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Interactions between Neuronal Nicotinic Acetylcholine Receptors, N-Methyl-D-Aspartate Receptors and β-amyloid in the Brain of Genetically Modified Mice

- Implications for Alzheimer's disease

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To Lasse with love

ABSTRACT

The amyloid hypothesis is one of the leading theories in the search for the cause of Alzheimer's disease (AD) and is based on the theory that hyperproduction and accumulation of amyloid β-peptide (Aβ) in the brain triggers the disruption of neuronal and synaptic function, thereby ultimately leading to neurodegeneration and dementia. Some of the crucial questions are if Aβ has a neuronal function in the brain and how it interacts with the neurotransmitter system in AD. The cholinergic α7 neuronal nicotinic receptors (nAChRs) have been suggested to have a close interaction with Aβ.

The aim of this thesis has been to investigate the potential neuropathological effect of Aβ on the α7 nAChRs, the N-Methyl-D-Aspartate (NMDA) receptors and their possible interactions, which may play an important role in understanding the cognitive and neuropathological mechanisms seen in AD.

To study the effect of Aβ on α7 nAChRs, the PC12 cell line and transgenic mice models (APPswe and APP/PS1) were used. In order to study the interaction between Aβ, α7 nAChRs and NMDA receptors, the APPswe transgenic mouse model was used. It was found that oligomeric Aβ could reduce the number of α7 nAChRs in the PC12 cell line, consistent with the observation in the human AD patients. In the APPswe transgenic mice, a biphasic effect on the α7 nAChRs was found, with a decrease in very young mice followed by an increase at 10 months. A persistent increase in NMDA receptors in cortex and hippocampus of APPswe transgenic mice was observed. The up-regulation of the NMDA receptors in young mice might reflect initial changes in response to the early, high levels of soluble Aβ observed, while the up-regulation at older ages might be due to more chronic exposure of Aβ.

The effect of galantamine, memantine and nicotine treatment on the neuropathological changes in the brain, with special focus on Aβ, α7 nAChRs and NMDA receptors, were investigated in APPswe transgenic mice. Subchronic treatment (10 days) with nicotine (0.45 mg/kg/day x 2) reduced the insoluble $A\beta_{1-40}$ and $A\beta_{1-42}$ levels by 46 % and 66 % respectively, while the intracellular Aβ levels remained unchanged. This also resulted in less GFAP (glial fibrillary acidic protein) immunoreactive astrocytes around the plaques and increased levels of both synaptophysin and the number of α7 nAChRs in the cortex of APPswe transgenic mice. Galantamine treatment (2 mg/kg/day x 2) caused a 2-fold increase in cortical synaptophysin levels in the APPswe mice. Memantine treatment (10 mg/kg/day x 2) reduced the total cortical levels of membrane-bound APP by 45 % and 55 % in transgenic and non-transgenic mice respectively, which eventually may decrease the level of Aß.

The α7 nAChRs and NMDA receptors are important in mediating synaptic plasticity in the brain. A persistent exposure to Aβ in the brain of APPswe transgenic mice causes an increase in both the α7 nAChRs and the NMDA receptors. Treatment with galantamine, memantine or nicotine showed different effects on Aß processes, α7 nAChRs and NMDA receptors in APPswe mice. These different effects might have therapeutic relevance and this knowledge might be applicable to the development of new effective therapeutic strategies in AD.

LIST OF PUBLICATIONS

This thesis is based on the following papers, which will be referred to in the text by their roman numbers:

I. Guan ZZ, Miao H, Tian JY, **Unger C**, Nordberg A and Zhang X
Suppressed expression of nicotinic acetylcholine receptors by nanomolar beta-amyloid peptides in PC12 cells.
Journal of Neural Transmission, 2001, 108(12), 1417-1433

II. Marutle A, **Unger C**, Hellström-Lindahl E, Wang J, Puoliväli J, Tanila H, Nordberg A and Zhang X
Elevated levels of $Abeta_{1-40}$ and $Abeta_{1-42}$ do not alter the binding sites of nicotinic receptor subtypes in the brain of APPswe and PS1 double transgenic mice.
Neuroscience Letters, 2002, 328(3), 269-72

III. **Unger C,** Hedberg MM, Mustafiz T, Svedberg MM and Nordberg A
Early Changes in Aβ Levels in the brain of APPswe transgenic mice - Implication on synaptic density, α7 neuronal nicotinic acetylcholine- and N-Methyl-D-Aspartate receptor levels
Molecular and Cellular Neuroscience, 2005, 30 (2), 218-227

IV. **Unger C,** Svedberg MM, Schutte M, Bednar I and Nordberg A
Effect of memantine on the alpha7 neuronal nicotinic receptors, synaptophysin- and low molecular weight MAP-2 levels in the brain of transgenic mice over-expressing human acetylcholinesterase.
Journal of Neural Transmission, 2005, 112(2), 255-68

V. **Unger C,** Svedberg MM, Yu W-F, Hedberg MM and Nordberg A
Effect of Subchronic Treatment of Memantine, Galantamine and Nicotine in the Brain of APPswe Transgenic Mice
Submitted to Journal of Pharmacology and Experimental Therapeutics

CONTENTS

LIST OF ABBREVIATIONS

Aβ	Amyloid β-peptide
ACh	Acetylcholine
AChE	Acetylcholinestrase
AChEI	Acetylcholinestrase inhibitor
AD	Alzheimer's disease
AICD	APP intracellular domain
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazole propionate
ApoE	Apolipoprotein E
APP	Amyloid precursor protein
BACE	β-site APP cleaving enzyme
BuChE	Butyrylcholinesterase
CDK	Cyclin-dependent kinase
CFN	Swedish national board for laboratory animals
ChAT	Choline acetyltransferase
CNS	Central nervous system
ECE	Endothelin-converting enzyme
ERK	Extracellular-signal-regulated kinase
FAD	Familiar Alzheimer's disease
GAG	Glucosaminoglycan
GSK	Glucogen-synthase kinase
hAChE-Tg	Human Acetylcholinesterase overexpressing mice
HPLC	High performance liquid chromatography
IDE	Insulin-degrading enzyme
LTD	Long-term depression
LTP	Long-term potentiation
mAChR	Muscarinic acetylcholine receptor
MALDI-MS	Matrix-associated laser desorption/ionisation-mass spectrophotometry
MAPK	Mitogen-activated protein kinase
MCI	Mild cognitive impairment
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5, diphenyl tetrazolium bromide
nAChR	Nicotinic acetylcholine receptor
NEP	Neprilysin
NFT	Neurofibrillary tangles
NGF	Nerve growth factor
NMDA	N-methyl-D-aspartate
NSAID	Non-steroid anti-inflammatory drug
PET	Positron emission tomography
PS1	Presenilin 1
PS2	Presenilin 2
PKC	Protein kinase C
RPA	RNase protection assay
SAP	Serum amyloid P

INTRODUCTION

The brain is probably the most complex structure in the known universe. It is a network of more than 10^{12} neurons with each neuron receiving about 10^4 to 10^5 synaptic contacts in systems that construct our perceptions of the external world, fix our attention, and control the machinery of our actions. Adaptive reorganization of neuronal connectivity, which allows for the acquisition of new epigenetic information both during development and in the mature brain, is based upon the strengthening of existing synapses, the formation of new synapses and the destabilization of previously established synaptic contacts.

Alzheimer's disease (AD) is a progressive, neurodegenerative disorder, affecting a large number of individuals around the world. It is a devastating disease, affecting not only the afflicted individual but also close relatives and friends. During the past decade there has been a rapid progress in this research field. However, there are still numerous questions about this disease that need to be resolved. Since the amyloid β-peptide (Aβ) is thought to be one of the main players in AD pathology, a special focus was here made on the interaction between Aβ, the α7 neuronal nicotinic receptors (nAChRs) and the N-Methyl-D-Aspartate (NMDA) receptors, with the aim to get a better understanding of the disease.

ALZHEIMER'S DISEASE

In November 1906, Alois Alzheimer described the first case of the disease that now bears his name (Alzheimer, 1907). This was a 51-year old woman, Auguste D, who developed memory deficits and progressive loss of cognitive abilities. She later became institutionalized in a psychiatric hospital and died within five years after the onset of illness.

Today, AD is the most common dementia disorder in the world. There is an exponential increase of the disease with increasing age, causing a 2-fold increase every five years, from 1% at 65 years of age to over 20% at 85 years of age (Jorm et al., 1987). Common, early symptoms of the disease are short-term memory impairment. At later stages there is a general cognitive decline, accompanied by deterioration of language, spatial and motor abilities as well as psychiatric hallucinations. Clinical diagnosis of AD is currently based on the outcome of several investigations, including medical history, neurological and psychiatric investigations, lab analysis, radiological analysis and cognitive testing. During recent years the concept of mild cognitive impairment (MCI) has been a focus of interest. The amnestic MCI patients, when of a degenerative etiology, progress to AD at a greatly accelerated rate (Petersen et al., 1999).

The major risk factor for AD is age. Although most cases of AD appear to be sporadic, there are a small number of cases that result from genetic mutations, familial AD (FAD). Mutations in three genes which are considered to be connected with early-onset FAD have been identified; the amyloid precursor protein (APP) on chromosome 21, presenilin 1 (PS1) on chromosome 14 and presenilin 2 (PS2) on

chromosome 1, all leading to an increase in Aβ production ($A\beta_{1-40}$ and $A\beta_{1-42}$) (Hardy, 1996).

The presence of one or two E4 alleles of apolipoprotein E (ApoE) predisposes to AD and confers an earlier age of onset of the disease (Strittmatter et al., 1993, Huang et al. 2004).

The amyloid hypothesis

"The amyloid hypothesis" is one of the leading theories behind AD pathogenesis. It is based on the theory that hyper-production of Aβ, ultimately leads to a cascade of neurodegenerative events due to the toxic effect of APP metabolism on neuronal structures (Hardy and Higgins, 1992). Proteolytic processing of APP involves cleavage of several different proteases and may lead to the generation of Aβ. The two major processing pathways identified are described in Figure 1.

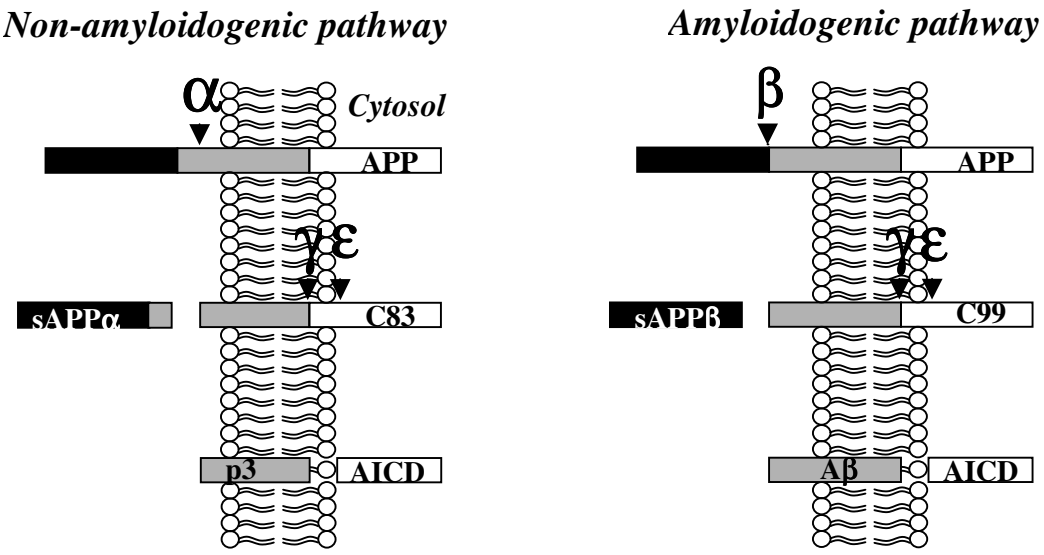

Figure 1: Schematic illustration of the proteolytic processing pathways for APP. The non-amyloidogenic processing of APP prevents the formation of Aβ, while amyloidogenic processing of APP generates Aβ.

In the non-amyloidogenic pathway α-secretase cleavage leads to the generation of a soluble extracellular fragment of APP (sAPPα) and a membrane-bound 83 amino acid residue fragment (C83). In the amyloidogenic pathway, APP is first cleaved at the β-secretase site by β-site cleaving enzyme (BACE) (Hussain et al., 1999). β-secretase hydrolysis generates a soluble extracellular fragment of APP (sAPPβ) and a membrane-bound 99 amino acid residue C-terminal fragment (C99). C83 and C99 are further processed by γ-secretase, within the transmembrane domain, to generate p3 and Aβ, respectively. There are two variants of Aβ ($A\beta_{1-40}$ and $A\beta_{1-42}$). 90 % of the Aβ produced is $A\beta_{1-40}$, but $A\beta_{1-42}$ is more prone to aggregate as fibrils (Selkoe, 2001). In addition, a soluble cytosolic fragment, APP intracellular domain (AICD), is generated. All AICD fragments analyzed to date have been shown to start at a position a few residues distal to the sites for γ-secretase cleavage (Sastre et al., 2001;

Yu et al., 2001; Weidemann et al., 2002). This indicates that there is an additional cleavage site in APP, the ε-cleavage site. The γ- and ε-secretase cleavages are closely linked to each other and are dependent on factors required for γ-secretase activity (Chen et al., 2002; Schroeter et al., 2003). Thus, γ- and ε- secretase cleavage may be performed by the same enzymatic activity.

The mutant APP in families with early onset AD supports the amyloid hypothesis (Hardy, 1996). Aβ has been shown to be toxic in vitro, suggesting that it could be involved in the neuronal cell death in AD. Although genetic and biochemical studies have suggested a cardinal role for Aβ, the underlying mechanism(s) of how Aβ induces degeneration in the central nervous system is still unclear. The aggregated form of Aβ, i.e. insoluble, soluble as well as intra/extracellular deposition, seems to be an important factor. The exact identity of the active species is still unclear. Fibrils, smaller peptide oligomers, water-soluble non-filamentous forms of Aβ, dimeric and trimeric species have all been suggested to be the toxic form (Terry, 1996; Davis and Chisholm, 1997; Hartley et al., 1999; Hsia et al., 1999; Klein et al., 2001). Observations from post-mortem brain suggest that a slow, age-related accumulation of Aβ deposit exist in normal aging, but is much more profound in AD.

It is still an open question whether Aβ has a physiological role in the brain. Under normal conditions, Aβ occurs as a soluble fragment, the concentration of which is normally tightly controlled below the threshold for its self-aggregation into β-sheet fibrils (Burdick et al., 1992). Aβ is actively degraded by several enzymes, including neprilysin (NEP), insulin-degrading enzyme (IDE), and endothelin-converting enzyme (ECE) (Carson and Turner, 2002). Although these Aβ-degrading enzymes have been well characterized, very little is known about the regulatory mechanisms that govern their expression and/or activity. Nevertheless, under normal physiological circumstances, the balance between the rates of production and clearance of Aβ is likely to be delicately regulated. A recent study has shown that although γ-secretase cleavage produces Aβ, the other product of γ-secretase cleavage, AICD, specifically upregulates the transcription of NEP, which in turn, accelerates the degradation of Aβ (Pardossi-Piquard et al., 2005). This transcriptional signalling pathway therefore provides a simple and elegant physiological mechanism for the regulation of Aβ levels following physiological activation of γ-secretase cleavage of βAPP. Aβ has also been found to be secreted from unaffected neurons in response to activity, and the peptide down-regulates excitatory synaptic transmission in organotypical slices from APPswe transgenic mice (Kamenetz et al., 2003). This negative feedback loop may provide the physiological homeostatic mechanism, which keeps the levels of neuronal activity in check, and that disruption of this feedback system could contribute to disease progression in AD. Although low levels of Aβ may be required for certain normal physiological functions, large numbers of studies have shown that excessively produced Aβ is critical to the complex pathogenesis of AD (Selkoe, 1994). A recent review suggests that APP fragments, including Aβ itself, serve a physiological role under normal circumstances, but that the increased levels of Aβ may trigger the neuropathology in the AD scenario (Cuello, 2005).

Neuropathology of AD

Functional brain imaging with positron emission tomography (PET) have revealed and increased the understanding of early pathological changes in the AD brain (Nordberg, 2004). The clinical symptoms of AD are caused by neurodegeneration in the hippocampus, cerebral cortex and entorhinal cortex (Braak and Braak, 1998). Characteristic changes include cortical atrophy, neuronal degeneration, loss of synapses and the presence of two classical lesions: intracellular neurofibrillary tangles (NFT) and extracellular amyloid plaques. The NFTs are intra-neuronal remains of the hyperphosphorylated forms of the microtubule-associated protein tau. Amyloid plaques are extracellular deposits with the main component Aβ (Glenner et al., 1984). There are two types of plaques that can be morphologically distinguished from each other, namely neuritic and diffuse plaques. Neuritic plaques are extracellular deposits of amyloid surrounded by dystrophic neuritis as well as the processes of astrocytes and microglia (Mattson, 2004). Diffuse plaques are premature, contain non-structured amyloid and are not surrounded by dystrophic neurites. Diffuse plaques may develop into neuritic plaques.

Damage to several neurotransmitter systems has been implicated in AD, with the cholinergic system being especially affected. Destruction or degeneration of particular nerve terminals and impairment in neurotransmission ultimately leads to dysfunction of related neuronal circuits. Neurotransmitter receptors are one of the decisive links in the synaptic information-processing chain. They can markedly respond to alterations in neuronal activity by adaptive mechanisms like sub/supersensitivity or down-regulation (Schwartz et al., 1983), which makes them an appropriate tool to monitor interactive changes in the neuronal activity of a particular transmitter system.

There is also growing evidence for a chronic immune response in the AD brain that significantly may contribute to the damage and degeneration of neurons leading to dementia. Several factors involved in inflammatory responses have been identified in the AD brain (McGeer and McGeer, 2001).

Also there are data indicating that the AD brain is under increased oxidative stress and this may have a role in the pathogenesis of neuronal degeneration and death in this disorder (Butterfield et al., 2001; Guan et al., 2003; Yu et al., 2003).

The cholinergic and glutamatergic neurotransmittersystems in AD

Cholinergic innervation

The vast majority of the cholinergic innervation of the primate cerebral cortex originates in the basal forebrain. The hippocampus receives most of its cholinergic input from the medial septal nucleus and the vertical limb of the diagonal band of Broca, whereas the rest of the cerebral cortex and the amygdala receive their cholinergic input from the nucleus basalis of Maynert (Mesulam, 2004). In contrast to primates, the rodent cerebral cortex contains intrinsic cholinergic interneurons, which may supply up to 30% of the local cholinergic innervation (Levey et al., 1984).

The depletion of acetylcholine (ACh) and cholinergic markers has long been thought to occur early in the course of the disease and be related to the impairment of memory (Winkler, 1998). This view is now challenged by reports that the

concentrations of the choline acetyltransferase (ChAT) and acetylcholinesterase (AChE) do not drop until the later disease stages (Davis et al., 1999; DeKosky et al., 2002). The cholinergic deficits in early stages of AD may therefore relate to cholinergic signal transduction defects (Mufson et al., 2000).

At later stages in the disease, loss of cholinergic innervation is most severe in the temporal lobes, including the entorhinal cortex, and is associated with a severe neurofibrillary degeneration and cell loss in the Ch4-nucleus basalis complex (Mesulam and Geula, 1988). In contrast to the destruction of cholinergic innervation in the cerebral cortex, the cholinergic innervation of the striatum (originating from striatal interneurons) and the thalamus (originating from the brainstem) remains relatively intact.

nAChRs

The nAChR subtypes play a major role in cognitive processes and are severely affected in AD (Nordberg, 1992; Court et al., 2001). Neuronal nAChRs belong to the same family of ligand-gated ion channels as glutamate, GABA and 5-HT and are believed to have a pentameric structure consisting of five membrane-spanning regions around a central ion channel. Presently, nine α-subunits (α_2-α_{10}) and three β-subunits (β_2-β_4) are known. They are generally in a 2α3β stoichiometry; where each combination appears to dictate particular pharmacological and physiological functions. However, α_7, α_8 and α_9 subunits are known to form homo-oligomers consisting of a single subunit subtype. α4β2 and α7 are the most common functional nAChRs in the brain. They are widely distributed in the brain, particular in thalamus, cortex and striatum (Paterson and Nordberg, 2000). A consistent loss of nAChRs is well documented in AD, with a widespread reduction in the α4 receptor subtype, but also in the α3 and α7 nAChR subtypes (Hellström-Lindahl et al., 1999; Guan et al., 2000), while there is an increase in α7 nAChRs in astrocytes (Teaktong et al., 2003; Yu et al., 2005). PET-studies of AD patients indicate that the loss of nAChR represent an early phenomenon in the course of the disease (Nordberg, 2001). Further mechanistic studies of the association between Aβ and α_7 nAChRs *in vitro*, have suggested that $A\beta_{1\text{-}42}$ can bind to α_7 nAChRs in hippocampus, leading to an activation of the extracellular-signal-regulated kinase/mitogen-activated protein kinase (ERK/MAPK) (Wang et al., 2000a; Wang et al., 2000b; Dineley et al., 2001).

Cholinesterase

AChE is the main cholinesterase in the central nervous system (CNS), while butyrylcholinesterase (BuChE), also known as pseudo or non-specific cholinesterase, is more common in serum and originated in glia (Nordberg and Svensson, 1998). Over the course of AD, AChE activity decreases while BuChE activity stabilizes and even increases, probably in relation to glial proliferation (Perry et al., 1978; Arendt et al., 1992). Interestingly, a study in AChE-KO mice showed normal cholinergic transmission due to the fact that BuChE compensated for the function of AChE (Mesulam et al., 2002). Cortical AChE and BuChE activities are also associated with Aβ deposits in the AD brain (Wright et al., 1993).

Pre-clinical studies have shown that the co-localization of AChE and Aβ represents an early step in the development of the senile plaques (Inestrosa and Alarcon, 1998) and further studies indicate that AChE acts by promoting the assembly of Aβ peptides into amyloid fibrils and also to form stable complexes with small Aβ peptide fragments (De Ferrari et al., 2001). These complexes change the biochemical and pharmacological properties of the enzyme and cause an increase in the neurotoxicity of the Aβ fibrils, suggesting that AChE may play a pathogenic role in AD by influencing the process leading to amyloid formation (Alvarez et al., 1998).

Glutamatergic innervation

The amino acid L-glutamate is recognized as the major excitatory neurotransmitter in the central nervous system (Collingridge and Lester, 1989). The glutamate transmitter system is organized at a high level of sophistication. The prominent glutamatergic pathways are: the cortico-cortical pathways, the pathways between the thalamus and the cortex and the extrapyramidal pathways (projections between the cortex and the striatum). Damage to glutamatergically-innervated cortical and hippocampal neurons are observed in AD (Albin and Greenamyre, 1992).

NMDA receptors

Glutamate receptors mediate most of the excitatory neurotransmission in the mammalian CNS. They also participate in plastic changes in the efficacy of synaptic transmission underlying memory and learning, and the formation of neural networks during development. The ionotropic glutamate receptors are widely distributed throughout the CNS, including the cerebral cortex, limbic regions and basal ganglia (Moriyoshi et al., 1991; Hollmann and Heinemann, 1994; Scherzer et al., 1998). The NMDA receptor is found at high density in the cortex and hippocampus. The subunits can be classified into two sub-families by amino acid sequence: NR1 and NR2 (A-D) (Lynch and Guttmann, 2001). Although the structure and stoichiometry of the native NMDA receptor channel is uncertain, in vivo receptors are assembled as tetrameric or pentameric complexes containing an obligatory NR1 subunit and one or more of the four NR2 subunits (Dingledine et al., 1999).

Changes in glutamatergic neurotransmission, including altered glutamate levels and loss of NMDA receptors have been reported in post-mortem evaluations of brain tissue from AD patients, indicating that cortical glutamate-association pathways are disrupted (Greenamyre and Young, 1989; Penney et al., 1990). Pre-clinical studies have shown that glutamate seems to be involved in the pathophysiological mechanism of neurodegenerative diseases in which increasing concentrations of glutamate cause NMDA channels to remain open for a longer period of time, resulting in an increase of intracellular Ca^{2+} (Meldrum and Garthwaite, 1990). This intracellular accumulation of calcium then initiates a cascade of events that results in further neuronal death (Greenamyre and Young, 1989; Greenamyre and Porter, 1994). Better understanding of the molecular mechanism of glutamate neurotoxicity and of possible mechanisms to prevent it would therefore be of great interest for the treatment of these neurodegenerative diseases.

Aβ-nicotinic-glutamatergic interactions

In pre-clinical studies, it has been shown that nicotinic–glutamatergic interactions are important for the synaptic plasticity underlying cognitive functions (McGehee et al., 1995; Mansvelder and McGehee, 2000; Ji et al., 2001) and NMDA mechanisms account for the actions of nicotine in many brain regions (Shoaib et al., 1994; Aramakis and Metherate, 1998; Schilström et al., 1998; Mansvelder and McGehee, 2000). Nicotinic–glutamatergic interactions have been best characterized at the α7 nAChR level. Activation of α7 nAChRs triggers action potential-independent glutamate release from axon terminals (McGehee et al., 1995; Alkondon et al., 1996; Mansvelder and McGehee, 2000; Ji et al., 2001). Further, in rats and mice, α7 nAChR activation enhances field stimulation-evoked glutamate currents, leading sometimes to long-term potentiation (LTP) (Aramakis and Metherate, 1998; Mansvelder and McGehee, 2000; Ji et al., 2001; Santos et al., 2002). Only a few reports have indicated that non-α7 nAChRs regulate glutamatergic synaptic transmission (Vidal and Changeux, 1993; Gil et al., 1997; Guo et al., 1998). Interneurons are crucial for sustaining the excitability and firing pattern of principal neurons and are thus critical for pacing rhythmic oscillations that set the window for learning. Although modulating glutamatergic inputs to hippocampal interneurons can have serious consequences to the hippocampal function, only limited information is available on the modulatory role of nAChRs on glutamatergic activity to the interneurons (Alkondon and Albuquerque, 2002).

In relation to AD, several lines of evidence suggest that the Aβ toxicity might be related to elevated levels of glutamate and/or overactivity of the NMDA receptors. The observation that Aβ reduces LTP and facilitates long-term depression (LTD) suggests a role for Aβ in regulating trafficking of glutamate receptors. A signalling pathway, where $A\beta_{1\text{-}42}$ by binding to α7 nAChRs may impair glutamatergic transmission, compromise synaptic function and reduce LTP and thereby promoting endocytosis of NMDA receptors in cortical neurons, was recently reported (Snyder et al., 2005). Neuronal cell cultures from APPswe transgenic mice showed a reduction in surface-expressed NMDA receptors, while no change was observed in total receptor amounts (Snyder et al., 2005).

Treatment strategies in Alzheimer's disease

Different treatment strategies have been developed in order to prevent or to slow down the progression of AD. Although a large amount of primary basic and clinical research has been performed already, it appears very difficult to identify appropriate targets, which would promise fast, effective and safe strategies to combat the disease onset and progression.

Neurotransmitter therapy

In the past decade, treatment for AD has largely involved replacement of neurotransmitters that are known to be lacking, mostly based on the "cholinergic hypothesis" of AD. To improve cholinergic neurotransmission, different strategies

have been investigated including: increasing of ACh synthesis, augmentation of presynaptic ACh release, stimulation of cholinergic postsynaptic muscarinic and nicotinic receptors and reduction of ACh synaptic degradation with AChEIs. AChEIs have beneficial effects on cognitive, functional, and behavioural symptoms of AD (Rogers et al., 1998; Cummings, 2000; Farlow et al., 2000; Tariot et al., 2000). Donepezil and galantamine selectively inhibit AChE, while rivastigmine in addition also inhibits BuChE (Darreh-Shori et al., 2002). It has been suggested that galantamine also improves cholinergic neurotransmission by acting as an allosteric ligand on the nAChRs to increase presynaptic ACh release and postsynaptic neurotransmission (Maelicke, 2000). Although the underlying pharmacological mechanisms for these AChEIs are somewhat different, the clinical effects appear to be comparable (Feldman, 2002; Reichman, 2003).

Memantine, a non-competitive NMDA receptor antagonist, is approved for symptomatic treatment of moderate to severe AD. Clinical studies have shown that patients receiving memantine had significantly less deterioration in cognitive and functional measurements (Reisberg et al., 2003; Tariot et al., 2004). The neurobiological basis for the therapeutic activity of memantine is not fully understood. Blockade of NMDA receptors by memantine may theoretically exert disease-modifying activity in AD by inhibiting the NMDA receptor-dependent excitotoxicity that has been hypothesized to play a role in the progressive neuronal loss that underlies the evolving dementia. In addition, there is pre-clinical evidence that memantine acts as an open channel blocker at several ligand-gated ion channels (Buisson and Bertrand, 1998; Oliver et al., 2001; Maskell et al., 2003).

Other drugs, modulating different neurotransmitter systems have also been suggested as a potential therapy in AD, based on results from pre-clinical studies. Modulators of the α-amino-3-hydroxy-5-methyl-4-isoxazole propionate (AMPA) receptor increase glutamatergic neurotransmission in brain and may thereby improve memory function (Lynch, 2002). The selective M1 muscarinic acetylcholine receptor (mAChR) agonist provides protection against the neuronal damage caused by Aβ toxicity via activation of the Wnt signalling pathway (Farias et al., 2004). Selective nAChR agonists are candidates for symptomatic and neuroprotective AD therapy (Nordberg, 2003). nAChR stimulation has been shown to increase neurotrophic factors in the brain, to increase the expression of nerve growth factor (NGF) receptors (Terry and Clarke, 1994), and to protect neural cells against glutamate-induced toxicity (Akaike et al., 1994). nAChR stimulation also offers protection against trophic factor-deprivation-induced toxicity (Marin et al., 1994; Yamashita and Nakamura, 1996) and Aβ-induced toxicity (Kihara et al., 1997).

NGF has been proposed as a treatment strategy in AD since there might be a lack of trophic support and NGF is an important factor for the basal forebrain cholinergic neurons (Blesch and Tuszynski, 1995; Eriksdotter Jönhagen et al., 1998).

Anti-amyloid therapies

One strategy for the development of anti-amyloid drugs is to block the production of Aβ. This effect might be obtained by inhibiting β- or γ-secretase activities, or by stimulating α-secretase activity (Citron, 2000; Wolfe, 2002). Blocking the

aggregation of Aβ or enhancing the clearance of Aβ are other possible therapeutic strategies. Metal chelators (Cu^{2+} or Zn^{2+}), Serum Amyloid P component (SAP) inhibitors, glucosaminoglycan (GAG) mimetics, cholesterol-lowering drugs and nicotine-like substances have all been suggested as possible therapies (Botto et al., 1997; Refolo et al., 2001; Nordberg et al., 2002; Ritchie et al., 2003; Bieler and Soto, 2004; Citron, 2004).

Another approach for both preventing formation and enhancing clearance of Aβ in the brain is active and passive immunization therapy. Immune-based therapies directed against the Aβ peptide have been shown to ameliorate Aβ pathology and reverse cognitive behavioural deficits in transgenic mice models (Schenk et al., 1999; Bard et al., 2000; Janus et al., 2000; Morgan et al., 2000; DeMattos et al., 2001). Based in part on the successful clearance of Aβ, active immunization trials were performed in AD patients. However, due to the development of an aseptic, vasocentric meningoencephalitis in a subset of patients, the trial was terminated (Orgogozo et al., 2003). Neuropathological investigations of post mortem brain tissue from AD patients, who had participated in the study, showed a decreased number of Aβ plaques compared to controls, but an increase in the number of NFT (Nicoll et al., 2003; Ferrer et al., 2004). Passive immunization with anti-Aβ monoclonal anti-bodies has been suggested to be a potentially safer alternative. However, a recent study showed meningoencephalitis subsequent to peripheral and intracerebral passive immunization of APPswe transgenic mice (Lee et al., 2005).

Anti-tau drugs

Hyperphosphorlation of tau is involved in the tau pathology seen in AD. Therefore, intervention of tau hyperphosphorylation by inhibition of the responsible kinases, such as cyclin-dependent kinase 5 (CDK5) and glucogen-synthase kinase-3β (GSK3β), might also be a possible therapeutic target (Wolfe, 2002).

Other therapies

Recent research, both epidemiological and in animals, suggests that keeping mentally and physically active when young and middle-aged decreases the risk of developing AD (Marx, 2005). Epidemiological studies have shown that the prevalence of AD is decreased in patients that have received long-term treatment with non-steroidal anti-inflammatory drugs (NSAIDs) (McGeer et al., 1996), cholesterol-lowering drugs (Jick et al., 2000), and estrogen treatment (Waring et al., 1999). However, pre-clinical studies have shown that altered expression of different inflammatory factors can either promote or counteract neurodegenerative processes. Since many of the inflammatory responses are beneficial, it might be a better therapeutic target to direct and instruct the inflammatory machinery than to suppress it (Wyss-Coray and Mucke, 2002).

Growing evidence suggests that oxidative processes in the brain may be involved in the underlying pathophysiological mechanism of AD. Long-term treatment with vitamin E in AD patients prolonged the time until institutionalization and death, but did not improve cognition (Sano et al., 1997). Similar findings were observed in AD patients following treatment with selegiline (Sano et al., 1997).

The concept of cell replacement by stem cells to compensate for cell loss and restore functionality has entered into the treatment possibilities of several diseases, including neurodegenerative disorders. However, the practical use of these types of techniques in patients might not be possible in the near future. Regarding gene therapy, preimplantation diagnosis for early AD, caused by V717L mutation, was performed with the result of a healthy child free of the predisposing gene mutation (Verlinsky et al., 2002).

Perspectives

During the last 10-15 years, the molecular details of the pathogenesis of AD have become clear enough to outline strategies for therapeutic intervention. Most approaches to developing therapeutics involve interfering with the production, aggregation and the toxic effects of Aβ. Unfortunately, these drugs might not help patients in the end-stage of the disease due to the fact that the extent of neurodegeneration could be beyond the point of no return. However, therapeutics might stop or slow down the disease process in patients with early or mid-stage AD. The potential of these agents to prevent the disease from occurring at all, must also be investigated. Continuing efforts in both academic and pharmaceutical laboratories to reveal the molecular mechanisms of the disease might also yield new therapeutic drugs that can be used in the clinic. Figure 2 summarizes the main hypothesized pathological processes and the related therapeutic approaches.

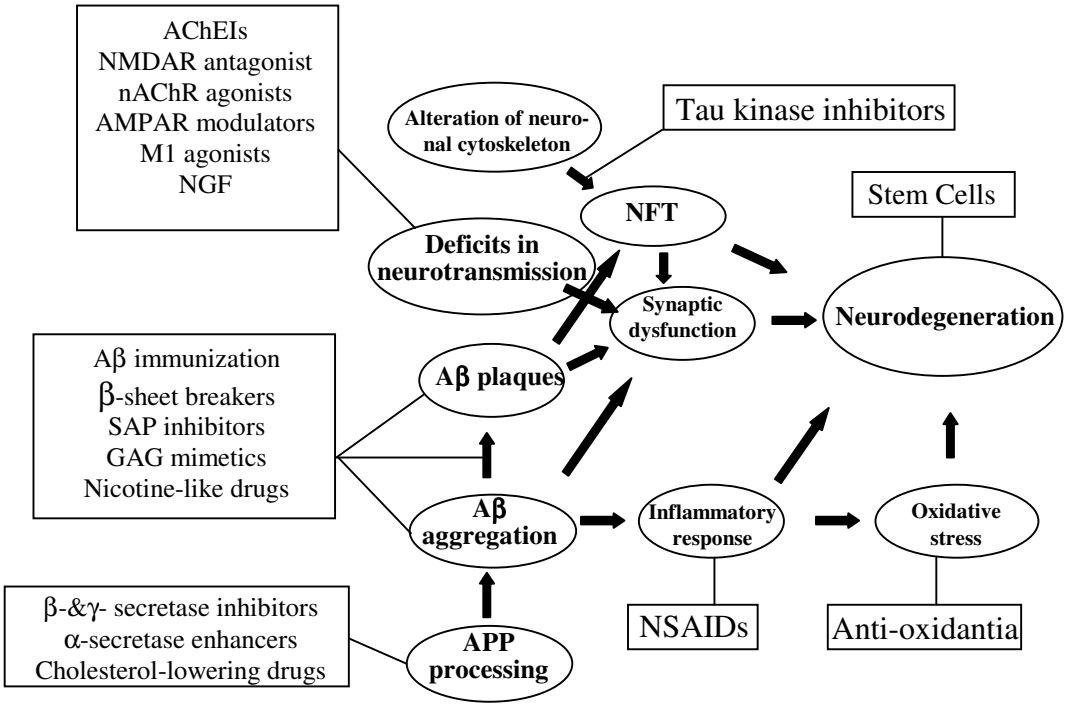

Figure 2: Schematic illustration of the present and future therapies for AD. The circles represent neuropathological changes, while the rectangles represent therapies aiming at preventing these changes

TRANSGENIC MOUSE MODELS

During the last decade, the availability of stable transgenic mouse strains has helped the understanding of the pathology of AD. The transgenic mice do not display the full neuropathology of AD, but they may provide a useful tool to investigate some of the mechanisms of the biochemical and pathological processes, which are not possible to study in human patients. There are no animal models for AD which adequately reproduce the underlying neurodegenerative process, which occurs spontaneously only in higher primates. However, many of the transgenic mice show Aβ deposition and neuritic plaques. Depending on factors such as the mutation of APP, gene promoter, copy number and integration site, as well as the mouse's genetic background; Aβ plaques develop between 3 and 15 months. The main characteristics and neuropathological features of several transgenic mouse models are summarized in Table 1.

Aβ plaque formation in the brain of transgenic mice can be considered to mimic the AD brain regarding staining and morphological characteristics. A recent study has shown significant differences in the inflammatory response in the brain of transgenic mice compared to human AD patients (Schwab et al., 2004). Human AD lesions had strongly activated microglia associated with the plaque core, while the mice showed weakly activated microglia in the periphery of the deposits. In AD patients, there is a massive neuronal loss, tangle formation and significant reduction in mainly the cholinergic system, while APP transgenic mouse models show little or no evidence of these neuropathological changes (Games et al., 1995; Irizarry et al., 1997; Holcomb et al., 1998). This is important to take into consideration when trying to interpret the data obtained from these transgenic mice models.

Table 1: Characteristics and neuropathological features of transgenic mice models

Line	Transgene	Aβ dep.	NFT	Neuron loss	Gliosis	Reference
Tg2576	APP695 (K670N/M671L)	9 mo	No (but AT8 IR)	No	Yes	(Hsiao et al., 1996)
APP23	APP751 (K670N/M671L)	6 mo	No (but AT8 IR)	Yes	Yes	(Sturchler-Pierrat et al., 1997)
APP/Ld	APP695 (V642I)	12-15 mo	No (but AT8 IR)	No	Yes	(Moechars et al., 1999)
TgCRND8	APP695 (K670N/M671L + V717F)	3 mo	Not reported	Not reported	Yes	(Chishti et al., 2001)
PDAPP	APP695, 751 and 770 (V717F)	6-9 mo	No (but AT8 IR)	No	Yes	(Games et al., 1995)
PSAPP	APP695 (K670N/M671L) + PS1 (M146V)	3 mo	Not reported	No	Yes	(Holcomb et al., 1998)
APP/PS1	APP695 (K595N/M596L) + PS1 (A246E)	9-10 mo	Not reported	Not reported	Yes	(Borchelt et al., 1997)
TAPP	APP695 (K670N/M671L) + tau (P301L)	6 mo	9-10 mos	Yes	Yes	(Lewis et al., 2001)
3 x Tg-AD	APP695 (K670N/M671L) + PS1 (M146V) + tau (P301L)	6 mo	15 mos	No	Yes	(Oddo et al., 2003)
hAChE-Tg	Overexpress hAChE	No	No	Attenuated dendritic branch, ↓ spine number	Not reported	(Beeri et al., 1995)
hAChE-Tg//APPswe	Overexpress hAchE + APP695 (K670N/M671L)	6 mo	Not reported	Not reported	Yes	(Rees et al., 2003)

AIMS OF THE THESIS

Aβ accumulation, loss of nerve cells and changes in the neurotransmitter systems are consistent features of AD. The general aim of this thesis was to gain further insight into how pathological processes related to AD, created in different transgenic animal models, influence the synapses, dendrites, cholinergic and glutamatergic receptors, especially the nAChRs and the NMDA receptors. Since Aβ is thought to be one of the main players in AD pathology, a special focus has been on the interaction between Aβ, the α7 nAChRs and the NMDA receptors. Figure 3 schematically illustrates the three main object and model systems that was used to investigate their interactions.

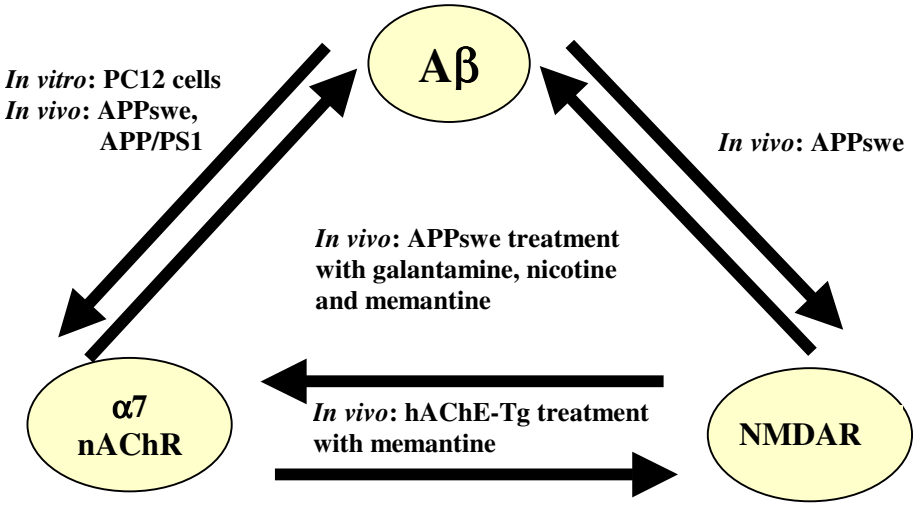

Figure 3. General focus of this thesis

The specific goals were:

- To investigate the effect of Aβ on the nAChRs in PC12 cells and in the brain of APP/PS1 transgenic mice *(Paper I and II)*
- To study early effects of Aβ on synaptic density, α7 nAChRs and NMDA receptors in the brain of APPswe transgenic mice, and to investigate if these effects are mediated through the ERK/MAPK pathway *(Paper III)*
- To study the nAChRs, NMDA receptors, the synapses and the dendrites in the brain of transgenic mice overexpressing human AChE (hAChE-Tg) and the effect of memantine *(PaperIV)*
- To study the effect of treatment with galantamine, nicotine and memantine on Aβ, nAChRs, NMDA receptors, the synapses, dendrites and glial cells in the brains of APPswe transgenic mice *(Paper V)*

MATERIALS AND METHODS

CELL CULTURE (Paper I)

PC12 cells were grown at 37° C in RPMI-1640 medium containing 10% horse serum, 5% fetal bovine serum, 25 U/ml penicillin-streptomycin (Gibco BRL Life Technologies AB, Sweden) in dishes pre-coated with collagen.

Aβ peptides and treatment (Paper I)

$A\beta_{1-40}$, $A\beta_{25-35}$ and $A\beta_{35-25}$ were obtained from the Department of Medical Biochemistry and Microbiology, Uppsala University, Sweden. The peptides were purified by reversed phase high performance liquid chromatography (HPLC) and analysed by matrix-assisted laser desorption/ionisation-mass spectrophotometry (MALDI-MS). $A\beta_{1-40}$ was dissolved in autoclaved distilled water at 25 µM and incubated at 37°C for 24 hr before use. $A\beta_{25-35}$ and $A\beta_{35-25}$ were dissolved in autoclaved distilled water at 1mM and stored at -20° C until use. Since $A\beta_{25-35}$ aggregates within a few hours under cell culture condition (Forloni et al., 1993), it was not pre-aged before treatment.

Apoptosis and Cell Viability assays (Paper I)

Annexin-V-FLUOS assay

Assay for apoptosis was performed with Annexin-V-FLUOS (Roche) according to the manufacturer's instructions. Briefly, 5 x 10^5 cells were incubated in 100 µl of labelling solution containing 1 µl Annexin-V-FLUOS and 1 µl propidium iodide for 15 minutes at room temperature. The cells were then analysed by a FACScan flow cytometer (Becton&Dickinson) using software of Cell Quest.

3-(4,5-dimethylthiazol-2-yl)-2,5, diphenyl tetrazolium bromide (MTT) reduction assay

Exponentially growing PC12 cells were plated at a density of ~5,000 cells in 100 µl medium containing 10% dialysed fetal bovine serum per well in 96-well plates. The day after plating, culture medium was replaced by the same medium but containing different concentrations of $A\beta_{1-40}$, $A\beta_{25-35}$ and $A\beta_{35-25}$ and the culture was continued for 7 days. MTT stock (10 µl, 5 mg MTT/ml) was then added per well and the incubation continued for 4 hr. 100 µl of a solution containing 20% SDS and 50% dimethylformamide pH 4.8 was then added to each well. After overnight incubation, absorption values at 570 nm were determined.

TRANSGENIC MOUSE MODELS (Paper II, III, IV and V)

The Principles of Laboratory Animal Care (NIH publication No. 86-23, revised 1985) were followed. All animal experimental protocols were approved by the local Ethics Committee and carried out in accordance with the guidelines of the Swedish National Board for Laboratory Animals (CFN). All mice that were born and bred in our own colony and housed under the same conditions with enriched environment, controlled temperature and humidity. The mice had access to food and water *ad libitum* and were maintained on a 12 h light/dark cycle. Offspring of the matings were weaned at 21 days and housed individually or with 1-4 siblings of same sex in standard laboratory Plexiglas cages (30 x 20 x 15 cm), with wood shavings provided as bedding material. The cages and bedding material were changed twice a week. All mice used in these studies were sacrificed by decapitation during daytime.

Paper II

Transgenic mice (APP/PS1) co-expressing a FAD mutant PS1-A246E and a chimeric mouse/human APP695 harbouring a Hu Aβ domain and mutations (K595N, M596L) linked to Swedish FAD pedigrees (APPswe). The mice included in this study were generated as previously described (Borchelt et al., 1997) at the university of Kuopio, Finland. The mice were sacrificed by decapitation and brain regions of interest were dissected and frozen at –85° C. The dissected frozen brains were obtained from the Department of Neuroscience and Neurology, University of Kuopio, Finland.

Paper III and V

Two female transgenic mice overexpressing APP_{695} and containing a KM670/671NL mutation driven by a hamster prion protein gene promoter in a C57B6 X SJL F1 hybrid mouse were back-crossed to C57BL/6J mice (Hsiao et al., 1996) were kindly provided as a gift from Dr. Karen Hsiao-Ashe. C57BL/6J mice (Bomice & Mollegard Breeding Laboratories, Denmark) were used to breed a colony of APPswe animals.

Paper IV

A transgenic mouse model, hAChE-Tg, with a two-fold increase in enzyme levels in comparison to control mice, show behavioural disturbances, early onset loss of learning and memory capacities, progressive dendritic depletion, neuropathology related to stress and modified anxiety responses (Beeri et al., 1995; Andres et al., 1997; Sternfeld et al., 2000; Erb et al., 2001). Two female and two male FVB/N mice carrying the human AChE cDNA under the control of 586 bp of the authentic human AChE promoter (Beeri et al., 1995) were kindly provided as gifts from Dr. Hermona Soreq. These mice were used to breed a colony of hAChE-Tg colony. FVB/N mice (Bomice & Mollegard Breeding Laboratories, Denmark) served as controls.

Drug treatment *(Paper IV and V)*

Ten five-months old hAChE-Tg mice and FVB/N mice were randomized into treatment s.c. with memantine HBr 10 mg/kg twice a day for 14 days. Memantine hydrobromide, dissolved in 0.9 % NaCl (pH 5.5-6), was freshly prepared for each injection session. Corresponding age- and sex-matched randomised control groups received 0.9 % NaCl s.c. The mice were weighed at day 0, 7 and 14. The animals were sacrificed by decapitation 36 hours after the last injection.

Ten-months old APPswe mice and non-transgenic control mice were randomized into treatment s.c. with memantine hydrochloride (10 mg/kg), (-)-nicotine (the dose of nicotine was gradually increased from 0.25 mg/kg (free base) on day 1 to 0.30 mg/kg on day 2 and 0.45 mg/kg on days 3-10) or galantamine hydrobromide (2 mg/kg) at 8.00–9.00 and 16.00–17.00 for 10 days (Table 1). Memantine hydrocloride, (-)-nicotine or galantamine hydrobromide, dissolved in 0.9% NaCl (pH 5.5–6), was freshly prepared for each injection session. Corresponding age-matched control groups of APPswe and non-transgenic control mice received 0.9% NaCl (s.c.). The mice were weighed at day 0 and day 10. Mice were killed by decapitation 1 h after drug withdrawal.

MEASUREMENT OF mRNA (Paper I)

Rnase protection Assay

The mRNA levels of nAChR α3, α7 and β2 subunits were measured with RNase protection assay (RPA) using ^{32}P-labelled riboprobes (Zhang et al., 1998). Briefly, RPA was carried out with 30 µg total RNA using the RPA IITM kit (Ambion, USA). The protected samples were separated by polyacrylamide gel electrophoresis. The gel was exposed to Kodak X-OMAT AR5 film.

RECEPTOR SUBTYPE EXPERIMENTAL PROCEDURES (Paper I, II, III, IV and V)

Membrane preparation *(Paper I and II)*

PC12 cells were homogenized in binding buffer, and the homogenates were centrifuged at 10,000 rpm for 15 minutes at 4° C. The resulting pellets were re-suspended in binding buffer and centrifuged again. The final pellets were suspended in binding buffer ready for receptor binding assay.

The mouse brain tissue was quickly removed and dissected into the parietal cortex and hippocampus, frozen on dry ice and maintained at –85° C until use. The same brain regions from two individual mice were pooled together, yielding each group with 6–8 samples. Membrane preparations (P1) were prepared from cortical and hippocampal regions by homogenisation in 20 mM Tris-HCl buffer (pH 8.5) with protease inhibitor cocktail (Complete, Roche Diagnostics) and centrifuged at 11,000 rpm at 4° C for 15 minutes. The final pellets were re-suspended in the appropriate binding buffer.

Protein content in all preparations was measured according to the method of Lowry, using bovine serum albumin (BSA) as the standard (Lowry et al., 1951).

Receptor binding assays (*Paper I and II)*

[^{125}I]αBungarotoxin binding

Membrane preparations (0.2 mg protein) were pre-incubated in the binding buffer (10 mM Na-phosphate buffer, pH 7.4, containing 50 mM NaCl and 0.1% BSA) with (for non-specific binding) or without (for total binding) 1µM (final concentration) of cold αbungarotoxin at 37°C for 30 minutes. Then, [^{125}I] αbungarotoxin (specific activity >125-152 Ci/mmol), final concentration 2 nM was added into the binding mixtures with the final concentration of 2nM and the incubation was continued for 30 minutes. The final binding volume was 200µl. The assay was terminated by addition of 1ml cold binding buffer, followed by centrifugation at 11,000 rpm and 4°C for 5 minutes. The pellets were washed with the binding buffer by centrifugation at the same condition as above two more times. The radioactivity enwrapped in the pellets was counted with a γ-counter.

[^{3}H]Epibatidine binding

Membrane preparations (0.2 mg protein) were incubated with [^{3}H]epibatidine (specific activity 52 Ci/mmol), final concentration 0.46 nM, in 50 mM Tris-HCl buffer (pH 7.4) at 25°C for 3 hr. The samples were then filtered through Whatman GF/C glass filters pre-soaked with 0.3% polyethyleneimine solution for 3–4hr and washed three times with assay buffer.

Non-specific binding was determined in the presence of 0.1 μM (-)-nicotine. The samples were counted with a scintillation counter.

[^{3}H]Cytisine binding

Membrane preparations (0.2 mg protein) were incubated with [^{3}H]cytisine (specific activity 35.2 Ci/mmol), final concentration 2 nM, in 50 mM Tris-HCl buffer (pH 7.4), containing 120 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$ and 2.5 mM $CaCl_2$, final volume 1 ml, at 4° C for 90 minutes. The samples were then filtered through Whatman GF/C glass filters pre-soaked with 0.3% polyethyleneimine solution for 3–4hr and washed three times with assay buffer. Non-specific binding was determined in the presence of 0.1 μM (-)-nicotine. The samples were counted with a scintillation counter.

Displacement binding assays of either [^{3}H]epibatidine or [^{125}I]αbungarotoxin by different concentrations of $A\beta_{1–42}$, $A\beta_{1–40}$ and $A\beta_{25–35}$ were performed by using the same experimental protocols as above. Since it has been reported that thoroughly washed membranes must be used in order to obtain significant displacement of [^{3}H]epibatidine and [^{125}I] αbungarotoxin binding by Aβ fragments (Wang et al., 2000; Wang et al., 2000), the homogenates of PC12 cells were washed by centrifugation in binding buffer at 11,000 rpm and 4°C for 5 times, each 15 minutes, immediately before the binding assays were performed. All of the Aβ fragments were pre-aged by incubation at 37°C for 24 hr before the binding assays.

Receptor autoradiography *(Paper III, IV and V)*

Frozen mouse brains were sectioned sagittally on a cryostat at –20° C to 8 μm (Paper IV) or 10 μM (Paper III and V) thickness; sections were thaw-mounted on to poly-L-lysine coated slides and directly re-frozen in the cryostat. The slides were stored at –80° C until use.

[^{3}H]MK-801 autoradiography

Frozen sections were thawed at room temperature for 30 minutes, followed by a 15 minutes pre-incubation at room temperature in 50 mM Tris-Acetate buffer, pH 7.4. The sections were then incubated in the same buffer, containing 10 nM [^{3}H]MK-801 with an addition of 100 mM of glutamic acid and 100 mM of glycine, at room temperature for 120 minutes. Non-specific binding was determined in the presence of 10 mM unlabelled MK-801. The incubation was terminated with two washes of 10 minutes each in binding buffer at room temperature, followed by a rinse in distilled water at 4° C.

The sections were dried in room temperature over night and then placed together with [^{3}H]microstandards to 3H-Hyperfilm for 8 weeks.

[^{3}H]Cytisine autoradiography

Frozen sections were thawed at room temperature for 30 minutes, followed by a 60 minutes pre-incubation at room temperature in binding buffer, 50 mM Tris-HCl buffer pH 7.4 containing 120 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$ and 2.5 mM $CaCl_2$. The sections were then incubated in the same buffer containing 2 nM [^{3}H]Cytisine at room temperature for 60 minutes. Non-specific binding was determined in the presence of 100 mM (-)-nicotine. The incubation was terminated by three washes of 3 minutes each in binding buffer at room temperature, followed by a rinse in distilled water at 4° C. The sections were dried in room temperature and then placed together with [^{3}H]microstandards and screens (BAS TR2025, Fuji film) in phospho-imaging cassettes (BAS Cassette, Fuji film) for 8 weeks.

[^{125}I]αBungarotoxin autoradiography

Frozen sections were thawed at room temperature for 30 minutes, followed by a 30 minutes pre-incubation at room temperature in binding buffer, 50 mM Tris-HCl buffer pH 7.4 containing 1 mg/ml BSA. The sections were then incubated in the same buffer containing 2 nM [^{125}I] αbungarotoxin at room temperature for 120 minutes. Non-specific binding was determined in the presence of 1 mM αbungarotoxin. The incubation was terminated by four washes of 5 minutes each in 50 mM Tris-HCl buffer pH 7.4 at 4° C, followed by a rinse in distilled water at 4° C. The sections were dried at room temperature and then placed together with [^{125}I]microstandards to ^{3}H-Hyperfilm for two weeks.

Receptor binding autoradiography films were developed in D-19 developer at room temperature for 5 minutes, rinsed in water and fixed for 2 minutes Kodak fixer. Films were then washed in running water for approximately 15 minutes.

Western blot (Paper I)

Homogenates of PC12 cells were prepared with ice-cold 50 mM sodium phosphate buffer (50 mM sodium phosphate, 50 mM NaCl, 2 mM EDTA, 2 mM EGTA and 1 mM phenylmethylsufonyl fluoride, pH 7.4), and then washed twice by centrifuging at 60 000 × *g* for 60 minutes at 4°C. The resulting pellets were re-suspended in ice-cold buffer containing 2% Triton X-100 and protease inhibitors (aprotinin, pepstatin, bestatin and leupeptin, 5 μg/ml each). The suspension was mixed for 2 h at 4° C, and then centrifuged at 100 000 × *g* for 60 minutes at 4°C. The supernatants were used for Western blot analysis and protein levels in the fraction were assayed by DC Protein Assay kit (Bio-Rad Inc.). The PC12 cells extracts (25 μl containing 30 μg protein) were submitted to electrophoresis on 10% SDS-polyacrylamide gel. After electrophoresis the separated proteins were blotted on to PVDF membrane (Amersham) that was incubated with goat polyclonal anti-α3, anti-α7 and anti-β2 antibodies, 0.25 μg/ml each (Santa Cruz Biotechnology Inc.). Immunoreactivity was detected using anti-goat antibody conjugated to horse radish peroxidase (Santa Cruz Biotechnology Inc.) and Enhanced Chemiluminescence (ECL) Plus kit (Amersham). The PVDF membrane was exposed to Hyper Performance Chemiluminescence film (Amersham).

APP PROTEIN LEVELS (Paper III and V)

Cortical homogenates were prepared with ice-cold 20 mM Tris-HCl, pH 8.5, containing Roche Diagnostics protease inhibitor cocktail and then centrifuged at 60,000 x g for 20 minutes. The supernatant was used to measure total sAPP and human sAPPα. The resulting pellets were re-suspended in the ice-cold homogenate buffer with addition of 2% Triton X-100. The suspension was mixed for 2 h at 4°C and then centrifuged at 100,000 x g for 1 hour. The supernatant was used for Western blot analysis of total membrane-bound APP.

Soluble APP Levels (Paper III)

Homogenates (30 μg/well) were loaded on 10% gradient minigels and the gels were run at 100 mV constant voltages at room temperature for 60-90 minutes. The protein bands were blotted onto a PVDF-membrane (RPN 303 F; Amersham Life Science) at 4°C for 90 minutes. Non-specific binding sites were blocked overnight at 4°C. The blots were then incubated with 22C11 (antibody to total sAPP (1:1000)) (Chemicon International) or 6E10 (human sAPPα (1:1000)) (Chemicon International) for 60 minutes at room temperature. Then, each

membrane was incubated with secondary antibody, donkey anti-mouse (1:1000) (SantaCruz) for 1 hour at room temperature. The blots were incubated with ECL Plus reagents, exposed to film and developed according to standard procedures.

Total membrane-bound APP Levels (Paper V)

Homogenates were loaded on 4-20 % gradient minigels and the gels were run at 100 mV constant voltages at room temperature for 60-70 minutes. The protein bands were blotted onto a PVDF-membrane (RPN 303 F; Amersham Life Science) at 4°C for 90 minutes. Non-specific binding sites were blocked overnight at 4°C. The blots were then incubated with the 22C11 antibody (1:500) (Chemicon International) for 60 minutes at room temperature. Then, each membrane was incubated with secondary antibody, donkey anti-mouse (1:1000) (Santa Cruz) for 1 hour at room temperature. The blots were incubated with ECL Plus reagents, exposed to film and developed according to standard procedures.

$A\beta_{1-40}$ AND $A\beta_{1-42}$ LEVELS (Paper II, IV and V)

For Aß measurements, the tissues were extracted in 7 volumes 20 mM Tris-HCl buffer, pH 8.5, containing protease inhibitor cocktail (Complete, Roche Diagnostics Scandinavia AB, Bromma, Sweden). After centrifugation (100,000 x g for 1 h at 4°C), the supernatant was diluted 1:4 with phosphate-buffered saline (PBS) including 0.5% bovine serum albumin, 0.05 % Tween 20 and protease inhibitors (standard buffer) and represented the Tris-extracted (soluble) Aß fraction. The pellet was extracted in 10 volumes of 5 M guanidium-HCl in 20 mM Tris-HCl, pH 8.0, for 1.5 h at room temperature and then diluted 1:5 with standard buffer and centrifuged at 13,100 x g for 20 minutes at 4°C. The resulting supernatant represented the guanidinium-extracted (insoluble) Aß fraction. The levels of $A\beta_{1-40}$ and $A\beta_{1-42}$ peptides were analysed by using the Signal Select$^{TM.}$ Human β-Amyloid $_{1-40}$ and $_{1-42}$ colorimetric sandwich ELISA kits (BioSource International Inc. CA, USA) according to the manufacturer's protocol. The Aβ values were calculated by comparison with a standard curve of synthetic human $A\beta_{1-40}$ and $A\beta_{1-42}$, respectively. The C-terminal-specific ELISAs use a monoclonal capture antibody directed against the first 16 amino acid residues of the N-terminal region of human Aβ and two other antibodies specific for $A\beta_{1-40}$ and $A\beta_{1-42}$. These antibodies were specific for human $A\beta_{1-40}$ and $A\beta_{1-42}$ and did not recognize those in the mouse. The manufacturer has characterized the specificity and sensitivity of the ELISA. All samples were analysed in the linear range of the ELISA.

Aβ AND GFAP IMMUNOHISTOCHEMICAL STAINING (Paper V)

Double immunofluorescence labelling of Aβ and glial fibrillary acidic protein (GFAP) was performed by pre-treating the frozen sections with concentrated formic acid for 5 minutes, followed by incubation of the sections in 5 % normal goat serum in TBS. Then, the sections were incubated with a cocktail of primary antibodies (anti-6E10/anti-GFAP) overnight at 4°C, followed by a secondary antibody cocktail consisting of Cy2-conjugated goat anti mouse and Cy3- conjugated goat anti-rabbit antibodies (each 20 µg/ml, Chemicon, CA, USA). For control staining, the primary serum was omitted and resulted in no detectable labelling.

The APPswe transgenic mice start to develop plaques around 9 months of age (Hsiao et al., 1996). Therefore, only a few plaques/slide were found in the cortex of these mice. These plaques were examined to investigate the effect of the different treatment on aggregated Aβ.

LEVELS OF SYNAPTIC AND DENDRITIC MARKERS (Paper III, IV and V)

Cortical homogenates were prepared with ice-cold 50 mM sodium phosphate buffer (50 mM sodium phosphate, 50 mM NaCl, 2 mM EDTA, 2 mM EGTA and 1 mM phenylmethyl sulfonyl fluoride and Roche Diagnostics proteinase inhibitor cocktail, pH 7.4) (Paper IV) or ice-cold 20 mM Tris-HCl, pH 8.5, containing Roche Diagnostics protease inhibitor cocktail (Paper III and V) and then centrifuged at 60.000 x g for 20 minutes. The resulting pellets were re-suspended in the ice-cold homogenate buffer with addition of 2 % Triton X-100. The suspension was mixed for 2 h at 4° C and then centrifuged at 100.000 x g for 1 hour. The supernatant were used for Western blot analysis. DC protein assay kit (Bio-Rad) was used to measure the protein content in the fractions.

Synaptophysin levels (Paper III, IV and V)

Synaptophysin was quantified by immunoblotting after resolution by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) under non-reducing conditions. For SDS-PAGE, 4–20% gradient minigels (12–15 wells, 20–15 ml, BioRad Ready Gel) were run at 100 mV *(Paper III and V)* or 150mV *(Paper IV)* constant voltages at room temperature for 60–70 minutes. The protein bands were blotted on a PVDF-membrane (RPN 303 F; Amersham Life Science) at 4° C for 90 minutes. Incubating the blots in blocking buffer for 1 hour at room temperature or overnight at 4° C blocked non-specific binding sites. The blots were then incubated with antibody to synaptophysin (1:2000) (DAKO) for 45 minutes at room temperature followed by secondary antibody, goat anti-rabbit (1:1000) (Santa Cruz) for 1 hour at room temperature. The blots were incubated with ECL Plus reagents, exposed to film and developed according to standard procedures.

MAP-2 levels (Paper IV)

MAP-2 was quantified by immunoblotting after resolution by SDS-PAGE under non-reducing conditions. For SDS-PAGE, 4–20% gradient minigels (12–15 wells, 20–15 ml, BioRad Ready Gel) were run at 150 mV constant voltages at room temperature for 60–70 minutes. The protein bands were blotted on a PVDF-membrane (RPN 303 F; Amersham Life Science) at 4° C for 150 minutes. Incubating the blots in blocking buffer for 1 hour at room temperature or overnight at 4° C blocked non-specific binding sites. The blots were then incubated with antibody to MAP-2 (1:500) (DAKO) for 45 minutes at room temperature followed by secondary antibody, donkey anti-mouse (1:2000) (Santa Cruz) for 1 hour at room temperature. The blots were incubated with ECL Plus reagents, exposed to film and developed according to standard procedures.

LEVELS OF ERK AND PHOSPHO-ERK (Paper III)

Cortical homogenates were prepared with ice-cold homogenate buffer [containing mM: 20 Tris, 1 EG/EDTA, 1 $Na_4P_2O_7$, and 1 Na_3VO_4, 0.1 PMSF, PNPP-PP2b, 1μM Microcystin-LR and Roche Diagnostics protease inhibitor cocktail and then centrifuged at 60,000 x g for

20 minutes. The supernatant was used for Western blot analysis. DC protein assay kit (Bio-Rad) was used to measure the protein content of the fractions. In the case of anti-phospho-Thr202/Tyr204 antibody directed against ERK MAPK, this dually phosphorylated form of ERK MAPK is the active kinase; therefore, this antibody directly measures ERK MAPK activity (Bell et al., 2004). For Western blot, 15 µg of each sample (in duplicate) was loaded per gel. Samples were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis in 10% Tris-HCl gels, transferred to PVDF-membrane for 75 minutes, blocked in 0.1% TBS-T containing 3% BSA for 1 hour at room temperature, washed 4 x 10 minutes in 0.1% TBS-T and then incubated with phospo-ERK antibody (1:3000) in blocking buffer for 1 hour. The membranes were washed 4 x 10 minutes in 0.1% TBS-T and then incubated with donkey anti-rabbit (1:3000) in blocking buffer for 1 hour. The blots were incubated with ECL Plus reagents, exposed to film and developed according to standard procedures. The membranes were then stripped according to the manufacturers instructions and then blocked in 0.1% TBS-T containing 3% BSA over night at 4° C. The following morning, the membranes were washed 4 x 10 minutes, incubated with total ERK antibody (1:1000) in blocking buffer, washed 4 x 10 minutes and finally incubated with the same secondary antibody as for phospo-ERK (1:3000) for 1 hour. The blots were incubated with ECL Plus reagents, exposed to film and developed according to standard procedures.

IMAGE ANALYSIS (Paper I, III, IV and V)

Paper I

The resulting RPA and Western blot signals on films were analysed using a video camera (CCD-72, Dage-MTI, Michigan City, IN) coupled to a Macintosh computer equipped with a video card and NIH-Image software (written by Wayne Rasband at the US National Institutes of Health).

Paper III, IV and V

The autoradiograms were analysed with a video camera (CCD-72. Dage-MTI, Michigan City, IN) coupled to a Macintosh computer with a video card and the public domain NIH-Image software (written by Wayne Rasband at the US National Institutes of Health). OD values were converted into fmol/mg tissue based on the standard curve derived from the [^{3}H]microstandards or [^{125}I]microstandards. The regions analysed were cortex (frontal cortex, retrosplenial gr. cortex and motor cortex), hippocampus (CA1, CA2 and CA3), thalamus and caudate nucleus. The specific binding was calculated by subtracting the non-specific binding from the total binding.

The films from the Western blots were scanned using a Sharp JX-325 scanner. The OD values of the bands were calculated as a product of contour OD and the area of the contour using Image Master 1D software (version 1.10; Pharmacia Biotech). Cortical membrane preparations obtained from each animal in one group were loaded onto one gel in duplicate with a duplicate of a pooled sample of all groups as a reference. Phospho-ERK1/2 band intensity was first normalized to the band intensity detected with total ERK. All samples were then standardised to the pooled sample, allowing for the comparison between groups.

For Aβ and GFAP, immunoreactive material in the brain sections was observed using an Olympus. Microscope equipped with a digital camera (Fuji digital camera HC-2500 3CCD, Fujifilm, Japan). Fluorescence was observed using a laser-scanning microscope (Olympus, Japan).

STATISTICAL ANALYSIS

Paper I

Saturation binding data were analyzed by non-linear curve fitting analyses (LIGAND) in order to determine the total number of binding sites (B_{max}) and affinity constants (Kd). A one factor ANOVA followed by Student-Newman-Keuls test was used to analyze the effect of treatment with $A\beta_{1-40}$, $A\beta_{25-35}$ and $A\beta_{35-25}$ on the mRNA levels, protein levels and receptor binding sites of the α3, α7 and β2 nAChR subunits.

Paper II

Student´s unpaired t-test was used for comparison within age group data and Bonferroni/Dunn-All means test for comparison between age groups of transgenic and wild-type mice.

Paper III and Paper V

Statistical analysis of the autoradiographic data and Western blot data was carried out using the non-parametric Kruskal-Wallis test followed by Mann-Whitney to calculate the significance between APPswe and non-transgenic controls as well as the age-related changes in APPswe and non-transgenic controls, respectively *(Paper III)* and also to calculate the significance between APPswe and non-transgenic controls as well as the significance between treatment with galantamine, nicotine and memantine on APPswe and non-transgenic mice, respectively *(Paper V)*. Simple regression analysis was used to detect relationships *(Paper III)*.

Paper IV

Statistical analysis of the autoradiographic data was carried out using two-way ANOVA followed by Scheffe Post-Hoc Test to calculate the significance between hAChE-Tg and FVB/N as well as the significance of treatment with memantine on hAChE-Tg mice and FVB/N mice compared to saline treatment. For the Western blot data, the non-parametric Kruskal-Wallis test was used followed by Mann-Whitney to calculate the significance between hAChE-Tg and FVB/N as well as the significance of treatment with memantine on hAChE-Tg mice and FVB/N mice, respectively.

RESULTS AND DISCUSSION

Aβ LEVELS IN APP_{SWE} AND APP/PS1 TRANSGENIC MICE

The availability of different transgenic mouse models of AD carrying human APP and/or PS mutants has made it possible to study the mechanisms and time course of the most early biochemical and pathological processes, which are not possible to follow in AD patients.

Detectable levels of Aβ was found at 7 days (Paper III), confirming that single transgenic APPswe mice produce human Aβ peptides from birth (Table 2). The levels at 7 days of age were almost 2-fold higher that at 90 days of age. Since we observed detectable guanidinium-extracted $Aβ_{1-40}$ and $Aβ_{1-42}$, (which are supposed to represent aggregated fibrillar Aβ levels), already at 7 days of age, this might indicate that Aβ probably starts to aggregate already from birth.

Soluble APP levels (total sAPP and human sAPPα) were increased in these mice in an age-dependent manner, suggesting that the young mice (7 days old) have impaired ability to degrade Aβ, and therefore, more Aβ is accumulated in the cortex. In agreement with earlier studies in these mice, an age-dependent increase in $Aβ_{1-40}$ and $Aβ_{1-42}$ was observed (Paper III and Paper V).

Table 2. Levels of Aβ in the cortex of APPswe transgenic mice

Age	Soluble $Aβ_{1-40}$ (pmol/g tissue)	Insoluble $Aβ_{1-40}$ (pmol/g tissue)	Soluble $Aβ_{1-42}$ (pmol/g tissue)	Insoluble $Aβ_{1-42}$ (pmol/g tissue)
7 days	2.28 ± 0.01	0.66 ± 0.09	n.d	0.68 ± 0.03
21 days	0.52 ± 0.08^{aaa}	0.45 ± 0.06	n.d	0.55 ± 0.07
90 days	1.25 ± 0.11$^{aaa, bb}$	0.48 ± 0.08	n.d	0.58 ± 0.03

Values are expressed as mean ± SEM. n.d = not detected.
$^{aaa}p < 0.001$ compared to 7 days old APPswe transgenic group, $^{bb}p < 0.01$ compared to 21 days old APPswe transgenic group (Kruskal-Wallis followed by Mann-Whitney Post-hoc test)

In the APP/PS1 transgenic mice (Paper II), we observed soluble and insoluble $Aβ_{1-40}$ levels at 3 weeks of age, with an age-dependent increase up to 17 months (Fig. 4). We also detected soluble $Aβ_{1-42}$ in hippocampus at the age of 12 months, increasing with age. In the hippocampus, a more marked significant increase in insoluble $Aβ_{1-42}$ levels (~870-fold) was observed between 3 weeks and 17 months compared with that of insoluble $Aβ_{1-40}$ levels (~120-fold), thus indicating a favourable accumulation of $Aβ_{1-42}$ in these transgenic mice. This is in agreement with earlier studies in other strains of double transgenic mice, reporting a selective increase in $Aβ_{1-42}$ levels, preceding Aβ deposition (Borchelt et al., 1997; Holcomb et al., 1998).

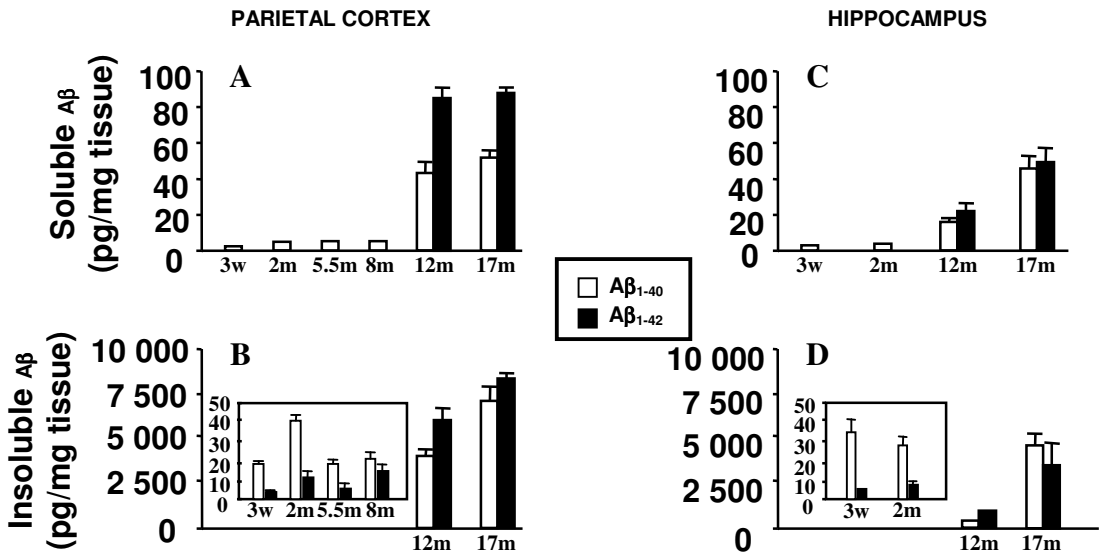

Figure 4. Time course for soluble and insoluble $A\beta_{1-40}$ and $A\beta_{1-42}$ levels in the parietal cortex (A, B) and the hippocampus (C, D) of APP/PS1 transgenic mice. Mean +SEM values for each group (n = 5-6).

When comparing these two different transgenic mice models, both show an age-dependent increase in Aβ levels. The most striking difference is the selective increase of insoluble $A\beta_{1-42}$ levels in the brain of the APP/PS1 transgenic mice, while the APPswe transgenic mice have an increase in both $A\beta_{1-40}$ and $A\beta_{1-42}$.

THE EFFECT OF Aβ ON THE α7 nAChRs

Research has revealed consistent deficiencies in nAChRs, and especially in the α4 nAChRs, in several regions of the brains of patients with AD (Nordberg and Winblad, 1986). However, diverse results with respect to alternations in the levels of α7 nAChRs in the AD brain have also been reported (Martin-Ruiz et al., 1999; Burghaus et al., 2000; Guan et al., 2000). The explanation for the differences in results obtained is unknown, but by using cellular systems and transgenic animals it is possible to study the effect of Aβ on the nAChRs. In our studies, we have seen that Aβ influences the α7 nAChRs. In PC12 cells (Paper I), we found a decrease in α7 nAChR mRNA levels, protein levels as well as receptor binding levels, which indicates a decrease in gene transcription and consequently, a decrease in protein levels and binding sites (Fig. 5). These results are consistent with the observation in the human AD patients.

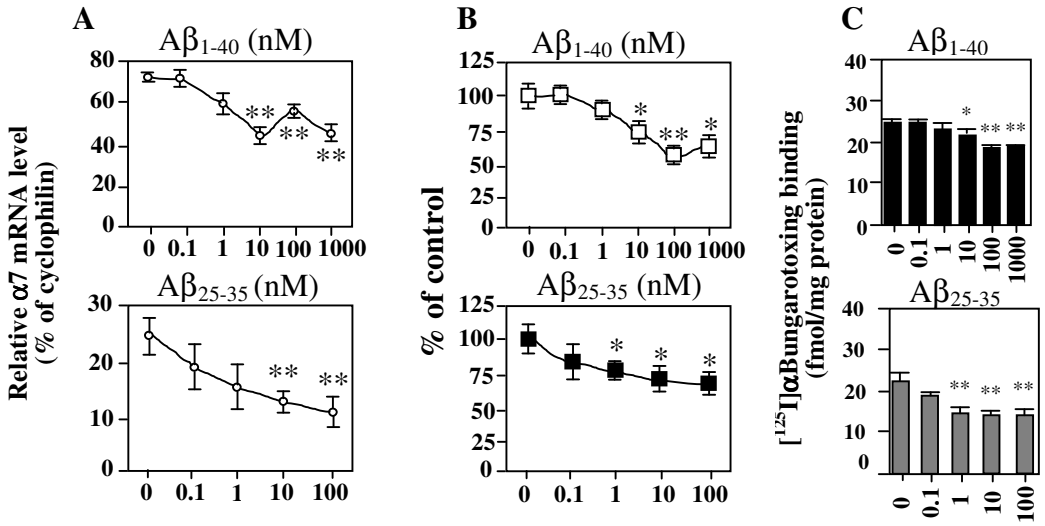

Figure 5. The effect of $A\beta_{1-40}$ and $A\beta_{25-35}$ on the mRNA levels (A), protein levels (B) and receptor binding levels (C) in PC12 cells. The values are expressed as mean +SEM for each group.

Similar to the findings in the PC12 cells, there was a significant decrease in number of α7 nAChRs in motor cortex in the APPswe transgenic mice at 7 days of age (Paper III). At 21 days of age, the levels of α7 nAChRs had returned to those of the non-transgenic control mice. Studies in these mice at later time-points have shown significant increases in α7 nAChRs, mainly in the hippocampus (Dineley et al., 2001; Bednar et al., 2002) as well as and in temporal/parietal cortex (Paper V).

Although elevated levels of both soluble and insoluble Aβ were observed in the APP/PS1 transgenic mice (Paper II), no alterations in the α7 nAChRs was observed in the parietal cortex or hippocampus compared to non-transgenic controls. Elevated levels of Aβ and amyloid plaques are common features of both transgenic mouse lines, nevertheless they differ largely in the extent of α7 nAChR binding. Possible explanations for these differences might be strain background, choice of promoters and level of APP overexpression, including other possible factors such as difference between brain regions. Although there are different results in cell lines and the two transgenic mouse models, it seems clear that the interaction between Aβ and the α7 nAChRs are able to modulate the plasticity and dynamics of the cells.

THE EFFECT OF Aβ ON THE NMDARs

Several lines of evidence suggest that Aβ toxicity may be related to elevated levels of glutamate and/or overactivity of NMDA receptors. Cellular damage in the brains of AD patients is also found in areas that display glutamatergic synaptic plasticity (Arendt et al., 1998). Other studies have shown reductions in the concentration of glutamate (Lowe and Bowen, 1990). Reductions in NMDA receptor binding in the hippocampus and neocortex have also been reported (Procter et al., 1990; Greenamyre and Maragos, 1993). In the APPswe transgenic mice, the NMDA receptors have earlier been reported to be preserved at 4 and 15 months of age (Cha et

al., 2001). However, there was a significant increase in NMDA receptors in cortex and hippocampus, both at 21 days of age (Paper III), as well as at 10 months of age (Paper IV) (Fig. 6). A recent study showed that Aβ intermediates, formed during fibrillogenesis, can alter glutamate neurotransmission, and that polymeric assemblies of distinct sizes, protofibrils versus fibrils, may differ in their disruption of biological processes (Ye et al., 2004). Thus, the up-regulation of the NMDA receptors at 21 days of age in the APPswe transgenic mice might reflect initial changes in response to the early, high levels of soluble Aβ observed in these mice, while the up-regulation at 10 months might be due to the effect of more chronic exposure to Aβ.

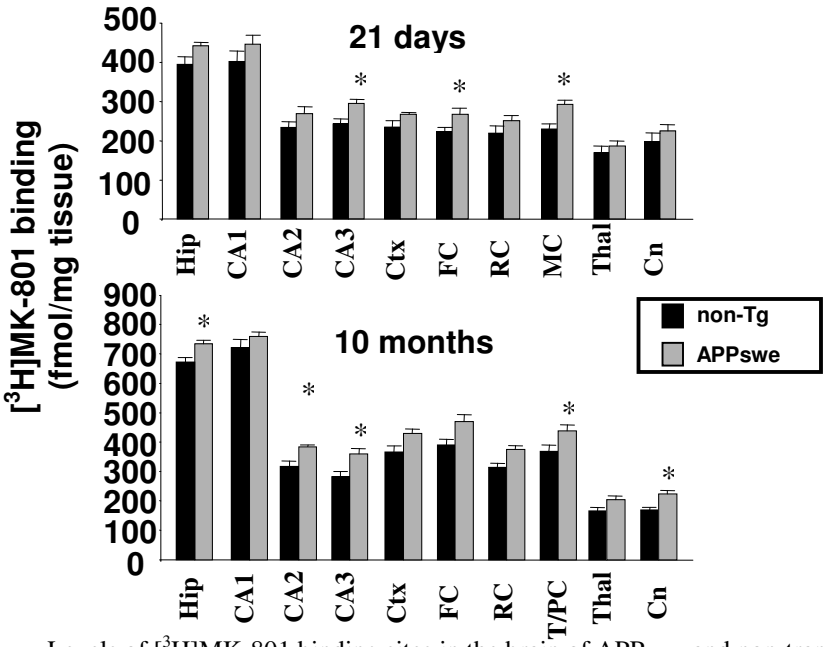

Figure 6. Levels of [^{3}H]MK-801 binding sites in the brain of APP_{swe} and non-transgenic control mice at 7 days and 10 months of age. * $p<0.05$ indicates significant differences between APP_{swe} and non-transgenic controls. The values are expressed as mean + SEM

INTERACTIONS BETWEEN Aβ, α7 nAChRs AND NMDARs

In pre-clinical studies, interactions with picomolar affinity of $A\beta_{1-42}$ and the α7 nAChRs have been reported using [^{3}H]MLA as a competitive ligand (Wang et al., 2000; Wang et al., 2000). However, several studies, including (Paper I) in this thesis, have failed to detect Aβ-induced inhibition of [^{125}I]αbungarotoxin binding. It is possible that the different radioligands used in the present and former studies may explain differences in the experimental results. It should be noted that [^{125}I]αbungarotoxin has greater selectivity for α7 nAChRs in rodent brain and its selectivity has been much more clearly established than the selectivity of [^{3}H]MLA. By washing extensively, it is possible that Wang et al. (Wang et al., 2000; Wang et al., 2000) instead of uncovering high affinity Aβ binding sites on the α7 nAChRs, have uncovered non-α7 nAChRs for [^{3}H]MLA and Aβ. However, there is evidence of interactions between the α7 nAChR and Aβ (Dineley et al., 2001; Liu et al., 2001; Pettit et al., 2001; Dineley et al., 2002; Grassi et al., 2003), although it might be

possible that Aβ binds to a different site on the α7 nAChRs than αbungarotoxin. The functional consequences of this interaction have been that Aβ controls AChE expression through α7 nAChRs in cortical neurons (Fodero et al., 2004), as well as that Aβ acts through α7 nAChRs to trigger elevations of presynaptic calcium in synaptic terminals from the hippocampus and neocortex (Dougherty et al., 2003).

nAChRs are widely distributed over many cell types and at different cellular locations (Wonnacott, 1997). Presynaptic nAChRs can enhance release at excitatory glutamatergic neurons, and postsynaptic receptors can add to the postsynaptic depolarization and calcium signal. Several studies have shown that Aβ can alter glutamate signalling via alterations of LTP (Wu et al., 1995; Cullen et al., 1997). There is a significant negative correlation between the Aβ and α7 nAChRs as well as Aβ and NMDA receptors at early ages in the APPswe transgenic mice (Paper III), which indicates that Aβ decrease the number of NMDA and α7 nAChR binding sites (Fig. 7A-D)

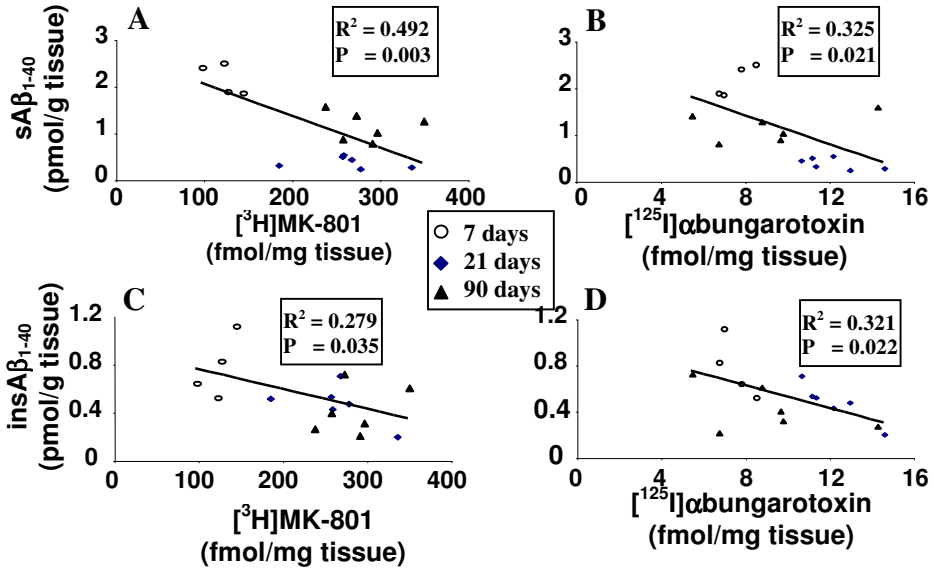

Figure 7. Correlation of (A and B) Tris-extracted $Aβ_{1–40}$ and (C and D) guanidinium-extracted $Aβ_{1–40}$ versus [^{3}H]MK-801 or [^{125}I]αBungarotoxin binding sites in the cortex of APP_{swe} mice at 7 to 90 days of age. Each point represents data from one mouse.

EFFECT OF Aβ ON SYNAPTOPHYSIN

Aβ accumulates in plaques near dystrophic neurites and nerve endings (Brendza et al., 2003). A large body of evidence supports the hypothesis that the memory failure of AD, particularly early impairment, is a result of synaptic dysfunction and loss, which is usually measured by synaptophysin immunoreactivity (Terry et al., 1991; Sze et al., 1997). On the other hand, presynaptic markers have also been shown to increase in early AD (Mukaetova-Ladinska et al., 2000). As yet, the effects of Aβ on the presynaptic nerve terminal have not been defined clearly. Analysis of the synaptic vesicle membrane protein, synaptophysin, in the cortex (Paper III) revealed that the APPswe transgenic animals displayed high levels of synaptophysin at 7 days

compared to non-transgenic controls, as well as in older transgenic mice (Fig. 8). Earlier studies have shown an increase in synaptophysin levels as an early response to increased levels of APP or Aβ (Mukaetova-Ladinska et al., 2000; King and Arendash, 2002; Hu et al., 2003). However, the fact that Aβ decreased the levels of synaptophysin in APPswe transgenic mice from the age of 21 days is in agreement with earlier studies (Mucke et al., 2000), indicating that that plaque-independent Aβ toxicity might play an important role in the synaptic deficits in AD. The decrease in synaptophysin seems to persist also at older ages (Paper V), since there was a significant decrease in synaptophysin at 10 months of age in the APPswe transgenic animals compared to non-transgenic controls (Fig.8).

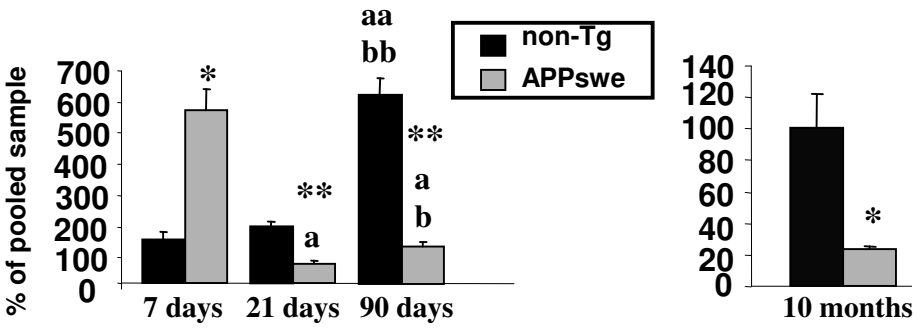

Figure 8. Levels of synaptophysin in the brain of APP_{swe} and non-transgenic control mice at 7, 21 and 90 days as well as 10 months of age. ** $p<0.01$ and * $p<0.05$ indicate significant differences between APP_{SWE} and age-matched controls. $^{aa}p<0.01$ and $^{a}p<0.05$ represent significant differences between 7 days old mice and 21 days or 90 days old mice. $^{bb}p<0.01$ and $^{b}p<0.05$ represent significant differences between 21 days and 90 days old mice. The values are expressed as mean +SEM

A significant positive correlation was observed between both Tris-extracted $Aβ_{1-40}$ and guanidinium-extracted $Aβ_{1-40}$ and synaptophysin levels in the cortex of APPswe transgenic mice (Paper III), suggesting that high levels of $Aβ_{1-40}$ may promote increased synaptic density (Fig. 9A, B). This effect might be a consequence of: (1) the high concentrations of $Aβ_{1-40}$, causing altered synaptic function and thereby increasing the synaptic terminals as a compensatory mechanism, or (2) an initial neurotrophic effect by APP or Aβ. Since sAPPα has been shown in several studies to have a neurothropic effect (Saitoh et al., 1989; Bhasin et al., 1991), it was also investigated if the levels of total sAPP and human sAPPα are higher in the transgenic mice at 7 days of age than at 21 or 90 days of age and if this could explain the increased levels of synaptophysin at 7 days of age (Paper III). This was shown not to be the case, since the highest levels of total sAPP and human sAPPα were found at 90 days of age. These findings support the assumption that it must be the Tris-extracted $Aβ_{1-40}$ that is responsible for the up-regulation of synaptophysin in the APPswe transgenic mice at 7 days of age.

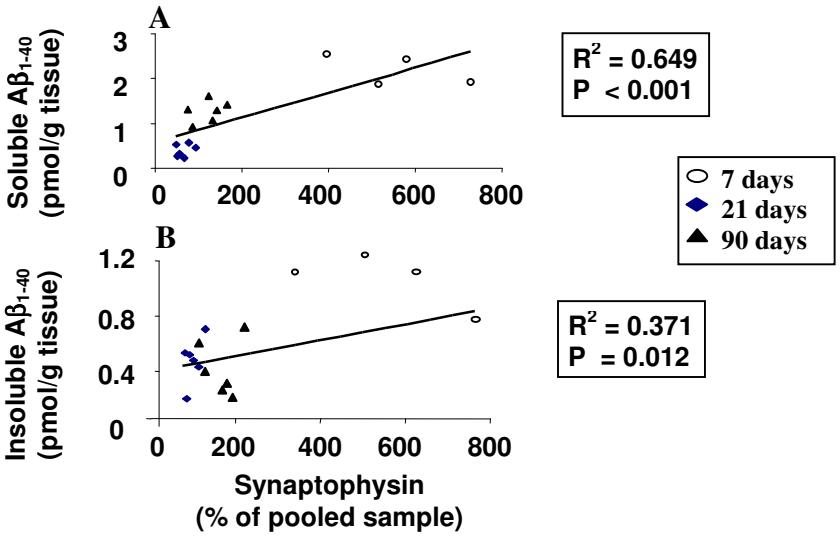

Figure 9. Correlation of levels of (A) Tris-extracted Aβ $_{1\text{-}40}$ and (B) guanidinium-extracted Aβ$_{1\text{-}40}$ versus synaptophysin levels in the cortex of APP$_{swe}$ mice at 7 to 90 days of age. Measurements were performed as described in the text. Each point represents data from one mouse.

Aβ AFFECTS THE ERK/MAPK PATHWAY

Signal transduction mechanisms are integral components of the neuronal information processing machinery. Signalling through cellular protein kinase cascades impinges upon targets at the neuronal membrane, in the cytoplasm, and within the nucleus in order to effect changes in synaptic function and connectivity. The ERK/MAPK pathway is activated in response to various growth and differentiation factors, and plays a significant role in differentiation and early embryonic development (Marshall, 1995; Doherty et al., 2000). It is also important in synaptic plasticity and memory formation (Dash et al., 2002). Recent studies have shown that when hippocampal slices were treated acutely with oligomeric Aβ$_{1\text{-}42}$, which binds to the α7 nAChRs, the ERK/MAPK cascade is activated while chronic exposure to oligomers or high molecular weight aggregates led to ERK/MAPK down-regulation (Bell et al., 2004).

A significant decrease of ERK2 activity was observed in the brain of APPswe transgenic mice at 7 days of age (Paper III), which might indicate that Aβs´ actions in the brain are not mediated through ERK activation. The increased ERK activity in the APPswe transgenic mice at 21 and 90 days of age is in agreement with earlier results obtained in hippocampus in this mouse model, suggesting a chronic activation of the ERK/MAPK cascade as a consequence of the increased Aβ burden (Dineley et al., 2001).

OVER EXPRESSION OF AChE IN hAChE-Tg MICE

The hAChE-Tg mice express human synaptic AChE in CNS neurons with an enhancement of two-fold higher enzyme levels in comparison to control mice (Beeri et al., 1995). A significant increase in AChE activity has also been observed in the

striatum, cortex and hippocampus of the hAChE-Tg mice, while no major alterations in BuChE activity were found compared to controls (Svedberg et al., 2003). The hAChE-Tg mice display progressive impairments in spatial learning and memory; increasing with age. Morphologically, they show reduced dendrite branching in the neocortex (Beeri et al., 1997) in addition to structural and functional abnormalities in neuromuscular junctions (Andres et al., 1997). Increased [^{3}H]hemicholinium-3-binding (Beeri et al., 1997) and acetylcholine release (Erb et al., 2001) have been observed, which probably reflect the cholinergic deficits due to increased AChE activity. Studies of the cholinergic activity in the hAChE-Tg mice have revealed increased α4 and α7 nAChRs as well as muscarinic M2 binding sites in brain, suggesting that these receptor subtypes play an important role in the compensatory mechanisms facilitating the impaired cholinergic neurotransmission (Svedberg et al., 2002; Svedberg et al., 2003).

Since studies of the glutamatergic neurotransmitter system have not been performed in these mice and little is known about how the excess of AChE activity influences other neurotransmitter system than the cholinergic, we focused on the NMDA receptor because this receptor is ideally suitable for mediating plasticity changes in the brain. A significant increase in the number of α7 nAChRs in the frontal cortex (82%) and a significant decrease in the number of NMDARs in the CA1 of the hippocampus (16%) and the thalamus (31%) was observed in the hAChE-Tg mice compared to non-transgenic controls (Fig. 10) (Paper IV). Several studies indicate that there exist a complex relationship between the cholinergic and the glutamatergic neurotransmission (Ohno et al., 1993; Maurice et al., 1994; Aigner, 1995). The α7 nAChR are not only located in cholinergic synapses, but also present presynaptically on glutamatergic axon terminals. Presynaptic nAChRs can enhance neurotransmitter release, and somatic or postsynaptic nAChRs may thus reduce the responsiveness of NMDA receptors and thereby influence the ability of NMDA receptors to induce long-term synaptic plasticity (Gray et al., 1996; Albuquerque et al., 1997; Radcliffe and Dani, 1998; Radcliffe et al., 1999; Fisher and Dani, 2000). The observed decrease in NMDA receptor binding sites in the CA1 of the hippocampus and the thalamus of hAChE-Tg (Paper IV) might be a consequence of the disrupted cholinergic transmission in these mice. The results may therefore provide important information about interactions between the cholinergic and the glutamatergic systems.

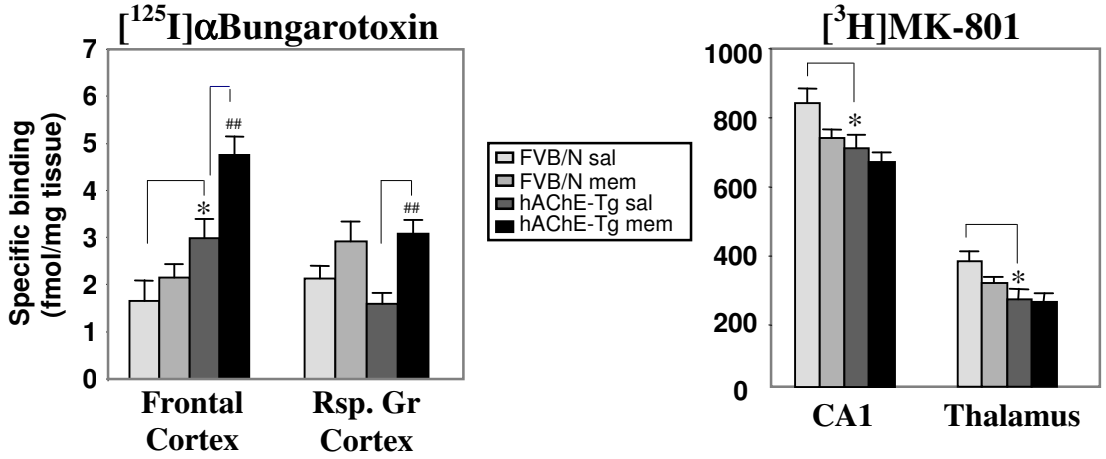

Figure 10. [^{125}I]αBungarotoxin binding sites in the frontal-and retrosplenial gr. cortex as well as [^{3}H]MK-801 binding sites in the CA1and the thalamus of hAChE-Tg mice and FVB/N mice. *$p < 0.05$ for saline treated hAChE-Tg mice compared to saline treated FVB/N mice. $^{\#\#}$ $p < 0.01$ for memantine treated hAChE-Tg compared to saline treated hAChE-Tg mice. Values are expressed as mean + SEM.

No difference in the levels of synaptophysin was observed in the cortex of hAChE-Tg compared to FVB/N mice (Fig.11) (Paper IV). The cholinergic hypofunction does not seem to alter the number of presynaptic terminals in the cortex although the numbers of α7 nAChRs were increased.

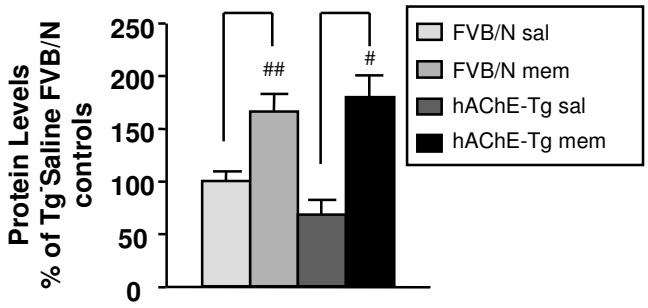

Figure 11. Levels of Synaptophysin in the cortex of hAChE-Tg mice and FVB/N mice. $^{\#}p < 0.05$ and $^{\#\#}$ $p < 0.01$ for memantine treated hAChE-Tg and FVB/N mice compared to saline treated hAChE-Tg and FVB/N mice, respectively. Values are expressed as mean + SEM.

In mature neurons MAP-2 is specifically localized in dendrites and has been proposed to play a role in neuritogenesis, synapse formation, and dendritic remodelling (Johnson and Jope, 1992). It has been demonstrated that HMW MAP-2 localizes to dendrites and soma, and is almost absent in axons, and therefore, HMW MAP-2 is known as a dendro-somatic protein (Binder et al., 1984; Riederer and Matus, 1985; Matus, 1988; 1994). LMW MAP-2 is generally expressed in embryonic and early postnatal brains, and disappears dramatically with the maturation of the central nervous system (Binder et al., 1984; Riederer and Matus, 1985). Therefore, it

is postulated that LMW MAP-2 is responsible for construction of neuronal and glial architectures in the developing brain (Matus, 1988; Tucker, 1990; Johnson and Jope, 1992; Matus, 1994). However, in regions of the adult brain that undergoes continuous neuronal remodelling, such as the olfactory system, LMW MAP-2 levels remain elevated. We observed that the hAChE-Tg mice displayed significantly reduced levels of LMW MAP-2 in the cortex compared to FVB/N, which might be in agreement with earlier findings of reduced dendritic branching in these mice (Beeri et al., 1997) (Fig. 12). Interestingly enough, there were no differences in HMW MAP-2, suggesting that deficits in the cholinergic neurotransmitter system may only influence the LMW MAP-2 isoforms (Fig. 12).

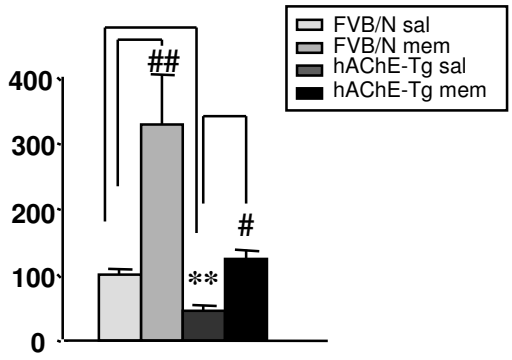

Figure 12. Levels of LMW MAP-2 in the cortex of hAChE-Tg mice and FVB/N mice. **$p < 0.01$ for saline treated hAChE-Tg mice compared to saline treated FVB/N mice. . $^{\#}p < 0.05$ and $^{\#\#}p < 0.01$ for memantine treated hAChE-Tg and FVB/N mice compared to saline treated hAChE-Tg and FVB/N mice, respectively. Values are expressed as mean + SEM.

MEMANTINE TREATMENT IN hAChE-Tg MICE

Blockade of NMDA receptors by memantine may theoretically inhibit the NMDA receptor-dependent excitotoxicity, which has been hypothesized to play a role in the progressive neuronal loss that underlies AD. Another possible action of memantine might be via the α7 nAChRs. There is evidence that memantine acts as an open channel blocker on several ligand-gated ion channels, for instance the human α7 nAChR (Maskell et al., 2003).

No changes in the number of NMDARs were detected in any brain region of hAChE-Tg mice or non-transgenic mice following treatment with memantine (Fig. 10) (Paper IV). However, memantine treatment caused a significant increase in the α7 nAChR binding sites in the frontal- (59 %) and retrosplenial gr. cortex (65 %) compared to saline treated animals, while no effect was observed in the non-transgenic mice treated with memantine (Fig. 10). An up-regulation of the α7 nAChR in the cortex of hAChE-Tg mice following treatment with memantine may, in part, be a compensatory mechanism caused by the cholinergic and glutamatergic alterations in this mouse model. These results may also further support the notion that the NMDA and α7 nAChRs may interact in the brain. It is important to remember that memantine is able to act as an open channel blocker on several other ligand-gated ion channels, for instance, the 5-HT3 receptors and the α4β2- and α7 nAChRs (Buisson and

Bertrand, 1998; Rammes et al., 2001; Maskell et al., 2003), and the changes observed following treatment with memantine may therefore have nothing to do with its interaction with the NMDA receptor, but instead reflects a direct interaction with the nAChRs or some other receptor.

Memantine treatment significantly increased the presynaptic terminal density in the cortex of both hAChE-Tg and FVB/N mice, which might indicate that memantine may affect neurogenesis (Fig. 11) (Paper IV). NMDA receptor blockade by MK-801 has also been shown to enhance neurogenesis in the adult rat dentate gyrus (Okuyama et al., 2004).

Memantine treatment increased the levels of LMW MAP-2 in both hAChE-Tg and FVB/N, suggesting that memantine can influence neuronal plasticity (Fig. 12) (Paper IV). It has been reported that activation of the NMDA receptors can induce activation of the calcium-dependent protein calpain, which selectively degrades MAP-2 (Felipo et al., 1993). Antagonism of the NMDA receptor by memantine might thus decrease the proteolysis of MAP-2 by calpain. Activation of the α7 nAChR, leading to altered phosphorylation of MAP-2, might also be of importance (Minana et al., 1998). Phosphorylation of MAP-2 by cAMP-dependent protein kinase protects MAP-2 from cleavage by calpain (Minana et al., 1998).

THE EFFECT OF CHOLINERGIC AND GLUTAMATERGIC DRUG TREATMENT IN APP_{swe} TRANSGENIC MICE

Increasing number of studies suggests that the present clinical therapy used in AD, in addition to having a symptomatic effect, also may interact with the ongoing neuropathological processes in the brain**.** Affecting the production, aggregation or clearance of Aβ, which is the constituent protein of the plaques, may well have a modifying effect on disease progression.

A significant decrease in total APP levels by 45 % and 55 % was observed following memantine treatment in APPswe transgenic mice and non-transgenic controls, respectively (Paper V). Since APP is expressed by glutamatergic neurons (Ouimet et al., 1994), blockade of the NMDA receptors may influence the production of total APP. The levels of Aβ may eventually decrease due to a lower production of APP following long-term treatment with memantine. Nicotine treatment significantly lowered the total APP levels by 35 % in the cortex of non-transgenic mice compared to saline treated animals, while no change was observed in the APPswe transgenic mice.

Accumulation of Aβ in the brain is believed to play a key role in the pathological feature of AD. In this study, we confirmed our observation that treatment with nicotine dramatically reduces the levels of insoluble $A\beta_{1-40}$ and $A\beta_{1-42}$ in APPswe transgenic mice (Nordberg et al., 2002; Hellström-Lindahl et al., 2004) (Table 3).

Table 3. Levels of Aβ in the cortex of APPswe transgenic mice

Treatment	sol. $A\beta_{1-40}$ (pmol/g tissue)	insol. $A\beta_{1-40}$ (pmol/g tissue)	sol. $A\beta_{1-42}$ (pmol/g tissue)	insol. $A\beta_{1-42}$ (pmol/g tissue)
Saline	0.80 ± 0.15	6.82 ± 1.03	n.d	0.29 ± 0.07
Nicotine	1.00 ± 0.30	3.68 ± 0.59$^{\#}$	n.d	0.10 ± 0.02$^{\#}$
Galantamine	0.76 ± 0.18	8.80 ± 1.51	n.d	0.33 ± 0.11
Memantine	0.65 ± 0.09	5.51 ± 1.16	n.d	0.23 ± 0.08

Values are expressed as mean ± SEM.
n.d = not detected
$^{\#}p < 0.05$ compared to saline treated Tg+ group (Kruskal-Wallis followed by Mann-Whitney Post-hoc test). Abbreviations: sol. = soluble, insol. = insoluble

Immunohistochemical studies (Paper V) revealed that short-term nicotine treatment, besides eliminating the plaques in the cortex of APPswe transgenic mice; also markedly reduced the GFAP immunoreactive astrocytes surrounding the plaques (Fig. 13). To determine if nicotine's action on Aβ might, for instance, be due to β-sheet breaking activity or the activation of astrocytes, leading to increased clearance of the peptide has to be further evaluated. Since the levels of intracellular Aβ were not reduced (Fig. 13) it is tempting to suggest that the action of nicotine might mainly be via degradation of insoluble Aβ. It was recently demonstrated that L-(-)-nicotine not only inhibits the aggregation of $A\beta_{1-40}$ and $A\beta_{1-42}$, but also disaggregates fibrils preformed from both of these peptides (Ono et al., 2002). In addition, there is evidence that nicotine reduces the Aβ associated with cerebral vessels (Hellström-Lindahl et al., 2004), which has not been reported following vaccination against Aβ.

Although memantine or galantamine treatment did not alter the levels of Aβ in the brain of APPswe transgenic mice, it does not exclude that these drugs may interact with Aβ processes.

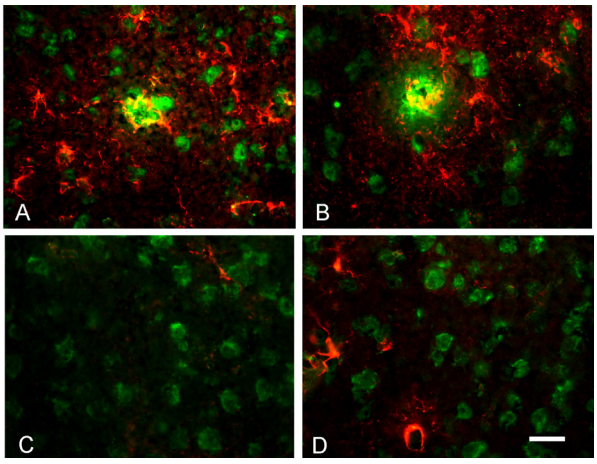

Figure 13. Aβ and GFAP immunohistochemistry in the cortex of 10 months old APP_{swe} transgenic mice following treatment with saline (A, B) and nicotine (C, D). Scale bar 30 μM. (For colour figures see paper V)

The Aβ deposit is hypothesized to disrupt neural and synaptic function leading to neuronal degeneration (Cuello, 2005). Analysis of the synaptic vesicle membrane

protein, synaptophysin revealed a 5-fold decrease in the cortical levels in APPswe transgenic mice compared to the non-transgenic controls (Paper V), which is in agreement with earlier observations in young APPswe transgenic mice in Paper III (Fig.14). The finding that treatment with galantamine and nicotine both caused a 2-fold increase in synaptophysin levels in the APPswe transgenic mice may suggest a neurotrophic effect by influencing the possible interaction between Aβ and the α7 nAChRs.

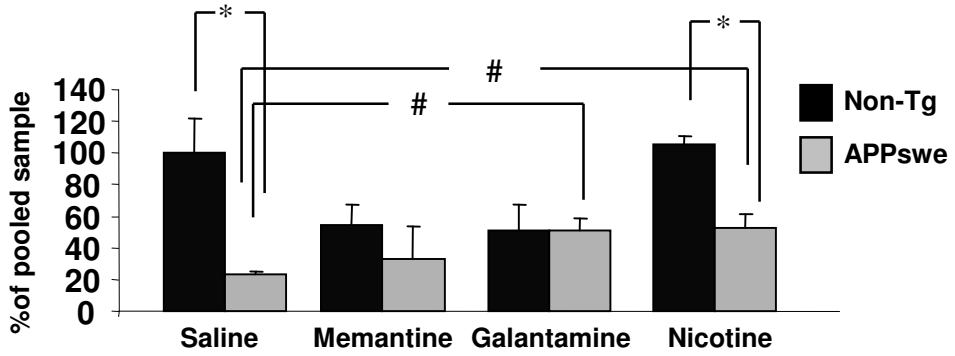

Figure 14. Levels of synaptophysin in the cortex of 10 months old APP_{swe} and non-transgenic control mice following treatment with saline, memantine, galantamine or nicotine. *p<0.05 indicate significant differences between APP_{swe} and non-transgenic controls. #p<0.05 compared to saline treated non-transgenic or transgenic group, respectively. The values are expressed as mean + SEM.

The α7 nAChRs are characterized by a rapid desensitisation following exposure to nicotinic agonists (Alkondon et al., 1994). Similarly, it is well known that treatment with nicotinic agonists, such as nicotine itself, up-regulates the nAChRs, mainly the α4 nAChRs, while the α7 nAChRs are more resistant to an up-regulation (Benwell et al., 1988; Breese et al., 1997; Flores et al., 1997; Bednar et al., 2002; Nguyen et al., 2003; Hellström-Lindahl et al., 2004). An increase in the α7 nAChRs in the CA3 of the hippocampus and in temporal/parietal cortex was observed in nicotine treated APPswe transgenic animals compared to saline treated mice (Paper V), suggesting that Aβ might potentiate the nicotine-induced desensitization of the α7 nAChRs and thereby causing an up-regulation of the α7 nAChRs (Fig. 15).

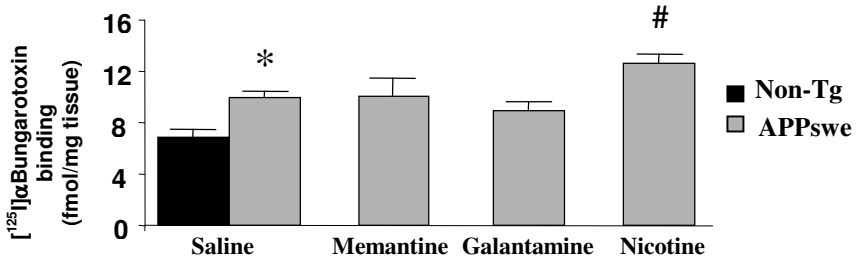

Figure 15. [^{125}I]αBungarotoxin binding sites in the temporal/parietal cortex of 10 months old APP_{swe} and non-transgenic control mice following treatment with saline, memantine, galantamine or nicotine. *p<0.05 indicate significant differences between APP_{swe} and non-transgenic controls. #p<0.05 compared to saline treated transgenic group. The values are expressed as mean + SEM.

Several lines of evidence suggest that the Aβ toxicity might be related to elevated levels of glutamate and/or overactivity of the NMDA receptors. The cellular damage in AD brains is observed especially in areas that display glutamatergic synaptic plasticity (Arendt et al., 1998). The observation that Aβ reduces LTP and facilitates LTD suggests a role for Aβ in regulating trafficking of glutamate receptors. A significant increase in the number of NMDA receptor binding sites was found in the CA2, CA3, temporal/parietal cortex and caudate nucleus of the APPswe transgenic mice (Paper V). This might reflect compensatory changes in response to the high Aβ levels in these mice. A signalling pathway, where $A\beta_{1-42}$ binds to α7 nAChRs, impairs glutamatergic transmission, compromise synaptic function and reduce LTP, thereby promoting endocytosis of NMDA receptors in cortical neurons, was recently reported (Snyder et al., 2005). Neuronal cell cultures from APPswe transgenic mice showed a reduction in surface-expressed NMDA receptors, while no change was observed in total receptor amounts (Snyder et al., 2005). The increase in NMDA receptors observed (Paper V) might therefore be the result of a compensatory response due to a persistent activation of the α7 nAChRs by Aβ, which consequently will affect the NMDA receptors. This mechanism needs to be further investigated. Treatment with galantamine caused a significant decrease in the number of NMDA receptor binding sites in the APPswe mice compared to saline treated mice (Fig. 16). Galantamine may potentiate the activity of NMDA receptors via protein kinase C (PKC) (Moriguchi et al., 2004), and interact with the α7 nAChRs (Kihara et al., 1997), but the exact mechanism needs to be further evaluated.

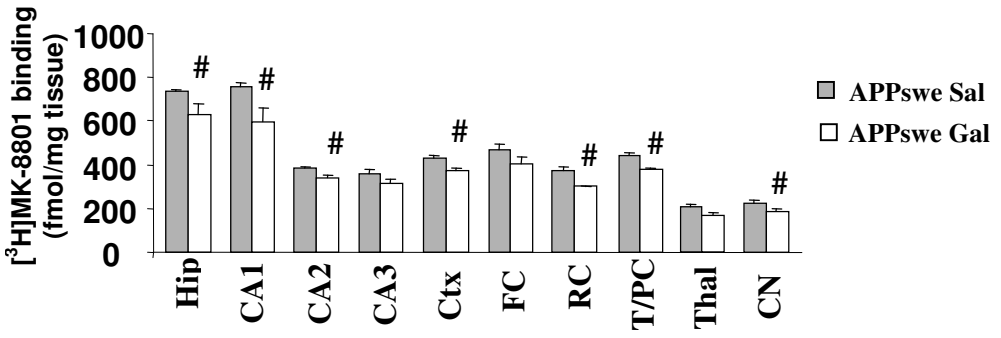

Figure 16. [^{3}H]MK-801 binding sites in the temporal/parietal cortex of 10 months old APP_{swe} transgenic mice following treatment with saline or galantamine. $^{\#}p<0.05$ compared to saline treated transgenic group. The values are expressed as mean + SEM.

CONCLUDING REMARKS

In the search for AD therapy, it is important to understand the pathological mechanisms behind the disease. Over the past decade, research on neurodegenerative diseases has revealed a common pathogenic mechanism: aggregation and deposition of misfolded proteins leading to progressive CNS amyloidosis. Thus, highly soluble proteins are gradually converted into insoluble, filamentous polymers with characteristic crossed-β-pleated sheet structures that accumulate in a disease and protein-specific manner as amyloid deposits in the cytosol or the nuclei of affected brain cells or in the extracellular space. In AD, Aβ is the protein that is suggested to initiate and/or influence the pathologic changes in the brain, leading to the cognitive decline seen in this disease.

Accumulating data indicate that Aβ may have a physiological role in the brain by interacting with neurotransmission. Furthermore it is also possible that Aβ might have a neuroprotective role in the brain. It remains to be elucidated if Aβ has a physiological function and the reason to why Aβ aggregation is one of the pathological hallmarks in AD. It is plausible that there are other components than Aβ that play a role in the underlying mechanisms of AD. Since AD has a subclinical phase that may be ongoing for years before clinical symptoms appear (Braak et al., 1999), it is possible that Aβ is a secondary event of the initial pathological mechanisms. It is of great importance to identify these mechanisms. The APP overexpressing transgenic mice provide a model to study the mechanisms that are caused by the excess of Aβ.

Neurotransmitter receptors are one of the decisive links in the synaptic information-processing chain and are relatively impaired in AD. They can markedly respond to alterations in neuronal activity by adaptive mechanisms like sub/supersensitivity or down-regulation (Schwartz et al., 1983), which makes them an appropriate tool to monitor interactive changes in the neuronal activity of a particular transmitter system. The utilization of the APPswe transgenic mice to mimic AD-related changes revealed selective alterations in nAChRs and NMDA receptor density. There was a biphasic effect on the α7 nAChRs with an initial decrease in the cortical levels at early ages, followed by an increase at later ages, while there was a persistent increase in the number of NMDA receptors already from 21 days of age. The increase in α7 nAChRs and NMDA receptors probably reflects compensatory mechanisms in response to the Aβ burden. The results may provide important information about the onset and consequences of Aβ pathology in this transgenic mouse model. The increase in α7 nAChRs might also reflect the non-cholinergic distribution of the α7 nAChRs in other neurotransmitter systems, such as the glutamatergic system (Paterson and Nordberg, 2000) and in glial cells (Yu et al., 2005).

Two of the current drugs clinically used in AD therapy, the AChEI galantamine and the NMDA receptor antagonist memantine appear to have different effects in the APPswe transgenic mice. Since galantamine influences the α7 nAChRs and the NMDA receptors, it is plausible that it mediates plastic changes in the brain. This might partly explain the neuroprotective effect seen following treatment with galantamine. Since subchronic treatment with memantine decreased the levels of total membrane bound APP, it is plausible that prolonged treatment may decrease the levels of Aβ. Neither galantamine nor memantine had similar effects as nicotine, which markedly reduced insoluble Aβ levels in the APPswe transgenic mice. By reducing plaque and activated astrocytes, nicotine seems to reduce the extracellular deposits, while the intracellular Aβ is unchanged. Further studies of the interaction of intracellular and extracellular Aβ at synapses may lead to a better understanding of the interactive effect of nicotine on AD pathology and may promote new therapeutic strategies for the disease.

To develop new treatment strategies, it is important to understand the mechanisms behind AD. Since AD remains subclinical for years and clinical symptoms are observed only late in the course of the disease, it must be of great importance to identify these patients early. The results in this thesis will hopefully contribute to the understanding of the mechanisms behind the disease and thereby improve the treatment of AD in order to give patients an active and as rich a life as possible.

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