40 levels and the amyloid plaques with total Aβ40 and soluble Aβ42 levels in the hippocampus. But APP and PS1 single transgenic mice had no amyloid plaques at this age.

Taken together, these results showed that by comparing double mutant mice to single APP or PS1 mice, the contribution of Aβ accumulation on behavioral and electrophysiological measures can be separated from the contribution of the transgene by itself.

6.2. AGE-DEPENDENT MEMORY IMPAIRMENT CORRELATES WITH HIPPOCAMPAL Aβ42

This study investigated spatial learning of A/P mice at the age of 3-4 months and 11-12 months, before and after the first amyloid plaques developed in the hippocampal formation. At both ages, the mice were tested in two cognitive tasks. First, normal water maze protocol with five days acquisition training followed by retention trial was used to measure hippocampal dependent spatial

memory. Then the mice were tested for position discrimination in a T-maze task, which is independent of normal hippocampal functioning. The results show for the first time that the age-dependent impairment in traditional water maze retention of double transgenic A/P mice correlates with the amount of Aβ accumulation in the hippocampus.

6.2.1. Middle-aged A/P mice are impaired in a hippocampal-dependent task

The phenotype of the double transgenic APPswe+PS1(A246E) mouse line has not been previously characterized behaviorally. As reported before (Borchelt et al. 1997), at 11-12 months of age (middle age) these mice had numerous Aβ deposits in the hippocampus, subiculum, and the neocortex but few deposits in other brain areas. However, at the age of 3-4 months (young adult age) no Aβ deposits were detected in these mice. The 11-12-month-old A/P mice were impaired in hippocampal-dependent spatial memory in the water maze when compared with nontransgenic littermates. In contrast, A/P mice were unimpaired in a position discrimination task in the T-maze, a task that measures learning of a response habit and does not depend on hippocampal function for its execution (Oliveira et al. 1997). Importantly, the young adult A/P mice did not differ from control mice in these two tasks. The hippocampal Aβ levels varied in a wide range among the middle-aged A/P mice, giving an excellent opportunity to relate individual parameters of hippocampal Aβ pathology to individual performance in the spatial learning task that is dependent on the hippocampal function.

An important finding in this study was that the middle-aged but not the young adult A/P mice were cognitively impaired both in water maze acquisition and retention compared to control mice. In previous studies, a similar behavioral pattern was initially reported for the APPswe (Tg2576) mice at 9 months of age (Hsiao 1996), but could not be confirmed in a later study (King´et al. 1999). Transgenic mice with combined APPswe (Tg2576) and PS1(M146L) mutations and accelerated amyloid accumulation compared with single APPswe (Tg2576) mutation, were found to be unimpaired in the water maze at 5-9 months of age (Holsomb et al. 1998, Arendash et al. 2001). However, at 15-17 months of age these mice showed impaired acquisition but normal retention compared to controls (Arendash et al. 2001). In contrast, transgenic mice carrying the APP(V717F) mutation (PDAPP mice) were impaired in both acquisition and retention of the standard water maze task already at a young age (Chen et al. 2000). The general tendency of different transgenic mice lines carrying mutated APP to be impaired in spatial learning tasks is consistent with the fact that the amyloid pathology is most severe in the hippocampus in all these mouse lines (Hsiao et al. 1996,

Borchelt et al. 1997, Dodart et al. 2000, Arendash et al. 2001). Conversely, contrasting results between these mouse lines may be due to the various extent of extrahippocampal pathology. For instance, unlike our mouse line the A/P double mutant of Tg2576 line expresses notable Aβ deposition also in the striatum (Holcomb et al. 1998, Gordon et al. 2001).

Intact performance in the position discrimination task in the T-maze is also consistent with hippocampal pathology and relative sparing of the striatum in our A/P mice. Namely, this kind of task requiring egocentric memory for the animal's own turning responses requires intact function of the striatum (Oliveira et al. 1997). Furthermore, C57BL/6J mice with fimbria-fornix lesion, resulting in deafferentation of the hippocampus from its cholinergic innervation, are as good or even slightly better than control mice in performing this task (Liu et al. 2002a). The A/P mice of the present study as well as the APPswe Tg2576 mice (Hsiao et al. 1996) were both impaired also in the visible platform version of the water maze, however, this deficit was independent of age. Otherwise, we did not observe any motor abnormalities in the transgenic mice during the task, and their swimming speed was similar to that of controls.

Impaired performance in the visual platform task could arise from visual problems in these mice but it is unlikely for several reasons. First, the A/P mice did not behave visually impaired; they found the visible platform more than eight times out of ten trials. Second, those mice that failed in the visual platform task often swam towards the platform, but went on swimming after they hit it. However, during the hidden platform task such behavior was never observed, maybe because the mice were pretrained with the submerged platform. It is possible that the A/P mice were worse than the controls in adapting to the new test situation when the visible platform was introduced instead of the submerged one. Alternatively, the A/P mice were simply more afraid in the new test situation. In support of this notion, the A/P mice spent less time than wild type mice in the open arms of the elevated plus maze, which speaks for increased level of anxiety in these animals. Third, impaired performance in the visual platform task can also result from hippocampal dysfunction. Notably, in the original description of water maze deficit in rats with hippocampal lesions, Morris et al. (1982) reported an initial learning deficit in the visual platform version of the task. Also in a recent study from our laboratory (Liu et al. 2002a), mice with fimbria-fornix transections were impaired in locating both the hidden and visible platform in the water maze.

6.2.2.   Impaired hippocampal-dependent task performance in middle-aged A/P mice correlates with hippocampal Aβ42

Central to our understanding of the pathophysiology of Alzheimer's disease is to determine whether amyloid accumulation is a cause or a product of the disease process. Although initial studies on post-mortem brains of AD patients did not find a correlation between amyloid plaque counts and cognitive impairment (Braak and Braak 1994), recent evidence suggest that Aβ load may indeed correlate with cognitive decline (McLean et al. 1999, Näslund et al. 2000). Controversy still exists as to which form of Aβ plays the most important role in pathogenic events. One recent post-mortem human study found significant correlations with both total Aβ40 and Aβ42 levels and cognitive decline (Näslund et al. 2000), whereas another post-mortem study reported such correlation for soluble but not insoluble Aβ (McLean et al. 1999). In transgenic animal models, two recent studies reported significant correlations between spatial learning impairment and Aβ burden (plaque surface area) in the hippocampus and/or frontal cortex (Chen et al. 2000, Gordon et al. 2001). Furthermore, in the latter study, amyloid burden estimated with a non-selective Aβ antibody, selective Aβ1-40 antibody and congo red staining yielded similar correlations. Our results further support the idea that increased Aβ load is associated with cognitive impairment, but in contrast to above mentioned animal studies, we did not find a correlation between the number of amyloid plaques and impaired spatial learning. However, our study differed also greatly from these studies in the extent of amyloid pathology present in the mouse brains. Notably, Chen et al. (2000) and Gordon et al. (2001) found the correlation in their oldest age group (16-22 months and 15-17 months, respectively) and used a mouse model with much faster rate of Aβ accumulation than ours. In fact, the amyloid burden reported in these mice (5-25% and 10-40% in the hippocampus) is an order of magnitude higher than in our mice (less than 1%) and even higher than in the brains of AD patients (2-16%) (Rogers and Morrison 1985, Kraszpulski et al. 2001). It is obvious that these animal studies examined Aβ pathology at very different stages, and correspondingly, different mechanisms may account for neuronal dysfunction and consequent learning impairment at very early and very late stage of the process.

This study is the first systematic attempt to correlate a critical learning parameter with the soluble Aβ42 levels in the brain. It is worth noticing that for technical reasons we had to measure soluble Aβ42 from the dorsal cortical sample while other measures were from the hippocampus. However, our previous measures have shown a high correlation between the levels of soluble Aβ42 in the hippocampus and overlying cortex, so we can assume that also in this material soluble Aβ42 as measured from the cortex represents hippocampal levels as well. Furthermore, soluble Aβ42 weakly correlated with total Aβ40 and Aβ42 as was expected, but not with amyloid burden or

plaque counts. In the present study, soluble Aβ42 did not correlate with the learning parameter at all, whereas the total Aβ42 did so. These findings suggest that aggregation of Aβ into insoluble fibrils may be the key step in the pathogenic process resulting in dysfunction of the affected brain area.

In summary, earlier studies looking at a possible correlation between Aβ load in the brain and memory performance in neuropsychological tests either in Alzheimer patients or in transgenic mice with AD-like amyloid accumulation have analyzed brains with advanced pathology. In contrast, animals of the current study represent early stages of pathology. Our finding that the total amount of Aβ1-42 in the hippocampus correlates with spatial learning impairment at a stage when only a few plaques are visible gives further evidence that Aβ1-42 is among the key factors resulting in neuronal dysfunction in AD. Whether spatial learning deficit is caused or merely correlated with hippocampal Aβ1-42 levels needs to be address in further studies.

6.3. AGE-INDEPENDENT ALTERATIONS OF EEG AND ERPs IN A/P MICE

This study is the first report on EEG and ERP changes in transgenic mice with Aβ-related pathology. Technically, this approach proved to be capable of long-term studies up to 6 months, since few mice had to be discarded because of the loss of electrode implant or deteriorating signal. In addition, the signal amplitude was remarkably stable over the entire follow-up period. We detected significant differences in the cortical EEG power spectrum, habituation and latency of the N35 response between transgenic and non-transgenic mice, but these changes were not age-dependent as would have been predicted on the basis of underlying progressive Aβ accumulation. In a control experiment with both double and single transgenic mice, we were able to associate altered cortical EEG and prolonged N35 latency (corresponding human P50) with the presence of APPswe transgene, whereas impaired auditory gating was associated with the presence of Aβ.

6.3.1. EEG alterations and prolonged ERP latency are age-independent and potentially linked to the APP transgene

EEG The observed EEG changes were obviously not caused by amyloid deposition in plaques. First, the differences were present at the age of 7 months, before the appearance of the first amyloid deposition, and did not change when plaques started to appear. Second, the number of plaques was small even at the age of 13 months, except in some layers of the hippocampus. Third, the cortical theta activity was found to be different between A/P and non-transgenic mice, whereas

the genotypes did not differ with regard to the hippocampal theta activity, although the hippocampal formation had more amyloid depositions. Similarly, these arguments speak against the role of Aβ42 levels as the cause of the EEG changes.

The control experiment with both double and single mutant mice clearly suggests that the observed EEG changes are linked to the APPswe genotype. This association indicates that either elevated levels of Aβ40 or the presence and/or overexpression of mutated human APP protein could be the underlying factor. The APP protein levels in these mice are about twice as high as endogenous mouse APP levels, and remain relatively constant over the entire age span of the present study (Liu et al. 2002a). In contrast, like Aβ42, Aβ40 accumulates in the brain as the mice age. Therefore, the presence of mutated human APP remains the most likely factor underlying the EEG changes. However, the role of soluble Aβ40 cannot be ruled out either, as we did not have a sensitive Aβ40 assay at our disposal.

Increased cortical beta has been reported in association with visual attention in cats (Wrobel 2000) and higher gamma power has been reported with movement and attention/arousal compared to quiet wakefulness in rats (Maloney et al. 1997). Hence one may argue that primary genotype differences in arousal levels or mobility could have contributed to secondary inter-group differences in EEG measures. Increased motility in APP or A/P mice was unlikely to contribute to EEG changes, however. First, all data samples during observable movement were rejected. Second, the frequency and power of hippocampal theta peak always increases with motility, but the frequency was same for all groups, and the power was actually lower in the APP and A/P mice compared to controls. Also the role of increased arousal as a major underlying factor for the group differences is unlikely. Namely, increased arousal is usually accompanied by a decreased power in all but gamma frequencies (Foote et al. 1991), but in mice carrying the APPswe mutation, only the theta peak was consistently decreased compared with NT mice. Therefore, these findings rather speak for the notion that the group differences in EEG are primarily due to functional differences in the cortical networks. On the other hand, an age or adaptation related decrease in the level of arousal may well contribute to the finding that the power in both beta and gamma frequencies significantly attenuated with age in all genotypes.

ERPs The observed changes in cortical and hippocampal AEPs in A/P mice were obviously not caused by the amyloid deposition in plaques. Namely, the group differences in auditory gating and N35 latency remained the same between 7 and 11 months of age, although the mouse brains

showed dramatic increase in the Aβ load and the formation of first amyloid plaques during this time. Furthermore, the prolongation of N35 latency was unlikely to be related to Aβ1-42 at all, as it was equally present in A/P and APP mice despite 20 to 30 fold difference in the levels of soluble Aβ1-42. Interestingly, cortical EEG abnormalities in our study III were also similar in magnitude in A/P and APP mice as compared with NT or PS1 mice (Publication **III**).

The most plausible link to prolonged N35 latency is the overexpression of mutated APP. Interestingly, the delayed auditory ERP latency was restricted to mid-latency component, while the subcortical N8 component did not differ between the groups. Consistent with this observation, we did not detect the transgene protein in the subcortical auditory relay nuclei, although it was abundantly present in the auditory cortex and in the hippocampus. It remains open whether this change is specific to the APPswe mutation or whether it is related to increased APP levels in general. Namely, in this transgenic mouse the transgenic APP levels are about twice as high as the endogenous APP protein levels (Liu et al. 2002b).

6.3.2. Impaired auditory gating is potentially linked to increased Aβ42 levels

The association between impaired auditory gating and A/P genotype is less straightforward. The fact that the deficit of auditory gating was age-independent over the critical age for plaque formation does not rule out other possible consequences of Aβ1-42 formation. Although the levels of soluble Aβ1-42 increased dramatically with age, it is possible that some of its biological interactions saturate at much lower concentrations. An interaction between soluble Aβ1-42 and the α7 nicotinic receptors is one possible mechanism. Aβ1-42 has been shown to bind to α7 nicotinic receptors at picomolar concentrations (Wang et al. 2000b), and to noncompetitively block these receptors in cultured hippocampal neurons at nanomolar concentrations (Liu et al. 2001). Interestingly, in the latter study, the blockade of whole-cell response was never greater than 80%, which level was obtained at around 30nM. The levels of hippocampal soluble Aβ1-42 in the mice of our study were around 10-15 nM, and the cortical levels were even higher. Taking into account the fact that the distribution of Aβ1-42 in the hippocampus is layer specific and concentrated on the dorsal hippocampus (Liu et al. 2002b) (from which all present recordings were made), while the entire hippocampus was used for ELISA, the local concentrations in the recorded area are likely to reach as high levels as 100 nM. Therefore, it is possible that a maximal blockade of hippocampal and cortical α7 nicotinic receptors by Aβ1-42 was already present in 7-month-old mice. On the

other hand, changes in the α7 nicotinic receptor number due to the expression of the APPswe transgene, as reported in some recent studies (Dineley et al. 2001), is an unlikely explanation for the difference in auditory gating, since no difference in α-bungarotoxin binding was observed between A/P and nontransgenic mice of our colony at any age between 3 weeks to 17 months (Publication **V**).

6.3.3. EEG and ERP findings in transgenic mice and AD patients

EEG The EEG changes in A/P mice are clearly very different from EEG changes observed in AD patients. The most common finding in AD patients is a general slowing of the EEG: specifically, the alpha and beta activity decrease, while an increase can be detected in delta and theta frequencies (Stigsby et al. 1981, Penttila et al. 1985). Although the functional correlates and underlying neural circuitry of the EEG frequency bands (especially the alpha) may differ between humans and rodents, similar slowing of EEG can be induced in humans and rats by anticholinergic drugs (Herz 1959, Longo 1966), or in rats by destruction of cholinergic nucleus basalis (Riekkinen et al. 1990). Conversely, EEG slowing in nucleus basalis lesioned rats can be alleviated by muscarinic agonists or cholinesterase inhibitors (Riekkinen et al. 1991a). To further support the role of cholinergic degeneration behind EEG changes in AD patients, slowing of occipital alpha (and corresponding shift to theta) correlates inversely with the frontal ChAT activity (Soininen et al. 1992). On the other hand, the cortical and hippocampal ChAT levels do not differ between our A/P and non-transgenic mice (Liu et al. 2002b). Therefore, it is likely that the EEG changes in AD can largely be attributed to the cholinergic neurodegeneration. In contrast, the common pathological feature between human AD and transgenic mouse models, the accumulation of Aβ, does not seem to significantly contribute to the EEG signal.

ERPs Several studies have compared the amplitude, latency and habituation of human middle-latency potentials termed P50 or P1 between patients with Alzheimer's disease and age-matched elderly controls. The findings have been controversial. Whereas two earlier studies reported absence or reduction of P50 in AD patients, but no difference in its latency when present (Buchwald et al. 1989, Green et al. 1992), a later study did not find any difference in P50 amplitude (Jessen et al. 2001). Jessen et al. (2001) reported deficit in P50 habituation, that was not found by Fein et al. (1994). Furthermore, a recent study reported increased P50 amplitude and latency in MCI (mild cognitive impairment) patients (Golob et al. 2001). Both differencies in recording techniques and the definitions of middle-latency positive component may contribute to this difference. Most

above mentioned studies employed a passive test condition (Buchwald et al. 1989, Green et al. 1992, Jessen et al. 2001), whereas the P50 component was measured in the context of an oddball detection paradigm in the MCI study (Golob et al. 2001). The filtering of the signal has varied greatly (Buchwald et al. 1989, Golob et al. 2001) as well as the definition of the P50 (or P1) component (Buchwald et al. 1989, Green et al. 1992). The status of the cholinergic system could have varied greatly between subjects depending on the severity of the disease and the use of anticholinergic medication. The latter aspect is noteworthy, since manipulation of the cholinergic system by scopolamine increased the P50m (magnetic correspondent to P50) response amplitude in a recent study (Pekkonen et al. 2001).

Our current experiments provided, in part, a useful animal model to elucidate the mechanism that underlies changes in P50 response and its habituation in AD patients. Unlike human studies in which the P50 component is small in amplitude and not easy to detect in standard scalp recordings, the corresponding N35 response in mice was sharp and prominent in intracortical recordings. In human studies, impaired habituation of P50 has been ascribed to cholinergic deficit and reduced number of α7 nicotinic receptors (Jessen et al. 2001). However, impaired habituation of N35 in A/P mice despite no loss of cholinergic presynaptic markers (Liu et al. 2002a) and nAChRs (Publication **V**) indicates that increased levels of Aβ1-42 may also directly influence N35/P50 habituation.

Taken together, the currently described EEG and AEP alterations in transgenic mice carrying mutated human APP gene are robust and repeatable over an period of several months. Although they are of no use in following the progression of Aβ accumulation in the brain as we originally hoped for, they may provide an important clue to elucidate the hitherto poorly understood normal function of the APP protein in the brain. On the other hand, habituation of N35 (corresponding to human P50) appears to index functional changes of soluble Aβ levels or impaired nAChR function in mice brain.

6.4. NO CHANGES IN nAChRs IN A/P MICE AND NO CORRELATIONS BETWEEN Aβ ACCUMULATION AND nAChRs

The current study did not show any correlation between Aβ accumulation and the number of nAChRs in A/P double transgenic mice. The lack of changes in nAChRs as measured by [^{3}H]cytisine and [^{125}I]α-BTX binding in the brains of A/P mice in this study is concordant with preserved cholinergic innervation of the cortex and hippocampus in this transgenic mouse line as

indicated by ChAT (Liu et al. 2002b). However, [³H]cytisine binding was also normal in another A/P transgenic mouse line expressing Aβ deposits as early as 3 months of age (Hernandez et al. 2001), although abnormalities in cholinergic synapses have been reported in the frontal cortex and hippocampus of these mice (Wong et al. 1999). By contrast, earlier study with the single mutant APPswe transgenic mouse of the Tg2576 line and a different genetic background found selective increases in α7 nAChRs (at 4 and 17-19 months) and α4β2 nAChRs (at 17-19 months) in many subregions of the cortex and hippocampus (Bednar et al. 2002). Increased α7 nAChR subunit protein level in the brain of the same mouse line has also recently been reported (Dineley et al. 2001). Because elevated levels of Aβ peptides and amyloid plaques are common features of all three transgenic mouse lines, their differences in the extent of nAChR binding is likely to be totally unrelated to amyloid accumulation or the presence of high concentrations of Aβ42 peptide.

A rather complex relationship emerges between expression of mutated FAD genes, the resultant Aβ burden and changes in cholinergic plasticity in the brains of transgenic animals created for AD studies. The observed differences in cholinergic parameters between various transgenic mice models of AD could be due to strain background, choice of promoters and level of APP overexpression, including other possible factors. An obvious difference between the two APPswe mouse lines used in these studies is their background. Our mice were practically pure C57BL/6J after backcrossing up to 10 generations, while the APPswe mice in both earlier studies (Dineley et al. 2001, Bednar et al. 2002) were of hybric origin. Secondly, the promoter used to generate two mouse lines differed as well as the extent of APP overexpression. Our APPswe transgenic mice showed only 2-fold overexpression of APP (Liu et al. 2002b), whereas the APPswe (Tg2576) mice have 6-7 fold overexpression (Hsiao et al. 1996). Impairment of the cholinergic neurotransmitter system including a marked loss of cortical and hippocampal nAChRs is a well established feature in the AD brain (Nordberg et al. 1994) and is not apparent in either A/P or APP transgenic mice. It could be that other factors (not present in transgenic mice) emerge during aging in the human brain that also influence the nAChRs during the neurodegenerative processes occurring. One obvious difference between human AD patients and mice with FAD mutations, is the severe cholinergic degeneration in AD patients, whereas degenerative changes in cholinergic terminals are modest in transgenic mice.

In summary, from the findings of this study it is clear that elevated levels of either soluble or insoluble Aβ, *per se*, are insufficient to cause any alterations in the number of binding sites of the two major nAChR subtypes in the brains of A/P mice.

7. SUMMARY AND CONCLUSIONS

The biological effects of Aβ accumulation are subject to intensive research based on the amyloid cascade hypothesis of the pathogenesis of Alzheimer's disease. Using transgenic mice that accumulate large amounts of Aβ in their brains as they age, the current study investigated whether Aβ accumulation can be attributed to four common clinical observations in Alzheimer patients. 1) We investigated whether higher incidence of AD among women than men in older age groups can be related to higher production of Aβ in females in general. 2) We determined whether age-dependent spatial memory impairment correlates with hippocampal Aβ levels. 3) We tested whether reported EEG and ERP alterations in AD patients could be directly related to Aβ accumulation. 4) We assessed whether reduction in the number of the nAChRs in AD patients is related to Aβ accumulation.

First, the Aβ40 and Aβ42 levels increased robustly with age, and the Aβ40 and Aβ42 levels, plaque count and Aβ burden were significantly correlated. There was a significant sex difference in the accumulation of Aβ between female and male A/P mice with age. Female mice showed higher Aβ40 and Aβ42 levels, more plaques and heavier amyloid burden than the males. No plaques were found in APP and PS1 single transgenic mice. These results suggest that A/P transgenic mice develop age-dependent AD-like amyloid pathology and can be a useful tool to study pathogenesis and treatment of AD.

Second, the A/P mice demonstrated age-dependent impairment in the water maze acquisition and retention, and this impairment in spatial memory retention in the water maze was correlated with the total Aβ42 levels in the hippocampus. These results suggest that with Aβ accumulation in the hippocampus, performance of hippocampus-dependent tasks is impaired in A/P mice, and support the notion that Aβ1-42 plays a pivotal role in the development cognitive deficits in AD.

Third, the alterations of EEG and ERPs were largely age-independent in the follow-up experiment and thus did not correlate with Aβ accumulation. However, the alterations of EEG and ERPs in A/P double and APP single transgenic mice were significantly different from PS1 and NT mice. These results suggest that Aβ accumulation contributes little to EEG and ERPs changes in A/P mice, and most of these changes are related to the presence of the APPswe transgene. However, habituation of the N35 response (which corresponds P50 in humans), or so-called auditory gating

was associated with the presence of increased levels of Aβ42. Future studies will show whether recording of P50 will be of help in the early diagnosis and the follow-up of treatment efficacy in AD patients.

Fourth, there was no correlation between Aβ levels and the number of α4β2 and α7 nAChRs in A/P mice from the age of 3 weeks to 17 months. Furthermore, no difference was observed between A/P and NT mice in the number of α4β2 and α7 nAChRs despite dramatic differences in the Aβ levels. These findings clearly demonstrate that accumulation of Aβ alone is not able to lead to reduced number of nAChRs as observed in AD patients or increased number of nAChRs as reported for some other mouse lines with APPswe transgene.

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