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β-Amyloid Processing and α7 Neuronal Nicotinic Acetylcholine Receptors in Alzheimer's Disease-related Mouse Models

- Interactive Mechanisms with Focus on New Drug Targets

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Cover picture: Photo of an APPswe transgenic mouse. Courtesy of Michael Schöll.

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Tillägnat min älskade familj
amor vincit omnia

ABSTRACT

Accumulation of beta-amyloid (Aβ), neuronal loss and changes in neurotransmitter systems, in particular the cholinergic system, are consistent features of Alzheimer's disease (AD). Aβ is thought to play a critical role in the pathogenesis of AD and it has therefore become a target of interest as regards a therapeutic approach. The aim of this work was to investigate how different Aβ peptides influence neurotransmission in the brains of AD-related mouse models, as well as to evaluate how drugs, acting via the cholinergic and glutamatergic neurotransmitter systems, affect these processes.

Three different transgenic mouse models were utilized: APPswe, hAChE-Tg//APPswe and 3xTg-AD mice, which revealed diverse brain Aβ pathologies. The hAChE-Tg//APPswe mice exhibited accelerated plaque pathology and showed, even at early ages, increased levels of insoluble Aβ in their brains compared with age-matched APPswe mice, whereas the latter expressed increased soluble Aβ. The 3xTg-AD mice showed no extracellular Aβ plaques, while intraneuronal APP/Aβ immunostaining was evident in the cortex and the hippocampus. Whereas APPswe mice showed a more pronounced Aβ pathology, with high Aβ levels both in the cortex and the hippocampus, the 3xTg-AD mice showed detectable Aβ peptides solely in the hippocampus.

A biphasic effect was found on cortical α7 neuronal nicotinic acetylcholine receptors (nAChRs) in APPswe mice, with an initial decrease at early ages, followed by an increase at later ages, while the *N*-methyl-D-aspartate (NMDA) receptors showed a persistent increase from a young age. The increased receptor levels probably reflect compensatory mechanisms in response to a high Aβ burden. No changes were found in α7 nAChRs in hAChE-Tg//APPswe mice or 3xTg-AD mice.

At a very early age, the APPswe mice expressed increased cortical synaptophysin levels, followed by a persistent decrease. In 3xTg-AD mice, the observed reduction in cortical synaptophysin might be ascribed to the presence of intraneuronal Aβ.

The choice of transgenic mouse model, as well as the stage of Aβ pathology, was shown to strongly affect the outcome of drug treatment. While decreasing cortical insoluble Aβ1–40 and Aβ1–42 in APPswe mice, L(-)-nicotine increased cortical insoluble Aβ1–40 and soluble Aβ1–42 (both enantiomers) in hAChE-Tg//APPswe mice. However, in both mouse models nicotine reduced glial fibrillary acidic protein (GFAP) immunoreactive astrocytes. In APPswe mice, L(-)-nicotine increased hippocampal and cortical α7 nAChRs, while D(+)-nicotine treatment resulted in an increase in these receptors in hAChE-Tg//APPswe mice. Huprine X decreased hippocampal insoluble Aβ1–40, the levels of α7 nAChRs in the caudate nucleus, and increased cortical synaptophysin levels in 3xTg-AD mice, while only increasing the levels of α7 nAChRs in the hippocampi of APPswe mice. Treatment with the current AD drug galantamine increased cortical synaptophysin levels and affected the NMDA receptors, indicating plastic changes in the brain, while memantine reduced cortical levels of membrane-bound amyloid precursor protein (APP), which eventually may decrease Aβ levels.

In conclusion, the diverse forms of Aβ displayed in the mouse models studied gave rise to differences in brain neuropathology, with diverging results regarding synapses and neuronal receptors as well as the outcome of drug treatment. The results in this work clearly highlight the fact that the choice of transgenic mouse model, as well as the stage of Aβ pathology, contribute importantly to the outcome of drug treatment. This emphasizes the importance of using different transgenic mouse models for evaluating the effects of new drug candidates for this devastating disease.

LIST OF PUBLICATIONS

This thesis is based on the following papers, which are referred to in the text by their roman numerals.

I. 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, α 7 neuronal nicotinic acetylcholine- and *N*-methyl-D-aspartate receptor levels
Molecular and Cellular Neuroscience, 2005, 30(2), 218-227

II. 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
Journal of Pharmacology and Experimental Therapeutics, 2006, 317(1):30-36

III. **Hedberg MM**, Svedberg MM, Mustafiz T, Yu W-F, Mousavi M, Guan Z-Z, Unger C and Nordberg A

Transgenic mice overexpressing human acetylcholinesterase and the Swedish amyloid precursor protein mutation: effect of nicotine treatment
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IV. **Hedberg MM**, Clos MV, Ratia M, Gonzalez D, Unger Lithner C, Camps P, Muñoz-Torrero D, Badia A, Giménez-Llort L and Nordberg A

Effect of huprine X on Aβ, synaptophysin, and α7 neuronal nicotinic acetylcholine receptors in the brain of 3xTg-AD and APPswe transgenic mice
Manuscript

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LIST OF ABBREVIATIONS

Aβ	β-amyloid
ACh	Acetylcholine
AChE	Acetylcholinesterase
AChE-E	Erythrocyte AChE
AChE-R	Readthrough AChE
AChE-S	Synaptic AChE
AChEI	Acetylcholinesterase inhibitor
AD	Alzheimer's disease
ADAM	A disintegrin and metalloproteinase
AICD	APP intracellular domain
Apo E	Apolipoprotein E
APP	Amyloid precursor protein
BACE	β-site cleaving enzyme
BuChE	Butyrylcholinesterase
CAA	Cerebral amyloid angiopathy
CDK5	Cyclin-dependent kinase 5
ChAT	Choline acetyltransferase
ChEs	Cholinesterases
CNS	Central nervous system
CSF	Cerebrospinal fluid
ERK	Extracellular-signal-regulated kinase
FAD	Familial Alzheimer's disease
GAG	Glucosaminoglycan
GFAP	Glial fibrillary acidic protein
GSK3β	Glucogen-synthase kinase 3β
Hup A	Huperzine A
HX	Huprine X
LTD	Long-term depression
LTP	Long-term potentiation
mAChR	Muscarinic acetylcholine receptor
MAPK	Mitogen-activated protein kinase
MCI	Mild cognitive impairment
MRI	Magnetic resonance imaging
nAChR	Nicotinic acetylcholine receptor
NMDA	*N*-methyl-D-aspartate
NFTs	Neurofibrillary tangles
NGF	Nerve growth factor
NSAID	Non-steroid anti-inflammatory drug
PAS	Peripheral anionic site
PET	Positron emission tomography
PKC	Protein kinase C
PS	Presenilin

INTRODUCTION

ALZHEIMER'S DISEASE

Alzheimer's disease (AD) is a progressive neurodegenerative disorder and the most common form of dementia, representing 50–70% of all cases[1]. The disease was first described in 1907 when the German psychiatrist Alois Alzheimer (1864–1915) reported the characteristic pathological changes in *post mortem* brain tissue from his 56-year-old patient Auguste D, who suffered from dementia[2]. AD is devastating because of the way it affects the mind and the personality of the patient, causing enormous emotional stress to affected families. The disease is characterized by cognitive impairments beginning with a decline in episodic memory. As the disease progresses, the cognitive impairments extend to the domains of language (aphasia), skilled movements (apraxia), recognition (agnosia), as well as executive functions such as decision-making and planning. Other symptoms such as changes in personality and behavior also become apparent.

The clinical diagnosis of AD is currently based upon the outcome of several investigations including medical history, neurological and psychiatric investigations, lab analyses, imaging analyses and cognitive testing. During recent years, the concept of mild cognitive impairment (MCI) has been a focus of interest. Amnestic MCI is thought to be a transitional state before progression to AD, and patients with amnestic MCI have indeed shown an increased risk of developing AD[3].

The prevalence of dementia is below 1% in individuals aged 60–64 years, but it shows an almost exponential increase with age, so that in people aged 85 years or older, the prevalence is between 24–33% in the Western world[4]. In 2006, the number of AD patients throughout the world was 26.6 million. By 2050, it is expected that the prevalence will quadruple, by which time 1 in 85 persons worldwide will be living with this disease[5].

Neuropathological features

Definitive diagnosis of AD is based upon the presence of the two major pathological hallmarks, neurofibrillary tangles (NFTs) and neuritic plaques (also called senile plaques). The former are intraneuronal inclusions, which accumulate in neuronal perikarya and consist of abnormally hyperphosphorylated tau, a microtubule-associated protein which self-assembles into paired helical filaments, building up the

tangles[6,7]. Neuritic plaques are insoluble extracellular aggregates of β-amyloid (Aβ) peptide fibrils[8].

The temporal and regional distribution of plaques and tangles differs in the brain. Braak-staging is a commonly used method for describing the pathological process as regards the two pathological hallmarks of AD. Neurofibrillary tangle pathology starts in the medial temporal lobe (entorhinal cortex and the hippocampus) and spreads to limbic areas and finally to neocortical association areas and the primary cortex. The plaques are first observed in neocortical areas of the temporal lobe and orbitofrontal cortex, and then they further spread to the parietal cortex and finally throughout the entire neocortex[9]. The cerebellum is usually spared from pathological lesions.

In the AD brain, numerous other structural and functional alterations have also been identified, including loss of neurotransmission, increased inflammatory responses[10], oxidative stress[11-13] and mitochondrial dysfunction[14,15]. The combined consequences of all these pathological changes, including the effects of the Aβ and tau pathologies, are severe neuronal and synaptic dysfunction and loss in the cerebral cortex and in certain subcortical areas. The greatest neuronal loss can be observed in the temporal and parietal lobes, in frontal lobe areas, and in the cingulate gyrus. At the time of death, the brain of an AD patient may weigh one third less than the brain of an age-matched non-demented individual.

β-amyloid is generated by processing of amyloid precursor protein

Amyloid precursor protein (APP) is a type I transmembrane glycoprotein, widely expressed in cells throughout the body, with a small cytosolic C-terminal domain and a large luminal N-terminus. It is proteolyzed via two mutually exclusive pathways (Fig. 1). In the non-amyloidogenic pathway, the formation of intact Aβ peptides is precluded and this is the major APP processing pathway in most cell types[16]. Here, α-secretase cleaves APP and releases a soluble extracellular fragment of APP (sAPPα), as well as generating a membrane-bound 83-amino-acid residue fragment (C83), which is then further processed by γ-secretase, generating a short p3 peptide, unable to form aggregates, and the APP intracellular domain (AICD). In the amyloidogenic pathway, which is associated with AD, APP is first cleaved at N-terminal position I of the Aβ sequence (β-site) by β-secretase, generating a soluble extracellular fragment of APP (sAPPβ), and a membrane-bound 99-amino-acid C-terminal fragment (C99).

Next, γ-secretase cuts the C99 fragment to release AICD and the Aβ peptides (38–43 amino acids in length).

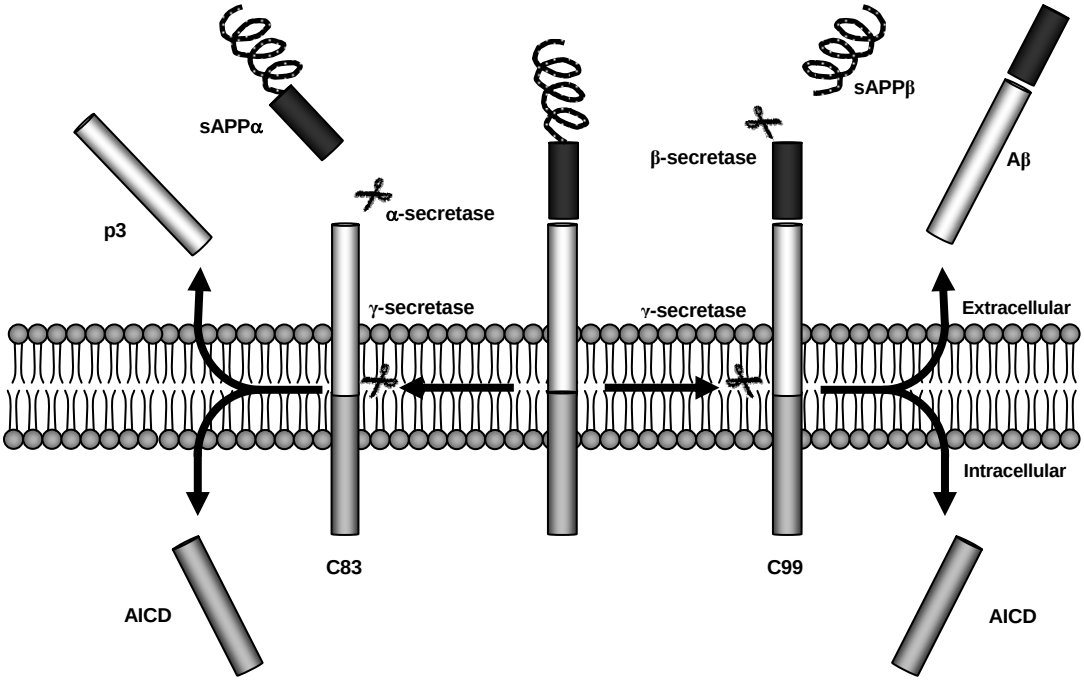

Figure 1. Schematic overview of the general processing of amyloid precursor protein. *In amyloidogenic processing, amyloid precursor protein is sequentially cleaved by β- and γ-secretase, generating intact Aβ. In non-amyloidogenic processing, formation of Aβ is precluded by sequential cleavages of amyloid precursor protein by α- and γ-secretase.*

The molecular identities of the three APP-cleaving secretases have now been proposed. β-secretase activity is performed by β-site cleaving enzyme (BACE) 1 and 2[17]. γ-secretase is a protein complex with presenilin (PS) 1 or 2 as the catalytic subunit[18]. The identity of α-secretase is less well understood. However, enzymes with α-secretase activity have been identified within the A disintegrin and metalloproteinase (ADAM) family, of which at least ADAM9, ADAM10 and ADAM17 are suggested to be responsible for α-secretase cleavage[19].

The β-amyloid peptide

In the AD brain, the predominant forms of Aβ peptides are the Aβ1–40 and Aβ1–42 isoforms[20], of which Aβ1–40 is the most abundant (90%), while Aβ1–42 is more prone to oligomerize and form amyloid fibrils, as well as being essential for seeding the deposition of Aβ[21-23].

The fibrillization process of Aβ peptides is a multistep reaction where soluble Aβ monomers are thought to undergo a conformational change into high β-sheet content,

rendering them prone to aggregate into soluble oligomers that polymerize into proto-fibrils, which mature into larger insoluble Aβ fibrils, found in the plaques[24].

There are two forms of plaque in the AD brain, in which the Aβ peptides are either arranged in insoluble fibrillar β-pleated sheets as dense-cored plaques (classical neuritic plaques) or deposited amorphously, forming diffuse plaques. The neuritic plaques are intimately surrounded by dystrophic neurites (abnormal growth of axonal or dendritic terminals), also found within the plaques, and activated glial cells, whereas the diffuse plaques lack the fibrillar compacted core and the dystrophic neurites[21]. The diffuse plaques are proposed to be immature precursors of the neuritic plaques. Rapid development in functional imaging techniques has now resulted in the possibility to measure fibrillar Aβ in the brains of living AD patients by means of positron emission tomography (PET)[25]. It is not known how long it takes to develop neuritic plaques in the human brain, but it is likely that the process takes months or even years. However, there is evidence that plaque formation is an early event in the pathogenesis of AD[26-28].

The amyloid cascade hypothesis and the detrimental role of β-amyloid

To date, the cause/causes of AD is/are still not known but the disease is believed to be multifactorial, with the involvement of both environmental and genetic factors. Many theories have been proposed over the years to explain the pathogenesis of AD. One of the leading theories is the "amyloid cascade hypothesis", which states that the gradual accumulation and aggregation of Aβ triggers a slow but deadly cascade of neurodegenerative events including synaptic alterations, inflammatory changes, the modification of the normally soluble tau protein into oligomers and subsequent formation of NFTs, and progressive neuronal loss associated with multiple neurotransmitter deficiencies and cognitive failure[29,30].

Several pieces of evidence support a causative role of Aβ in the pathogenesis of AD:

- Mutations implicated in familial forms of AD are present in genes encoding both the substrate (APP) and the enzymes (presenilins) required for Aβ production[31-33].
- The APP gene is localized on chromosome 21 and individuals with Down's syndrome (trisomy of chromosome 21) overproduce Aβ and develop AD-like neuropathology as a result of increased APP expression[34,35].

- Synthetic Aβ peptides are toxic to hippocampal and cortical neurons[36-40].
- APP transgenic mice show a time-dependent increase in extracellular Aβ and develop certain neuropathological and behavioral changes similar to those seen in AD patients[41,42].
- Injection of synthetic Aβ into the brains of tau transgenic mice, or co-expression of mutant APP with mutant tau accelerates tau hyperphosphorylation and induces NFT formation[43-46].

Although Aβ is suggested to play a cardinal role in the pathogenesis of AD, the molecular mechanisms by which Aβ exerts its neurotoxic effects are not yet fully understood. Much attention is also being focused on the form(s) of Aβ that has/have the most deleterious effect. Evidence indicates that protofibrillar and oligomeric forms of Aβ, rather than plaque-associated fibrillar Aβ, contribute to early dendritic and synaptic injury and thereby to neuronal dysfunction[47,48]. Moreover, in several recent studies intraneuronal Aβ has been implicated in the pathogenesis of AD as well[49,50]. Nevertheless, at the current stage of research, it is not clear if large insoluble Aβ deposits, or small soluble oligomers represent the sole neurotoxic entity. Indeed, a continuous dynamic exchange between these forms may well be detrimental.

Even though the amyloid cascade hypothesis is convincing, several observations suggest that it is lacking in detail. One persistent concern is that this theory does not explain why many apparently healthy people start to accumulate Aβ in the brain upon aging[51,52].

Function of amyloid precursor protein and β-amyloid in the brain

A number of possible physiological functions have been ascribed to APP and/or its major secreted derivative (sAPPα). Expression of APP has been found to be up-regulated during neuronal maturation, differentiation and synaptogenesis[53-55], as well as after head injury[56,57], suggesting an important functional role in neuronal development and neuroprotection. Full-length APP also seems to play a critical role in appropriate migration of cortical progenitor cells during brain development[58]. Soluble APPα has been shown to play important roles in neurogenesis, neurotropic and neuroprotective functions, synaptogenesis and memory processes[59-62].

There is no dispute that Aβ is toxic to neurons, but since it is also produced during normal metabolism[63-65], it has been suggested that the peptide may play important

roles in normal cell function that may differ either quantitatively or qualitatively from its effects when levels are elevated during disease conditions. A recent study showed that a low (picomolar) concentration of Aβ42 caused a marked increase in hippocampal long-term potentiation (LTP), whereas a high (nanomolar) concentration lead to its well established reduction[66]. Interestingly, a negative feedback role has been suggested for Aβ, as the peptide has been found to be secreted from unaffected neurons in response to activity, but it down-regulates excitatory synaptic transmission in organotypical slices from APPswe transgenic mice[67]. This negative feedback loop may provide a physiological homeostatic mechanism, which keeps the levels of neuronal activity in check, and 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, the result of many studies have shown that excessively produced Aβ is critical to the complex pathogenesis of AD[68].

GENETICS AND RISK FACTORS OF ALZHEIMER'S DISEASE

AD can appear as a hereditary disorder, familial AD (FAD), or as a sporadic form, of which the latter is the most common since known genetic cases represent less than 2% of all AD cases[69]. FAD generally shows a relatively early onset (< 60 years of age), while sporadic AD has a later onset[70].

FAD is attributable to dominant missense mutations in genes encoding APP on chromosome 21[31], presenilin 1 (PS1) on chromosome 14[33] and presenilin 2 (PS2) on chromosome 1[32]. A common characteristic of these mutations is that they alter the proteolytic processing of APP, leading to increased production of either total Aβ or Aβ1–42[71].

Most of the known pathogenic APP mutations are located close to the major APP processing sites[72], either adjacent to the Aβ domain (the β- and γ-secretase sites) or within the Aβ domain itself (the α-secretase site). Mutations near the β-site, such as the Swedish APP mutation (KM670/671NL)[73], increase the absolute levels of both Aβ1–40 and Aβ1–42. Mutations close to the γ-site, such as the London mutation (V717I)[31] selectively enhance the production of fibrillogenic Aβ1–42. Most of the mutations within the Aβ sequence result in hemorrhages or strokes caused by unusually severe cerebral amyloid angiopathy (CAA) that also accompanies parenchymal Aβ deposition. The Dutch (E693Q), Flemish (A692G), Italian (E693K) and Arctic

(E693G) mutations are all located within the Aβ sequence of APP. These mutations are generally believed to cause Aβ accumulation by augmenting aggregation or protofibril formation[74-76].

Biochemically, mutations in the PS1 or PS2 genes lead to a selective increase in Aβ1–42 levels[77,78]. Mutations in the PS1 gene, which account for more than 70% of all FAD mutations, cause the most aggressive forms of AD, in some cases with onset younger than 30 years of age[33,79].

Sporadic AD is heterogeneous and multifactorial in its pathogenesis and the cause is thought to be a combination of both biological and environmental risk factors. The most prominent of these risk factors is old age, but family history, low levels of formal education and a history of head trauma are also important risk factors[80]. In addition, the inheritance of one or two ε4 alleles of apolipoprotein E (ApoE), a protein important in cholesterol transport, uptake and distribution, is directly linked to an increased risk of late onset sporadic AD[81].

THE CHOLINERGIC NEUROTRANSMITTER SYSTEM IN ALZHEIMER'S DISEASE

Cholinergic neurotransmission is mediated by the key endogenous neurotransmitter acetylcholine (ACh), which is the neurotransmitter that best correlates with cognitive functions. It elicits its effect by binding to two different types of receptors: the nicotinic acetylcholine receptors (nAChRs) and the muscarinic acetylcholine receptors (mAChRs).

The most severe and consistent neurochemical abnormality in the AD brain is loss of cholinergic innervation. Selective degeneration in basal forebrain cholinergic neurons, innervating the cortex, amygdala and the hippocampus, has been demonstrated[82,83]. The cholinergic cell loss is paralleled by reductions in cholinergic markers such as choline acetyltransferase (ChAT), high affinity choline uptake, nAChR number and ACh levels[84].

Nicotinic acetylcholine receptors

Neuronal nAChRs are widely distributed in the human brain, particularly in the thalamus, cortex and the striatum[85]. They are cation selective ligand-gated ion channels, which flux the pluripotent second messenger Ca^{2+}, and they are composed of individual subunits in a pentameric structure around the central ion channel. Twelve

nAChR subunit genes with a common ancestor have so far been cloned and classified into two subfamilies of nine α (α2–α10) and three β (β2–β4) subunits. Generally, nAChRs are composed of α and β subunits in a 2 to 3 ratio, each combination defining their particular pharmacological and physiological functions. However, the α7, α8 and α9 subunits are known to form functional homomeric receptors consisting of a single subunit type, although a novel heteromeric α7β2 nAChR was recently reported[86]. The most abundant functional nAChR subtypes in the human brain are the α4β2 and the α7 subtypes.

The nAChRs are involved in several important physiological functions in the brain, including cognitive functions such as learning and memory[85].

A consistent loss of nAChRs is well documented in AD, with significant decreases of different receptor subtypes in specific regions of the brain, particularly in the cortex and the hippocampus[87-89]. The predominant nAChR subtype lost in the AD brain is the α4 subtype, but there are also reductions in the α3 and α7 subunits[89,90], while an increase in the α7 subunit has been found in astrocytes[91,92]. *In vivo* PET studies have further indicated that the nAChR deficits in fact is an early phenomenon in the course of AD[93], and that these receptor deficits are significantly correlated with cognitive impairments[94,95].

Nicotinic acetylcholine receptors and β-amyloid

Several findings imply an interaction between the cholinergic system and Aβ in the brain. The α7 nAChR is highly expressed on the basal forebrain cholinergic neurons, which project to the hippocampus and cortex, correlating well with brain areas that display Aβ plaques[85].

Different mechanisms implicating nAChRs have been proposed to explain the toxic effects of Aβ in the brain. One of them suggests that the formation of the stable α7 nAChR-Aβ1–42 complex on neuronal cell membranes might represent an important first step in intracellular Aβ1–42 accumulation and toxicity[96]. In addition, prolonged *in vitro* exposure to Aβ led to cell toxicity in cells that expressed α7 nAChRs, an effect found to be blocked by α7 nAChR antagonists and absent in preparations devoid of α7 nAChRs[96,97]. Aβ peptides have also been found to functionally block both human and rodent α7 nAChRs, suggesting that the blockade importantly contributes to the long-term behavioral consequences of AD[98-100].

On the other hand, other studies have suggested that the activation of nAChRs/α7 nAChRs can alleviate Aβ toxicity. Stimulation of these receptors has been found to inhibit Aβ plaque formation both *in vitro* and *in vivo*[101], to activate α-secretase cleavage of APP[102] and to mediate neuroprotective effects of nicotine[103,104]. The protection against Aβ toxicity has been shown to be proportional to the number of α7 nAChRs expressed by cultured cells[105]. Interestingly, it has also been proposed that Aβ itself acts as an agonist on α7 nAChRs[106], mediating activation of the extracellular-signal-regulated kinase/mitogen-activated protein kinase (ERK/MAPK) signalling cascade that has a neuroprotective role[107,108]. Whether Aβ acts as an agonist or antagonist on α7 nAChRs appears to depend on the concentration of Aβ. Low concentrations (picomolar) might activate α7 nAChRs while higher concentrations (nanomolar) may lead to their desensitization[108]. As Aβ levels become pathologic in the course of AD, it might therefore be useful to develop ways in which interruption of Aβ-nAChR interaction is achieved, especially if this interaction is involved in the accumulation of intracellular Aβ or mediates neurotoxic signalling cascades.

Cholinesterases and their role in Alzheimer's disease

Cholinesterases (ChEs) are ubiquitous constituents of cholinergic pathways and necessary for terminating the synaptic action of ACh through catalytic hydrolysis of ACh into choline and acetate. In humans the ChEs exist in two distinct forms: acetylcholinesterase (AChE) and butyrylcholinesterase (BuChE)[109,110]. The former is the primary ChE in the central nervous system (CNS), while BuChE, also known as pseudo- or non-specific ChE, is the major ChE in the circulation, originating from glial cells[111]. AChE and BuChE are generally considered to be independently regulated, but AChE-knockout mice have demonstrated normal cholinergic transmission, as a result of compensation of the function of AChE by BuChE[112].

In humans, three different natural splice variants of AChE have been demonstrated[113]: the membrane-associated synaptic AChE (AChE-S), which is the main AChE isoform in the brain and muscles; erythrocyte AChE (AChE-E), located in red blood cells; and the soluble readthrough AChE (AChE-R), expressed in embryonic cells and tumor cells. The last has been suggested to be induced by stress or anticholinesterase exposure[114].

During the course of the disease, decreased activity of AChE and stable or increased activity of BuChE has been reported in AD patients[115]. PET studies have also

revealed lower AChE activity in cortical brain regions of MCI patients who later converted to AD[116]. Interestingly, AChE-S may have a direct effect on plaque formation[117-119], while BuChE, instead, has been shown to delay the onset and decrease the rate of Aβ fibrillization *in vitro*[120].

THE GLUTAMATERGIC NEUROTRANSMITTER SYSTEM IN ALZHEIMER'S DISEASE

The primary excitatory neurotransmitter of the CNS is glutamate, which is used by approximately two thirds of all synapses in the neocortex and hippocampus[121]. Glutamate is involved in most, if not all, aspects of cognition and higher mental function. Increasing evidence implicates a malfunction in glutamatergic activity in AD. Pyramidal neurons of the neocortex forming corticortical and corticofugal connections together with those of the entorhinal and hippocampal CA1 region are damaged in AD[122,123], and these changes have been shown to be strongly correlated with cognitive impairment[122,124].

The *N*-methyl-D-aspartate receptor

The *N*-methyl-D-aspartate (NMDA) receptor is a major subtype of ligand-gated ionotropic glutamate receptors, highly represented throughout the brain, especially in the neocortex and hippocampus, where it is involved in excitatory synaptic transmission[125]. It is composed of two types of subunits: NR1 and NR2, each of which can exist in many different isoforms and splice variants.

Normal (physiologic) glutamate stimulation of the NMDA receptors is considered essential to learning and memory processes, and is likely to involve LTP[126]. However, owing to their high Ca^{2+} permeability, activation of NMDA receptors is often the first event in glutamate-induced neuronal injury[127]. Accordingly, excitotoxic neuronal death facilitated by excessive glutamate in the synaptic microenvironment, and persistent Ca^{2+} influx through the NMDA receptor, is believed to play a critical role in the neurodegenerative processes seen in AD. *Post mortem* studies in brain tissue from AD patients have revealed both altered glutamate levels and loss of NMDA receptors[128,129].

The *N*-methyl-D-aspartate receptor and β-amyloid

Several findings support the view that NMDA receptors play a central role in Aβ-induced neurotoxicity. In rat magnocellular nucleus basalis, Aβ1–42 and Aβ1–35 promoted an excitotoxic pathway including astroglial depolarization, extracellular glutamate accumulation and NMDA receptor activation, culminating in intracellular Ca^{2+} overload and cell death, effects that were effectively reduced by the NMDA receptor antagonist MK-801[130]. However, the role of NMDA receptors in AD pathology might be more than just mediating excitotoxicity. Interestingly, the finding that Aβ has the ability to reduce LTP and facilitate long-term depression (LTD)[131] suggested a role for Aβ in regulating the trafficking of glutamate receptors. Application of Aβ1–42 to cortical slices has been reported to promote the endocytosis of some NMDA receptors through a mechanism that involves initial binding of Aβ1–42 to α7 nAChRs[132]. In addition, neuronal cultures from APPswe transgenic mice showed a reduced amount of surface NMDA receptors, while no change was observed in total receptor amounts[132]. More recently, it was also shown that soluble Aβ oligomers strongly bind to hippocampal neurons grown in culture, and this binding significantly altered neuronal morphology, as well as decreasing membrane expression of NMDA receptors[133]. These studies suggest that Aβ-induced reduction in surface NMDA receptors might impair the function of the receptors and thereby depress synaptic glutamatergic transmission.

THERAPEUTIC STRATEGIES FOR ALZHEIMER'S DISEASE

Knowledge of the neurotransmitter disturbances in AD has led to the development of drugs with symptomatic effects. Research advances in the molecular pathogenesis of AD have also resulted in other treatment strategies aiming to slow down or prevent the progression of the disease by targeting Aβ, taupathies or other pathological consequences of the disease (Fig. 2).

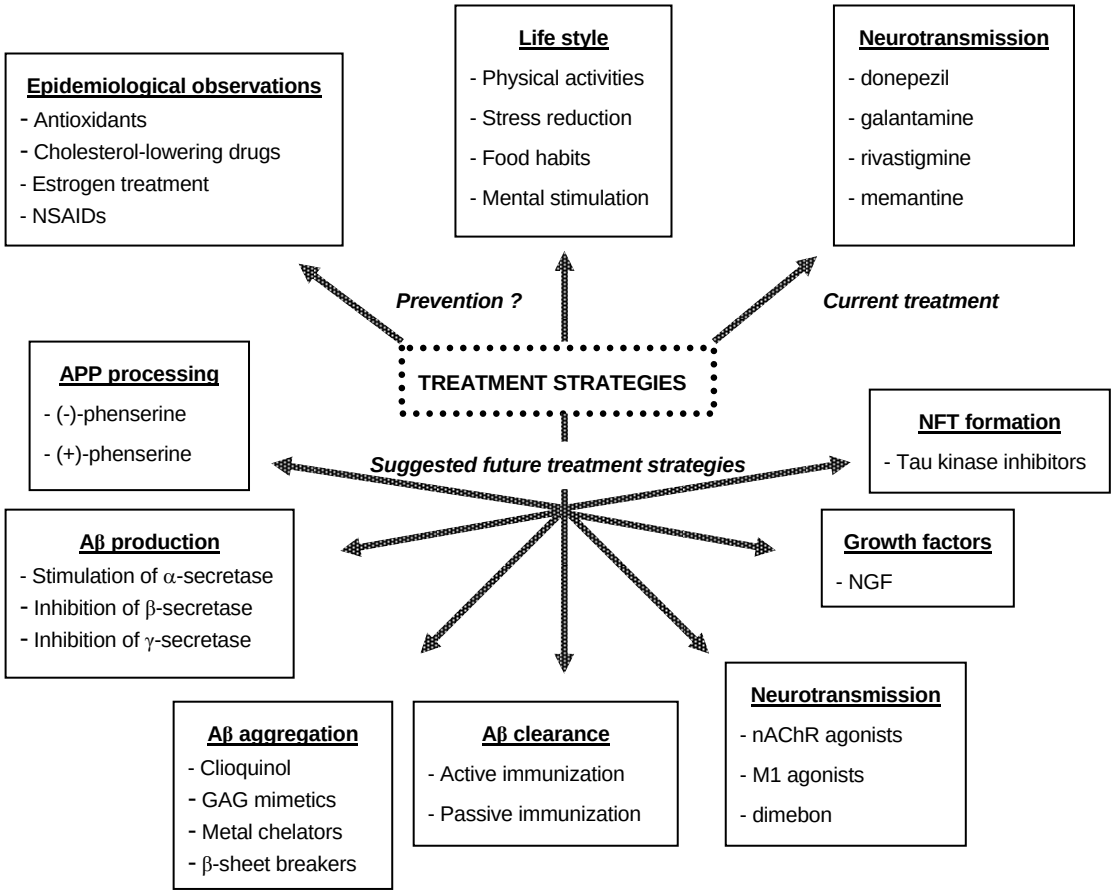

Figure 2. Schematic illustration of present and future therapies for Alzheimer's disease. *Treatment possibilities for Alzheimer's disease can be symptomatic, curative or disease-modifying.*

Neurotransmitter therapy

The presently available treatments for AD are symptomatic cholinergic neurotransmitter therapy with AChE inhibitors (AChEIs) and the non-competitive partial NMDA receptor antagonist memantine.

Three different AChEIs are approved for treatment of mild to moderate AD; donepezil, rivastigmine and galantamine, which all act by increasing the synaptic concentration of ACh, thereby enhancing and prolonging the actions of ACh on the mAChRs and nAChRs[134,135]. These AChEI drugs show different pharmacological properties with diverse selectivity, inhibition and bioavailability[134,135]. Donepezil and galantamine selectively inhibit AChE, whereas rivastigmine also inhibits BuChE with similar affinity[136]. Interestingly, galantamine has also been found to allosterically modulate presynaptic nAChRs, thereby increasing presynaptic ACh release and postsynaptic neurotransmission[137]. The AChEI drugs have been shown to delay the rate of cognitive and functional decline for up to 1[138-140] or 2 years[141,142] in AD patients. In-

terestingly, increasing evidence suggests that the effects of AChEI treatment extend beyond symptomatic benefits[143].

Memantine, currently the only symptomatic treatment approved for moderate to severe AD, is believed to protect neurons from glutamate-mediated excitotoxicity without preventing the physiological NMDA receptor activation needed for cognitive function[144]. In addition, there is pre-clinical evidence that memantine also acts as an open channel blocker for several nAChR subtypes[145-147]. Randomized double-blind clinical trials have shown modest positive effects on cognitive and behavioral symptoms, and improved the ability to perform activities of daily living at 6 months in patients with moderate to severe AD[144]. The durability of clinical improvements associated with memantine treatment is however currently not known.

Other potential therapeutic strategies involving the neurotransmitter systems might involve compounds that are selective muscarinic and nicotinic agents. Agonists partially selective for the M1 subtype of the mAChR have been reported to increase secretion of sAPPα and thereby decrease Aβ production[148,149], effects that appear to occur via activation of downstream signalling pathways involving protein kinase C (PKC) and MAPK[150]. Activation of the same receptor subtype has also been shown to provide protection against neuronal damage caused by Aβ toxicity via activation of the Wnt signalling pathway[151]. In addition, since *in vitro* studies have shown that stimulation of α7 nAChRs protects against Aβ-induced neurotoxicity[152-154], this receptor might also represent an important therapeutic target.

Anti-amyloid therapy

Since deposition of Aβ seems to be an early and crucial event in the pathogenesis of AD, it has been a target of interest as regards a therapeutic approach[155]. A number of therapeutic strategies are currently being examined both in the laboratory and in the clinic, aiming to reduce the Aβ load in the brain.

Targeting β-amyloid production

β-secretase is an excellent theoretical target to block the generation of Aβ, as it is the rate-limiting enzyme in Aβ production. It has been shown that BACE1 knockout mice are viable and have abolished Aβ production[156]. However, other reports suggest that these mice may have a harmful phenotype, raising concerns over the safety of β-secretase inhibitors[157,158]. Indeed, this enzyme has been proven to be a difficult phar-

macologic target. For γ-secretase inhibition, there is also concern about adverse effects due to cleavage of other vital substrates such as Notch by this secretase[159,160]. Both safety and pharmacokinetic problems hinder progress with secretase inhibitors[161].

Targeting the processing of amyloid precursor protein

Phenserine, a novel AChEI drug, has been shown to decrease Aβ levels by regulating APP translation[162], as shown both in cell cultures and in mouse brain[163,164]. The effect of long-term treatment with phenserine has also been evaluated in patients with mild AD by PET, using a ligand, PIB, which allows detection of amyloid deposits[165]. A reciprocal change was found between reduction in PIB retention in the brain and an increased level of Aβ40 in the cerebrospinal fluid (CSF), suggesting that phenserine can influence Aβ content both in the brain and in the CSF, together with an improvement in cognition[165].

Targeting β-amyloid aggregation

Blocking the assembly of Aβ into oligomers and fibrils is another potential treatment strategy. An essential role of metals, particularly copper and zinc, in Aβ aggregation[166] led to the discovery of small molecule chelators that perturb Aβ-metal binding. Clioquinol (PBT-1) has been found to reduce Aβ deposition in AD-related transgenic mice[167], which led to clinical trials in AD patients[168]. However, it is not clear from those trials whether clioquinol actually shows any positive clinical result in AD patients, since statistically significant positive results in cognitive tests and as regards plasma Aβ were seen only among the more severely affected subgroup of patients and the effects were not maintained at the 36-week end-point.

Glycosaminoglycans bind Aβ and can promote its aggregation[169]. The drug candidate tramiprosate (NC-531) is a glycosaminoglycan mimetic designed to interfere with the association between glycosaminoglycans and Aβ[170]. A phase II clinical trial of tramiprosate demonstrated a good safety profile, indicated a reduction of Aβ1–42 levels in CSF, and stabilized cognitive decline[171]. However, the North American phase III clinical trial did not provide a clear enough benefit in a large population. The clinical data gathered in connection with this trial are currently being subjected to secondary statistical analysis.

Targeting clearance of β-amyloid by immunization

An approach to prevent the formation of Aβ and to enhance its clearance from the brain, is immunization therapy. Both active immunization with fibrillar Aβ, and passive immunization with antibodies against Aβ, have been shown to reduce the Aβ load and plaque development in AD-related transgenic mice[172,173]. These results were the basis for initiating clinical trials with active immunization in AD patients, using synthetic Aβ42 (AN1792)[174]. Unfortunately, the trials had to be halted as a result of the development of aseptic vasocentric meningoencephalitis in a subset of patients[175]. However, *post mortem* examinations of immunized AD patients demonstrated clearance of Aβ plaques from the brain, although an increase in the number of NFTs was also evident[176,177]. Volumetric magnetic resonance imaging (MRI) of the patients revealed an increased loss in brain volume among the antibody responders[178]. Moreover, a decrease in CSF tau was found and the patients showed significant effects on some memory functions[179]. New active and passive vaccines have been developed, some of which are now undergoing clinical trials[180].

Anti-tau therapy

Reducing tau phosphorylation via inhibition of kinases is a major therapeutic strategy based on the presence of hyperphosphorylated tau in the AD brain. Inhibition of glucogen-synthase kinase 3β (GSK3β) and cyclin-dependent kinase 5 (CDK5) has emerged as regards interesting therapeutic targets[181]. However, this effort is complicated by the large number of tau phosphorylation sites, the unknown role of individual phosphorylation sites in disease etiology, and the ability of multiple kinases to phosphorylate individual sites[182].

Other therapies

Administration of nerve growth factor (NGF) has been proposed as a treatment strategy in AD, since there might be a lack of tropic support and NGF stimulates the growth of cholinergic neurons[183,184]. Studies with *ex vivo* NGF gene delivery into the forebrain of patients with mild AD have been carried out and the results suggested improvement in the rate of cognitive decline[185].

Dimebon, originally launched as an antihistamine, has been shown to block AChE and BuChE as well as the NMDA receptor[186].Clinical trials in AD patients

showed significant improvements in memory, global function, cognition, activities of daily living and behavior[187].

Epidemiological studies have shown that the prevalence of AD is decreased in subjects receiving long-term treatment with non-steroidal anti-inflammatory drugs (NSAIDs)[188], cholesterol-lowering drugs[189] and estrogen treatment[190].

The results of epidemiological studies suggest that keeping both mentally and physically active when young and middle-aged, decreases the risk of developing AD[191]. Life-style alterations that are thought to promote improved brain health include physical and mental stimulation, stress reduction and prevention of vascular insults.

ALZHEIMER'S DISEASE-RELATED MOUSE MODELS

Transgenic mice harboring mutations found in familial AD are now widely used in the investigation of critical AD-related mechanisms, inaccessible in humans, as well as the behavioral features associated with AD. These mouse models also play a major role in the evaluation of novel therapeutic approaches. Transgenic mice do not adequately reproduce all aspects of human AD, but several models that overproduce Aβ mimic many facets of the disease. Many transgenic mouse models develop brain Aβ deposition and plaques similar to those found in the human brain, in an age-dependent fashion. Depending on factors such as the type of mutant APP, the gene promoter, the copy number and integration site, as well as the genetic background of the mice, Aβ plaques develop between 2.5 and 15 months. They initiate as aggregates consisting mainly of Aβ1–42, develop a dense core of Aβ1–42 and then incorporate Aβ1–40, as well as many other non-Aβ components such as ubiquitin and α-synuclein[192]. The majority of AD-related transgenic mouse models also exhibit memory impairments in various cognitive tests[193].

In order to model NFTs, it has been necessary to develop transgenic mice that, in addition to mutated APP, express further gene alterations such as mutated human tau[43,44]. These multigenic mouse models develop NFTs similar to those seen in the human AD brain, and have aided investigation of the relationship between Aβ and tau[194]. The main characteristics and neuropathological features of a number of transgenic mouse models are shown in Table 1.

Although the existing mouse models provide valuable tools to investigate some of the neuropathological features characteristic of AD, there are differences in Aβ pa-

thology between human AD subjects and AD-related transgenic mice[195]. In addition, the inflammation seen in the AD brain is not adequately reproduced in transgenic mice, as there are differences between the species with respect to the nature and severity of the inflammation[196,197]. In AD patients, there is massive neuronal loss, formation of NFTs and significant reductions in (mainly) the cholinergic neurotransmitter system, while AD-related transgenic mouse models show little or no evidence of these neuropathological changes[198-200]. These differences are important to bear in mind when interpreting data obtained from these transgenic animal models.

Name	*Transgene*	*Aβ dep.*	*NFT*	*Neuron loss*	*Gliosis*	*Reference*
Tg2576	APP695 (K670N/M671L)	9 months	No (but AT8 IR)	No	Yes	[201]
hAChE-Tg	Overexpression of human AChE	No	No	Attenuated dendritic branching, reduced spine numbers	Not reported	[202]
hAChE-Tg //APPswe	Overexpression of human AChE + APP695 (K670N/M671L)	6 months	Not reported	Not reported	Yes	[118]
APP23	APP751 (K670N/M671L)	6 months	No (but AT8 IR)	Yes	Yes	[203]
APP/Ld	APP695 (V642I)	12-15 months	No (but AT8 IR)	No	Yes	[204]
TgCRND8	APP695 (K670N/K671L + V717F)	3 months	No	Not reported	Yes	[205]
PDAPP	APP695, 751 and 770 (V717F)	6-9 months	No (but AT8 IR)	No	Yes	[199]
PSAPP	APP695 (K670N/K671L) + PS1 (M146V)	3 months	Not reported	Minor	Yes	[198]
APP/PS1KI	APP751 (K670N/M671L, V717I) + PS1 (M233T, L235P)	2.5 months	No	Yes	Yes	[206]
TAPP	APP695 (K670N/M671L) + tau (P301L)	6 months	9-10 months	Yes	Yes	[44]
3xTg-AD	APP695 (K670N/M671L) + PS1 (M146V) + tau (P301L)	6 months	15 months	Not reported	Yes	[43]

Table 1. *Characteristics and neuropathological features of several transgenic mouse models*

AIMS OF THE THESIS

GENERAL AIMS

Accumulation of Aβ, loss of nerve cells and changes in neurotransmitter systems, in particular the cholinergic system, are consistent features of AD. Aβ is thought to play a critical role in the pathogenesis of AD and it has therefore become a target of interest as regards a therapeutic approach. To develop new effective drugs, an increased understanding of the complex mechanisms related to Aβ neuropathology is obligatory. By using different AD-related transgenic mouse models, the overall aim of the present work was to gain further insight into how different Aβ peptides influence the synapses, glial cells, and cholinergic and glutamatergic receptors, as well as to gain insight into how different drugs, acting via the cholinergic and glutamatergic neurotransmitter systems, affect these processes. A special focus has been on the interaction between Aβ processing and the α7 nAChRs since a functional association has been suggested.

SPECIFIC AIMS

- To investigate early effects of Aβ on synapses, α7 nAChRs and NMDA receptors, as well as to evaluate whether these effects are mediated via the ERK/MAPK signalling pathway in APPswe mice (Paper I)

- To study the effects of treatment with galantamine, nicotine and memantine on Aβ, α7 nAChRs, NMDA receptors, synapses and glial cells in APPswe mice (Paper II)

- To elucidate the effects of Aβ, under the constant influence of AChE, on α7 nAChRs, synapses and glial cells in hAChE-Tg//APPswe mice (Paper III)

- To analyze and compare the effects of both enantiomers of nicotine, D(+)- and L(-)-nicotine, on Aβ, α7 nAChRs, synapses and glial cells in hAChE-Tg//APPswe mice (Paper III)

- To study the effects of treatment with huprine X on Aβ, α7 nAChRs and synapses in the brains of 3xTg-AD mice and APPswe mice, as well as to compare the effects of huprine X and huperzine A on the above parameters in 3xTg-AD mice (Paper IV)

MATERIALS & METHODS

Transgenic mouse models (Papers I, II, III and IV)

The principles of Laboratory Animal Care (National Institutes of Health, publication 86-23, revised 1996) 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) (Dnr S82/01, S128/04, S81/01, S129/04, S43/07, S73/07). All mice that were born and bred in our own colony at the animal housing facilities at Karolinska Institutet, Karolinska University Hospital, Huddinge, were housed under the same conditions with an enriched environment, controlled temperature and humidity and a 12 h light/dark cycle. The mice had access to food and water *ad libitum.* Offspring were weaned at 21 days and housed alone or with 1–4 siblings of the same sex in standard laboratory Plexiglas cages (30 × 20 × 15 cm), with wood shavings provided as bedding material. The cages and bedding material were changed twice a week. All mice used in the studies were sacrificed by decapitation during the daytime.

APPswe mice (Papers I, II, III and IV)

Two female transgenic mice overexpressing APP695, containing a KM670/671NL mutation driven by a hamster prion protein gene promoter in a C57B6 × SJLF1 hybrid mouse, back-crossed to C57B6 mice[201], were kindly provided by Prof. Karen Hsiao-Ashe. C57B6 mice (Bomice & Mollegard Breeding Laboratories, Denmark) were used to breed a colony of experimental animals (Papers I, II and III).

C6/SJL F1 females (Jackson Laboratories, Bar Harbor, Maine, USA) and male APPswe mice (C6/SJL background) were used to breed a colony of APPswe mice (Paper IV). Non-transgenic littermates served as control animals.

hAChE-Tg//APPswe mice (Paper III)

Male APPswe transgenic mice and female FVB/N mice carrying human AChE cDNA under the control of 586 bp of authentic human AChE promoter[202] (hAChE-Tg mice: two females kindly provided by Prof. Hermona Soreq, used to breed our own colony of hAChE-Tg mice) were used to breed a colony of double transgenic hAChE-Tg//APPswe mice. Offspring of C57B6 mice crossed with FVB/N mice (Bomice & Mollegaard Breeding Laboratories, Ejby, Denmark) served as control animals.

3xTg-AD mice (Paper IV)

The 3xTg-AD mice were originally generated by co-microinjection of the human APP-swe and tauP301L genes, both under transcriptional control of a modified Thy1.2 promoter, into single-cell homozygous mutant PS1M146V knock-in mouse embryos[43]. Homozygous 3xTg-AD mice, used in this study, were bred at the Medical Psychology Unit, Universitat Autònoma de Barcelona, Spain. 129/Sv × C57BL/6 mice served as control animals.

Drug treatment (Papers II, III and IV)

Memantine, galantamine and nicotine (Paper II)

Ten-month-old APPswe mice and non-transgenic littermates were randomized to treatment (s.c.) with memantine hydrochloride (10 mg/kg), L(-)-nicotine (dose gradually increased from 0.25 mg/kg (free base) on day 1 to 0.30 mg/kg on day 2 and finally to 0.45 mg/kg on days 3–10) or galantamine hydrobromide (2 mg/kg) at 8.00–9.00 and at 16.00–17.00 for 10 consecutive days. The drugs, dissolved in sterile 0.9% NaCl, were freshly prepared for each injection session. Corresponding age-matched control groups of APPswe and non-transgenic control mice received injections (s.c.) of sterile 0.9% NaCl. The mice were weighed at day 0 and day 10. The mice were sacrificed by decapitation 1 h after drug withdrawal.

The two enantiomers of nicotine (Paper III)

Fourteen-month-old hAChE-Tg//APPswe mice and non-transgenic control mice were randomized to treatment (s.c.) with L(-)-nicotine or D(+)-nicotine at 8.00–9.00 and at 16.00-17.00 for 10 consecutive days. The doses of D(+)- and L(-)-nicotine were gradually increased from 0.20 mg/kg (free base) on day 1, 0.30 mg/kg on day 2 and 0.45 mg/kg on days 3–10. The drugs, dissolved in sterile 0.9% NaCl, were freshly prepared for each injection session. Corresponding age-matched control groups of hAChE-Tg//APPswe mice and non-transgenic control mice received injections (s.c.) of sterile 0.9% NaCl. The mice were weighed at day 0 and day 10. The mice were sacrificed by decapitation 18 h after the last drug injection.

Huprine X and huperzine A (Paper IV)

Six- to seven-month-old 3xTg-AD mice were randomized to treatment (i.p.) with huprine X (0.12 μmol/kg) or huperzine A (0.8 μmol/kg) once daily for 21 consecutive days. The drugs, dissolved in sterile 0.9% NaCl, were freshly prepared for each injec-

tion session. Corresponding age-matched non-transgenic control mice (129/Sv × C57BL/6) received injections (i.p.) of sterile 0.9% NaCl.

Six- to seven-month-old APPswe mice and non-transgenic littermates were randomized to treatment (i.p.) with huprine X (0.12 μmol/kg) or sterile 0.9% NaCl, once daily for 21 consecutive days.

All mice were weighed at day 0 and day 21. The mice were sacrificed by decapitation 24 h after the last drug injection.

Receptor autoradiography

[^{3}H]MK-801 autoradiography (Papers I and II)

Frozen brain sections (10 μm) were thawed at room temperature for 30 minutes, followed by 15 minutes of pre-incubation with binding buffer (50 mM Tris-acetate buffer, pH 7.4) at room temperature. The sections were then incubated with the same buffer, containing 10 nM [^{3}H]MK-801 with 100 mM glutamic acid and 100 mM glycine, at room temperature for 120 minutes. Non-specific binding was determined in the presence of 10 μM unlabelled MK-801. The incubation was terminated with two washing steps, 10 minutes each, with binding buffer at room temperature, followed by a rinse in distilled water at 4ºC. The sections were dried at room temperature overnight and were then placed together with [^{3}H]microstandards on ^{3}H-Hyperfilm for 8 weeks.

[^{125}I]α-bungarotoxin autoradiography (Papers I, II, III and IV)

Frozen brain sections (10 μm) were thawed at room temperature for 30 minutes, followed by 30 minutes of pre-incubation with binding buffer (50 mM Tris-HCl, 1 mg BSA/ml, pH 7.4) at room temperature. The sections were then incubated in the same buffer containing 2 nM (Paper I), 1.79 nM (Paper II), 1.55 nM (characterization: Paper III), 1.94 nM (treatment: Paper III), or 1.92 nM (Paper IV) [^{125}I]α-bungarotoxin. Non-specific binding was determined in the presence of 1 μM unlabelled α-bungarotoxin. The incubations were terminated by four washes, 5 minutes each, with 50 mM Tris-HCl buffer (pH 7.4) at 4ºC, followed by a rinse in distilled water at 4ºC. The sections were dried at room temperature and then placed together with [^{125}I]microstandards (Papers I, II and III) or standards of different concentrations of [^{125}I]α-bungarotoxin (Paper IV) on [^{3}H]-Hyperfilm (Papers I and II), Hyperfilm MP (Paper III) or Kodak Biomax MR film (Paper IV). The sections were incubated for 8–29 days.

Post-processing and image analysis

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

The autoradiograms were either analyzed with a video camera (CCD-72: Dage-MTI, Michigan City, IN, USA) coupled to a Macintosh computer with a video card and public domain NIH Image software (written by Wayne Rasband at the US National Institutes of Health) (Papers I and II), or first scanned by using a high-resolution scanner (Canon Cano-Scan 5200F) and then analyzed by using with Image J (version 1.37v, Paper III) or ImageQuant (version 7.0, Paper IV).

Optical density values were converted into fmol/mg tissue based on the standard curve derived from the [^{3}H]microstandards, the [^{125}I]microstandards or the standards composed of different concentrations of [^{125}I]α-bungarotoxin. The regions analyzed were the cortex (frontal cortex, retrosplenial granular cortex and motor cortex), hippocampus (regions CA1, CA2 and CA3), thalamus and caudate nucleus. Specific binding was calculated by subtracting non-specific binding from total binding.

Western blot

Tissue extraction

Homogenates of brain cortex tissue were prepared in ice-cold 20 mM Tris-HCl (pH 8.5), containing Roche Diagnostics protease inhibitor cocktail (for ERK and phospho-ERK 1 mM EG/EDTA, 1 mM $Na_4P_2O_7$, 1 mM Na_3VO_4, 0.1 mM PMSF, 0.1 mM PNPP-PP2b and 1 μM Microcystin-LR was added), followed by centrifugation at 60,000 × *g* for 20 minutes. The obtained supernatant represented the cytosolic fraction. The remaining pellets were re-suspended in ice-cold homogenate buffer with the addition of Triton X-100 (2%). The suspension was mixed for 2 h at 4 ºC followed by centrifugation at 100,000 × *g* for 1 h. The obtained supernatant represented the membranous fraction.

A DC protein assay kit (BioRad) was used to measure the protein content in the different fractions.

Levels of APP (Papers I and II)

For investigation of APP, cytosolic fractions of cortical homogenates (total soluble APP or human soluble APPα levels) were loaded onto 10% gradient minigels, and membranous fractions of cortical homogenates (total membrane-bound APP) were

loaded onto 4–20% gradient minigels which were run at 100 mV constant voltage at room temperature for 60–90 minutes. The protein bands were blotted onto a PVDF membrane (RPN 303 F, Amersham Life Science, Uppsala, Sweden) at 4ºC for 90 minutes. Non-specific binding was blocked overnight at 4ºC. The membranes were then incubated for 1 h with 22C11 (total sAPP, 1:1000, total membrane-bound APP, 1:500: Chemicon International), 6E10 (human sAPPα, 1:1000: Chemicon International) at room temperature. Each membrane was then incubated for 1 h with secondary antibody (donkey anti-mouse, 1:1000: Santa Cruz) at room temperature.

Levels of synaptophysin (Papers I, II, III and IV)

Membranous fractions were used to measure levels of the synaptic marker synaptophysin (Papers I–IV) by running 4–20% gradient minigels (BioRad Ready Gel) at 100 mV constant voltage for 60–70 minutes at room temperature. The protein bands were blotted onto a PVDF membrane at 4ºC for 90 minutes. Non-specific binding was blocked in blocking buffer for 1 h at room temperature or overnight at 4 ºC. The blots were then incubated for 45–60 minutes with an antibody against synaptophysin (1:2000: DakoCytomation, Denmark) at room temperature, followed by incubation for 1 h with secondary antibody (goat anti-rabbit, 1:1000: Santa Cruz: Papers I–III and donkey anti-rabbit: 1:2000: Vector Laboratories Inc., Burlingame, CA, USA: Paper IV).

Levels of ERK and phospho-ERK (Paper I)

For investigation of ERK and phospho-ERK, cytosolic fractions of cortical homogenates were loaded onto 10% gradient minigels which were run at 100 mV constant voltage at room temperature for 90 minutes. The protein bands were blotted onto a PVDF membrane at 4 ºC for 75 minutes. Non-specific binding was blocked for 1 h at room temperature. The blots were incubated for 1 h with phospho-ERK antibody (anti-phospho-Thr202/Tyr204, 1:3000: Cell Signaling Technology) followed by incubation for 1 h with secondary antibody (donkey anti-rabbit, 1:3000: Santa Cruz). The membranes were then stripped according to the manufacturer's instructions, blocked overnight at 4ºC followed by 1 h incubation with total ERK antibody (1:1000: BioSource) and 1 h incubation with the same secondary antibody as indicated for phospho-ERK (1:3000).

All the blots were incubated with ECL Plus reagents, exposed to film and developed according to standard procedures.

Image analysis

The Western blot films were scanned using a Sharp JX-325 scanner (Papers I and II) or by using a high-resolution scanner (Canon CanoScan 5200F, Papers III and IV). 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: Papers I and II) or by using Image J (version 1.37v (Paper III), version 1.40g (Paper IV)).

Cortical homogenate fractions 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. As regards phospho-ERK1/2, band intensity was first normalized to the band intensity detected with total ERK, and then all samples were standardized to the pooled sample, allowing comparison between groups.

ELISAs

Levels of Aβ1–40 and Aβ1–42 (Papers I, II, III and IV)

Brain cortex (Papers I–IV) and hippocampus (Paper IV) were homogenized in 7 volumes of 20 mM Tris-HCl buffer (pH 8.5) containing Roche Diagnostics protease inhibitor cocktail. The homogenate solution was then centrifuged at 100,000 × *g* for 1 h at 4ºC. The supernatant was diluted with phosphate-buffered saline (PBS) including bovine serum albumin (0.5 %), Tween 20 (0.05%) and protease inhibitors (standard buffer) to appropriate concentrations and this represented the Tris-extracted (soluble) Aβ fraction. The remaining pellet was extracted in 10 volumes of 5 M guanidinium-HCl in 20 mM Tris-HCl (pH 8.0) for 1.5–2 h at room temperature, diluted 1:5 with standard buffer and centrifuged at 13,100 × *g* for 20 minutes at 4ºC. The obtained supernatant represented the guanidinium-extracted (insoluble) Aβ fraction. The levels of Aβ1–40 and Aβ1–42 peptides were analyzed by using Signal Select™ Human β-Amyloid 1–40 and 1–42 colorimetric sandwich ELISA kits (BioSource International Inc., Camarillo, CA, USA) according to the manufacturer's protocols. Concentrations of Aβ1–40 and Aβ1–42 were calculated by comparison with standard curves of synthetic human Aβ1–40 and Aβ1–42, 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 Aβ1–40 and Aβ1–42. These antibodies were specific for human Aβ1–40 and Aβ1–42 and did not recognize those in the mouse. The manufacturer has characterized the specificities and sensitivities of the ELISAs. All samples were analyzed in the linear range of the ELISA.

GFAP immunoreactivity (Paper III)

For measurement of GFAP immunoreactivity, hippocampal mouse brain tissue was homogenized in 1% sodium dodecyl sulfate (SDS). A DC protein assay kit (BioRad, Stockholm, Sweden), using bovine serum albumin as a standard, was used to measure the protein content in the fractions.

A polyclonal GFAP antibody (1:400: Z0334, DakoCytomation, Glostrup, Denmark) was used to coat the wells of microtiter plates. After blocking non-specific binding with non-fat dried milk, aliquots of the SDS homogenates were diluted and added to the wells. Following appropriate blocking and washing steps, a monoclonal GFAP antibody (1:500: IF03L, Calbiochem, USA) was added to the "sandwich". An alkaline phosphatase-linked IgG antibody (1:3000) was then added and a colored reaction product was obtained by subsequent addition of enzyme substrate. Spectrophotometric absorption at 405 nm was measured and expressed as GFAP immunoreactivity/mg total protein.

Immunohistochemical staining (Papers II, III and IV)

Immunofluorescence labelling of Aβ and GFAP (Papers II and III)

Immunofluorescence labelling of Aβ and GFAP was performed by pre-treating frozen brain sections with concentrated formic acid (for Aβ) during 5 minutes, followed by 1 h incubation with 5% normal goat serum in Tris-buffered saline (TBS). Brain sections were then incubated overnight at 4 ºC with the primary antibody (1:100: 6E10, MAB 1560, Chemicon, Temecula, CA, USA) for Aβ deposits, or polyclonal rabbit anti-GFAP (1:300: Z0334, DakoCytomation, Glostrup, Denmark) to label astrocytes. The sections were then incubated with Cy2-conjugated goat anti-mouse or Cy3-conjugated goat anti-rabbit secondary antibodies (1:200 each: Chemicon, Temecula, CA, USA). Double immunofluorescence labelling of Aβ and GFAP was performed by incubating the sections with a cocktail of the primary antibodies (6E10 (1:100) and polyclonal rabbit anti-GFAP (1:300)) followed by a cocktail of the secondary antibodies as indicated for the single fluorescence staining. For control staining, the primary serum was omitted and this resulted in no detectable labelling.

Immunohistochemical staining of APP/Aβ (Paper IV)

For immunohistochemistry of Aβ, frozen serial brain sections were post-fixed in 4% formaldehyde for 10 minutes, allowed to dry at room temperature and then processed by using the immune-peroxidase method. Antigen retrieval was performed using con-

centrated formic acid for 10 minutes, the sections were washed in 0.1 M PBS and then they were treated with 0.3% H_2O_2/methanol to eliminate endogenous peroxidase activity. After washing, the slides were blocked with 1% horse serum buffer for 20 minutes at room temperature to prevent non-specific protein binding. The slides were then incubated with primary antibody 6E10 (Covance Research Products, Denver, PA, USA: 1:1000) in 3% BSA, 1% goat serum, overnight at 4 °C. Then the sections were rinsed in 1x PBS and incubated with biotinylated secondary antibody (anti-mouse IgG (1:200) in 1% horse serum) for 60 min at room temperature. Bound antibodies were detected using a standard avidin-biotin peroxidase complex method (Vectastain Elite ABC kit; Vector Laboratories, Burlingame, USA) according to the manufacturer's protocol. For negative controls, slides were processed without primary antibody.

Image analysis

Images were generated by using an Anxiophot microscope (Carl Zeiss AG, Göttingen, Germany) equipped with a digital camera (Hamamatsu Photonics, Hamamatsu City, Japan) and a computerized imaging system (Openlab, version 2.1 for Mac OS, Improbvision, Conventry, England: Papers I–III) or by using a Leica microscope (Leica Mikroskopie & Systeme GmbH, Wetzlar, Germany) equipped with a digital camera (Leica DC 480, Microsystems AG, Heerbrugg, Switzerland) and a Leica computerized imaging system (Leica IM50, version 1.20: Paper IV).

Statistical analysis

Statistical analyses in all cases involved the non-parametric Kruskal–Wallis test followed by the Mann–Whitney post hoc test to assess the significance of differences between groups. In Paper III, one-way ANOVA followed by the Bonferroni/Dunn post hoc test was performed to calculate the significance of differences between hAChE-Tg//APPswe mice and FVB/N//C57B6 controls, APPswe mice and non-transgenic controls, as well as the significance of differences between hAChE-Tg//APPswe mice and APPswe mice in the GFAP ELISA experiment. The same tests were also used to calculate the significance of differences between saline treatment and L(-)- or D(+)-nicotine treatment as regards autoradiographic and Aβ data. In addition, simple regression analysis was used to detect relationships between different parameters (Paper I).

RESULTS & DISCUSSION

BRAIN β-AMYLOID IN DIFFERENT TRANSGENIC MOUSE MODELS

Transgenic mice harboring mutations found in FAD have become widely used in *in vivo* approaches to elucidate critical disease-related mechanisms, inaccessible in humans. Numerous mouse models express mutations in human APP, thus exhibiting brain Aβ production. Besides mutations in APP, some models also express mutations in other AD-related proteins such as PS and tau, or they overexpress other proteins such as AChE, leading to genetic differences that might influence Aβ pathology.

High β-amyloid levels at a young age in APPswe mice

We have found that APPswe mice exhibit detectable Aβ peptides in their brain cortices as early as 7 days of age (Paper I), suggesting that these mice produce Aβ from birth. Detectable guanidinium-extracted Aβ (which represents aggregated Aβ) at the same age indicates that Aβ starts to aggregate from the time of birth (Fig. 1).

Levels of soluble Aβ1–40 were markedly higher at 7 days of age compared with other ages (Fig. 1). Therefore, it appears as if these very young mice are more susceptible to the overexpression of APP and hence more Aβ accumulates in the brain. The decreased levels of tris-extracted Aβ1–40 (which represents soluble Aβ) at 21 days of age might be ascribed to protective and compensatory mechanisms aimed at preventing the effect of APP overexpression.

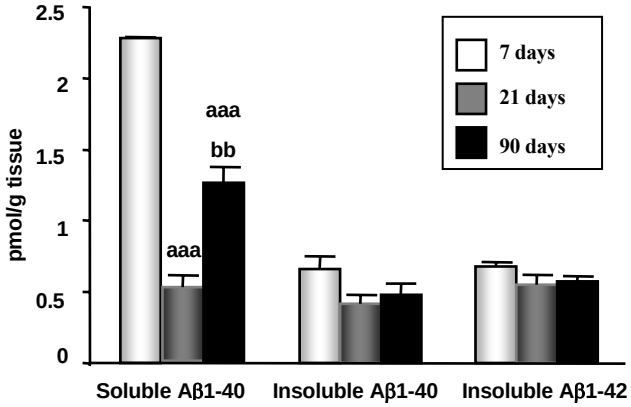

Figure 1. Levels of Aβ in the cortices of APPswe mice. *aaa $P < 0.001$ compared with APPswe mice at 7 days of age. bb $P < 0.01$ compared with APPswe mice at 21 days of age. Values are expressed as mean ± SEM.*

Accelerated β-amyloid plaque formation in hAChE-Tg//APPswe mice

In the AD brain, AChE-S has been shown to co-localize with Aβ and its activity is increased in the microenvironment surrounding the Aβ plaques, although the overall activity is reduced[207,208]. *In vitro* studies have shown that AChE-S facilitates fibril formation thereby accelerating Aβ deposition[209,210].

When we crossed APPswe mice with mice overexpressing human AChE-S (hAChE-Tg mice, Paper III) we found that the double transgenic hAChE-Tg//APPswe mice exhibited Aβ deposition in the hippocampus and cortex as early as at 7 months of age (Fig. 2), which is well before the onset of plaque formation in APPswe mice, which starts at 9 months of age[211]. This is in agreement with earlier studies showing that AChE-S may have a direct effect on plaque formation[117-119]. The Aβ plaques also appeared larger in 10-month-old hAChE-Tg//APPswe mice compared with the plaques in age-matched APPswe mice (Fig. 2), which also lends support to this interpretation.

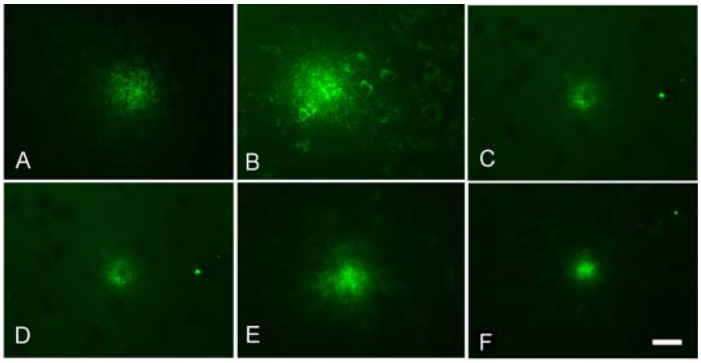

Figure 2. Aβ plaques in the brains of hAChE-Tg//APPswe and APPswe mice. *Plaques in the cortex (A) and hippocampus (D) of a 7-month-old hAChE-Tg//APPswe mouse. Plaques in the cortex (B) and hippocampus (E) of a 10-month-old hAChE-Tg//APPswe mouse. Plaques in the cortex (C) and hippocampus (F) of a 10-month-old APPswe mouse. Scale bar 30μm.*

Different β-amyloid patterns in hAChE-Tg//APPswe and APPswe mice

When comparing the hybrid hAChE-Tg//APPswe mice (Paper III) with single transgenic APPswe mice as regards Aβ peptides, the results showed different patterns in the cortices of these two mouse models. Whereas hAChE-Tg//APPswe mice exhibited increased levels of insoluble Aβ1–40 and Aβ1–42 peptides at 1 and 3 months of age, the levels of soluble Aβ1–40 were decreased at 1, 3 and 10 months of age, when compared with age-matched APPswe mice (Fig. 3). The presence of elevated levels of insoluble Aβ peptides, but decreased levels of soluble Aβ1–40, and lack of detectable soluble Aβ1–42 supports the assumption that AChE-S promotes the formation of Aβ

plaques[209,210], and also that Aβ1–42 peptides are more prone to aggregation compared with Aβ1–40[212].

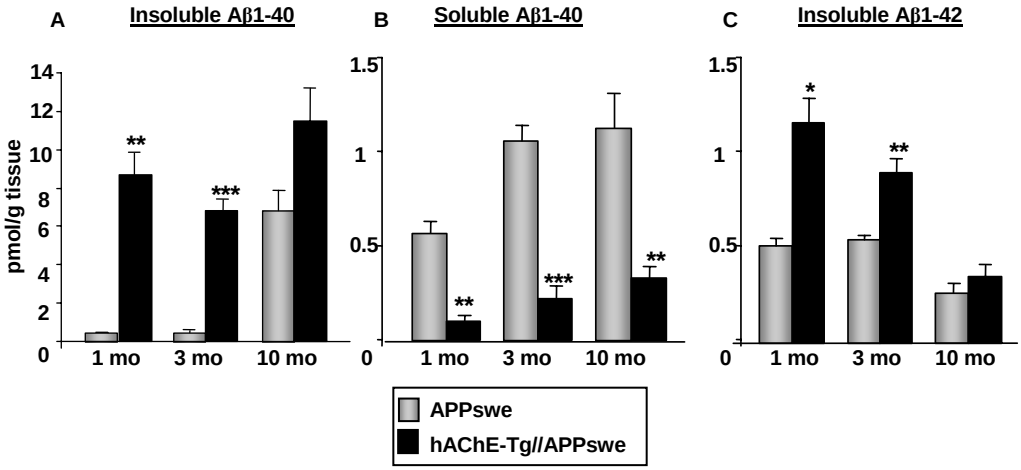

Figure 3. Levels of Aβ in the cortices of APPswe and hAChE-Tg//APPswe mice. ** P < 0.05, ** P < 0.01 and *** P < 0.001 compared with age-matched APPswe mice in the same Aβ group. mo = months. Values are expressed as mean ± SEM.*

Studies of *post mortem* AD brain tissue indicate that astrocytes may play an important role in AD pathogenesis. Astrocytes positive for GFAP are located in the vicinity of the Aβ plaques[92] and have been suggested to promote the formation and maturation of Aβ plaques[213]. When we compared hAChE-Tg//APPswe mice and APPswe mice in regard to GFAP immunoreactivity, we did not observe any significant differences at either 1 or 10 months of age (Fig. 4, Paper III). This might indicate that AChE-S, and not the astrocytes, is responsible for the increased levels of aggregated Aβ in the hAChE-Tg//APPswe mice, although the involvement of astrocytes in Aβ plaque formation cannot be excluded.

β-amyloid in 3xTg-AD mice

Transgenic 3xTg-AD mice express a progressive and age-related development of Aβ plaques, as well as NFTs, predominantly restricted to the hippocampus, amygdala and the cerebral cortex, closely mimicking disease progression in humans[214]. In these mice, Aβ pathology has been reported to start in the brain cortex, with Aβ plaques appearing at 6 months of age[43,214].

In our study (Paper IV), we detected intraneuronal APP/Aβ staining both in the cortex and the hippocampus of 6- to 7-month-old 3xTg-AD mice (Fig. 3, Paper IV), in agreement with other studies[215], while no extracellular Aβ plaques were evident. In these mice, the levels of cortical Aβ peptides were under the detection limit of the

ELISA, by which we successfully detected high cortical Aβ levels as early as 7 days of age in the APPswe mice (Paper I). Nevertheless, soluble and insoluble Aβ1–40, as well as insoluble Aβ1–42 peptides, were detectable in the hippocampus of 3xTg-AD mice (Fig. 4).

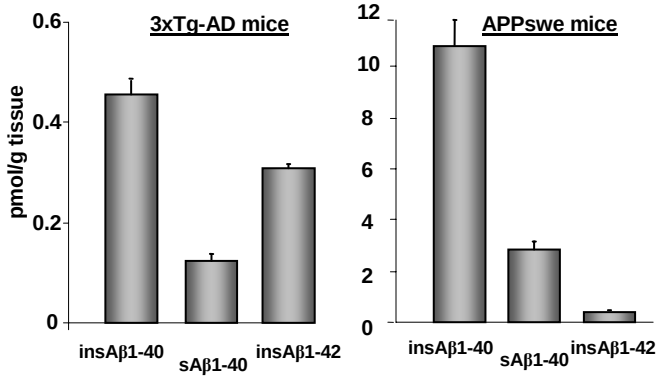

Figure 4. Levels of Aβ in the hippocampi of 3xTg-AD and APPswe mice.
ins = insoluble and s = soluble. Values are expressed as mean ± SEM.

When comparing 6- to 7-month-old 3xTg-AD mice with age-matched APPswe mice, it was evident that the levels of insoluble and soluble Aβ1–40 were ~20-fold higher in the APPswe mice (Fig. 4). Taken together, these results possibly reflect genetic differences and thus diverse neuropathology in the two mouse models. However, the strain background, choice of promoters and level of APP overexpression, among other possible factors, might also play an important role as regards differences in pathology.

EFFECTS OF β-AMYLOID ON SYNAPTOPHYSIN

Reduced synaptophysin levels, as a sign of synaptic loss, are consistently observed in AD patients, with the synapse loss better correlated to cognitive impairment than either Aβ plaques or NFTs[216-218]. The role of Aβ in neuronal degeneration is a matter of controversial debate, but it is hypothesized that Aβ deposits might disrupt neuronal and synaptic function, leading to neuronal degeneration[219].

In our studies, we detected significant reductions in cortical synaptophysin levels in APPswe mice at 21 and 90 days (Paper I), as well as at 10 months of age (Paper II) compared with age-matched non-transgenic controls (Fig. 5).

The 3xTg-AD mice also exhibited a marked reduction (Paper IV) in cortical synaptophysin levels compared with age-matched non-transgenic controls (Fig. 5). Accumulating evidence implicates intraneuronal Aβ in AD neurodegeneration[220]. A recent study showed that transient intraneuronal Aβ, rather than extracellular plaque pathology, cor-

related with neuronal loss in APP/PS1KI mice[221]. As we did not detect any extracellular Aβ plaques, but intraneuronal APP/Aβ in the cortices of these mice (Fig. 3, Paper IV), intraneuronal Aβ might account for the decreased synaptophysin levels. However, this needs to be further evaluated by using an antibody not cross-reactive to APP. Dysfunction in synaptic plasticity, due to early intraneuronal Aβ accumulation, has earlier been reported in 3xTg-AD mice[43].

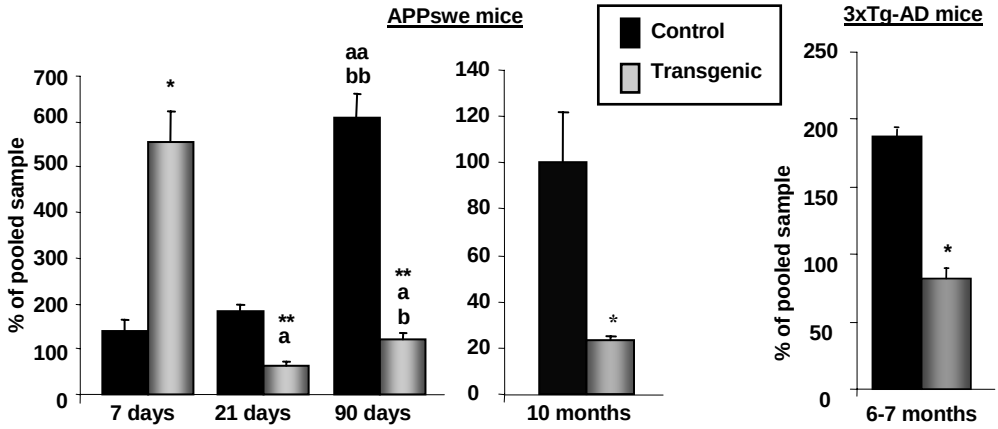

Figure 5. Levels of synaptophysin in the cortices of APPswe mice, 3xTg-AD mice and non-transgenic control mice. ** P < 0.05 and ** P < 0.01 compared with age-matched non-transgenic mice. a P < 0.05 and aa P < 0.01 compared with APPswe mice or non-transgenic mice at 7 days of age. b P < 0.05 and bb P < 0.01 compared with APPswe mice or non-transgenic mice at 21 days of age. Values are expressed as mean ± SEM.*

On the other hand, studies have also shown increased synaptophysin levels in response to increased levels of APP or Aβ[222-224]. We found that 7-day-old APPswe mice exhibited markedly increased levels of synaptophysin compared with non-transgenic controls (Fig. 5). The levels of synaptophysin correlated positively with both soluble (tris-extracted) Aβ1–40 (r^2 = 0.649) and insoluble (guanidinium-extracted) Aβ1–40 (r^2 = 0.371) (Paper I, Fig. 7), suggesting that high Aβ levels might promote increased synaptic density. The elevated synaptophysin levels could either be a compensatory response to early high Aβ levels, or be a result of neurotropic effects of APP or Aβ. Several studies have suggested that sAPPα plays an important role in neurotropic/neuroprotective functions and synaptogenesis[60-62,225]. Opposite to what we expected, the highest levels of total sAPP and human sAPPα were found at 90 days, and not at 7 days of age, in the APPswe mice (Paper I, Figs. 1 and 2, respectively), thereby supporting the assumption that soluble Aβ1–40 contributed to the increased synaptophysin levels at 7 days of age.

INVOLVEMENT OF THE ERK/MAPK SIGNALLING CASCADE IN β-AMYLOID PATHOLOGY

The ERK/MAPK signalling cascade is activated in response to various growth and differentiation factors, and plays a significant role in differentiation and early embryonic development[226,227]. This signalling pathway is also involved in neuroprotective mechanisms[228], which is why we investigated it at early ages in APPswe mice (Paper I), with the rationale that increased activity of ERK/MAPK signalling would be a marker of tropic receptor activation, consistent with our tropic hypothesis. However, there was a significant decrease in ERK/MAPK activity at 7 days of age, which may indicate that the effects of Aβ in the brain are not mediated through ERK/MAPK activation (Paper I, Fig. 4). At 21 and 90 days of age, we instead found a significant increase in ERK/MAPK activity in the APPswe mice, suggesting chronic activation of this signal transduction cascade as a consequence of the increased Aβ burden (Paper I, Fig. 4), in agreement with earlier studies[107].

EFFECTS OF β-AMYLOID ON α7 NEURONAL NICOTINIC ACETYLCHOLINE RECEPTORS

In the AD brain, a consistent loss of multiple nAChR subtypes, mainly the α4 but also α3 and α7 subtypes, is well documented[90,94], whereas α7 nAChRs instead are up-regulated in astrocytes[92]. Cholinergic dysfunction and Aβ accumulation are central components in the pathogenesis of AD, although the mechanism linking these two remains to be established. However, in the human brain, regions that are more susceptible to AD neuropathology, such as the hippocampus and cortex, have been shown to express the highest levels of α7 nAChRs[229]. A functional association between α7 nAChRs and Aβ has been suggested[96,97,107].

In young APPswe mice (Paper I), we found significant negative correlations in the cortices of the mice mice between [^{125}I]α-bungarotoxin binding sites and soluble (tris-extracted) Aβ1–40 (r^2 = 0.325) and insoluble (guanidinium-extracted) Aβ1–40 (r^2 = 0.321), suggesting that high levels of Aβ decrease the number of α7 nAChR receptor binding sites (Fig. 6).

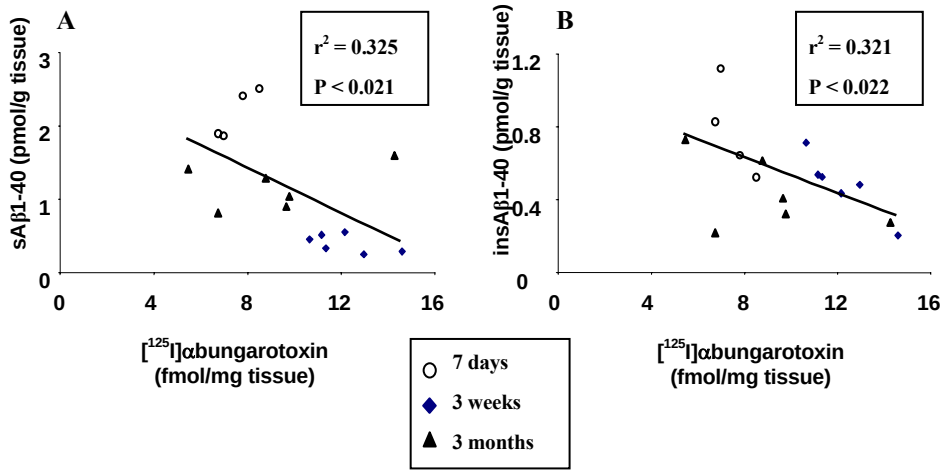

Figure 6. Correlation of Aβ1–40 versus [^{125}I]α-bungarotoxin binding sites in the cortices of APPswe mice. *Correlations with soluble Aβ1–40 are shown in A and correlations with insoluble Aβ1–40 are shown in B. Each point represents data from one mouse. s = soluble and ins = insoluble.*

Interestingly, in a study involving the use of hippocampal cultures, exposure to nanomolar concentrations of Aβ1–42 led to up-regulation of α7 nAChRs, comparable to the effects of chronic nicotine exposure[107]. Likewise, we found an increase in α7 nAChRs in the cortices of APPswe mice at 10 months of age (Paper II, Table 2), probably due to prolonged exposure and increased production of Aβ in the brain. This finding is also consistent with earlier findings in APPswe mice, suggesting an interaction between Aβ and α7 nAChRs[97,107,211].

Even though hAChE-Tg//APPswe mice express high Aβ levels with a forced Aβ pathology in their brains, we detected no differences in α7 nAChRs between transgenic mice and non-transgenic controls (Paper III). However, previous studies have shown that these mice exhibit a significant increase in mRNA levels coding for cortical α7 nAChRs at 3 and 7 months of age, when compared with non-transgenic controls[230], suggesting that changes indeed are ongoing, but it remains to be studied whether the modulation is located in neurons or in astrocytes.

In the 3xTg-AD mice (Paper IV), we observed no significant differences in α7 nAChRs in comparison with non-transgenic mice in any brain region studied (Paper IV, Fig. 5), despite the presence of both guanidinium-extracted (aggregated) Aβ, as well as intraneuronal APP/Aβ at least in the hippocampus. Decreased levels of α7 nAChRs have earlier been reported in brain regions displaying intraneuronal Aβ1–42 accumulation in 6-month-old hemizygous 3xTg-AD mice[231]. It might be possible that the outcome would have been different with an increased number of animals.

EFFECTS OF β-AMYLOID ON *N*-METHYL-D-ASPARTATE RECEPTORS

Several findings support the view that NMDA receptors play a central role in Aβ induced neurotoxicity[130]. Aβ has been shown to alter glutamate signalling via alterations of LTP[131,232,233] and the cellular damage of AD brains has been found to be especially prominent in areas that display glutamatergic synaptic plasticity[234].

In the AD brain, reductions in NMDA receptors have been shown in the hippocampus and neocortex[235,236], while these receptors have been reported to be preserved in the brains of APPswe mice (4 and 15 months of age)[237]. However, in our studies we found significant up-regulation of this receptor in several brain regions at 21 days of age (Paper I, Fig. 5), as well as at 10 months of age (Paper II, Table 1) in APPswe mice compared with non-transgenic controls. Intermediates of Aβ, formed during Aβ fibrillogenesis, have been shown to alter glutamate neurotransmission. In addition, assemblies of distinct sizes (protofibrils versus fibrils), may vary in their disruption of biological processes[238], suggesting that the up-regulated NMDA receptors at 21 days of age in APPswe mice might reflect changes in response to the early, high levels of soluble Aβ1–40, whereas the up-regulation at an older age might be due to a more chronic Aβ exposure. Additionaly, we also found a significant negative correlation between both soluble Aβ1–40 (r^2 = 0.492) and insoluble Aβ1–40 (r^2 = 0.379) versus [^{3}H]MK-801 binding sites in the cortices of APPswe mice (Paper I, Fig. 8), which might reflect the fact that high Aβ levels decrease NMDA receptor binding sites.

EFFECTS OF CHOLINERGIC AND GLUTAMATERGIC DRUG TREATMENT IN DIFFERENT TRANSGENIC MOUSE MODELS

While current treatments are focused on targeting symptoms with synaptic agents, the ultimate goal of research into AD therapies is disease-modifying treatment. Affecting the production, aggregation or clearance of Aβ may well have a modifying effect on disease progression.

Effects of nicotine in APPswe and hAChE-Tg//APPswe mice

Despite being somewhat controversial, the results of epidemiological studies have indicated that smoking is associated with a decreased risk of AD[239]. *Post mortem* studies in former smokers with AD have shown diminutions of brain Aβ plaques[240].

Different effects on β-amyloid levels

Treatment of 10-month-old APPswe mice with L(-)-nicotine for 10 days (Paper II), markedly decreased the levels of cortical insoluble Aβ1–40 and Aβ1–42 (Fig. 7), confirming the results of earlier studies by our group[241,242].

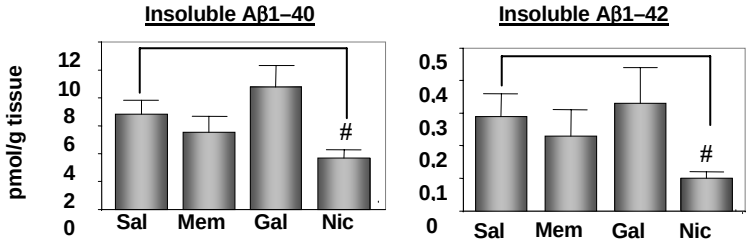

Figure 7. Levels of Aβ in the cortices of APPswe mice treated with saline, memantine, galantamine or nicotine. *# P < 0.05 compared with saline-treated APPswe mice in the same Aβ group. sal = saline, mem = memantine, gal = galantamine and nic = nicotine. Values are expressed as mean ± SEM.*

However, the mechanism of Aβ clearance by nicotine remains to be studied. An earlier study has shown that nicotine does not change the activities of cortical α-, β-, or γ-secretase in APPswe mice or non-transgenic controls[241], suggesting that nicotine (or one of its metabolites) may act by degrading insoluble Aβ deposits, rather than affecting accumulation of the peptide. This interpretation is also supported by the observation that the levels of intracellular Aβ seemed to be unaffected by nicotine treatment (Fig. 8). In addition, both enantiomers of nicotine (D(+)- and L(-)-nicotine) have been shown to affect early stages of Aβ aggregation, delaying oligomerization and fibril formation and thus maintaining less toxic Aβ species[243].

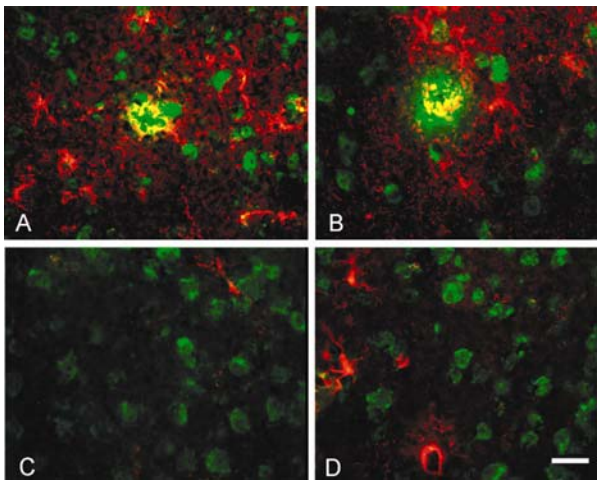

Figure 8. Aβ and GFAP in the cortices of APPswe mice. *Aβ and GFAP in the cortex of a saline-treated APPswe mouse (A, B) and a nicotine-treated APPswe mouse (C, D). Scale bar 30 μm.*

Our current knowledge on the degradation of Aβ is relatively limited compared with the information available concerning production of the peptides. To date, a number of potential Aβ-degrading enzymes have been identified, including neprilysin and insulin-degrading enzyme[244]. It remains to be elucidated which one of these enzymes

plays the physiologically most important role in mediating Aβ clearance, as well as whether they might be involved in the Aβ-reducing effect of nicotine.

Surprisingly, when 14-month-old hAChE-Tg//APPswe mice were treated for 10 days with L(-)-nicotine or D(+)-nicotine (Paper III), the levels of cortical insoluble Aβ1–40 and soluble Aβ1–42 were increased (Fig. 9).

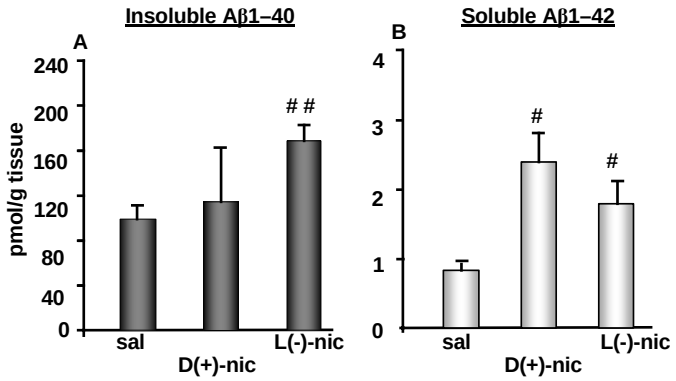

Figure 9. Levels of Aβ in the cortices of hAChE-Tg//APPswe mice following treatment with saline, D(+)-nicotine, or L(-)-nicotine. *# P < 0.05 and ## P < 0.01 compared with saline-treated hAChE-Tg//APPswe mice in the same Aβ group. sal = saline and nic = nicotine. Values are expressed as mean ± SEM.*

Studies have demonstrated that Aβ pathology is age-dependent, and in the treatment studies the hAChE-Tg//APPswe and APPswe mice were exposed to nicotine at different ages. The APPswe mice were treated at 10 months of age, when Aβ deposits begin to appear[201,211]. During early stages of deposition, Aβ may be comparatively unmodified and more amenable to disruption. The hAChE-Tg//APPswe mice received nicotine 8 months after Aβ deposits are first observed in these mice[118] and at this age, the nature of the Aβ deposit might result in difficulties with successfull Aβ clearance. This is supported by the results of immunization studies in APP transgenic mice, where efficient removal of Aβ plaques decreases with age[245,246].

Although being unable to remove plaques from the brains of hAChE-Tg//APPswe mice, nicotine might in some way interact with the plaques, leading to a release of Aβ peptides into a brain Aβ pool, detectable by ELISA. This interaction remains to be further evaluated.

The elevated Aβ levels following nicotine treatment in the hAChE-Tg//APPswe mice might in some way be linked to overexpression of AChE-S. Increased stress responses[247] and cholinergic hyperexcitation have been reported in the brains of single transgenic hAChE-Tg mice[248]. If this is also the case in hAChE-Tg//APPswe mice it might explain the increased Aβ levels following nicotine treatment, and thus needs

further investigation. Increased Aβ levels have been reported as a consequence of both acute and chronic stress in APPswe mice[249,250].

Effect on astrocytes

Nicotine has been shown to induce anti-inflammatory mechanisms that diminish local inflammatory responses[251]. Consistent with these findings, GFAP levels were reduced following nicotine treatment in both APPswe (Fig. 8) and hAChE-Tg//APPswe mice (Fig. 10). This reduction might play a beneficial role, especially if astrocytes promote the formation and maturation of Aβ plaques.

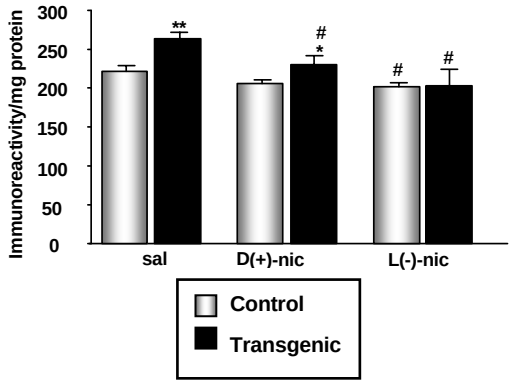

Figure 10. GFAP immunoreactivity in the hippocampi of hAChE-Tg//APPswe mice and non-transgenic control mice following treatment with saline, D(+)-nicotine, or L(-)-nicotine. ** $P < 0.05$ and ** $P < 0.01$ compared with non-transgenic controls in the same treatment group. # $P < 0.05$ compared with saline-treated hAChE-Tg//APPswe mice or non-transgenic mice. sal = saline and nic = nicotine. Values are expressed as mean ± SEM.*

Consequences of treatment on α7 neuronal nicotinic acetylcholine receptors

The α7 nAChRs are characterized by rapid desensitization following exposure to nicotinic agonists[252]. Again, 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[241,253-255]. We observed an increase in α7 nAChRs in the CA3 region of the hippocampus and in the temporal/parietal cortex of nicotine-treated APPswe mice compared with saline-treated APPswe mice (Paper II, Table 2), suggesting that Aβ might potentiate the nicotine-induced desensitization of the α7 nAChRs and thereby bring about up-regulation of the receptor.

As regards hAChE-Tg//APPswe mice, only transgenic animals treated with D(+)-nicotine showed an increase in cortical and hippocampal α7 nAChRs (Paper III, Table 1), an increase that might be linked to the elevated levels of Aβ in these mice, and thus increased interaction of Aβ with this receptor[97].

Effects of memantine and galantamine in APPswe mice

An increasing number of studies suggest that the present clinical therapy used in AD, in addition to having a symptomatic effect, may also interact with the ongoing neuropathological processes in the brain[143,256].

Treatment with memantine (Paper II) did not affect Aβ levels, but significantly lowered the levels of cortical total membrane-bound APP in both the APPswe mice and non-transgenic controls, when compared with saline treatment (Fig. 11). The results indicate, that with a longer treatment period, Aβ levels might eventually be affected, which indeed was recently proven, as long-term treatment (6 months) with memantine significantly decreased Aβ plaque deposition in APPswe mice[257]. The mechanism has to be further evaluated. However, APP is expressed by glutamatergic neurons[258] and a blockade of NMDA receptors by memantine might therefore influence the production of total APP.

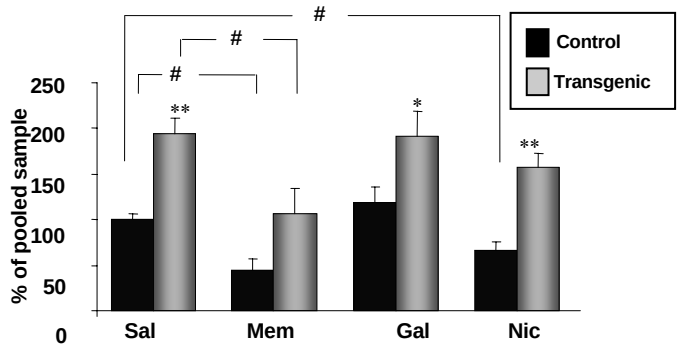

Figure 11. Levels of total APP in the cortices of APPswe mice and non-transgenic control mice following treatment with saline, memantine, galantamine or nicotine. ** P < 0.05 and ** P < 0.01 compared with non-transgenic controls in the same treatment group. # P < 0.05 compared with saline-treated APPswe mice or non-transgenic mice. sal = saline, mem = memantine, gal = galantamine and nic = nicotine. Values are expressed as mean ± SEM.*

Treatment with galantamine caused a significant decrease in the number of NMDA receptor binding sites in several brain regions of the APPswe mice compared with saline-treated mice (Paper II, Table 1). Galantamine may potentiate the activity of NMDA receptors via PKC[259] and interact with the α7 nAChRs[260], but the exact mechanism behind this needs to be further evaluated. Treatment with galantamine also caused an increase in synaptophysin levels in the APPswe mice (Fig. 12), suggesting a neurotropic effect by influencing the possible interaction between Aβ and the α7 nAChRs. Taken together, these results suggest that by affecting both α7 nAChRs and NMDA receptors, galantamine is able to mediate plastic changes in the brain, which partly might explain the neuroprotective effect that has been reported in connection with this drug[261,262].

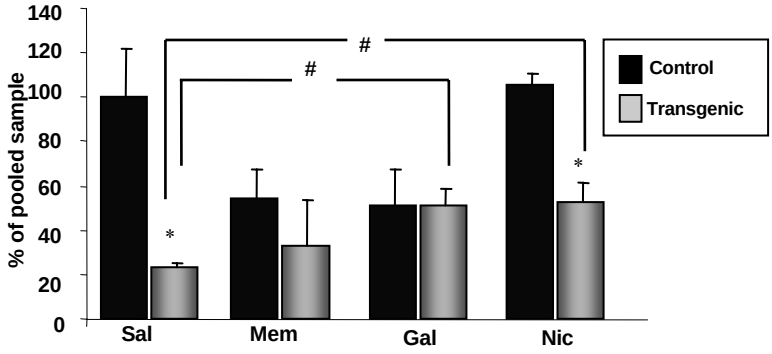

Figure 12. Levels of synaptophysin in the cortices of APPswe mice and non-transgenic control mice following treatment with saline, memantine, galantamine or nicotine. ** $P < 0.05$ compared with non-transgenic controls in the same treatment group. # $P < 0.05$ compared with saline-treated APPswe mice or non-transgenic mice. sal = saline, mem = memantine, gal = galantamine and nic = nicotine. Values are expressed as mean ± SEM.*

Effects of huprine X in 3xTg-AD and APPswe mice

The huprines belong to a class of potent and selective AChEIs designed from tacrine and huperzine A (Hup A) through a conjunctive approach[263,264]. Although the huprines have been shown to bind preferentially to the acylation site of AChE, experimental data and molecular modelling studies have demonstrated that the binding geometry of huprine X (HX) and its molecular volume result in a significant decrease in the affinity of ligands to the peripheral anionic site (PAS) of AChE[263]. These results have led to speculation concerning whether or not HX has the ability to affect Aβ levels by interfering with AChE-induced Aβ aggregation.

Different effects on β-amyloid levels

In our study (Paper IV), we found that chronic treatment with HX indeed reduced the levels of insoluble Aβ1–40 in the hippocampi of 3xTg-AD mice, while no significant changes were found in insoluble Aβ1–42 (Fig. 13), probably as a result of the more fibrillogenic nature of Aβ1–42 versus Aβ1–40[212]. The Aβ-reducing effect of HX might be ascribed to interaction of HX with PAS, but other mechanisms, not necessarily linked to inhibition of AChE, cannot be ruled out.

Hup A did not reduce the levels of Aβ in the brains of 3xTg-AD mice (Fig. 13). *Ex vivo* studies in mice have shown that Hup A inhibited mouse brain AChE by 30%, whereas HX exhibited 77% inhibition[265,266], which might explain the diverse effects of these drugs on Aβ levels. Hup A instead increased the levels of insoluble Aβ1–42 (Fig. 13), an effect that remains to be further evaluated, but could be linked to the different pharmacological profile of the drug compared with HX.

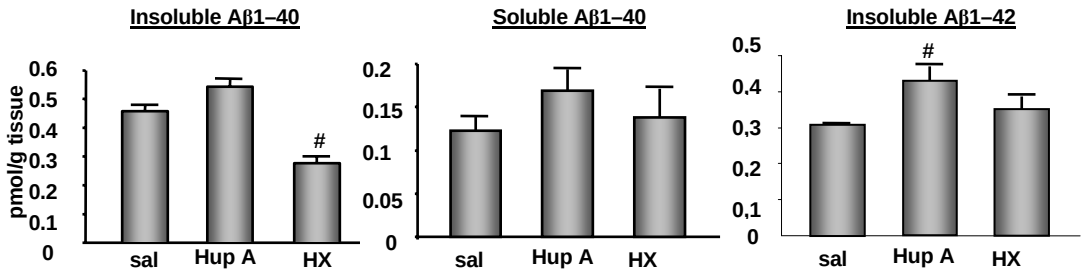

Figure 13. Levels of Aβ in the hippocampi of 3xTg-AD mice following treatment with saline, huperzine A or huprine X. *# P < 0.05 compared with saline-treated 3xTg-AD mice in the same Aβ group. sal = saline, Hup A = huperzine A and HX = huprine X. Values are expressed as mean ± SEM.*

In APPswe mice, HX did not alter Aβ levels in the brain. A plausible explanation might be that the levels of Aβ1–40 were ~20-fold higher in the APPswe mice compared with the 3xTg-AD mice (Fig. 4). Moreover, the APPswe mice seemed to have a different Aβ pathology compared with the 3xTg-AD mice since Aβ peptides were detected in both the cortices and the hippocampi of the mice, whereas the 3xTg-AD mice exhibited detectable levels of Aβ only in the hippocampus. Contradictory results concerning the effects of drug treatment (i.e. nicotine) on Aβ pathology have earlier been shown in different transgenic mice[231,241,242,267]. It might be possible that with a treatment period longer than 21 days, Aβ levels may eventually decrease in APPswe mice.

Different effects on synaptophysin

Interestingly, treatment with HX resulted in a marked increase in cortical synaptophysin levels in 3xTg-AD mice, reaching those of non-transgenic controls (Fig. 14), which might indicate an acute phase of synaptic plasticity in response to stimulation by AChEI therapy, which in turn could lead to remodelling of cholinergic and related neuronal networks in the brain.

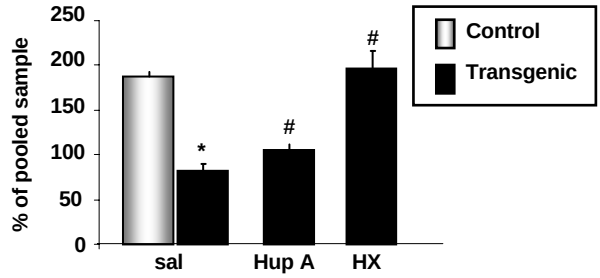

Figure 14. Levels of synaptophysin in the cortices of 3xTg-AD mice and non-transgenic control mice following treatment with saline, huperzine A or huprine X. ** P < 0.05 compared with non-transgenic controls. # P < 0.05 compared with saline-treated 3xTg-AD mice. sal = saline, Hup A = huperzine A and HX = huprine X. Values are expressed as mean ± SEM.*

Compared with HX, treatment with Hup A resulted in only a slight increase in synaptophysin levels in the 3xTg-AD mice (Fig. 14), which partly might be explained by its lower affinity for AChE[263,265] and/or lack of nAChR activation[268,269].

In the APPswe mice, HX did not show any significant effects on synaptophysin levels compared with saline treatment (Paper IV, Fig. 4), which is inconsistent with galantamine treatment in APPswe mice, where up-regulation was found in the transgenic mice (Paper II). However, since no baseline changes were detected between transgenic mice and controls, it might be possible that HX cannot further modulate these processes.

Consequences of treatment on α7 neuronal nicotinic acetylcholine receptors

Treatment with HX, as well as with Hup A, significantly decreased α7 nAChRs in the caudate nuclei of 3xTg-AD mice, compared with saline treatment (Paper IV, Fig. 5C). This brain region has been shown to exhibit high AChE expression/activity, as well as high ACh levels in mice[270-272]. As a result of chronic treatment with AChEIs, an even higher amount of ACh might be available in the synaptic cleft in this brain region, which might lead to over-activation of nAChRs. As a consequence, these receptors might subsequently be down-regulated.

In the APPswe mice, treatment with HX, on the other hand, significantly increased the levels of hippocampal α7 nAChRs compared with saline-treated APPswe mice (Paper IV, Fig. 6), an effect possibly linked to the higher Aβ levels in the brains of these mice, and thus the suggested interaction with α7 nAChRs[96,97,107]. Notably, a novel heteromeric α7β2 nAChR, with high sensitivity to Aβ peptides, was recently reported in rodent basal forebrain cholinergic neurons[86]. This receptor subtype might also be expressed in APPswe and 3xTg-AD mice, with diverse brain distributions, which also may explain the different effects involving α7 nAChRs in the two mouse models.

CONCLUDING REMARKS

In this work, the characteristic pathological features of AD, with focus on Aβ accumulation, were investigated in different AD-related transgenic mouse models. In addition, the consequences of the excess of human Aβ on the synaptic density, the neurotransmitter receptors: α7 nAChRs and NMDA receptors as well as inflammatory processes were studied in the brain of these mice. The results clearly show that Aβ is present early in the brain of the different transgenic mouse models, although the individual models exhibited different forms of Aβ. There was also a redistribution with age between the different forms of Aβ (soluble vs. insoluble) in the transgenic mouse models as well as compensatory changes between the α7 nAChRs and the NMDA receptors. The high levels of soluble Aβ in the APPswe mice were not found in the age-matched hAChE-Tg//APPswe mice, probably as a result of the overexpression of AChE in the latter, leading to increased aggregation of Aβ and consequently, earlier plaque formation. Compared with age-matched APPswe mice, which displayed high Aβ levels in both the cortex and the hippocampus, the 3xTg-AD mice showed detectable Aβ solely in the hippocampus. The observed differences in Aβ pathology between the APPswe, hAChE-Tg//APPswe and the 3xTg-AD mouse models are probably the result of genetic differences, although other factors including the mouse strain background, choice of promoters and level of APP overexpression might also play important roles. Our results clearly highlight the importance of using the proper transgenic mouse model or to utilize several types of transgenic mouse models when evaluating specific amyloid processes and their consequences in the brain. Longitudinal studies in subjects at increased risk of developing AD, regarding amyloid processes and their time frame, will shed light on the comparability between human AD patients and AD-related transgenic mice.

In this work, treatment studies were performed with substances such as nicotine, galantamine, memantine, huperzine A and huprine X to investigate the ability of these drugs to reduce the levels of Aβ in the brain of different AD-related transgenic mouse models. The results clearly demonstrated that the choice of transgenic mouse model, as well as the stage of Aβ pathology, importantly affected the outcome of drug treatment. Although nicotine markedly reduced cortical levels of insoluble Aβ peptides in APPswe mice, there was an increase in both soluble and insoluble Aβ in the hAChE-Tg//APPswe mice. The lack of reduction of Aβ levels in the hAChE-

Tg//APPswe mice following treatment with nicotine is probably explained by the more advanced stage of Aβ pathology in these animals. Similarly, treatment with the AChEI drug huprine X reduced the levels of insoluble Aβ in the hippocampi of 3xTg-AD mice, but it had no effect in APPswe mice. These results, again, might be ascribed to the more pronounced Aβ pathology in the latter mouse model.

The goal of using transgenic mouse models of neurodegeneration is to move us closer to an understanding of the underlying causes of AD, ultimately leading to the development of new effective therapies. However, one should never neglect the potential dangers of uncritical extrapolating data from mice to humans. At present, there is no animal model that recapitulates all aspects of human AD, which reflects the limitations of using a rodent system to model a human condition that takes decades to develop and mainly involves higher cognitive functions. Nevertheless, transgenic mice that overproduce human Aβ in their brains mimic many facets of AD. These mice are of great experimental value since they allow 1) the examination of the interrelationship between different pathways and AD-related lesions, 2) the dissection of specific pathways involved in AD pathogenesis, 3) studies of the significance of individual lesions such as Aβ plaques in the brain as well as to 4) test specific therapeutic compounds.

In conclusion, the diverse forms of Aβ peptides displayed in the different AD-related mouse models gave rise to differences in brain neuropathology, with diverging results regarding synapses and neuronal receptors. The results in this thesis clearly highlight the fact that the choice of transgenic mouse model, as well as the stage of Aβ pathology, importantly contribute to the outcome of drug treatment. This emphasizes the importance of using different transgenic mouse models for evaluating the effects of new drug candidates for AD treatment as well as when investigating specific amyloid processes. Therefore, by utilizing different AD-related transgenic mouse models, this thesis has hopefully provided valuable information in understanding the underlying AD-related mechanisms, which is obligatory for the development of new effective curative treatment strategies greatly needed for this devastating disease.

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