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LI LIU

Cholinergic Neurotransmission, Amyloid-β Peptide and the Pathogenesis of Alzheimer's Disease

A study in the APP and PS1 double transgenic mouse model

Doctoral dissertation

To be presented with assent of the Medical Faculty of the University of Kuopio
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ABSTRACT

Alzheimer's disease (AD) is characterized clinically by a progressive decline in cognition, and histopathologically by the presence of senile plaques (consisting mainly of amyloid-β, Aβ) and neurofibrillary tangles in the brain, and the degeneration of basal forebrain cholinergic neurons. Aβ, a 39-43 amino acid long peptide, is derived by proteolytic processing from its precursor, the amyloid precursor protein (APP). Growing evidence indicates that the accumulation and deposition of Aβ in the brain, which may result from overproducion due to APP mismetabolism, or/and a failure in the clearance, is central to the pathogenesis of AD. In recent years, a number of *in vitro* and *in vivo* studies have suggested that cholinergic neurotransmission may be involved in the regulation of APP metabolism. However, the two questions raised by these findings, i.e. whether cholinergic dysfunction would lead to an increased Aβ production and thus deposition of Aβ in AD brain, and whether this process could be prevented by restoring or enhancing cholinergic activity, have not yet been studied in an *in vivo* model of AD. In addition, the question regarding the pivotal role of Aβ overproduction and/or deposition in the development of cognitive impairments in AD needs to be further addressed in a suitable disease model.

In the present study we used APPswe and PS1(A246E) double transgenic mice (AP mice) as an AD model. These mice develop the first amyloid deposits around the age of 9 months and age-related cognitive deficits at a later age. In these mice, we manipulated the level of acetylcholine (ACh) by two methods, 1) by treatment with a cholinesterase inhibitor, metrifonate, to increase the level of ACh, or 2) by fimbria-fornix lesion, to lower the ACh level in the hippocampus, in order to investigate the consequences of these changes on APP metabolism and Aβ deposition. In addition, we investigated the relation between the cognitive deficits and Aβ overproduction and/or deposition in these mice.

We demonstrated that neither an increase nor a reduction in the level of ACh significantly affected APP metabolism, Aβ production and the deposition of Aβ. Our results do not support the idea that cholinergic dysfunction would promote the process of Aβ production and deposition, or that an enhancement in cholinergic activity would slow down the progression of amyloid pathology in AD; conversely they indicate that APP metabolism may not be regulated by the level of ACh, at least in the AP mice that were used in our studies. In the AP mice, the early amyloid deposits are largely restricted to the dorsal hippocampal formation; accordingly the mice showed a selective impairment in hippocampal-dependent long-term spatial memory. Furthermore, the impaired memory significantly correlated with the amount of insoluble Aβ42, but not that of soluble Aβ42 in the hippocampus, or the Aβ levels in the CSF and serum. Together, these findings support the role of insoluble Aβ42 in the development of memory impairments in AD.

National Library of Medicine Classification: WL 359, QV 126
Medical Subject Headings: Alzheimer disease/pathology; amyloid beta-protein precursor/ metabolism; amyloid-beta protein; brain/drug effect/pathology; hippocampus /pathology /physiology; acetylcholine; cholinesterase inhibitors/therapeutic use; memory; cognition; maze learning; disease models, animal; mice, transgenic

Knowledge is power.

Sir Francis Bacon (1561-1626)

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Kuopio, January 2003

Li Liu

ABBREVIATIONS

Aβ	amyloid-β peptide
ACh	acetylcholine
AChE	acetylcholinesterase
AD	Alzheimer's disease
AP	APPswe + PS1(A246E)
APP	amyloid precursor protein
ChAT	choline acetyltransferase
ChEI	cholinesterase inhibitor
CNS	central nervous system
CSF	cerebrospinal fluid
DDVP	dichlorovinyl dimethyl phosphate
FAD	familial Alzheimer's disease
FFX	fimbria-fornix
HDB	horizontal limb of the diagonal band of Broca
huAPP	human APP gene
mAChR	muscarinic ACh receptor
M1, 2, 3, 4	muscarinic ACh receptor subtype-1, -2, -3, -4
MS	medial septum
MTF	metrifonate
NbM	nucleus basalis of Meynert
NFT	neurofibrillary tangle
NT	non-transgenic
PKC	protein kinase C
PS1	presenilin-1
PS2	presenilin-2
RAM	radial arm maze
sAPP	secreted form of APP
Tg	transgenic
TM	transmembrane
VDB	vertical limb of the diagonal band of Broca
WM	Morris water maze

LIST OF ORIGINAL PUBLICATIONS

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

I. Liu L., Ikonen S., Heikkinen T., Tapiola T., van Groen T., Tanila H., The effects of long-term treatment with metrifonate, a cholinesterase inhibitor, on cholinergic activity, amyloid pathology, and cognitive function in APP and PS1 doubly transgenic mice. ***Exp Neurol*** 2002, 173(2): 196-204.

II. Liu L., Ikonen S., Tapiola T., Tanila H., van Groen T., Fimbria-fornix lesion does not affect APP levels and amyloid deposition in the hippocampus of APP + PS1 double transgenic mice. ***Exp Neurol*** 2002, 177(2): 565-74.

III. Liu L., Ikonen S., Heikkinen T., Heikkilä M., Puoliväli J., van Groen T., Tanila H**.**, Effects of fimbria-fornix lesion and amyloid pathology on spatial learning and memory in transgenic APP + PS1 mice. ***Behav Brain Res*** 2002, 134(1-2): 433-445.

IV. Liu L., Tapiola T., Herukka S-K., Heikkilä M., Tanila H., Aβ Levels in serum, CSF and brain, and cognitive deficits in APP + PS1 transgenic mice. ***NeuroReport*** 2003, 14(1): 163-166.

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1. INTRODUCTION

Alzheimer's disease (AD) is the most common cause of dementia in the elderly (Evans et al., 1989). The characteristic pathological features of AD include senile (i.e. neuritic) plaques, neurofibrillary tangles, and the degeneration of basal forebrain cholinergic neurons (Terry et al., 1999; Geula and Mesulam, 1999). The core of senile plaques consists mainly of amyloid-β (Aβ) (Glenner and Wong, 1984; Masters et al., 1985), a predominantly 39-43 amino acid long peptide derived by proteolytic processing from its precursor, the amyloid precursor protein (APP) (Robakis et al., 1987; Tanzi et al., 1987).

APP is proteolytically processed via alternative pathways. The cleavage of APP by the α-secretase occurs within the Aβ domain, generating a large secreted form of APP (sAPP-α) and leaving a 83 amino acid long membrane-retained C-terminal fragment (Esch et al., 1990), and this will therefore preclude the formation of Aβ. The Aβ peptides are produced when APP has undergone two sequential cleavages by the β- and γ-secretase, and this also produces a secreted APP derivative (sAPP-β) (Selkoe, 2001). Multiple lines of evidence indicate that an aberrant APP metabolism leads to overproduction and, eventually, deposition of Aβ in the brain, the process that is considered to be central to the pathogenesis of AD (Selkoe, 2001).

In recent years, a number of studies have suggested that cholinergic neurotransmission may be involved in the regulation of APP metabolism (reviewed by Roberson and Harrell, 1997, and Rossner et al., 1998). It has been demonstrated in *vitro* that stimulation of M1 and M3 acetylcholine receptors with muscarinic receptor agonists or treatment with cholinesterase inhibitor (ChEI) greatly increases the release of secreted APP from cultured cells or brain slices, thereby leading to reduced formation of Aβ, presumably by the activation of the α-secretase pathway (e.g. Nitsch et al., 1992; Mori et al., 1995b; Pakaski et al., 2000; Nitsch and Growdon, 1994). On the other hand, cholinergic deafferentation in animals was shown to result in an elevated APP expression or production in the cerebral cortex or hippocampus (e.g. Wallace et al., 1993; Leanza, 1998; Lin et al., 1998), though whether this would lead to increased production of Aβ, and Aβ deposition has not yet been clarified due to a lack of *in vivo* AD models (Beach et al., 2000; Boncristiano et al., 2002). Together, these findings

imply a role of cholinergic dysfunction in the promotion of Aβ production, and, further, a potential neuroprotective effect of ChEIs or muscarinic receptor agonists in preventing the disease progress by restoring or enhancing cholinergic activity; these questions warrant further investigation in a suitable *in vivo* model of AD.

Aggregated Aβ has been suggested to be neurotoxic by many studies (Lorenzo and Yankner, 1996; Harkany et al., 2000); however, the relation between amyloid deposition and cognitive impairments in AD is still controversial. It was shown by several studies that the loss of synapses but not the number of amyloid plaques correlates with cognitive deficits in AD (reviewed by Terry et al., 1999). In contrast, several recent studies have suggested that the number of amyloid plaques or the Aβ load does correlate with cognitive dysfunction (Cummings and Cotman, 1995; Cummings et al., 1996; Haroutunian et al., 1998; Kanne et al., 1998; Parvathy et al., 2001). In addition, the role of soluble Aβ behind the dementia symptoms was also emphasized by recent human neuropathological data (McLean et al., 1999). With the advent of new therapeutic approaches, such as immunization against Aβ (Schenk et al., 2001), treatment with amyloid β-sheet breakers (Soto et al., 1998), and with β- and γ-secretase inhibitors (Ghosh et al., 2002; Wolfe, 2002), it becomes essential to determine the role of Aβ in the development of cognitive impairments in AD.

During the last several years, many groups have generated transgenic mouse models that develop amyloid deposits and cognitive deficits with age (Janus and Westaway, 2001; Price and Sisodia, 1998), providing an opportunity to directly study the factors that are involved in amyloid deposition and cognitive deficits. In the present study, we have used APPswe and PS1(A246E) double mutant transgenic mice (AP mice) (Borchelt et al., 1997) as an AD model. We manipulated the levels of ACh by using metrifonate, a second generation ChEI, and further we transected the fimbria-fornix in these mice, to investigate the consequences on APP metabolism and amyloid deposition in the hippocampus. In addition, we also investigated the relation between Aβ overproduction, deposition and cognitive decline in these AP mice.

2. REVIEW OF THE LITERATURE

2.1 Alzheimer's disease (AD)

Alzheimer's disease (AD) was named after the German physician Alois Alzheimer, who reported in 1907 a case of a presenile dementia in a 51-year-old woman (Alzheimer, 1907). Since the late 1960s, AD has gradually been recognized as the most common cause of senile dementia (Selkoe, 2001). Currently, AD accounts for about 50 to 60% of the cases of cognitive impairment in elderly patients (Lobo et al., 2000; von Strauss et al., 1999). The prevalence of AD increases after the age of 60 from about 1% among the 60- to 64-years-old to up to 40% of those aged 85 years and older (von Strauss et al., 1999).

2.1.1. Clinical features of AD

Clinically, AD is characterized by a progressive loss of cognitive abilities. The majority of patients exhibit dementia after 60 years of age, while a small portion of patients with familial AD (FAD) often become demented in their midlife (Price and Sisodia, 1998). The memory impairment is selective as to the type and its time span. Declarative memory, i.e. the conscious recollection of facts and events, is affected; whereas procedural memory, i.e. the memory for skills and habits, is largely intact (Forstl and Kurz, 1999; Rossor et al., 1996; Squire and Zola, 1998). Further, memory for recent events that occurred in a particular spatial and temporal context, i.e. episodic memory, declines early in the course of the disease; whereas semantic memory for general facts is impaired only later in the disease (Almkvist, 1996; Rossor et al., 1996; Squire and Zola, 1998). Gradually, language impairments, visual perceptual and spatial deficits, agnosias and apraxias emerge as the disease progresses (Rossor et al., 1996). Meanwhile, the patients are also increasingly impaired in their daily living activities, and in late stages of AD, patients often become mute, incontinent, and bedridden (Price and Sisodia, 1998).

2.1.2. Neuropathological features of AD

The definite diagnosis of AD requires neuropathological examination of the brain at autopsy. The currently used diagnostic criteria of AD are based on either the assessment of amyloid plaque frequency (i.e. NIA criteria, Khachaturian, 1985 and

CERAD, Heyman et al., 1990), or neurofibrillary pathology stageing (Braak and Braak, 1991), or both (The National Institute on Aging, and Reagan Institute Working Group on Diagnostic Criteria for the Neuropathological Assessment of Alzheimer's Disease, 1997). Macroscopic changes found in late AD brains include shrinkage of the gyri and widening of the sulci especially in medial temporal lobes, and enlargement of the ventricles; and the two major microscopical lesions are extracellular amyloid plaques and intracellular neurofibrillary tangles (Esiri, 2001). In AD, progressive neurodegeneration starts from the medial temporal lobe, i.e. the entorhinal cortex, hippocampus and subiculum, and later extends to other neocortical regions, particularly association areas, as well as subcortical areas, such as the basal forebrain cholinergic system, and several brain stem monoaminergic nuclei (Price and Sisodia, 1998; Geula, 1998).

2.1.2.1. Amyloid plaques

Two major types of amyloid plaques are recognized in the brain of AD patients (Terry et al., 1999), i.e. neuritic and diffuse plaques. The classical neuritic plaque is a small, spherical structure with a 50 to 200 μm diameter, which has a dense amyloid core and is surrounded by dystrophic neurites (Cummings et al., 1998b). Reactive astrocytes and microglia are found in the plaque periphery and around the plaque (Cummings et al., 1998b). Most of the Aβ found in neuritic plaques is the 42 amino acid long form of Aβ (i.e. Aβ42), with the 40 amino acid long Aβ40 co-localized (Selkoe, 2001). In contrast, diffuse plaques, the second type of amyloid plaque, *do not* contain an amyloid core and are *not* surrounded by dystrophic neurites (Cummings et al., 1998b). The amyloid deposits in the diffuse plaques are non-fibrillar (Selkoe, 2001) and are almost comprised exclusively of Aβ42, with little or no Aβ40 present (Iwatsubo et al., 1994).

2.1.2.2. Neurofibrillary tangles

Many brain regions that are typically affected early in AD contain large numbers of neurofibrillary tangles, such as the entorhinal cortex, hippocampus, parahippocampal gyrus, and amygdala (Selkoe, 2001). These tangles consist of pairs of ~10-nm filaments twisted into the form of paired helical filaments, and they occupy much of the perinuclear cytoplasm and may extend into the dendrites but do not occur in axons (Selkoe, 2001). The filaments are composed of the microtubule-associated protein tau,

which is abnormally hyperphosphorylated (Grundke-Iqbal et al., 1986; Kosik et al., 1986; Wood et al., 1986). Since hyperphosphorylated tau binds poorly to microtubules and thus alters their stability, these modifications of tau could disrupt intracellular transport, cellular geometry, and neuronal viability, eventually causing cell death (Price and Sisodia, 1998). Large pyramidal cells are the neurons that mostly develop neurofibrillary tangles, particularly those with long cortico-cortical connections (Cummings et al., 1998b).

2.1.2.3. Neuronal loss and synaptic alterations

Neuronal loss has been observed in a number of cortical and subcortical regions (Cummings et al., 1998b); within the neocortex, the frontal and temporal lobes are typically involved. Further, in the early stages of AD pathology massive neuronal loss is regularly observed in the superficial entorhinal cortex (the major input of the hippocampus), and in the major hippocampal efferent area, i.e. the subiculum (Geula, 1998). Therefore, it has been suggested that the cognitive decline in AD is at least partially attributable to the functional isolation of the hippocampus (Geula, 1998). In addition, the cognitive decline can also be related to the disruption of the structural integrity of synapses (Terry et al., 1999). In the AD brain, presynaptic terminal density is significantly decreased in the neocortex, most remarkably in frontal cortex and hippocampus (Honer et al., 1992; Scheff et al., 1990; Terry et al., 1999). Further, synaptic density is greatly decreased in regions with many neuritic plaques, but not diminished in areas with diffuse plaques (Masliah et al., 1990).

2.2. Amyloid-β peptide and the pathogenesis of AD

Amyloid-β peptide (Aβ) is the main component of amyloid plaques (Glenner and Wong, 1984; Masters et al., 1985). Based on genetic findings in FAD and observations in Down's syndrome, the role of Aβ in the pathogenesis of AD has been formulated as the "amyloid cascade hypothesis" by Hardy and Higgins (1992) a decade ago. It proposes that a dysregulation in APP processing occurs early in the disease process, resulting in increased production of the highly self-aggregating Aβ42. The aggregated Aβ is then supposed to induce all subsequent pathology, including tau hyperphosphorylation, tangle formation, and neuronal death.

2.2.1. APP metabolism and Aβ production

Aβ is derived from its large precursor protein (i.e. APP) by proteolysis, and only a trace amount of Aβ can be detected under physiological contitions (Haass et al., 1992; Shoji et al., 1992; Seubert et al., 1992). However, an aberrant APP metabolism will lead to overproduction of Aβ, the process that has been suggested to be central to the pathogenesis of AD (Selkoe, 1997).

APP is a single transmembrane domain protein, and it is ubiquitously expressed in a variety of tissues, including brain (Selkoe, 1999). In human, there are three major isoforms that have 695, 751, and 770 residues, respectively (Figure 1A). The two longer forms, APP751 and APP770 contain a 56 amino acid domain (Sinha and Lieberburg, 1999) that is homologous to a Kunitz-type of serine protease inhibitor (KPI) (Figure 1A). APP751 and APP770 are widely expressed throughout the body, but in the brain it is mainly expressed by glial cells (Rohan de Silva et al., 1997). APP695, which lacks the KPI domain, is the most abundant isoform in the brain and expressed predominantly by neuronal cells, with a very low level of expression in non-neuronal cells.

APP has a short half-life (about 20-30 minutes) (Weidemann et al., 1989), and it is metabolized down two pathways after biosynthesis, either the non-amyloidogenic α-secretase pathway or the amyloidogenic β-secretase pathway (Hardy, 1997). Under normal circumstances the α-secretase pathway is predominant; it cleaves APP between amino acids 16 and 17 of the Aβ region, which therefore precludes the production of Aβ (Esch et al., 1990) (Figure 1A and B). This cleavage creates a large, soluble ectodomain fragment (sAPP-α) that is subsequently released, and a membrane-retained C-terminal fragment (CTF) consisting of 83 amino acids (i.e. C83) (Selkoe, 2001). A smaller amount of APP is processed by the β-secretase, which cleaves APP between residues 671 and 672 (Figure 1C). This releases a slightly truncated form of sAPP (sAPP-β) from the cell (Schubert et al., 1989) and leaves a 99-residue CTF (i.e. C99) at the membrane. The C99 can subsequently be cleaved by the γ-secretase at either residue 711 or residue 713 to create Aβ, and the site of cleavage by the γ-secretase determines whether the long or short form of Aβ (Aβ42 or Aβ40, respectively) is

produced (Hardy, 1997). The C83 CTF produced by α-secretase can also undergo cleavage by the γ-secretase to generate the p3 peptides (Figure 1B) (Selkoe, 2001).

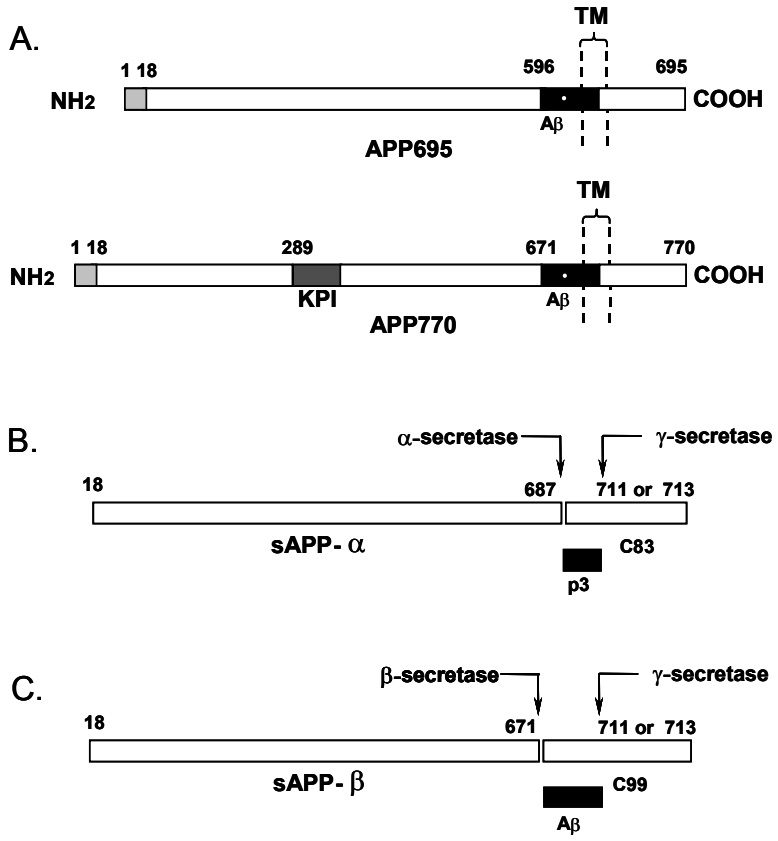

Figure 1. Schematic diagrams of the structure of the APP protein with the two pathways that process APP indicated. (A). APP has a single membrane-spanning domain (TM), and a 17-residue signal peptide at the N terminus. The diagram illustrates two APP alternate splice forms, which comprise 695 and 770 amino acids, respectively (i.e. APP695 and APP770). APP770 contains a serine protease inhibitor domain of the Kunitz type (KPI). (B) α-secretase cleaves APP after residue 687, resulting in the secretion of the large, soluble ectodomain of APP (sAPP-α) into the medium and retention of carboxy-terminal fragment C83 in the membrane. The C83 fragment can undergo cleavage by γ-secretase at residue 711 or residue 713 to release the p3 peptides. (C) Alternatively, β-secretase cleaves APP after residue 671, resulting in the secretion of the slightly truncated sAPP-β, and the retention of C99, which can be further cleaved by γ-secretase at residue 711 or residue 713 to release the Aβ40 or Aβ42 peptides. (modified from a figure by Selkoe, 1999).

Despite intensive study, the functions of APP and its fragments are not yet very well understood. Soluble sAPP-α appears to be capable of acting as an autocrine factor (Saitoh et al., 1989) and as a neuroprotective and neurotrophic factor (Mattson et al.,

1993). Further, APP has been suggested to function in cell-to-cell interactions (Qiu et al., 1995). However, most of these functions have not yet been confirmed *in vivo*. On the other hand, mice with a targeted deletion of the APP gene appear to be relatively normal, perhaps because of the fact that they still express the amyloid precursor-like proteins (APLPs), which are homologous to APP (Slunt et al., 1994).

2.2.2. Genetic mutations in AD

Since the early 1990s, it has been shown that AD can be caused by autosomal dominant mutations in three genes: **APP** on chromosome 21, **presenilin-1** on chromosome 14 (PS1) and **presenilin-2** on chromosome 1 (PS2) (reviewed by Selkoe, 2001). These mutations are estimated to account for about half of the early-onset familial AD cases (FAD), i.e. about 2.5% of all AD cases (Blacker and Tanzi, 1998; Price et al., 1998).

APP mutations typically lead to AD at about 45 to 60 years of age. All mutations are located closely to the cleavage sites of either β-secretase or γ-secretase (Selkoe, 2001). The "Swedish mutation", a double mutation that occurs in the two amino acids immediately prior to the β-secretase cleavage site, induces increased production of both Aβ40 and Aβ42 by enhancing β-secretase cleavage (Citron et al., 1992; Mullan et al., 1992; Selkoe, 2001). The APP mutations all have in common that they lead to the production of more Aβ42 or to a change in the aggregation properties of Aβ (Hardy, 1997), thereby predisposing to the development of AD. Studies of Down's syndrome strongly support the crucial role of Aβ in the pathogenesis of AD. These patients overexpress normal APP due to an extra copy of chromosome 21 (Petronis, 1999; Potter, 1991), and this leads to an overproduction of APP and Aβ (Tokuda et al., 1997). Almost invariably, Down's syndrome patients first develop diffuse plaques, which solely consist of Aβ42, in their teenage years (Lemere et al., 1996) and eventually develop neuropathology that is very similar to AD by middle age.

PS1 mutations account for most of the reported FAD cases and generally lead to an onset of AD before the age of 50 (Selkoe, 2001). In contrast to PS1 mutations, mutations in PS2 are relatively rare (Sherrington et al., 1996). PS1 and PS2 are transmembrane (TM) proteins with six to nine TM domains (Levy-Lahad et al., 1995; Sherrington et al., 1995), and they show a high degree of homology to each other,

especially in their putative TM domains. Similar to APP, presenilins are also expressed ubiquitously, and the majority of the presenilin protein is cleaved to yield a ~25 kDa N-terminal fragment and a ~19 kDa C-terminal fragment (Mercken et al., 1996; Thinakaran et al., 1996).

PS1 has been proposed to play a role in protein trafficking within the cell (Naruse et al., 1998). In addition, PS1 is needed for *Notc*h-mediated signaling during embryogenesis (Ray et al., 1999); it has been shown that PS1 knockout mice display abnormalities in body segmentation (Conlon et al., 1995; Wong et al., 1997). The effects of the presenilin mutations on APP metabolism have been well documented. Cells expressing the FAD mutant form of PS1 have an increased generation of Aβ42, thus increasing the potential for Aβ aggregation (Borchelt et al., 1996b; Citron et al., 1997). Correspondingly, transgenic mice expressing both the mutant forms of APP and PS1 demonstrate an accelerated rate of amyloide deposition presumably due to the increase of Aβ42 production (Borchelt et al., 1997; Holcomb et al., 1998).

Taken together, common to all these genetic mutations is the property to increase the production of Aβ, especially Aβ42, or enhance Aβ aggregation in the brain. These observations suggest the causative role of Aβ in the pathogenesis of AD (Selkoe, 2001).

*2.2.3. A*β *and the pathogenesis of AD*

The Aβ peptide mainly exists in two forms: as a soluble peptide or in an aggregated state as insoluble amyloid deposits, which can have the β-pleated sheet conformation (Selkoe, 2001). The deposition of Aβ42 is considered to be an early event in AD, followed by the deposition of Aβ40 (Jarrett et al., 1993). It has been suggested that the aggregated form of the Aβ peptide is the primary neurotoxic agent both *in vitro* (Pike et al., 1993; Lorenzo and Yankner, 1994) and *in vivo* (Giovannelli et al., 1998). The gradual accumulation and deposition of Aβ appear to activate adjacent microglia and astrocytes, with the concomitant release of cytokines and acute-phase proteins (McGeer and McGeer, 1995). Subsequently, local neurons and their processes could be injured by the inflammatory response. In addition, Aβ may further injure neurons by disrupting calcium homeostasis (Joseph and Han, 1992) or by inducing oxidative damage (Keller et al., 1997; Thomas et al., 1996). The accumulation of Aβ may also

induce or accelerate the phosphorylation of tau and thus the formation of paired helical filaments and NFTs (Selkoe, 2001). Eventually, these pathological events could lead to synaptic loss, shrinkage of neuronal perikarya, neuronal dystrophy, and selective neuronal loss, as well as neurotransmitter deficits, including the basal forebrain cholinergic system (Selkoe, 2001).

Although the neurotoxicity of aggregated Aβ has been suggested by many studies (Lorenzo and Yankner, 1996; Harkany et al., 2000), the correlation between amyloid plaques and cognitive impairments in AD is still controversial. It has been shown by earlier studies that the loss of synapses but not the number of amyloid plaques correlates with cognitive deficits in AD (reviewed by Terry et al., 1999). In contrast, several recent studies have shown that amyloid plaques do correlate with cognitive dysfunction (Cummings and Cotman, 1995; Cummings et al., 1996; Haroutunian et al., 1998; Kanne et al., 1998). However, in some transgenic AD mouse models, cognitive deficits occur in the absence of amyloid deposits (D'Hooge et al., 1996; Koistinaho et al., 2001; Moechars et al., 1999). Further, Mucke et al. (2000) observed that mutant or wildtype human APP (huAPP) transgenic mice show loss of synapses regardless of whether amyloid deposits were present. Together, these findings suggest that plaque-independent Aβ neurotoxicity could also play an important role in the neuropathology of AD. Recent studies suggest that such toxic effects might be attributed to the neurotoxicity of Aβ oligomers and protofibrils (Klein et al., 2001). Further, it has been suggested that Aβ per se is only a marker for proteolytic cleavage of APP and that it is the transcriptionally active carboxy-terminal of APP itself that is involved in the pathogenesis of AD (Cao and Sudhof, 2001).

2.2.4. Transgenic mouse models of AD

Before the identification of AD-causing gene mutations, animal models of AD were limited to reproduce other aspects of neuropathology, such as neuronal loss or cholinergic depletion (Chapman et al., 2001). Now, multiple lines of transgenic AD model mice have been created that have Aβ deposition and amyloid plaques, the key pathological hallmark of AD, as well as age-related learning and memory deficits (Janus and Westaway, 2001; Seabrook and Rosahl, 1999). These transgenic mouse models offer new opportunities to investigate the biochemical and cellular mechanisms of this

disease, and also provide systems to test potential therapeutics (Price and Sisodia, 1998). APP transgenic mice include mice expressing wild-type huAPP751 on the mouse APP background (Buxbaum et al., 1993a), the truncated C-terminus of APP (Kammesheidt et al., 1992), and mutations associated with FAD including APP(V717F) (PDAPP mouse)(Games et al., 1995), APP695(K670N, M671L) (Tg2576 mouse) (Hsiao et al., 1996), and APP751(K670N,M671L) (Sturchler-Pierrat et al., 1997), etc. The PDAPP and the Tg2576 transgenic mice are most widely used APP single mutant AD models (see Table 1). Most of these mice age-dependently overproduce Aβ and develop amyloid plaques, but none of these mice have been shown to display remarkable neuronal loss and formation of NFTs, which are also characteristic of AD (Chapman et al., 2001).

PS1 transgenic mice overexpressing mutant forms of human PS1 transgene on the mouse PS1 background (Borchelt et al., 1996b; Citron et al., 1997; Duff et al., 1996) do not exhibit amyloid deposition, despite the increase of Aβ1-42/43 production and an elevated ratio of Aβ42 to Aβ40. However, in APP and PS1 double mutant mice, the increase in the ratio of the highly amyloidogenic Aβ42 relative to Aβ40 is greatly enhanced compared with transgenic lines overexpressing either mutant APP or mutant PS1 alone (Borchelt et al., 1997; Citron et al., 1997; Holcomb et al., 1998). Accordingly, the neuropathology is greatly accelerated in double transgenic mice. Mice expressing both APPswe and the PS1(M146L) mutation (Holcomb et al., 1998) show amyloid deposits in the cerebral cortex and hippocampus already at 13-16 weeks of age in comparison to 9-12 months in the single APPswe mouse line. In the APPswe mouse line expressing a chimeric mouse/human (mo/hu) APP695 harboring a human Aβ domain and Swedish AD mutations, amyloid deposition was accelerated from 18 months of age to 9 months of age when PS1(A246E) mutation is also present (Borchelt et al., 1997). In this APP+PS1 mouse line (i.e. AP mice that we used), the formation of amyloid plaques starts in the hippocampus, subiculum and caudal cortex, and later extends to all cortical areas (Borchelt et al., 1997).

Among the two APP+PS1 mouse lines, the APPswe and PS1(M146L) line has been extensively studied both histopathologically and behaviourally. Aβ deposition started primarily in the cingulate cortex of APP+PS1 mice at approximately 10 weeks of age, and by the age of 6 months extensive Aβ depostion can be detected in the

hippocampus and cortex (McGowan et al., 1999). The early Aβ deposits were found to be primarily composed of fibrillar Aβ and resembled compact amyloid plaques (Gordon et al., 2002). As the mice aged, the fibrillar Aβ deposits remained restricted to cortical and hippocampal structures, while non-fibrillar Aβ deposits increased in number and spread to regions, such as striatum and cerebellum, that not typically associated with amyloid plaques in AD. In these doubly transgenic mice, the diffuse deposits are primarily composed of Aβ42, while the compact deposits contain both C-terminal forms of the peptide. The fibrillar deposits were associated with dystrophic neuritis, and the activation of microglia and astrocytes (Gordon et al., 2002). From the age of 5-7 months to 15-17 months, a progressive cognitive impairment was observed in transgenic mice for both water maze acquisition and radial arm water maze working memory (Arendash et al., 2001). In the radial arm water maze task, the performance of individual mice within the transgenic group was inversely correlated with the amount of Aβ deposited in the frontal cortex and hippocampus (Gordon et al., 2001), implying that APP+PS1 transgenic mice develop deficits in cognitive ability as Aβ deposits increase. However, transgenic mice did not show impairment in performing the tasks, such as Y-maze alternations, circular platform performance, standard water maze retention, or visible platform recognition at either age (Arendash et al., 2001). Compared with the APP+PS1(M146L) mice, the pathological and behavioural characteristics of the APP+PS1(A246E) mice (i.e. our AP mice) have been much less studied (Borchelt et al., 1997).

A comparison of histopathological and behavioural features of several representative transgenic mouse models of AD is shown in Table 1.

Table 1. Representative transgenic mouse models of Alzheimer's disease

Tg mouse line	Construct	Neuropathology	Learning and memory
APP Tg mouse			
Hsiao et al., 1996 (Tg2576 mouse)	HmPrP: APP695swe (K670N+M671L)	Amyloid plaques ($\geq$ 12 months) Astrocytosis Microgliosis No cell loss and NFT	Deficits in Morris water maze Reduced spontaneous alternation performance in Y-maze
Games et al., 1995 (PDAPP mouse)	PDGF: APPV717F	Amyloid plaques ($\geq$ 6 months) Astrocytosis Microgliosis Synapse loss No cell loss and NFT Hippocampal atrophy	Deficits in modified water maze Deficits in a spatial discrimination learning task in radial arm maze Deficits in a spontaneous object recognition task
Borchelt et al., 1997	MoPrP: APP695swe (K670N+M671L)	Amyloid plaques ($\geq$ 18 months) Astrocytosis Microgliosis No cell loss and NFT	Not studied
PS1 Tg mouse			
Duff et al., 1996	PDGF: PS1M146L and M146V	Elevated Aβ42 level without amyloid plaque	No cognitive deficits
Borchelt et al., 1996	MoPrP: PS1A246E	Elevated Aβ42 level without amyloid plaque	No cognitive deficits
APP+PS1 Tg mouse			
Holcomb et al., 1998 (APP+PS1M146L mouse)	HmPrP: APP695swe (K670N+M671L) x PDGF: PS1M146L	Amyloid plaques ($\geq$ 3 months) Astrocytosis Microgliosis No cell loss and NFT	Deficits in Morris water maze acquisition Deficits in radial arm water maze working memory
Borchelt et al., 1997 (APP+PS1A246E mouse)	MoPrP: APP695swe (K670N+M671L) x PS1A246E	Amyloid plaques ($\geq$ 9 months) Astrocytosis Microgliosis No cell loss and NFT	Not studied (subject of the present study)

APP: amyloid precursor protein; PS1: presenilin 1; HmPrP: hamster prion protein promoter; MoPrP: mouse prion protein promoter; PDGF: platelet-derived growth factor promoter

2.3. Cholinergic system and AD

2.3.1. Cholinergic degeneration in AD and cholinergic hypothesis

In human, the basal forebrain cholinergic system consists of four cell groups that are located in the medial septum (MS-Ch1), the vertical and horizontal limb of the diagonal bands of Broca (VDB-Ch2 and HDB-Ch3, respectively), and the nucleus basalis of Meynert (NbM-Ch4) (Geula and Mesulam, 1999). In the non-human primate (rhesus monkey), the MS-Ch1 and VDB-Ch2 neurons provide the major cholinergic innervation of the hippocampus, the HDB-Ch3 neurons provide the major cholinergic innervation of the olfactory bulb, and the NbM-Ch4 neurons provide the major cholinergic innervation of the entire cortical mantle and the amygdala (Mesulam et al., 1983).

A consistent feature of the AD brain at autopsy is the loss of basal forebrain cholinergic neurons (Davies and Maloney, 1976) that has been reported for all four cholinergic cell groups, with the NbM-Ch4 neurons affected most severely (Geula and Mesulam, 1999). The loss of cholinergic neurons in the basal forebrain causes a dramatic reduction of ChAT activity (up to 95%) in the neocortex (e.g. Davies and Maloney, 1976 and Bowen et al., 1976) and hippocampus (e.g. Araujo et al., 1988), and it has been speculated that this would likely result in a marked decrease of acetylcholine (ACh) levels in above regions (Bartus et al., 1982). Acetylcholinesterase (AChE), the enzyme responsible for the degradation of ACh, is also reduced in AD (Perry et al., 1978). In addition, changes in cholinergic receptors in AD have been reported. Muscarinic M1 receptors are relatively preserved in AD, whereas M2 and nicotinic receptors are significantly decreased (Geula and Mesulam, 1999). Furthermore, the cognitive deficits induced by centrally active anticholinergic compound (i.e. scopolamine) in young human subjects somewhat resembled that seen in AD (Drachman and Leavitt, 1974). Together, these observations formed the basis of the "Cholinergic hypothesis of AD", which stated that the cholinergic dysfunction plays an important role in the memory loss and related cognitive problems (Bartus et al., 1982). However, later studies have suggested that the cognitive deficits in AD are unlikely to be caused solely by one neurotransmitter system, because selective lesioning of the cholinergic neurons in the basal forebrain by immunotoxin 192IgG-saporin does not

lead to significant learning and memory impairment but rather attention deficits in rats (Muir, 1997). Other neurotransmitter systems that are affected by the disease, such as the glutamatergic, serotonergic and noradrenergic systems (Procter et al., 1988; Palmer et al., 1987; Mann, 1983), may also contribute to the cognitive deficits in AD.

2.3.2. Cholinergic therapeutic strategy of AD

The cholinergic hypothesis of AD has provided the rationale for the current major therapeutic approach to the disease, which is aimed to correct the cognitive decline by enhancing cholinergic neurotransmission. Of the multiple possible strategies for enhancing cholinergic neurotransmission in the brain, cholinesterase inhibition has been so far the most extensively used. Cholinesterase inhibitors (ChEI) prevent the hydrolysis of ACh, thus increasing cholinergic transmission by making more ACh available. Giacobini (2001) compared the therapeutic effects of six ChEIs (including metrifonate) based on the results of clinical studies in AD patients. All six ChEIs produced statistically significant but modest improvements in both cognitive and non-cognitive function. It is noteworthy that in the placebo group there is a continuing cognitive decline with little or no change in the drug-treated patients over the trial course. This observation suggests that ChEIs may possess cognitive stabilization effect via a mechanism of action other than symptomatic or directly cholinergic (Giacobini, 2001).

Metrifonate (MTF), a representative cholinesterase inhibitor of the second generation, is an organophosphorous compound (Figure 2), which is converted by a nonenzymatic process to the active AChE inhibitor 2,2-dichlorovinyl dimethyl phosphate (DDVP, Figure 2) (Hinz et al., 1996a; Hinz et al., 1996b). DDVP binds to the active site of AChE and irreversibly inhibits the enzyme; therefore, the recovery of AChE is dependent on synthesis of new enzyme (Hinz et al., 1996b). Although the elimination half-life of DDVP is 2-3 hours, the half-life of cholinesterase inhibition by DDVP is as long as 2 -6 days (Ringman and Cummings, 1999). Animal studies have demonstrated the efficacy of MTF in ameliorating learning and memory deficits in both rats (Riekkinen et al., 1997) and mice (Ikonen et al., 1999) that have lesions in the cholinergic system. In double-blind, placebo-controlled studies in AD patients, MTF led to improvements of cognition or a reduced rate of decline of cognition compared

with placebo (Cummings et al., 1998a), and it also benefited the global function of these patients (Morris et al., 1998). However, adverse effects were noted in clinical trials, specifically a number of cases involving respiratory paralysis, caused the withdrawal of MTF from clinical trials.

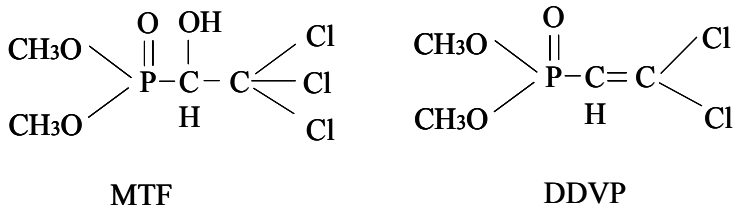

Figure 2. Schematic diagrams of the chemical structure of metrifonate (MTF), and its derivative dichlorovinyl dimethyl phosphate (DDVP). DDVP binds to the active site of the AChE and irreversibly inhibits the activity of the enzyme (re-drawn form a figure by Taylor, 1998).

2.3.3. Cholinergic neurotransmission and APP metabolism

Several lines of evidence suggest that modulating cholinergic neurotransmission can influence the metabolism of APP.

First, stimulation of M1 and M3 receptors with muscarinic receptor agonists or ChEI greatly increases the production of secreted APP with concomitant decreases in Aβ generation. It was first reported by Nitsch et al. (Nitsch et al., 1992), using cells that were stably transfected with human mAChRs (M1, M2, M3, M4), that carbachol, a non-selective muscarinic receptor agonist, increased the amount of secreted APP released in cells expressing M1 and M3 receptors, but not in cells expressing the M2 or M4 subtypes. The increase of APP secretion could be blocked by the muscarinic antagonist atropine or by protein kinase C (PKC) inhibitors, suggesting that PKC mediates the receptor-controlled APP secretion. In addition, increased APP secretion from rat brain slices and cell cultures was also observed after indirect activation of muscarinic ACh receptors via the inhibition of AChE (Mori et al., 1995b; Pakaski et al., 2000). Further, increased APP secretion has been demonstrated to be associated with a reduction of Aβ generation. Hung et al. (1993) have shown that in cell lines overexpressing mAChR and huAPP with AD-associated mutations, the increase in APP secretion after mAChR stimulation is accompanied by a 50% reduction in the release of soluble Aβ and by an

increase in the generation of the non-amyloidogenic p3 fragment. Based on these findings, it was postulated that the activation of M1/M3 mAChR-associated signaling pathways enhances α-secretase activity but decreases β-secretase processing of APP (Nitsch and Growdon, 1994). In addition, it should be noted that few studies reported that treatment with nicoitine, both *in vitro* and *in vivo*, could also modify APP processing by increasing the release of secreted form of APP (Kim et al., 1997; Lahiri et al., 2002).

Second, a reduction in cholinergic neurotransmission has been linked to enhancesed APP expression/production, which may potentially lead to an increase in Aβ production. In rats, excitotoxic lesions of the cholinergic basal forebrain or transection of fimbria-fornix have been reported to increase the levels of APP mRNA or APP immunoreactivity in the cerebral cortex, or hippocampus (e.g. Wallace et al., 1991; Wallace et al., 1993; Beeson et al., 1994). Similarly, selective cholinergic lesions of basal forebrain have also been shown to increase APP gene expression or protein level in rats (Leanza, 1998; Lin et al., 1998) and in marmosets (Ramirez et al., 2001). However, whether an enhanced APP expression/production would lead to increased production of Aβ, and further the deposition of Aβ has not yet been clarified due to a lack of *in vivo* AD models (Beach et al., 2000; Boncristiano et al., 2002). On the other hand, it should be noted that sevral studies have reported contrasting results showing a decrease or no changes in the APP levels after lesion of the cholinergic basal forebrain (Ramirez et al., 2001; Apelt et al. 1997; Rossner et al., 1997).

Together, these findings imply a role of cholinergic dysfunction in the promotion of Aβ production, and, further, support the notion that treatment with ChEIs may slow down the disease progression by modulating APP processing into the less amyloidogenic direction (Giacobini, 2001).

2.3.4. Hippocampus, septohippocampal cholinergic pathway, and fimbria-fornix lesion

One of the main outputs of the basal forebrain cholinergic system of rats is through the septohippocampal pathway, i.e. the projection from the medial septal nucleus and diagonal band of Broca to the hippocampal formation, the other main output are the projections to the neocortex.

We use the term hippocampal formation to encompass three subregions: the dentate gyrus, hippocampus proper, and subiculum (Amaral and Witter, 1995; Amaral and Witter, 1989). Typically, in the rat the hippocampus proper is subdivided into three subregions, i.e. CA1, CA2, and CA3 (Figure 3). The main population of neurons consists of a dense, thin layer of large pyramidal cells, which form stratum pyramidale (sp), the layer above stratum pyramidale is stratum oriens (so), the layer immediately below stratum pyramidale is stratum radiatum (sr), and the layer near the fissure is called the stratum lacunosum-moleculare (slm). The dentate gyrus is subdivided in a molecular layer (stratum moleculare, sm), the granule cell layer and a polymorphic cell layer (Figure 3). The subiculum has three principal layers, i.e. the molecular layer, an enlarged pyramidal cell layer containing the soma of the principal neurons, and a deep polymorphic layer (not illustrated). The principal cell layer of the subiculum is mainly populated with large pyramidal neurons; among these are many smaller neurons, which are considered the interneurons of the subiculum (O'Mara et al., 2001).

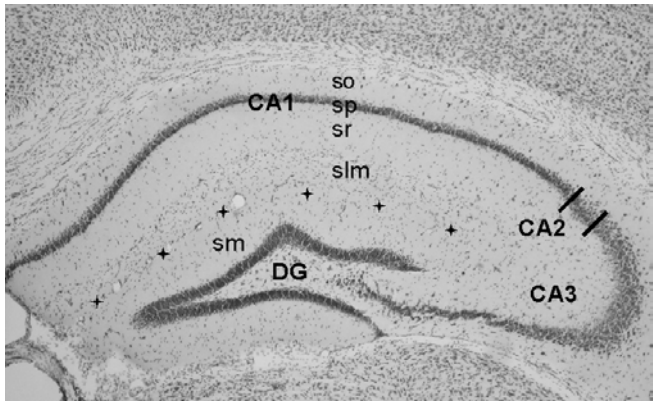

Figure 3. A photomicrograph of a Nissl stained coronal section through the dorsal hippocampus of the mouse shows the anatomy and the laminar organization of the hippocampus.

The septal complex is divided into two parts: the medial septum/diagonal band of Broca (MS/DB) complex and the lateral septum (LS) (Figure 4). The MS/DB complex is divided into medial septal nucleus (MS) and nucleus of the diagonal band of Broca (DB). The DB is composed of two parts: the vertical limb of DB (VDB) and the horizontal limb of DB (HDB). The cholinergic innervation of the hippocampus mainly

originates from the MS and the HDB and VDB, and reaches the hippocampal formation via three anatomically distinct routes (Cassel et al., 1997), i.e. the cingulum bundle, the fimbria-fornix, and a ventral pathway (Figure 4). The fimbria-fornix pathway carries the largest part of cholinergic afferents to the hippocampal formation (approximately 75%), and the supracallosal and ventral pathways contribute a smaller part of the cholinergic innervation (Storm-Mathisen and Guldberg, 1974). In addition, the fimbria-fornix pathway carries a GABAergic projection from the MS/DB to the hippocampus (Kohler et al., 1984), a monoaminergic projection to the hippocampus from the brainstem (Gage et al., 1983a and 1983b), and the descending projections from the hippocampal formation to the septum (i.e. the hippocamposeptal pathway) (Toth et al., 1993); therefore, a fimbria-fornix transection is not entirely cholinergic-specific as it also lesions above pathways.

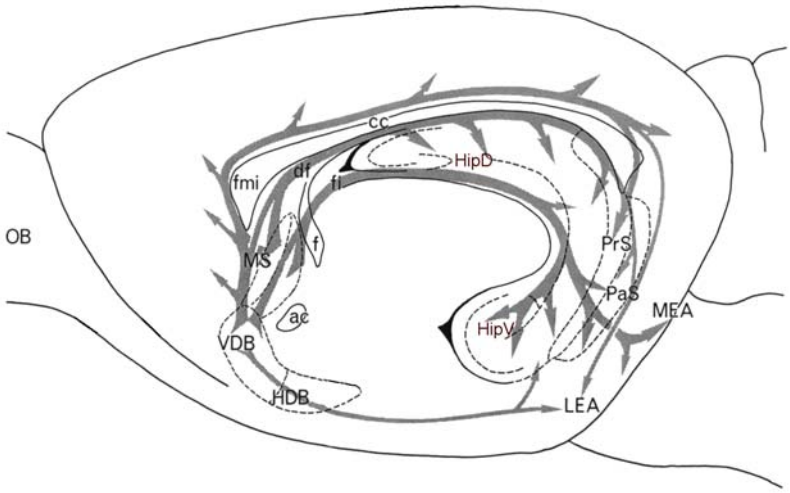

Figure 4. Schematic diagram of a sagittal view of the rat brain showing the main pathways of the septal nuclei innervation of the hippocampal formation. The fibers arising in the medial septal nucleus (MS) and vertical limb of the diagonal band of Broca (VDB) reach the hippocampal formation via the fimbria (fi), and the dorsal fornix (df), and the cingulum bundle (overlying the corpus callosum, cc). The ventral pathway to the hippocampal formation mainly originates from the nuclei of the diagonal band of Broca (i.e. both VDB and HDB). Abbreviations: VDB and HDB, nuclei of the vertical and horizontal diagonal band of Broca, respectively; HipD and HipV, dorsal and ventral portions of the hippocampus, respectively (modified from a figure by Gaykema et al, 1990).

Fimbria-fornix lesions had been used as an experimental animal model of AD in order to test potential therapeutical interventions. Fimbria-fornix lesion can be made by aspiration or transection, and this will result in a dramatic depletion of cholinergic markers in the hippocampus (Henderson, 1996). AChE staining disappears completely in the dorsal hippocampus after a transection of the fimbria-fornix, but the cholinergic innervation in the ventral hippocampus is partially retained because it receives part of its cholinergic input from the MS/DB via a ventral route (Gage et al., 1984; Gaykema et al., 1990) (Figure 4). Rats with a fimbria-fornix lesion have been shown to be significantly impaired in spatial learning and memory tasks, including the radial arm maze task and the Morris water maze (reviewed by Cassel et al., 1997).

3. AIMS OF THE STUDY

The deposition of amyloid-β peptides (Aβ) and neurofibrillary tangles are the two characteristic pathological features of Alzheimer's disease (AD), but cholinergic dysfunction is also a common feature of the disease. Both *in vitro* and *in vivo* studies have suggested that cholinergic neurotransmission may be involved in the regulation of APP processing. However, the role of altered levels of acetylcholine (ACh) in the regulation of APP metabolism and Aβ production has not yet been investigated in an *in vivo* model of AD.

The primary aim of our study was to examine the role of acetylcholine (ACh) levels in the regulation of APP metabolism and/or Aβ production in the APPswe+PS1(A246E) transgenic mouse model of AD. The second aim was to study the relation between Aβ overproduction and/or deposition, and the cognitive decline in these mice.

The specific aims of this study were:

– To study whether an increase in the level of ACh, induced by chronic treatment with a ChEI, would affect APP processing and decrease Aβ production, and thus slow down amyloid deposition in AP mice (**I**).

– To elucidate whether a decrease in the level of ACh, produced by fimbria-fornix transection, could induce APP production and increase Aβ levels in the brain, and thus accelerate amyloid deposition in AP mice (**II**).

– To behaviourally characterize the cognitive deficits in AP mice and study its potential relationship with amyloid deposition in the hippocampal formation (**III**).

– To investigate whether the levels of Aβ in the different compartments of the CNS and periphery correlate with impaired spatial learning and memory in AP mice (**IV**).

4. MATERIALS AND METHODS

4.1. Animals

Male APPswe and PS1(A246E) double transgenic mice (**AP** mice) and nontransgenic littermate controls (**NT** mice), aged from 4.5 months to 14 months, were used in the present study [study I: AP, n = 56, NT, n = 56; study II: AP mice, n = 124, NT mice, n = 116; study III: AP mice, n=100; NT mice, n=145; study IV: AP, n=37, NT, n=34). They were generated from matings between APPswe transgenic mice and PS1(A246E) transgenic mice. The fouder mice, i.e. APPswe Tg-mice expressing a chimeric mouse/human (mo/hu) APP695 harboring a human Aβ domain and mutations (K595N and M596L) linked to FAD (i.e. Swedish mutations), and PS1(A246E) Tg-mice expressing a mutated human PS1(A246E), were originally generated by Dr. Borchelt (1997) at Johns Hopkins University (Baltimore, MD, USA), and they are now bred locally on a C57BL/6J background. Throughout the period of the experiments the animals were housed individually in a controlled environment (temperature 22ºC, humidity 50-60%, light schedule from 0700-1900). Food and water were available *ad libitum*, except during the radial arm maze and the delayed alternation and position discrimination tasks in the T-maze testing period. During these tests the mice were kept on food restriction to limit their weights to 80-85% of the free-feeding weight, and the body weights were checked daily before the behavioural testing. 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. Drug treatment

Metrifonate (MTF, a donation from Bayer, Leverkusen, Germany) was freshly dissolved in 5% sodium citrate buffer (pH 5.5) on each experimental day. MTF (100 mg/kg/day) was given p.o. five days per week and continued for 7 months. The choice of this dose was based on a preliminary dose response study (unpublished findings) in which five groups of single PS1 mutant transgenic mice (n = 9 mice/group) were treated (p.o.) with MTF (5 days/week) for 6 weeks at doses of 0, 25, 50, 100 and 150 mg/kg. The dose of 100 mg/kg yielded the maximum inhibition of AChE activity, i.e. 81%. In

clinical trials, this level of AChE inhibition (but not 66% or less) has been shown to induce cognitive improvements in AD patients (Pettigrew et al., 1998).

4.3. Surgery procedures

4.3.1. Fimbria-fornix lesion

The lesion method was adapted from the method of Goss and Morgan (1995). In short, under general anesthesia (pentobarbital + chloralhydrate [50/50]; 36 mg/kg, i.p.) the mouse was placed in a stereotactic frame, and the skull was exposed. A 1.6 mm slot was drilled through the skull between 0.8 mm and 2.4 mm lateral, and 0.1 mm posterior to Bregma, according to mouse brain atlas (Franklin and Paxinos, 1996). In order to completely cut the fimbria-fornix, two cuts were made from the same starting point (0.8 mm lateral and 0.1 mm posterior to Bregma). For the first cut, a 1 mm-wide knife blade was lowered 3.5 mm from the surface of the brain through the slot at a 26^{o} angle and moved 1.6 mm laterally. The second cut was made as the knife was lowered 3.3 mm vertically and moved 1.0 mm laterally. Sham-operated animals underwent the same surgical procedure but without the knife insertion.

4.3.2. Cisterna magna puncture for cerebrospinal fluid (CSF)

CSF samples were taken from the cisterna magna according to a method that was adapted from the method of Meyding-Lamade et al (Meyding-Lamade et al., 1996). In short, the mice were anesthesized and placed prone on the stereotaxic instrument. A sagittal incision of the skin was made inferior to the occiput. Blunt separation of the subcutaneous tissue and neck muscles through the midline was performed without detachment from the skull under a dissection microscope. A microretractor was used to hold the muscles apart. Then the mouse was laid down such that a nearly 135^{o} between the body and the head was present. Under this angle the dura and spinal medulla are visible, they have a characteristic glistening and clear appearance, and the circulatory pulsation of the medulla and adjacent CSF space can be seen. The dura was then penetrated by a glass capillary, which was 6 cm long, and had a diameter of 1 mm. Following a noticeable change in resistance to the capillary insertion, the CSF flowed into the capillary. The average volume of CSF obtained was approximately 7 µl.

4.4. Behavioural testing

4.4.1. Water maze task

The Morris water maze (WM) has been developed to measure spatial learning and memory in the rat, and it has been shown to be particularly sensitive to the effects of hippocampal lesions (Morris et al., 1982; reviewed by D'Hooge and De Deyn, 2001). The apparatus was a black plastic pool with a diameter of 120 cm, and with 25 cm high walls. A black escape platform (square, 10 cm x 10 cm) was used, either 0.5 cm above (visible) or 1.0 cm below (hidden) the water surface. The water was kept at a constant temperature throughout the experiments (20.0 ± 1.0°C). (Figure 5)

First, the mice were pretrained to find and climb onto the submerged platform for two days by using an alley with high black walls leading to a large platform (14 x 14 cm). Mice were allowed to swim until they found the platform or for a maximum of 20 s before they were placed on the platform for 10 s. This procedure was repeated 8 times consecutively on each pre-training day.

During testing days 1-5, mice were trained to find a hidden platform that was constantly kept in the middle of the southwest quadrant throughout these 5 days (spatial version of WM task), and each mouse performed 5 trials/day. The mice were placed in the water facing the wall to begin the first daily trial from the starting point farthest from the platform (i.e. East or North); the other 4 trials were started in a semi-random starting point order. The latency to find the submerged platform was timed manually by the experimenter. A video camera was connected to an image analyzer (HVS Image, Hampton, UK), and a microcomputer was used to record the swim path. The mice were given 50 s to find the platform, and then were allowed to stay on the platform for 10 s. If the mouse failed to find the escape platform in the maximum time (50 s), the experimenter would gently place it there for 10 s. Mice were tested in groups of four with each mouse having one trial in turn, so that the intertrial interval was approximately 5 min. For *Study IV*, on the sixth day the platform was removed from the pool and the mouse was allowed to swim for 50 s as a probe trial for its search bias.

During testing days 6-7 (for *Study I and III*), a black curtain was hung around the swimming pool in order to conceal the extramaze visual cues. The mice were trained to find a visible platform (cued version of WM task), which had a 10 cm high

pole with a white flag and which was changed every trial to a novel position. Daily sessions were the same as in the hidden platform version. During the water maze training, swimming speed, latency to find the platform, escape length and the percentage of trials that each animal found the platform were measured.

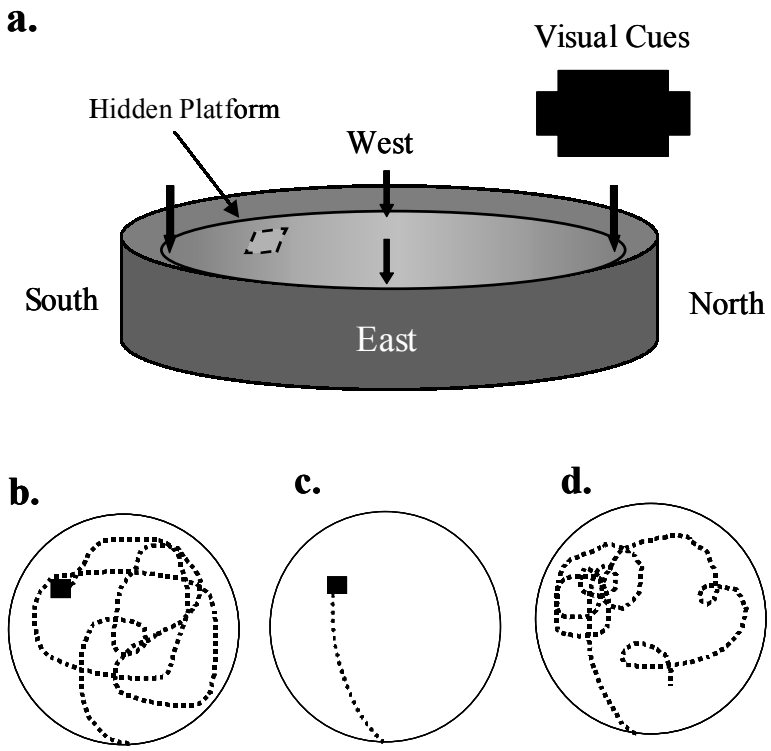

Figure 5. A schematic illustration of the Morris water maze test (spatial navigation version). (a). The water maze pool, an escape platform was placed in the southwest quadrant just below the surface of the water. Mice were trained to learn the position of the hidden platform by using the external visual cues around the maze. They were released into the water from one of four starting points (North, East, South, West. (b-d) Examples of swimming paths of a control mouse during the water maze training. (b) At the beginning, the mouse was randomly searching for the platform, and it found the platform only by chance. (c). After learning the position of the hidden platform, the mouse swam directly to the platform. (d). During the probe trial, the mouse mainly searched around the former location of the platform.

4.4.2. Radial arm maze tasks

The radial-arm maze (RAM) can be used to measure both long-term reference memory and short-term working memory (either spatial or nonspatial). This study used a design similar to the one developed by Olton et al. (1978) for rats and adapted to the mouse by Crusio et al. (1987). The 8 arms radiate out from an octagonal plexiglas center, 22 cm in diameter. The plexiglas arms were 25 cm long, 6 cm wide, and 6 cm high. The entrance to the arms can be blocked by removable guillotine doors. Rice crispies (Kellogg's) were placed at an inaccessible recess at the end of each arm behind a perforated wall to give all arms a uniform scent. Two versions of RAM task were employed in this study.

4.4.2.1. Win-shift task in the radial arm maze

Acquisition

The win-shift version of RAM task can be used to measure spatial learning and memory, which is sensitive to hippocampal lesion (Olton et al., 1978). Mice were trained to collect food rewards from every arm of the radial maze (see Figure 6-a). Prior to the experiment, mice were handled for 2 min daily for 4 days. A food restriction schedule was initiated simultaneously with handling. The body weight of mice was thus progressively reduced and subsequently maintained at 80-85% of the free feeding level throughout testing. The mice were first familiarized to the RAM by letting them explore it freely with all arms open for 10 min on two days, during which time food reward was available at the end of each arm. During the experimental phase, all the eight arms were baited. A single food reward (rice crispies, Kellogg's) was placed immediately in front of the perforated wall, but behind a low visual barrier (1 cm). Each trial began with the placement of the mice on the central platform, with all doors closed. After 5 s, all doors were opened. An entry was defined as all four paws entering the arm, and an entry into an arm from which it had already retrieved the food pellet was deemed an error. The mice were removed from the RAM after retrieval of all rewards, or after a total of 16 arm entries had been made or after 10 min had passed, whichever came first. After each return to the center from an arm, the doors were closed for 5 s. This discouraged the mouse from utilizing mediating strategies. Because of large inter-individual variability in the number of errors before finding the most

difficult 8th reward, the performance was evaluated on the basis of total number of errors made before the 7th correct choice. Learning of the RAM took 15 days, which were averaged into 5 blocks of 3 days in the analysis.

Retention

After a two-week interval, the mice were again tested in the RAM for two days. Thereafter, the same procedure was repeated every month until the end of the testing period (i.e. three months later).

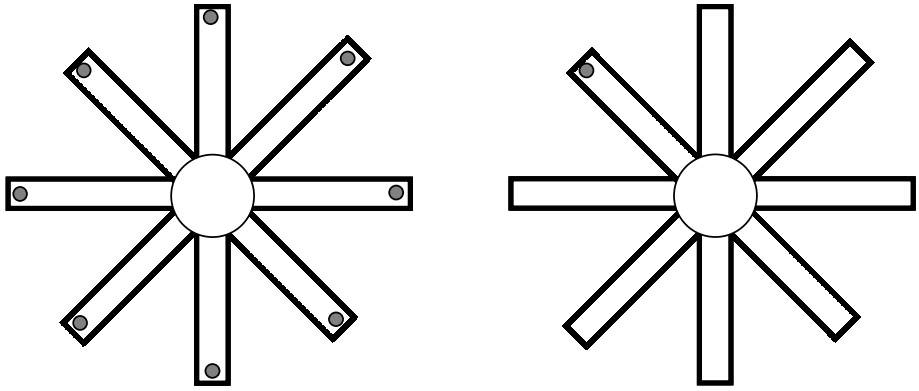

Figure 6. Schematic illustration of the two versions of the radial arm maze (RAM) test. (a). The win-shift version of RAM. All eight arms were baited with food rewards. The mouse had to visit each arm once in order to most efficiently find the food rewards. (b) The win-stay version of RAM. Only one arm was constantly baited with food reward. After repeated training, a mouse learnt to only visit the baited arm and ignore the unbaited arms.

4.4.2.2. Win-stay task in the radial arm maze

The win-stay version of RAM is a nonspatial learning and memory task (adapted from the method of Jarrard, 1978). In this task, one randomly selected arm was constantly baited throughout the 5 days of testing. The mice were trained to enter the one baited arm and to avoid the remaining seven non-baited arms (see Figure 6-b). Each trial began with the placement of the mice on the central platform with all doors closed. After 5 s, all doors were opened. Each trial was completed when the mouse reached the end of the baited arm and returned to the central platform after consuming the food reward. The doors, which were kept open during the trial, were then closed. A

30 s intertrial interval was used. The mice were given 8 daily trials and trained for five days. The total number of errors was recorded for each testing day.

4.4.3. T-maze Tasks

4.4.3.1. Delayed alternation in the T-maze

The delayed alternation task in the T-maze was used to measure working memory (Tanila et al., 1999), which is dependent on the functional integrity of the septo-hippocampal system (Brito et al., 1983; Murray and Fibiger, 1986).

Apparatus

The T-maze consists of a stem (38 x 7 cm) and two arms (35 x 7 cm). A sliding door separated the first 11 cm of the stem as the starting compartment, and a door at 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.

Pretraining

After four days of food restriction, the mice were familiarized with the maze by giving them a 10-min period of free exploration with about 10 rewards scattered on the floor of the maze. Each mouse was given repetitive daily sessions of 10 min until it ate most of the rice rewards during the session.

Training session

The mice were trained to visit one arm, eat the food reward, and return to the starting compartment. If the mouse did not leave the starting compartment in 5 s after the door was opened, the experimenter would gently push the mouse. After two visits into the same arm, that arm was blocked with the slide door forcing the mouse to visit the opposite arm. When the mouse spontaneously chose each arm successively, the actual alternation training began with 15 trials daily. On the first trial both arms were baited. After the mouse had chosen one arm and eaten the food reward, it was returned to the start compartment and confined there for 10 s. On the following trials the food reward was located in the arm that was not visited on the previous trial. After the mouse had entered one arm, it was confined there by a slide door for 20 s. Thereafter it

was given 10 s to return to the staring compartment, while the access to the opposite arm was blocked by a door. The baited arm remained the same until it was visited even if the mouse repeatedly visited the incorrect arm. The training was continued until the mouse make 11 or more correct choices of 15 trials in three consecutive days. If a mouse failed to reach the criterion by the end of 10 days, it was dropped for the mixed-delay testing session (Figure 7-a, -b and -c).

Mixed-delay testing sessions

The delayed alternation test session consists of 15 trials in which three delays (20, 60, 100 s) were varied in a pseudorandom order that was the same for all animals in a given session. This test phase took three days. The procedure was the same as in the training phase except for the delays. Choice accuracy was evaluated by counting the total number of errors.

4.4.3.2. Spontaneous alternation in the T-maze

Based on the animal's natural tendency to explore novelty, the spontaneous alternation in the T-maze task was designed to assess exploratory behaviour and working memory in an unbiased way (Gerlai, 1998). The apparatus consisted of a start stem and two goal arms (length of start and goal stems=75 cm, width=12 cm, height=20 cm). The walls of the maze were made of transparent acrylic and were glued to a matte black acrylic square bottom piece. The maze was equipped with three removable sliding doors. One separated a 24-cm compartment at the beginning of the start arm, and the other two were placed at the entrance of each goal arm and could be lowered to block entry. The procedure consisted of one forced and 14 choice trials. On the first trial, the mouse was allowed to explore the start arm and one of the goal arms of the maze. The entry was blocked to the other goal, which was chosen in a semi-random order. When the mouse left the explored goal arm and re-entered the start arm, it was confined there for 5 s by lowering the slide door. On the second trial, the door of the previously blocked arm was lifted and both goal arms were open to the mouse. After the mouse had chosen and entered one goal arm, the opposite goal arm was blocked by the door. The mice left the explored goal arm and eventually went back to the start arm again. It was confined there for 5 s and the testing circle started again with another free

choice trial. Consecutive choices made by the mouse were recorded and the total alternation rate during the 14 free choice trials was calculated.

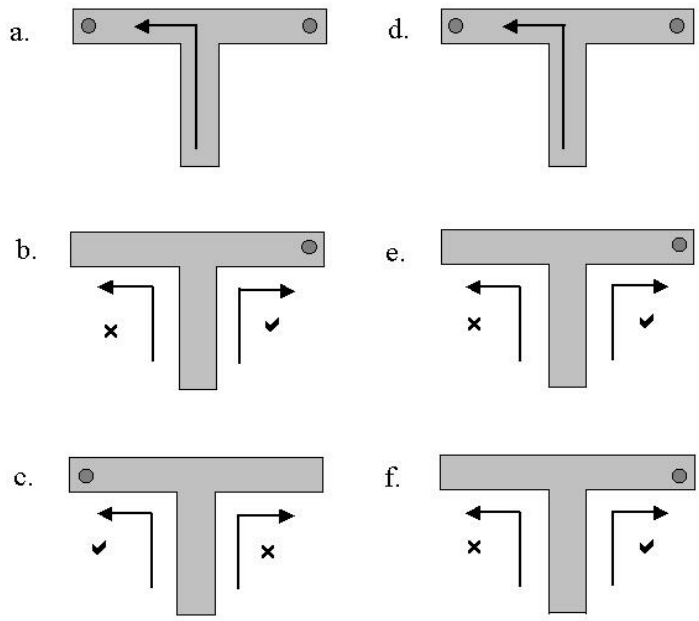

Figure 7. Schematic illustration of the two food-motivated learning and memory tasks in the T-maze. (a), (b), and (c) show the testing procedure of the delayed alternation task in the T-maze. In the first free trial, the mouse was allowed to make either a left or a right turn to retrieve the food reward (a). During the following trials, the mouse must make consecutive alternations in order to obtain the food rewards (b, c). Thus the correct choices are based on remembering the previous visits (i.e. working memory). (d), (e) and (f) show the testing procedure of the position discrimination task in the T-maze. The baited arm was selected during the first free trial, which was opposite to the mouse's preference (d). For the following trials, this arm was constantly baited (e, f). The performance in this task is largely based on habituation learning.

4.4.3.3. Position discrimination in the T-maze

The position discrimination task in the T-maze was used to measure egocentric memory. The apparatus was the same as that was used in the delayed alternation task. Food restriction was introduced in order to reduce the weights of mice to 80-85% of their normal body weight. Mice were handled and weighed daily, and they were familiarized to the T-maze by letting them freely explore it for 10 min until they repeatedly ate the rewards at both arms of the maze. 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 did not select was baited for for all three days (or four days, Study I) of testing. Each mouse was given 15 consecutive trials per day. A 1-min intertrial interval was introduced between the trials during which time the animal was confined to the starting compartment. For Study I, on the fifth day, the location of the food reward was changed to the other arm, and this arm remained rewarded for three days. The total number of errors over the 15 trials was recorded daily (Figure 7-d, -e, -f).

4.4.4. Other paradigms

Two additional tasks were run to detect possible alterations in motor function, exploratory behaviour and emotional behaviour (e.g. anxiety or fear) due to drug treatment, brain lesion or transgenic manipulation.

4.4.4.1. Open field

The open field test was used to assess both exploratory behaviour and locomotor activity. All mice were tested using automated TruScan system with infrared photodetectors (Coulbourn Co, USA). The system consists of four identical 26 x 26 x 39 cm plexiglas cages, an interface station and a microcomputer for data recording and analysis. Two photobeam sensor rings were employed to detect the animal's movement in two planes. Horizontal activity (XY-move time), vertical activity (rearing), center move time and stereotypic move time were measured for 10 min in the first testing day and repeated after 48 hours for another 10 min.

4.4.4.2. Elevated plus-maze

The elevated plus-maze was used to measure the anxiety level of the mice (Lister, 1987). The apparatus was made of black plastic, and consisted of two opposite, open arms (length 30 cm, width 5 cm, ledge 0.25 cm) and two opposite, enclosed arms (30 cm x 5 cm x 15cm). The arms were connected by a central platform (5 x 5cm), together forming a plus shape. The maze was elevated 40 cm from the floor, and it was illuminated by a 40-Watt light bulb. Each mouse was placed on the central platform facing an open arm and allowed to freely explore the maze for 5 min. At the end of each trial, the maze was thoroughly cleaned with a damp cloth. The time spent on the

open and enclosed arms was recorded semi-manually with a computer program. The percentage time spent on the open arms [open time/ (open + close time) x 100] was calculated. An increase in the percentage of time spent on the open arms is interpreted as an anxiolytic response, whereas a decrease indicates a higher level of anxiety.

4.5. Biochemical assays

4.5.1. APP Western-blot

The dissected hippocampi were homogenized in phosphate buffered saline (PBS, pH 7.2) containing 1 mM Complete™ protease inhibitor (Boehringer Mannheim). After centrifugation the supernatants were collected, and the remaining pellets were homogenized in 1% Triton X-100-PBS containing Complete™, and centrifuged. Protein samples (30 μg) were subjected to 7.5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to a nitrocellulose filter. Monoclonal anti-APP IgG (clone 22C11, Boehringer Mannheim), which detects both human and mouse APP, was used as primary antibody and alkaline phosphatase conjugated anti-mouse IgG (Jackson ImmunoResearch Laboratories, Inc.) as secondary antibody. APP immunoreactivity (in optical density units, OD) was visualized using ECF substrate for Western blotting (Amersham Pharmacia Biotech) and STORM™-fluoroimager (650 mV, 200 microns, Molecular Dynamics).

4.5.2. Aβ *ELISA*

The hippocampi were weighed and homogenized in phosphate buffered saline, pH 7.2, containing a mixture of protease inhibitors (Complete™, Boehringer Mannheim, Germany). After centrifugation at 218,000g for 2 h at 4° C the supernatants containing soluble Aβ42 were collected. The remaining pellets containing insoluble Aβ were homogenized in guanidine buffer (5.0 M guanidine-HCl/50 mM Tris-HCl, pH 8.0), incubated 3 hours at room temperature, centrifuged, and diluted to reduce the concentration of guanidine-HCl to 0.5 M (Johnson-Wood et al., 1997). All samples were stored at – 70° C until the analysis.

The samples, and the Aβ peptides that were used as standards, were prepared to contain 0.5 M guanidine-0.5% BSA-1 mM AEBSF in the final composition. The levels

of Aβ42 in CSF and soluble Aβ42 in the hippocampi were quantified using the Innotest β-amyloid1-42 High Sensitivity Test–ELISA kit (Innogenetics, Belgium), and the levels of Aβ40 in serum and insoluble Aβ40 and Aβ42 in the hippocampi were quantified using the Signal Select™ Beta Amyloid ELISA Kits (BioSource International Inc.). Both kits use antibodies that do not recognize mouse endogenous Aβ (Innogenetics, Johnson-Wood et al., 1997; BioSource, manufacturer's report). The Aβ42 and Aβ40 levels in hippocampi were standardized to brain tissue weight and expressed as ng (Aβ) / g (brain tissue), and the levels of Aβ42 in CSF and Aβ 40 in serum were expressed as pg/ml.

4.5.3. ChAT activity

Hippocampal and cortical tissues were homogenized in buffered (50μM sodium phosphate buffer, pH 7.4) 0.32 M sucrose (10v/w) using a Potter-Elvehjem homogenizer (1000 rpm, six strokes) in an ice bath. ChAT activity was measured in duplicates according to the method of Fonnum (1975).

4.6. Histology

Histology

Mice were anesthetized, and transcardially perfused with 0.9 % saline followed by 4% paraformaldehyde in 0.1 M Na-phosphate buffer (pH 7.4). The brains were removed and placed in the fixative for 2 hours, then transferred to a 30% sucrose solution, in which they were kept overnight on a shaker table. Following this procedure the brains were either cut, or stored in antifreeze. Six series of coronal sections (35 μm, 1 in 6 series) were cut through the brain using a sliding, freezing microtome. The first series of sections was stained with cresyl violet for general morphology, the second series was silver-stained to analyze amyloid plaques (Garvey et al., 1991), the third series was histochemically stained for AChE to visualize cholinergic fibers (van Groen and Wyss, 1992). The fourth series was immunohistochemically stained for Aβ using the W0-2 antibody, which was raised against the 1-16 amino acid sequence of human Aβ (Jensen et al., 2000). The other two series were stored in antifreeze in freezer for future use. For the W0-2 staining, the sections were rinsed overnight in Tris-buffered saline (TBS), and then the series of sections was transferred to a solution containing the

primary antibody (mouse anti-human Aβ, 1:20000, W0-2; this solution consists of TBS with 0.5 % Triton X-100 added [TBS-T]). Following incubation in this solution for 18 h on a shaker table at room temperature (20°C) in the dark, the sections were rinsed three times in TBS-T and transferred to the solution containing the secondary antibody (goat anti-mouse*biotin; Sigma). After two hours, the sections were rinsed three times with TBS-T and transferred to a solution containing mouse ExtrAvidin® (Sigma) for 2 hours. Following rinsing the sections were incubated for approximately 3 min with Ni-enhanced DAB (10 mg DAB in 20 ml 0.1 M phosphate buffer, 30 μl H_2O_2 [30%], pH 7.4 with 1 ml of a 15% ammonium Ni-sulfate solution added). All stained sections were mounted on slides and coverslipped.

Measurements

The number of amyloid plaques in the dorsal hippocampal formation (i.e. in CA1, dentate gyrus and subiculum) was counted using the silver-stained material for AP mice (I, II). Three sections (or seven, Study I) through the hippocampus beginning at approximately 200 μm posterior to bregma were used for counting plaque number. Similarly four sections (or seven, Study I) beginning at approximately 540 μm posterior to bregma were used for counting the plaque numbers in dorsal subiculum. The sections were digitized using a Nikon Coolpix 990 digital camera, and the images were converted to gray scale using the Adobe Photoshop 5.5 program. The size of each area of the hippocampal formation was measured using the Scion Image (NIH) program. The plaque counts were calculated per unit brain area and these values were normalized with reference to the dorsal subiculum. From the adjacent, W0-2 stained sections, the Aβ load in the hippocampal formation was measured. In short, the appropriate areas of the sections were digitized by the same procedure described above. The percentage of area covered by the reaction product to Aβ in CA1, dentate gyrus and dorsal subiculum was measured using the Scion Image (NIH) program.

4.7. Experimental design

Study I

Seven-month-old male APPswe and PS1(A246E) doubly transgenic mice (AP mice, n = 56) and nontransgenic littermate controls (NT mice, n = 56) were used in this study. Both AP and NT mice were randomly assigned to either MTF or vehicle groups

(n = 28 mice / group). Drug (100 mg/kg/day) or vehicle was given p.o. five days per week and continued for 7 months. The behavioural tests (open field, water maze, the position discrimination task in the T-maze) were conducted after 6 months treatment with MTF. At the end of the testing period, eight mice of each group were transcardially perfused for histopathology; the other 20 mice were sacrificed, and the hippocampi and cortices were dissected for measurement of ChAT activity and levels of APP, Aβ40 and Aβ42.

Study II

Male AP mice (n = 124) and non-transgenic littermate controls (NT mice, n = 116) were used in this study.

One short, preliminary experiment, and two long-term experiments were performed.

Preliminary Experiment

Thirty-nine AP mice and 32 NT mice were FFX lesioned or sham-operated at the age of 4.5 months (AP-FFX, n=19; AP-sham, n=20; NT-FFX, n=16; NT-sham, n=16). Following a two weeks recovery period, the mice were sacrificed and the hippocampi were dissected for measurement of ChAT activity and the levels of APP, Aβ40 and Aβ42.

Experiment 1

Forty-eight AP mice and 48 NT mice were FFX lesioned or sham-operated at the age of 5 months (AP-FFX, n=24; AP-sham, n=24; NT-FFX, n=24; NT-sham, n=24). Three months later (at 8 months of age), eight mice of each group were transcardially perfused for histopathology; the other 16 mice were sacrificed, and the hippocampi were dissected for measurement of ChAT activity and levels of APP, Aβ40 and Aβ42.

Experiment 2

Forty-one AP mice and 40 NT mice were FFX lesioned or sham-operated at the age of 7 months (AP-FFX, n=21; AP-sham, n=20; NT-FFX, n=20; NT-sham, n=20). After 11 months (at 18 months of age), eight mice of each group were transcardially perfused for histopathology; the other mice were sacrificed and the hippocampi were dissected for measurement of ChAT activity and Aβ 40 and Aβ42 levels.

Study III

Male AP mice (n = 100) and NT mice (n = 145) were used in this study. In total 127 mice (AP mice, n=40; NT mice, n=87) were randomly assigned to fimbria-fornix lesion (FFX-lesion) or sham-operated groups (all mice of experiment 2, study II were included.). Four experiments were performed: Experiments 1 and 2 consisted of a large battery of behavioural tasks that have earlier been reported to be sensitive to FFX lesion in rats or mice, in contrast, experiments 3 and 4 consisted of behavioural tasks in which hippocampal or FFX lesions have not been shown to result in impaired performance.

Experiment 1

Twenty-four AP and 24 NT mice received a FFX lesion or were sham operated at the age of 7 months (AP-FFX, AP-sham, NT-FFX, NT-sham, n = 12/group). After 7 months recovery the mice were tested in the win-shift task in the radial arm maze, elevated plus-maze, and spontaneous alternation task in the T-maze.

Experiment 2

Sixteen AP mice and 17 NT mice received a FFX lesion or were sham operated at the age of 7 months (AP-FFX, AP-sham, NT-FFX, NT-sham, n = 8-9/group). Ten months after the lesion, the mice were tested delayed alternation task in the T-maze and water maze.

Experiment 3

Separate groups of NT mice were FFX-lesioned (NT-FFX, n=24) or were sham operated (NT-sham, n=22) at the age of 5 months. After a 3 months recovery period, the mice were tested in the win-stay task in the radial arm maze and the position discrimination task in the T-maze.

Experiment 4

Separate groups of AP and NT mice (AP, n=25; NT, n=25) were tested at the age of 14 months in the win-stay task in the RAM. Another separate group of AP and NT mice (AP, n=17; NT, n=17) was tested at the age of 11 months in the position discrimination task in the T-maze.

Study IV

Fourteen-month-old male AP mice (AP, n=37) and nontransgenic littermate controls (NT mice, n=34) were used in this study. Following the completion of the

water maze task, the mice (AP mice, n=22; NT mice, n=22) were deeply anesthetized, and CSF and blood samples were taken from the cisterna magna, and left cardiac ventricle, respectively. Thereafter the mice were sacrificed, and the hippocampi were dissected. The levels of soluble and/or insoluble Aβ40 and Aβ42 in brain, CSF and serum were measured by ELISA (AP, n=22; NT=4).

4.8. Statistical analyses

All statistical analyses were carried out using SPSS for Windows software (version 8.0, SPSS Inc., USA). The effects of mouse genotype, drug treatment or lesion, training day or trials, and their interactions on locomotor activity and maze performance of mice were analyzed by univariate analysis of variance (ANOVA) or ANOVA for repeated measures. The effects of genotype and drug treatment or lesion upon APP level, Aβ levels, ChAT activity, density of AChE positive fibers, amyloid plaque counts, amyloid plaque density, Aβ load and hippocampal CA1 width were analyzed with ANOVA or independent-samples *t*- test. The correlations among Aβ levels in CSF, serum, brain and water maze learning were analysed with Pearson's correlation analysis.

5. RESULTS

5.1. Effects of metrifonate on cholinergic activity, amyloid pathology, and cognitive function in AP mice (I)

5.1.1. Changes in cholinergic activity

No genotype-related difference was found in the density of AChE positive fibers in the hippocampus or in the parietal cortex. In both genotypes, and brain areas, MTF treatment continued to inhibit AChE highly significantly. The average AChE activity in the MTF treated animals was 54.7 ± 2.6 % (mean ± SEM) in the hippocampus and 61.0 ± 5.0 % in the parietal cortex relative to that of vehicle treated animals (I, Figure 4 and 5A). However, the degree of AChE inhibition after 7 months of chronic MTF administration is considerably less than the 81% inhibition after 5 weeks of treatment. No genotype or treatment main effect was found for the ChAT activity in the hippocampus or parietal cortex; however, the ANOVA revealed a significant genotype x treatment interaction for the ChAT activity in the parietal cortex. The ChAT activity in the parietal cortex was slightly higher in untreated AP mice (1.05 ± 0.19 nmol/mg prot/h; mean ± SEM) than in NT mice (0.90 ± 0.17 nmol/mg prot/h) and tended to decrease after MTF treatment in AP mice while it was increased in NT mice (Figure 5B). ChAT activity in the hippocampus showed a similar, but non-significant trend.

5.1.2. Effects on amyloid metabolism

The hippocampal APP levels were approximately 100 % higher in AP mice than in NT mice at the age of 14 months. The genotype effect was highly significant but no treatment effect or genotype x treatment interaction on APP levels was found. Hippocampal Aβ40 and Aβ42 could not be detected in NT mice but reached high levels (compared to 5 and 8 month old AP mice, II) in the 14-month-old AP mice. The levels of Aβ42 were about three times higher than those of Aβ40. The treatment effect was highly significant for both Aβ40 and Aβ42 levels; but contrary to the hypothesis, the levels were higher in the MTF treated group (I, Figure 2). On the other hand, the Aβ42 to Aβ 40 ratio significantly decreased from 4.3 ± 0.4 (mean ± SEM) of the vehicle treated AP mice to 3.2 ± 0.3 in the MTF treated AP mice.

Amyloid plaques were most frequently detected in the dorsal hippocampal formation (hippocampus proper and subiculum; I, Figure 3). Table 1 of (I) summarizes the total plaque counts in 7 sections of the dorsal hippocampal formation in 8 vehicle-treated and 8 MTF-treated AP mice. The number of plaques did not differ between the treatments.

The width of the hippocampal CA1 area in the four groups of mice is also summarized in Table 1 of (I). No difference between the groups was detected indicating that amyloid pathology or the presence of mutated APP is not associated with shrinkage of the hippocampus, as has been reported in the PDAPP transgenic mice (Dodart et al., 2000).

5.1.3. Behavioural findings

In the open field, the ANOVA revealed genotype and treatment main effects but no significant interactions. The AP mice exhibited less horizontal locomotor activity and less rearing than the NT mice. Chronic treatment with MTF decreased horizontal locomotor activity and rearing in both genotypes.

In the water maze no genotype difference was observed in the swimming speed, but MTF treatment significantly reduced the swimming speed in both genotypes (I, Figure 1). Because of the robust MTF effect on swimming speed, the water maze performance was evaluated based on the path length and not on the escape latency. The path length over the 5 training days did not differ significantly between the genotypes or the treatments (I, Figure 1). However, the analysis over the last three days, when the performance reached an asymptote, did reveal a significant effect of the genotype (I, Figure 1) but no treatment effect. No significant interactions were observed in either analysis of the hidden platform task. The AP mice were worse than NT mice in finding the visible platform, but no treatment effect or interaction was revealed. However, on the second day of the visible platform task, the performance of the AP mice was comparable with that of the NT mice in both escape length and the percentage of platform finding.

In the position discrimination task in the T-maze AP mice performed better than NT mice, both in the initial acquisition and in the reversal learning phases. The MTF treatment did not affect performance of either group (Data not shown).

5.2. Effects of fimbria-fornix lesion on APP levels and amyloid deposition in the hippocampus of AP mice (II)

5.2.1. Verification of the FFX lesion

The preliminary experiments demonstrated that in most of the FFX mice, the lesions completely cut the fornix and the fimbria; however, many of these lesions slightly encroached on the medial part of the cingulate cortex and on the corpus callosum overlying the fornix. In experiment 1, three animals had incomplete lesions, and the data from these animals was not included in the analysis. In experiment 2, four animals showed ventricular enlargement, and the data from these animals were removed from the study.

In experiment 1, three months postlesion (i.e. at eight months of age), the FFX transection resulted in a nearly complete loss of the hippocampal AChE positive fibers (II, Figure 1). The decrease in AChE staining was higher in the hippocampus than in the subiculum, and, further, the decrease in AChE staining was higher in the dorsal compared to the ventral hippocampal formation. In experiment 2, eleven months postlesion, the number of hippocampal AChE positive fibers was also significantly reduced, and again, similar to experiment 1, the reduction in AChE staining was more severe in the dorsal hippocampus than in the ventral hippocampus. However, it should be noted that the reduction in AChE staining in both the dorsal and the ventral hippocampus was less marked 11 months postlesion (i.e. 18 months of age) than in 3 months postlesion (II, Figure 1).

ChAT activity in the hippocampal formation of the FFX mice was significantly lower than in sham-operated mice for all three age groups, and the hippocampal ChAT activity of AP mice did not differ from that of NT mice. Two weeks, 3 months and 11 months after lesion, the ChAT activity of the FFX mice was decreased by 76%, 72% and 52 % compared to age-matched sham-operated mice, respectively (II, Figure 2). The interaction between genotype and lesion was not significant for these groups.

5.2.2. APP and Aβ *levels in the hippocampus*

The total APP levels (soluble APP [sAPP] + membrane bound APP [mAPP]) were analyzed only for the preliminary experiment (5-month old) and experiment 1 (8-

month-old) mice. The total APP level of the AP mice (which overexpress huAPP) was significantly higher than that of the NT mice. There was no difference in total APP level between FFX mice and their sham-operated controls (II, Table 1), and the interaction of genotype and lesion was not significant. In order to detect any changes in APP processing after cholinergic denervation (Rossner et al., 1997), the levels of sAPP and mAPP were analyzed separately. In both 5- and 8-month-old groups, the sAPP and mAPP levels of AP mice were significantly higher than those of NT mice. Again there was no difference between FFX animals and their respective controls (II, Table 1). The genotype by lesion interaction was not significant between these two groups.

The data on the Aβ levels, and Aβ42 to Aβ40 ratio are summarized in Table 2 of (II). From the age of 5 months to 18 months, the Aβ40 and Aβ42 levels in the AP mice significantly increased. The Aβ42 to Aβ40 ratio also increased significantly over that time period. However, there were no differences between the AP-FFX and AP-sham mice in the Aβ40 or Aβ42 levels, nor in total Aβ levels (i.e. Aβ40 + Aβ42) at any of the three time points studied. The Aβ42 to Aβ40 ratio did not differ between AP-FFX and AP-sham mice.

5.2.3. Extent of amyloid deposition in the hippocampal formation

At eight months of age, no amyloid plaques were detected in the brain of AP-FFX mice or AP-sham mice, neither in the silver- nor in the Aβ-stained materials; and no amyloid pathology was present at any age in the NT mice. Therefore only the brains of the 18-month-old transgenic mice of Experiment 2 were analyzed for hippocampal amyloid neuropathology (II, Figure 3). In the 18-month-old AP mice, the density of silver stained plaques in the dentate gyrus, CA1 region and dorsal subiculum did not differ between AP-FFX and AP-sham mice (II, Figure 4A). Image analysis of the Aβ load in the hippocampal formation demonstrated that there were no significant differences between the AP-FFX and AP-sham mice. The Aβ load in AP-FFX mice averaged 5.4% in the hippocampal formation, ranging from 2.3 % to 9.1 %, while the average Aβ load in the AP-sham mice was 5.2 %, with a range from 1.1 % to 7.7 %. Further, an analysis of the separate hippocampal regions, i.e. dentate gyrus, CA1 region and dorsal subiculum also did not reveal any significant differences between AP-FFX and AP-sham animals (II, Figure 4B).

5.3. Characterization of learning and memory deficits in AP mice, and its possible relationship with amyloid deposition (III)

5.3.1. Behavioural findings

All the behavioural findings of study III have been summarized in Table 2.

Experiment 1

Win-shift task in the RAM

In the acquisition phase, all groups of mice showed learning as assessed by the total number of errors made until 7th correct choice. No interaction between trials and genotype or lesion was found. The ANOVA revealed a significant FFX lesion effect, but no genotype effect or genotype x lesion interaction (III, Figure 4). Retention of the task learning was reassessed after 2 weeks, and 1.5, 2.5, and 3.5 months, respectively. Two daily sessions were run a month apart, so we performed an ANOVA with both the month and the day as within-subjects factors. During the retraining phase, no further improvement was observed as testing progressed. The FFX mice made significantly more errors than the sham mice, and in contrast to the acquisition phase AP mice were also found to be worse than NT mice. The genotype x lesion interaction was not significant, but the ANOVA revealed a significant day x lesion interaction. This interaction was due to late sessions (2.5 and 3.5 months after the task acquisition), in which the AP-sham mice dramatically improved their performance from Day 1 to Day 2 while both FFX groups showed further impairment. This day-dependent variation in the task performance was further assessed by comparing the performance of AP-sham and both FFX groups to NT-sham groups separately on the first and second testing days. In the Day 1 comparison, only the AP-FFX mice differed from the NT-sham mice, whereas both the AP-FFX and NT-FFX differed from the NT-sham mice on Day 2 (III, Figure 4).

Elevated plus-maze

The FFX lesion significantly increased the percentage of time that the mice spent in the open arms, indicating a decreased level of anxiety in the FFX mice. The genotype effect was nonsignificant, as was the genotype x FFX lesion interaction (data not shown).

Spontaneous alternation task in the T-maze

In the initial experiment the ANOVA revealed a significant decrease in the alternation rate of the FFX mice compared with the sham mice (III, Figure 5A, black columns); on the other hand, the genotype did not affect the alternation rate significantly. However, the alternation rates were generally low in this task, so that only the NT-sham performed slightly above chance level and the AP-FFX mice clearly below the chance level. The mice were also sluggish to move, so that it took up to 30 min to complete the task. To rule out low activity and poor motivation-to-explore as confounding factors, we replicated the experiment with another group of mice that had been food deprived overnight. This procedure resulted in more active locomotion and better alternation rates, and both genotypes performed significantly above chance level (III, Figure 5A white columns). But again no difference was observed between the genotypes (III, Figure 5A).

Experiment 2

Delayed alternation task in the T-maze

The cumulative number of errors to the criterion made by the four groups of mice is shown in Figure 5B (III). FFX mice of both genotypes made significantly more errors and failed to reach the criterion by the 10th day. Except for one AP-sham mouse and one NT-sham mouse which were excluded for the next session, both groups of sham-operated mice reached the criterion within 10 days and did not differ from each other in the rate of acquisition. These mice were tested further in the mixed-delay session of the task. No difference was found between AP and NT mice in performing the task with mixed delays ranging from 20 to 100 s.

Water maze

The swimming speed of the mice was not affected by FFX lesion or genotype. In the overall ANOVA with lesion and genotype as between-subjects factors, the FFX lesion significantly increased the latency of the mice to find the platform, but the genotype effect on escape latency was not significant. However, the genotype x lesion interaction was significant; therefore this interaction was further examined with ANOVAs performed separately for FFX and sham groups. In this analysis, the AP-sham mice were found to be inferior to NT-sham mice, but the AP-FFX and NT-FFX

mice did not differ from each other. These results suggest that AP genotype itself can impair the task learning to a certain extent and that AP genotype and FFX lesion have no additive effects. The latency of the mice to find the visible platform was also analysed. FFX lesion significantly increased the latency of the mice to find the visible platform, but no main effect of the genotype was found nor any genotype x lesion interaction.

Short-term memory vs. long-term memory in the water maze

As above findings suggested, AP mice are impaired in some but not all tasks that are sensitive to lesions of the FFX. Most notably, the AP-sham mice were normal in delayed alternation in the T-maze, although FFX mice showed a robust impairment. In order to distinguish deficits between short-term memory (working memory) and long-term memory (reference memory), the water maze data were further analysed. The long-term memory component of water maze task was assessed by defining the mean latency to platform finding of the first 2 trials on the 2nd, 3rd, 4th and 5th day; while, the short-term memory component was assessed by subtracting the latency of the 5th trial from the 1st trial (same starting point) over all five testing days.

With regard to the long-term memory index, the effect of FFX lesion was shown to be significant by ANOVA, while the genotype effect was not. However, the genotype x lesion interaction reached significance. The subsequent separate ANOVAs for the lesion groups showed that the AP-sham group was worse than NT-sham mice, but the AP-FFX and NT-FFX did not differ from each other (III, Figure 6B). For the short-term memory index, the ANOVA revealed a significant effect of the FFX lesion, but no genotype effect or genotype x lesion interaction (III, Figure 6C).

Experiment 3

Win-stay task in the RAM and position discrimination in the T-maze

Both NT-sham and NT-FFX mice improved their performance in these two tasks over the training days as measured by the total number of errors. The FFX lesion did not affect the task acquisition (III, Figure 7A and 8A)

Experiment 4

Win-stay task in the RAM and position discrimination in the T-maze

Both AP and NT mice improved their performance over training days as measured by the total number of errors. The ANOVA did not reveal any effect of the genotype on acquisition of the task (III, Figure 7B and 8B).

Table 2. Summary of the behavioural findings of Study III

Tasks	NT-sham	AP-sham	NT-FFX	AP-FFX
Win-shift task in RAM: acquisition	✓	N	↓	↓
Win-shift task in RAM: retention	✓	↓ *	↓	↓
Spontaneous alternation in T-maze	✓	N	↓	↓
Delayed alternation in T-maze	✓	N	↓	↓
Water maze: hidden platform	✓	↓	↓	↓
Water maze: visible platform	✓	N †	↓	↓
Water maze: short-term memory	✓	N	↓	↓
Water maze: long-term memory	✓	↓	↓	↓
Win-stay task in RAM	✓	N	N	N
Position discrimination in T-maze	✓	N ǂ	N	N
Elevated plus-maze: time in open arms	✓	N	↑	↑

✓: Normal; N: no impairment/ difference vs. NT-sham; ↓: impaired vs. NT-sham;
↑: increased vs. NT-sham; *: see text; †: found impaired in Study I;
ǂ: found better in Study I.

5.3.2. Amyloid pathology

Since the Aβ level and Aβ load in the hippocampus did not differ between 18-month-old AP-FFX and AP-sham mice (II), the data from AP-FFX and AP-sham mice were combined for the analysis of the regional distribution and extent of amyloid deposition in the brain, which are summarized in Figure 2 of (III). The dorsal

hippocampal formation, more specifically the dorsal subiculum, has the highest density of Aβ depositions. Less severe but notable amyloid deposition was found in all cortical areas examined, with a tendency for posterior areas to have a larger amyloid burden than the anterior cortical areas. In contrast, the striatum and thalamus were nearly devoid of amyloid pathology even at the age of 18 months. Figure 3 of (III) demonstrates three coronal sections from an AP-sham mouse at different levels, illustrating the unequal distribution of amyloid deposits.

5.4. Correlation between Aβ levels in brain, CSF and serum, and cognitive deficits in AP mice (IV)

In the water maze test, the swimming speed did not differ between AP and NT mice. However, the average latency of AP mice to find the hidden platform was significantly longer than that of NT mice (not illustrated). A spatial learning impairment of AP mice was confirmed in the probe trial by their reduced search time over the previous platform location compared to NT mice (IV, Figure 1A).

The Aβ level in all samples of the NT mice (n= 4) was below the detection level, verifying the specificity of the used antibody to human Aβ. Therefore, the following correlation analysis was limited to AP mice only (n= 22). In serum, the Aβ42 level of the AP mice was below the detection level, but the Aβ40 could be measured. Due to the limited amount of CSF obtained, only Aβ42 was measured. The Aβ level in the serum and CSF, the soluble and insoluble Aβ levels in the brain (hippocampus), and the correlations among these Aβ levels and spatial learning are summarized in Table 1 of (IV). The insoluble Aβ40 and Aβ42 levels in the hippocampus were highly correlated, and these levels correlated with the level of soluble Aβ42 (IV, Figure 1C); however, only the insoluble Aβ42 level was significantly associated with spatial learning (IV, Figure 1B). Neither CSF nor serum Aβ was significantly correlated with spatial learning. The CSF Aβ42 correlated with the brain soluble Aβ42 level (IV, Figure 1D), but not with serum or insoluble brain Aβ levels. Serum Aβ40 level was not correlated with either CSF or brain Aβ level.

6. DISCUSSION

6.1. Methodological considerations of the study

6.1.1. The animal model

The mouse model of AD that we used, the APPswe and PS1(A246E) double transgenic mice, were developed and described by Borchelt et al. in 1997. These mice have elevated levels of the highly fibrillogenic Aβ42 peptide in the brain, and develop amyloid plaques starting around the age of 9 months (Borchelt et al., 1997). The formation of amyloid plaques starts in the subiculum and caudal cortex, and later extends to hippocampus and all cortical areas. This feature is somewhat similar to the early stages of AD, in which the pathology is also largely restricted to the medial temporal cortical structures (Braak and Braak, 1991).

Principally, we used these mice as the animal model of AD in these studies for two reasons:

1) They provide a good model for studying the metabolism of Aβ, since they have elevated levels and deposits of Aβ that are restricted to the cortex and the hippocampus.

2) They develop amyloid pathology at a relatively late age, i.e. around 9 months of age, which gives a good window of opportunity for testing potential therapeutics for AD.

6.1.2. Choice of metrifonate for the chronic treatment

Metrifonate (MTF) is a prodrug that is hydrolyzed non-enzymatically to 2,2-dimethyl dicholorovinyl phosphate (DDVP), which provides a sustained inhibiton of AChE by binding stably to the catalytic site of the enzyme (Reiner et al., 1975). As a second generation ChEI, metrifonate, has been shown to improve cognitive performance of AD patients in clinical trials (Cummings et al., 1998a; Farlow and Cyrus, 2000), and in preclinical studies it was demonstrated to raise brain ACh levels (Mori et al., 1995a) and to have beneficial effects on learning and memory, both in rats and mice (Riekkinen et al., 1997; Ikonen et al., 1999).

We chose to use metrifonate as ChEI to investigate whether chronic treatment with a ChEI could decrease Aβ production for three reasons:

1) Metrifonate provides a relatively stable and long-term inhibition of ChE with one daily dose, and its unique property of action allows for a well-tolerated inhibition of ChE over longer time period compared with other drugs that are already on the market (such as donepezil, rivastigmine) (Ringman and Cummings, 1999; Schmidt and Heinig, 1998).

2) Cell cultures studies have shown that metrifonate modulates APP metabolism into the less amyloidogenic direction (Pakaski et al., 2000).

3) Previous studies in our group have used this drug in mice with the same genetic background (C57BL/6J) (Ikonen et al., 1999).

6.1.3. Cholinergic depletion of the hippocampus by fimbria-fornix transection

In order to investigate the effects of cholinergic dysfunction on APP processing in the hippocampus, a fimbria-fornix lesion model was used. The fimbria-fornix carries the largest part of cholinergic afferents to the hippocampal formation (approximately 75%) (Storm-Mathisen and Guldberg, 1974). In rats, a fimbria-fornix lesion has been demonstrated to result in a dramatic depletion of cholinergic markers in the hippocampus (Henderson, 1996). It should be noted that AChE staining disappears completely in the dorsal hippocampus after transection of the fimbria-fornix, but the cholinergic staining in the ventral hippocampus is partially retained (Gage et al., 1984; Gaykema et al., 1990). Compared to other alternatives for the cholinergic depletion of the hippocampus, the FFX-transection model was chosen for two reasons:

1) The lesion effect of FFX-transection is similar to that induced by other alternative lesion methods, such as electrolytic or excitotoxic lesion of the cholinergic basal forebrain (Cassel et al., 1997), but the outcome is more consistent.

2) Producing selective cholinergic lesions with immunotoxin in mice was not possible at the time point when our experiment was carried out, as the available immunotoxin, i.e. 192 IgG-saporin, only works well in rats.

6.1.4. Choice of behavioural tests

For the behavioural phenotyping of the AP mice, we used a battery of behavioural tasks, which are varying both in nature and in the sensorimotor functions required. The open field test was used first, primarily as a preliminary screening in order to detect possible deficits of the transgenic mice in locomotor functions, and the elevated plus-maze test was used to test for changes in emotional status (i.e. anxiety). Thereafter, the mice were tested in different learning and memory tasks. Several appetite-motivated learning and memory tasks were used. Among these, the win-shift version of RAM task and delayed alternation task in the T-maze were used to measure spatial working memory, whereas the win-stay version of RAM and position discrimination in the T-maze task were employed to detect non-spatial learning and memory. In addition, spontaneous alternation task in the T-maze was conducted to assess exploratory behaviour and working memory, which is based on the animal's natural tendency to explore novelty. The Morris water maze task was used to specifically detect impairment in hippocampal-dependent spatial learning and memory (Morris et al., 1982), and this task is primarily aversively motivated. In order to minimize confounding factors such as carry-over or interference, we have divided the mice into subgroups and tested them with a limited number of tasks only.

6.2. Effects of cholinergic manipulation on APP and Aβ levels, and the deposition of Aβ

6.2.1. Inhibition of AChE by metrifonate failed to prevent the marked overproduction and deposition of Aβ, and the spatial memory deficits in AP mice

In this study, seven-month-old AP mice were chronically treated with metrifonate (MTF) for 7 months at a dose (100mg/kg) that has been found to lead to 81% inhibition in the activity of AChE after acute administration. At the end of the treatment period, the level of inhibition of AChE was found to be approximately 50%; however, the total APP levels in the hippocampus were not altered by MTF in both AP and NT mice. No reduction in the Aβ40 or Aβ42 level was observed in the MTF treated AP mice; in contrast to our hypothesis, MTF significantly increased Aβ levels in the hippocampus. These findings do not support the suggestion derived from *in vitro*

studies that ChEI treatment would slow down amyloid production in the brain. Our results appear at odds with recent *in vitro* findings that ChEIs (including MTF) (Pakaski et al., 2000) promote APP processing through the α-secretase pathway and thus limit the production of Aβ (Mori et al., 1995b). They also contrast with the findings that direct stimulation of muscarinic (Nitsch et al., 1992) or nicotinic (Kim et al., 1997) receptors leads to increases in the production of soluble APP *in vitro*. The discrepancy between these *in vitro* studies and the present *in vivo* study may be explained by several mechanisms. First, chronic treatment with ChEIs may not result in a sufficient increase in synaptic ACh level compared to short-term treatment. Chronic inhibition of AChE is likely to result in upregulation of the enzyme; the fact that AChE inhibition was decreased from 81% (short-term) to about 50% at the end of our experiment, and that increased staining of AChE could be seen in some neurons indicate that an increased AChE synthesis may be present. Further, the increased levels of ACh can reduce its own release through stimulation of M2 autoreceptors; indeed, it has been shown in brain slices that nonselective muscarinic stimulation with carbachol does not increase the secretion of sAPP unless M2 receptors are simultaneously blocked (Farber et al., 1995). Second, synthesis of ACh by ChAT is limited by the high-affinity uptake of choline, which in turn is partially derived from the extracellular breakdown of ACh by its esterase; prolonged inhibition of AChE could thus reduce ACh synthesis. Third, a chronic increase in the ACh level in the intact brain may have additional effects that could cancel the (beneficial) direct effects on APP metabolism; in other words, the APP processing *in vivo* may be only partially under the regulation of cholinergic activity. Finally, it is possible that the processing of the human APP was regulated differently from the endogenous mouse APP. To elucidate the relationship between APP processing and cholinergic activity, a study that specifically investigates the effects of cholinergic depletion on APP processing in AP mice is needed.

Although a significant increase in the total amount of Aβ40 and Aβ42 in the hippocampus was found in the MTF treated AP mice; however, the number of amyloid plaques in the hippocampus did not differ from the vehicle treated AP mice. This finding may be partly due to the fact that separate sets of animals were used for biochemical and neuropathological analysis, or that the number of animals in the neuropathological analysis was relatively small. An alternative explanation is that this

discrepancy may be related to the decreased Aβ42 to Aβ40 ratio in the MTF treated animals. A recent report (Mucke et al., 2000) comparing different APP transgenic mouse lines found that a high Aβ42 to Aβ40 ratio is more favourable for plaque formation. Interestingly, in an *in vitro* study, MTF reduced the amount of Aβ42 secreted into the conditioned medium but had no effect on the amount of Aβ40 (Lahiri et al., 2000). This was not observed in other equipotent ChEIs, suggesting that MTF may modify γ-secretase activity through a mechanism that is independent of its action on AChE.

The present study demonstrated that the AP mice were impaired in the spatial version of the water maze, which could indicate compromised functioning of the hippocampus (Morris et al., 1982). However, it should be noted that the AP mice were also impaired in the cued version of the water maze task, which is consistent with the original observation by Morris (1982) that rats with hippocampal lesions were also impaired in the initial learning in the visible platform version of the task. Notably, such deficits have been demonstrated to be independent of age in an earlier study (Puoliväli et al., 2002). Further, in this study no motor abnormalities were observed in the AP mice, and their swimming speed was similar to that of the controls. Since all transgenic AP mice could find the visible platform, their inferior performance in this task is unlikely caused by an impaired visual ability (Puoliväli et al., 2002), but more likely by a deficit in learning a new task. Similar to our previous study, AP mice that failed in the visible platform task often swam towards the platform, but went on swimming after they hit it (Puoliväli et al., 2002).

In contrast to water maze learning, AP mice performed the place discrimination task in the T-maze better than NT mice; the learning of this task requires egocentric memory, which has been suggested to require intact functioning of the striatum (Oliveira et al., 1997). A better performance of AP mice in such a striatum-dependent task might reflect a compensatory effect due to the impaired functioning of the hippocampal formation. On the other hand, our previous study demonstrated that younger AP mice (12-month-old) performed the same task equally to the NT control mice (Puoliväli et al., 2002).

The behavioural effects of chronic treatment with a relatively large dose of MTF in the AP mice were not very favourable, since it did not improve the performance in

any of the learning and memory tasks employed. In general, the drug was well tolerated in the mice, but it clearly decreased locomotor activity in the open field, and reduced swimming speed in the water maze. These effects most likely reflect muscle weakness due to peripheral effects of MTF at the neuromuscular junction; muscle weakness is a common symptom of excessive inhibition of AChE due to organophosphate poisoning (He et al., 1998; Singh and Sharma, 2000). It is possible that the decreased swimming speed could have masked a beneficial effect of MTF in the water maze learning.

Finally, it should be noted that one important difference between our mouse model and human AD is the extensive cholinergic degeneration in AD patients as opposed to the absent (or possibly, very mild) cholinergic dysfunction in our mice. In the AP mice, the brain ChAT activity, the density and distribution pattern of the AChE positive fibers, and the binding levels of $\alpha4\beta2$ and $\alpha7$ nAChRs are not different from their nontransgenic controls (I, II and Marutle et al., 2002), indicating that no obvious cholinergic changes exist in the AP brain. This might also explain the lack of beneficial effect of MTF on water maze performance of the AP mice, as in previous studies of our laboratory, MTF dose-dependently improved water maze performance only in mice with medial septal lesions but did not affect the performance of sham-operated mice (Ikonen et al., 1999). However, based on the currently available data, we cannot entirely exclude a possible role of cholinergic dysfunction in causing the memory impairment in our AP mice. In fact, cholinergic changes have been reported in several other transgenic mouse models. For instance, in the APP+PS1(M146L) mouse, a prominent decrease in the density of cholinergic synapses in the frontal cortex and a reduction of size of these synapses in the frontal cortex and hippocampus have been observed (Wong et al., 1999). In the Tg2576 mice, Apelt et al. demonstrated (Apelt et al., 2002) a significant reduction of high-affinity choline uptake in the hippocampus in 21-month-old mice, and reduced binding levels of M1- and M2-muscarinic cholinergic receptors in the cortex and hippocampus, but there was no difference in the level of AChE and ChAT activity in the cortex or hippocampus between transgenic mice and non-transgenic littermates, at any age between 7 and 24 months (Apelt et al., 2002). For our AP mice, besides the ChAT activity or AChE staining, other parameters that are related to the cholinergic neurotransmission, such as synaptic ACh level, binding levels of M1-

and M2-muscarinic cholinergic receptors, high-affinity choline uptake, and the possible changes of cholinergic synapses, etc., should also be studied in the future.

6.2.2. Fimbria-fornix lesion does not affect APP levels and amyloid deposition in the hippocampus of AP mice

The fimbria-fornix transection resulted in a substantial depletion of cholinergic markers in the hippocampus at all three time points studied, and the extent of cholinergic depletion was similar for both transgenic and control mice. A more than 70% decrease in ChAT activity was found 2 weeks and 3 months postlesion, but the decrease was reduced to only 52% following an 11 months recovery period. The AChE staining density in the hippocampus was also higher in the 18-month-old animals compared to the 8 month-old mice (i.e. 11 vs. 3 months postlesion). The recovery of cholinergic markers after a long-term survival period has been observed in earlier studies in rats (e.g. Gage et al., 1983). They demonstrated a reinnervation of the hippocampus by the undamaged ventral afferent axons (Gage et al., 1984; Gasser and Dravid, 1987), and this likely also occurred in our mice.

Despite the marked cholinergic depletion (> 50-70 %) of the hippocampus, total APP or Aβ levels were not significantly changed in the hippocampus at either of the time points studied. Further, the lack of change in the level of either sAPP or mAPP indicated that probably APP processing was not altered by the lesion. Moreover, the extent of Aβ deposition did not differ between the 18-month-old AP-FFX and AP-sham mice (i.e. 11 months postlesion), as assessed by plaque counts and analysis of the Aβ load. Taken together, our data suggest that transection of the fimbria-fornix does not substantially alter APP processing in the hippocampus and, thus, does not accelerate or decrease Aβ production (or deposition) in the hippocampus of our AP mice.

The relationship between cholinergic denervation and APP metabolism is not clear yet; some studies have shown an increase in APP levels, whereas other studies demonstrated a decrease. Ramirez et al. (2001) reported that APP mRNA levels were reduced in the hippocampus after selective lesions of the cholinergic medial septum in rats. Similarly, Apelt et al. (1997) showed that 192-IgG-saporin induced basal forebrain cholinergic lesion moderately decreased levels of APP695 mRNA but not APP 751 or APP770 isoforms in the cortex and hippocampus in rats. In contrast, Rossner et al.

(1997) did not detect significant changes in total APP mRNA levels after selective lesions of the cholinergic basal forebrain in rats, although a significant reduction of secreted APP and a concomitant increase of membrane-bound APP were observed. However, these findings together with our findings are at odds with other previous studies demonstrating increased APP expression after lesions of the basal forebrain cholinergic system in different mammalian species. For instance, in rats cholinergic basal forebrain lesions, produced by NMDA injection, have been reported to increase APP mRNA and APP synthesis in the cortex (Wallace et al., 1991; Wallace et al., 1993). Similarly, selective lesions of the cholinergic neurons of the basal forebrain have also been reported to increase APP gene expression and protein level in various cortical and hippocampal regions (Leanza, 1998; Lin et al., 1998). The discrepancies between these results may likely be attributed to the different time points studied, the type of lesion, or the methods of measuring APP.

With regard to the alterations in Aβ levels and/or Aβ deposition after cholinergic lesion, two recent studies reported contradictory results. Beach et al. (2000) lesioned the nucleus basalis of magnocellularis (nbM) in 12-week-old rabbits, which resulted in a 2.5-and 8-fold increase in the levels of cortical Aβ40 and Aβ42, respectively. In contrast, Boncristiano et al. (2002) performed unilateral nbM lesions in adult, transgenic APP23 mice, and they found a significant *decrease* in Aβ levels (19%) in the ipsilateral frontal cortex compared with the contralateral side. These discrepant results may likely be attributed to the overexpression of APP in the transgenic mouse compared to the normal expression level of APP in the rabbit, but it is also possible that species differences have an influence on APP metabolism. For example, phorbol ester activation of PKC has been shown to increase the release of sAPP and decrease Aβ release in rat cell cultures or other cell lines (Buxbaum et al., 1993b; Caputi et al., 1997; Gabuzda et al., 1993); but in the brains of transgenic mice producing elevated levels of human Aβ, activation of PKC decreased both sAPP-β and Aβ levels without affecting sAPP-α levels (Savage et al., 1998). Alternatively, the overproduction of Aβ due to the APPswe mutation (Citron et al., 1992) may mask the possible mild alteration in Aβ levels induced by the fimbria-fornix lesion.

Taken together, our findings indicate that disruption of cholinergic neurotransmission does not seem to promote the progression of AD-like amyloid

pathology in the hippocampus in AP mice, contrary to what has been suggested by earlier studies (e.g. Wallace et al., 1993; Lin et al., 1998; Leanza, 1998 #163). Considering the non-specific nature of the fimbria-fornix lesion, further investigation is still needed to examine the effects of pure cholinergic dysfunction on APP metabolism and the progression of Aβ pathology, when the highly selective cholinergic lesion approach with IgG-saporin is available also for mice (Berger-Sweeney et al., 2001).

6.3. Learning and memory impairments in AP mice, and their relation to Aβ overproduction and deposition

6.3.1. Hippocampus-dependent long-term spatial memory is selectively impaired in AP mice that develop amyloid pathology mimicking early stages of AD

The aim of this study was to elucidate the behavioural consequence of amyloid deposition in the 17-month-old AP mice. The results of the histopathological study confirm earlier observations (Borchelt et al., 1997) that amyloid deposition in these mice is largely restricted to the hippocampal formation and the neocortex, with the earliest deposits in the dorsal subiculum. In contrast to the more widely used APP+PS1(M146L) double transgenic mouse (Arendash et al. 2001; Gordon et al., 2001; Gordon et al., 2002), very few amyloid deposits were found in the striatum and thalamus at any age in our AD mouse model. Thus, in some aspects, our AP mouse is a good model for the early stages of Alzheimer's disease in which the pathology is most prominent in medial temporal lobe structures (Braak and Braak, 1991); however, it should be noted that the mouse does not develop neurofibrillary tangles.

To test whether the amyloid pathology in the hippocampal formation would have a functional correlate, we tested AP mice in a large battery of learning and memory tasks, and, further, we compared the behavioural profile of AP mice with fimbria-fornix lesioned mice. The comparison between AP mice and fimbria-fornix lesioned mice revealed interesting similarities. First, both AP and FFX mice performed similar to their controls in two non-hippocampal dependent tasks, i.e. the win-stay task in the 8-arm radial maze and in the position discrimination task in the T-maze (Table 2). In rats, both tasks have been suggested to be sensitive to striatal lesions but not to hippocampal lesions (Oliveira et al., 1997; Packard et al., 1989). Normal performance

of the AP mice in these two tasks is consistent with the minimal pathology in the striatum in these animals. Second, both AP and FFX mice were impaired in the water maze task, which is hippocampus-dependent. Remarkably, this impairment is selective to the long-term memory component of the water maze task, as the AP mice improved their performance comparably to that of the NT control mice during each daily session. Interestingly, in addition to the hidden platform task, FFX mice also performed worse in the visible platform task than the sham-operated control mice. It is possible that the earlier experience in the hidden platform training interfered with the learning of the visible platform task in the FFX mice (Gerlai, 2001).

However, in sharp contrast to the impaired performance of FFX mice, AP mice (i.e. AP-sham) performed comparably to NT control mice in the win-shift version of the radial arm maze task, and in both spontaneous and food motivated delayed alternation tasks in the T-maze. Common to these tasks is that they all require intact spatial working memory and have been shown to be sensitive to damage of the hippocampal formation. One could argue that impaired performance of AP mice in the water maze, but relatively normal performance in dry mazes, is due to the aversive nature and the stress associated with swimming. However, two observations in this study strongly argue for a memory deficit in the AP mice rather than increased sensitivity to stress in the aversive water maze tasks. First, AP mice showed normal short-term memory in the water maze task, as their improvement within the day was not different from that of controls, but, in contrast, their memory retention for the platform location on the next day was clearly impaired. Second, AP mice were unimpaired in two different T-maze alternation (working memory) tasks that differed in the motivational aspect. Whereas the delayed alternation was food-motivated as were all radial arm maze tasks, the spontaneous alternation is based on the innate explorative behaviour of mice in a novel environment. Hence, the likely explanation of the behavioural profile of the AP mice is to conclude that they were impaired in hippocampal dependent tasks but only in those that tax long-term memory.

The only task in which the AP genotype and FFX lesion appeared to have additive effects was the retention phase of the win-shift version of the RAM task. In this task the AP-FFX group was clearly worse than any other group. The nature of this additive effect may be explained when we compare the performance of AP-sham and

NT-FFX groups over two testing days (III, Figure 4). In the last two sessions, the AP-sham mice made as many errors as AP-FFX mice on Day 1, but they dramatically improved their performance on Day 2. It looks as if these mice had forgotten the rule of the task after a pause of one month in the testing routine, but performed as controls on Day 2 after re-training in the task. Such rule learning may not depend on the hippocampus as much as on frontal and parietal cortices that were intact in NT-FFX mice but loaded with amyloid in the AP-sham and AP-FFX mice.

The selective impairment of hippocampal dependent long-term memory is at odds with other studies in APP mutant mice or APP+PS1(M146L) mice that reported impaired working memory in the tasks of radial arm water maze (Arendash et al., 2001), the win-shift version of the RAM (Dodart et al., 1999), and delayed alternation in the T-maze (Chapman et al., 1999). However, this discrepancy might be related to the differences in the mouse strain that was used to generate transgenic mice (Crawley et al., 1997), or differences in the type, amount, or localization of the amyloid pathology in these animal models. It is noteworthy that the densest amyloid deposition in our AP animals is found in the dorsal subiculum; based on selective lesions of either hippocampus or subiculum, Morris et al. (Morris, 1990) suggested that subicular lesions may cause an impairment of long-term spatial memory in the water maze navigation but little impairment in spatial short-term memory. Rats with subicular lesions have been reported to be impaired in the working memory version of the RAM, but the impairment is less severe than in rats with hippocampal lesions (Galani, 1998). It should be noted that the only study with selective subicular lesions in mice showed increased reference but not working memory errors in a modified RAM task (Cho and Jaffard, 1995). Further, different stages of amyloid pathology may have distinct effects on memory processes. All studies that have reported impaired working memory used transgenic animals with higher amounts of Aβ deposits compared to our mice (Arendash et al., 2001; Chapman et al., 1999; Dodart et al., 1999). For instance, the APP+PS1(M146L) mice at 15-16 months age have an average amyloid load of approximately 50 % in the frontal cortex, while that in our mice never exceeds 5% at the age tested (17 months). Large amyloid deposits with surrounding inflammatory reaction (Stephan et al., 2001) may have non-selective effects on nearby neurons, whereas a less robust accumulation of Aβ may more selectively interfere with neuronal plasticity. Several mechanisms

have been proposed by which Aβ may interfere with neuronal plasticity without direct neurotoxicity. For example, intracellular calcium signaling plays a pivotal role in learning induced plasticity, and Aβ has been shown to destabilize calcium homeostasis in neurons (Mattson et al., 1992). Elevated levels of Aβ may also downregulate the mitogen-activated protein kinase (MAPK) cascade and decrease phosphorylation of cAMP-response element binding protein (CREB) (Dineley et al., 2001; Tong et al., 2001), which plays an essential role in learning and memory processes (reviewed by Silva et al., 1998). In addition, both APP and its C-terminal fragment containing the Aβ peptide are axonally transported to the axon terminals where it may interfere with synaptic transmission (Buxbaum et al., 1998), or with critical signal transduction processes that mediate neuronal plasticity.

6.3.2. Cognitive deficits in AP mice correlate with insoluble Aβ42 level, but not with the soluble Aβ42 level in the brain, or with the Aβ levels in CSF and serum

The primary aim of this study was to investigate the relationship between Aβ levels of different forms (i.e. soluble and insoluble) and species (i.e. Aβ40 and Aβ42) in brain (hippocampus) and the spatial learning and memory deficits in AP mice. Our results demonstrated that it is Aβ42 but not Aβ40, and it is the levels of insoluble but not soluble form of Aβ42 that correlate with impaired spatial memory, as shown by a decreased search bias in the water maze. This result confirms the previous finding of our laboratory in AP mice that the concentration of total Aβ42 but not that of soluble Aβ42 in the hippocampus correlates with impaired spatial memory in the water maze (Puoliväli et al., 2002). This study also demonstrated that at the age of 12 months, the memory impairment was not correlated with Aβ load or plaque number in the hippocampus (Puoliväli et al., 2002). Our results substentiate the previous findings showing that aggregated form of the Aβ peptide is the primary neurotoxic agent both *in vitro* (Lorenzo and Yankner, 1994; Pike et al., 1993) and *in vivo* (Giovannelli et al., 1998). They are also in accordance with a very recent study that demonstrated that neuronal loss is associated primarily with thioflavin S-positive fibrillar Aβ deposits (Urbanc et al., 2002) in both AD patients and transgenic mice. However, the results of our studies are slightly different from an earlier study in AD patients demonstrating that

elevated levels of both total Aβ42 and Aβ40 in the neocortex are correlated with cognitive decline (Näslund et al., 2000). This may be explained by the difference in genetic background between transgenic mice and humans, or the expression of the mo/hu APP-695swe in the AP mice. Further, the ratio of Aβ42 and Aβ40 is quite different between humans and mice (Näslund et al., 2000). On the other hand, immunocytochemical studies of the AD brains have revealed that it is Aβ42 (and Aβ43) that deposits very early in the disease process, and this is associated with the cognitive decline (Parvathy et al., 2001), strongly indicating a higher neurotoxic effect for Aβ42 than for Aβ40. In contrast, McLean et al. reported that cognitive deficits correlated only with soluble but not insoluble Aβ (McLean et al., 1999), suggesting that more studies are needed to clarify the current discrepancies.

To achieve an early diagnosis of AD and monitor the disease progress, considerable attempts have been made to find a reliable biomarker for AD. Among the several candidates (such as CSF Aβ, tau, and plasma Aβ etc.) the relationship between Aβ levels in the cerebrospinal fluid (CSF) and the presence and/or severity of the disease has been most intensively studied. The results have been variable showing a decrease (Pirttilä et al., 1994), an increase (Nakamura et al., 1994) or no change (Motter et al., 1995; Nitsch et al., 1995; van Gool et al., 1995) in total Aβ levels. In longitudinal studies, CSF Aβ levels were shown to be stable during a follow-up period of one year (Andreasen et al., 1999), but to decrease during an extended follow-up (Tapiola et al., 2000). The decrease in the CSF Aβ level may reflect the deposition of Aβ in the brain (Kawarabayashi et al., 2001) and hence the reduced efflux of Aβ from brain to CSF. This notion is supported by a recent study in Tg2576 APPswe transgenic mice that demonstrated an inverse-correlation between the Aβ levels in CSF and brain as mice age, i.e. Aβ42 levels in the CSF decreased at the age when there is marked amyloid deposition in the brain (Kawarabayashi et al., 2001). However, the relationship between CSF Aβ and the severity of cognitive deficits in AD patients is currently unclear, showing either an inverse correlation (Hock et al., 1998; Nitsch et al., 1995) or no correlation (Andreasen et al., 1999; Nakamura et al., 1994). So far, in transgenic mouse models of AD, the possible correlation between the CSF Aβ level and memory deficits, and, more importantly, the relation between CSF Aβ level and brain Aβ burden have

never been studied. Therefore, we correlated CSF Aβ levels with those of the brain, as well as with the water maze learning. The results revealed a significant correlation between CSF Aβ42 level and brain soluble, but not insoluble, Aβ42 level; on the other hand, the CSF Aβ42 levels are not correlated with the spatial learning deficits in the water maze tasks (IV, Table 1). Together, these results suggest that the measurement of CSF Aβ42 may not be a good indicator for the cognitive decline. However, longitudinal studies are still needed to further elucidate the relationship between changes in the CSF Aβ42 level and the progression of amyloid deposition in AP mice.

Controversy still exists whether the Aβ that is present in the CSF is produced only by neurons. It may partly originate from blood cells, e.g. platelets release Aβ on activation (Smith et al., 2001) and intravenously injected Aβ can enter brain through the blood-brain-barrier (Maness et al., 1994). The situation is likely different in the present study. The prion protein promoter used in our mice directs transgene expression to practically all tissues, but at high levels only in brain and heart (Borchelt et al., 1996a); therefore, neurons are likely to be the main source of Aβ in the CSF of our AP mice, but the heart cannot be excluded.

In this study, we also examined the relation between Aβ levels in serum, CSF, or brain, and the spatial learning deficits. Since Aβ42 in serum was under the detection level of the ELISA kit we used, only Aβ40 was measured. Our results showed that the serum Aβ40 was not correlated with any of the above parameters (IV, Table 1), which is consistent with the study in PDAPP transgenic mice (DeMattos et al., 2002), as with a study in AD patients (Mehta et al., 2000). The poor relation between serum and CSF Aβ levels, or serum and brain Aβ levels may arise from a difference in the rate of clearance of Aβ or a compromised (i.e. decreased) permeability of Aβ across the blood-brain barrier. Another possibility is a significant contribution from a non-neuronal source, such as the heart, to the total Aβ in serum. Since we measured Aβ40 in the serum and soluble Aβ42 in the CSF and brain this could play a role; however, this is likely not the case since the Aβ40 and Aβ42 levels in the brain of our AP mice are highly correlated. Together, these results suggest that the measurement of serum (or plasma) Aβ40 may not be a useful biomarker for the diagnosis of AD.

The possible relationships in the distribution of soluble and insoluble Aβ among different compartments, i.e. brain, CSF and blood were schematically summarized in Figure 8.

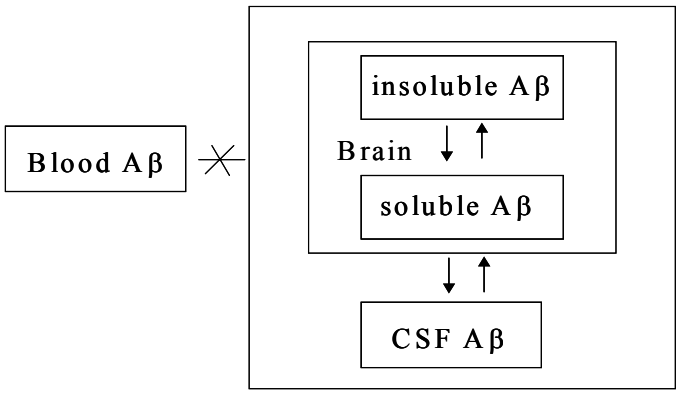

Figure 8. A schematic diagram showing the putative relationships in the distribution of soluble and insoluble Aβ among the brain, CSF, and blood compartments.

6.4. General discussion

Using the APPswe and PS1(A246E) double transgenic mouse model of AD that have Aβ deposition in their brains as they age, the present studies were aimed to investigate the potential role of altered levels of ACh in the regulation of APP metabolism and the progression of amyloid pathology, the role of Aβ overproduction or/and deposition in causing cognition decline, and, finally, the correlation between cognitive impairments and Aβ levels in different compartments (i.e. in brain, CSF, or blood).

In the *first* study, we showed that an increase in the levels of ACh, induced by long-term treatment with metrifonate, did not affect the APP levels in the brain. However, presumably via a mechanism that is independent of its action on AChE, metrifonate significantly increased both Aβ40 and Aβ42 levels in the hippocampus, but reduced the Aβ42 to Aβ40 ratio, without affecting the number of amyloid plaques in the hippocampus (I). Correspondingly, in the *second* study we demonstrated that a sustained decrease in ACh levels in the hippocampus, produced by a fimbria-fornix lesion, did not significantly affect hippocampal APP levels and Aβ production, and Aβ deposition (II). Taken together, our results do not support the postulation that

modulating ACh levels could significantly alter APP metabolism and Aβ production, or the putative role of cholinergic dysfunction in the promotion of Aβ production and deposition, which are contrary to previous *in vitro* or *in vivo* studies (e.g. Nitsch et al., 1992; Pakaski et al., 2000; Wallace et al., 1993; Leanza, 1998; Lin et al., 1998). Although our results do not support the hypothesis of a causative role of cholinergic dysfunction in the pathogenesis of AD, one should be cautious when extrapolating these results to human studies, as our AP mice express a chimeric mo/hu APP-695swe cDNA on the background of the mouse APP gene. Thus, the expression of mo/hu APP and the regulation of its processing in these mice should be different from humans. Further, considering the non-specific nature of the fimbria-fornix lesion and the possible differences in APP regulation in different species, more studies are needed to examine the effects of cholinergic dysfunction on APP metabolism and the progression of Aβ pathology in a higher mammalian species (e.g. monkey) that have the same APP sequence as humans.

The *third* study of the thesis demonstrated a selective impairment of hippocampus-dependent long-term spatial memory of the AP mice in the water maze task (III). A more recent study of our lab has demonstrated that such a learning impairment is possibly related to a more rapid decay of long-term potentiation (LTP) in the hippocampus (K. Gurevicius et al., unpublished data), but the exact mechanism behind the cognitive deficits in our AP transgenic mice is not clear yet. Based on the currently available data, we cannot entirely exclude a possible role of cholinergic dysfunction in causing the memory impairment in our AP mice, as some cholinergic deficits have already been observed in several other AD model mice (e.g. Apelt et al., 2002; Wong et al., 1999). It has been demonstrated by a number of *in vitro* studies that the soluble form of Aβ can reduce ACh synthesis and/or ACh release at a lower concentration, while higher concentrations of Aβ can be directly neurotoxic to the cholinergic neurons by disrupting intracellular ion concentrations (e.g. Ca2+) and by generating reactive oxygen species (reviewed by Auld et al., 1998). Further, it has been demonstrated that microinjection of fibrillar Aβ causes degeneration of basal forebrain cholinergic neurons and a concomitant decrease in ACh release in rats (Harkany et al., 1995a; Harkany et al., 1995b). On the other hand, it should also be noted that the "cholinergic hypothesis" as such is being challenged now by several recent studies. For

example, two postmortem studies reported that ChAT activity was upregulated in subjects with mild cognitive impairment (DeKosky et al., 2002), and unchanged in early stage AD compared with control subjects (Davis et al., 1999), respectively, while a decrease in ChAT activity was only found in the relatively late stage of AD (Davis et al., 1999; DeKosky et al., 2002). Together, these studies suggest that cholinergic dysfunction is likely not responsible for the early cognitive deficits that occur in AD.

Furthermore, in the *fourth* study we found that the impaired spatial memory in AP mice is significantly correlated with the level of insoluble Aβ42, but not that of soluble Aβ42, in the hippocampus (IV), suggesting that the age-related cognitive deficits in AP mice are likely related to the overproduction and deposition of Aβ. Whereas several studies have shown that there is not a clear relation between the amount of deposited Aβ and memory disturbances (e.g. D'Hooge et al., 1996; Holcomb et al., 1998; Koistinaho et al., 2001; Moechars et al., 1999), our study (and studies of others, e.g. Gordon et al., 2001; Chen et al., 2000) demonstrated that memory impairments correlate with insoluble Aβ levels (and deposits) in the brain in middle-aged AP mice (as discussed in section 6.2.1 and 6.2.2). Although it is still controversial which form of Aβ (soluble or insoluble) is pivotal for the dementia in AD, earlier studies have focused on the neurotoxic effects of insoluble Aβ. Now it becomes gradually more accepted that different forms of Aβ may play distinct roles in AD pathogenesis. Increasing evidence suggests that it is soluble oligomer form of Aβ, but not the insoluble fibrillar form of Aβ, that causes the prominent hippocampal synaptic dysfunction seen in the earliest stages of AD (Selkoe, 2002). In AD, the monomers of Aβ undergo a complex process of fibril formation, during which they exist in the forms of soluble oligomers, protofibrils, and fibrils (Walsh et al., 2002b). Walsh et al. (2002a) demonstrated that intracerebroventricular microinjection of soluble oligomers of Aβ, but not monomers or insoluble Aβ fibrils, can potently inhibit hippocampal long-term potentiation in adult rats, supporting the role of soluble oligomers of Aβ in causing the cognitive decline in the early stages of AD. However, it should be noted that it is difficult to test this hypothesis in transgenic mice because a mixture of Aβ forms (including monomers, soluble oligomers, insoluble amyloid fibrils, etc) are present in the brain (Selkoe, 2002), and they cannot be differentiated by the currently available

ELISA for Aβ. This could also possibly explain the lack of correlation between soluble Aβ42 and spatial learning deficits of the AP mice found in the present study (IV). On the other hand, the later forms of Aβ, mainly insoluble Aβ fibrils, are proposed to be principally responsible for the neuronal dysfunction and neuronal loss in the late stage of AD (Selkoe, 2002; Walsh et al., 2002b).

Regardless of the different roles of soluble and insoluble Aβ, it seems that therapeutics targeted to both Aβ forms could prevent the cognitive decline as demonstrated by several recent studies showing that immunization against Aβ can protect transgenic mice from learning and memory impairments (Janus et al., 2000; Morgan et al., 2000; Dodart et al., 2002). The results of Janus et al. (2000) and Morgan et al. (2000) indicate that the cognitive improvements are largely related to the reduction of the Aβ burden; whereas Dodart et al. (2002) reported that passive immunization could rapidly reverse memory deficits in PDAPP mice without altering brain Aβ burden, suggesting a role for soluble Aβ in the cognitive impairments. Nevertheless, more studies are still needed to understand better how these different Aβ species injure neurons and synapses *in vivo*.

Finally, it should also be noted that the current transgenic mouse models of AD do not develop the full range of pathological features of AD, and that the mouse is a different species; thus one must be cautious when extrapolating the animal data to humans. One good example is also from the Aβ vaccination studies. Whereas transgenic AD model mice tolerated the Aβ vaccination well, some AD patients developed brain inflammation after this treatment (however, in a recent study Aβ vaccination, under certain circumstances, was also shown to induce CNS inflammation in *normal* mice, Furlan et al., 2003). Similarly, the results obtained from the present study may not entirely be applicable to AD patients; however, as an *in vivo* system, transgenic mice are still invaluable in exploring the pathological mechanisms of the disease and in testing novel therapeutics for treatment of AD.

7. CONCLUSIONS

– An increase in the levels of ACh, induced by long-term treatment with metrifonate, failed to inhibit the marked overproduction and deposition of Aβ in AP mice (I).

– Hippocampal APP and Aβ levels, and the extent of Aβ deposition were not increased by a non-selective cholinergic depletion resulting from the transection of the fimbria-fornix (II).

Together, these findings do not support the notion derived from *in vitro* studies that ChEI treatment would slow down amyloid accumulation in the brain, or a putative role of cholinergic dysfunction in the promotion of Aβ production and deposition, but conversely they indicate that APP metabolism may not be regulated by or related to the levels of ACh, at least in the AP mice used in our studies. However, considering the differences between AD mouse model and human patients, the potential role of altered ACh levels in the regulation of APP metabolism still need to be more carefully investigated in future studies.

– In AP mice, amyloid deposits are largely restricted to the hippocampal formation and the neocortex; few plaques, if any, are present in the thalamus, brainstem or cerebellum. The earliest and densest depositions are present in the dorsal subiculum, which may underlie the selective impairment of hippocampal-dependent long-term spatial memory in AP mice (III).

– The impairment of spatial memory in AP mice is significantly correlated with the level of insoluble, but not that of soluble Aβ42, in the hippocampus. The CSF Aβ42 level appears not to be a suitable indicator of the cognitive decline, because it only correlates with the level of hippocampal soluble Aβ42 (IV).

Taken together, these findings indicate an important role for insoluble Aβ42 in the memory dysfunctions in our AP mice. As the characteristics of amyloid neuropathology and memory impairment in AP mice resemble the phenotype of early AD, this transgenic mouse line may provide a good model to examine the pathophysiological events leading to memory impairments in AD.

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