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AGE DEPENDENT BETA-AMYLOID ISOFORMS AND IMPLICATIONS OF DIFFERENT DRUG TREATMENTS AS STUDIED IN DIFFERENT TRANSGENIC MOUSE MODELS AND CELL LINES

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Dedicated to
My grandfather & My family

ABSTRACT

The Amyloid-β (Aβ) peptide is the main component of the the amyloid plaques in Alzheimer's Disease (AD) and has been implicated to be the cause of the disease. During the last decade it has become increasingly evident that soluble, oligomeric forms of Aβ are more toxic to neurons than the plaques and might play an important role in the disease pathogenesis. The aim of this thesis was to investigate the time course of different Aβ isoforms and species and how these forms affects the neuropathological changes seen in AD and how different cholinergic drugs can modulate Aβ and it's processing.

A translational approach ranging from transfected human neuroblastoma SH-SY5Y/APPswe cells, APPswe and hAChE-Tg//APPswe transgenic mouse models of AD to postmortem AD brain tissue were used to study how changes of different levels of Aβ influence the brain and related processes.

APPswe transgenic mice showed already at 7-days of age, high levels of soluble form of Aβ, as a sign for that Aβ starts to aggregate from birth. Between 7 to 90-days of age, the major Aβ isoforms in brain were shorter forms than $Aβ_{1-40}$. The levels of $Aβ_{1-40}$ were high and remained fairly constant up to 15-months of age while $Aβ_{1-42}$ showed an age-dependent consistent increase from 7-days up to 15-months of age. High levels of Aβ oligomers but low levels of synaphtophysin were observed in 90-days-old APPswe mice probably due to the toxicity of the oligomers. Low levels of α7 neuronal nicotinic acetylcholine receptors (nAChRs) compared to non-transgenic mice were measured in 7-days-old APPswe mice; while an increased number *N*-methyl-D-aspartate (NMDA) receptors binding sites were found at 21-days of age probably reflecting compensatory mechanisms in response to a high Aβ burden. Epigenetic studies showed increased levels of acetylated (AcH3), and di-methylated (2MeH3) histone H3 at 4-months-old APPswe mice. When a γ-secretase inhibitor reduced Aβ, there was a reduction in AcH3 in SH-SY5Y/ APPswe cells. nAChR agonists showed to influence the Aβ levels in hAChE-Tg//APPswe transgenic mice and in SH-SY5Y/ APPswe cells.

LIST OF PUBLICATIONS

This thesis is based on the following papers, which will be referred to their Roman numbers:

I. Unger C, Hedberg MM, **Mustafiz T**, Svedberg MM, Nordberg A.
Early changes in Abeta levels in the brain of APPswe transgenic mice-implication on synaptic density, alpha7 neuronal nicotinic acetylcholine- and N-methyl-D-aspartate receptor levels.
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Transgenic mice overexpressing human acetylcholinesterase and the Swedish amyloid precursor protein mutation: effect of nicotine treatment.
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III. **Mustafiz T**, Portelius E, Gustavsson MK, Hölttä M, Zetterberg H, Blennow K, Nordberg A, Unger Lithner C.
Characterization of the brain β-amyloid isoform pattern at different ages of Tg2576 mice.
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IV. Unger Lithner C, **Mustafiz T**, Nordberg A, Sweatt DJ, Hernandez CM
Aβ increases histone acetylation in the brain; epigenetic changes in Alzheimer's disease.
Manuscript

V. **Mustafiz T**, Unger Lithner C, Nordberg A
Different modulation of amyloid processing mechanism by cholinergic substances in SH-SY5Y/APPswe cells
Manuscript

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ABBREVIATIONS

Aβ	β-Amyloid
ACh	Acetylcholine
AChE	Acetylcholinesterase
AChE-E	Erythrocyte AChE
AChE-R	Read-through AChE
AChE-S	Synaptic AChE
AChEI	Acetylcholinesterase inhibitor
AcH3	Acetylated histone 3
AD	Alzheimer's disease
AICD	APP intracellular domain
APOE	Apolipoprotein E
APP	Amyloid precursor protein
APPswe	APP Swedish KM670/671NL
BACE	β-site APP cleaving enzyme
CDK5	Cyclin-dependent kinase-5
CNS	Central nervous system
CTRL	Control
ELISA	Enzyme linked immune sorbent assay
ERK1/2	Extra cellular regulated kinase 1/2
GFAP	Glial fibrillary acidic protein
GSK3β	Glycogen synthase kinase 3β
HEK	Human embryonic kidney
H3	Histone 3
H4	Histone 4
nAChRs	Nicotinic acetylcholine receptors
NFTs	Neurofibrillary tangles
NMDA	*N*-methyl-D-aspartate
NPs	Neuritic plaques
α7 nAChRs	alpha 7 nAChRs
PET	Positron emission tomography
PRF	Pre-frontal cortex
PS	Presenilin
sAPPα	Soluble APP-α
sAPPβ	Soluble APP-β
Ser	Serine
TH3	Phosphorylated H3
Thr	Threonin
2MeH3	Di-methylated H3

INTRODUCTION

ALZHEIMER'S DISEASE

Alzheimer's disease (AD) is the most common neurodegenerative disease among the elderly population, causing insidious and progressive damage to the brain, leading to memory loss. According to the recent WHO report in April 2012, the present number of patients with dementia is 35.6 million and is expected to increase to 106 million in 2050. It is estimated that the global prevalence of dementia will be 1 in 85 persons by 2050[1]. This means that every year around 7.7 million people are affected with dementia, estimating one new case in every four seconds[2]. AD is the most common form of dementia disorder representing 50-70% of cases over 65 years of age. The incidence rate of AD increases exponentially with increasing age. Every 5-6 years of time, the number of AD patients is duplicated . The prevalence of AD is 1% at 60-65 years of age; thereafter, it increases to 24-33% at the age of 85[3]. A recent study showed that AD affected 34 million people worldwide in 2009 and the estimated cost was $422 billion, which had increased by 34% between 2005 and 2009[4].

AD is characterized initially with subtle impairment in episodic memory, which also starts to involve other memory domains. Besides memory impairments, this disease also includes damages of other functions in the brain resulting in impairments of personality and behavior, speaking as well as executive functions such as decision-making and planning which seriously interfere with daily life. AD affects not only the afflicted patients but also close relatives as well as the society. The cause of death is usually due to secondary conditions such as pneumonia or other infections and malnutrition.

Risk factors and genetics

The AD cases are categorized as: sporadic AD (sAD) or late onset AD (irregular and amorphous occurrence of AD), and familial AD (FAD) or early onset AD (inherited as autosomal-dominant disorder). sAD is the most common since FAD represents less than 2% of all AD cases[5]. FAD generally has a relatively early onset (< 60 years of age), while sAD has a later onset [6].

sAD is diverse and its causal factor(s) that triggers the disease process are not fully understood. However a combination of both biological and environmental risk factors may attribute to the cause[7]. Advancing age is the greatest risk for developing AD[8].

In addition to family history[9,10], stroke [11], low education levels[12], female gender[13], and diabetes[14,15] are also risk factors. The inheritance of one or two ε4 alleles of apolipoprotein E (ApoE) gene on chromosome 19q13, a protein involved in cholesterol metabolism contributes to the risk of late onset AD[16-18].

There are three genes that have been identified in which mutations lead to the development of FAD: presenilin 1 (PS1) on chromosome 14[19], presenilin 2 (PS2) on chromosome 1[20] and amyloid-β protein precursor (APP) on chromosome 21[21]. All these mutations alter the proteolytic processing of APP, resulting in increased production of either total Aβ or $A\beta_{1-42}$[22].

More than 70% of FAD cases are due to the mutations in the PS1 gene. These mutations cause the most aggressive forms of AD, in some cases with onset younger than 30 years of age[19,23]. Mutations in the PS1 or PS2 genes cause a selective increase in $A\beta_{1-42}$ levels[24,25]. It was suggested that PS mutations selectively elevate the levels of highly amyloidogenic $A\beta_{1-42}$ peptides by shifting the cleavage site in APP[26].

A number of mutations in the APP gene have been identified causing different phenotypes of onset and progression of the disease processes: Dutch[27], Flemish[28], London [29], Arctic[30], Australian[31], Belgian[32], German[33] and Swedish mutation[34]. The Swedish APP mutation (KM670/671NL) occurs near the β-site adjacent to the Aβ domain, increasing the production of APP which in turn leads to elevation of the absolute levels of both $A\beta_{1-40}$ and $A\beta_{1-42}$[35]. All these mutations in the APP gene are generally believed to cause Aβ accumulation by formation of protofibrils[30,36,37].

Clinical diagnosis

AD is diagnosed according to the criteria of the *Diagnostic and Statistical Manual of Mental Disorders,* fourth edition (DSM-IV) [38], National Institute of Neurological and Communication Disorders and Stroke-Alzheimer's disease and Related Disorders Associations (NINCDS-ADRDA)[39]. For the assessment of memory problems, the patient undergoes investigations including disease history, neurological and psychiatric assessments, blood analysis, cognitive testing and brain imaging (CT or MRI). At more specialized memory clinics the investigations might also include cerebrospinal fluid (CSF) sampling for analysis of biomarkers and positron emission tomography (PET) or single photon emission tomography (SPECT) imaging. Recent progress in the development of new diagnostic biomarkers suggesting the incorporation of biomarkers such as CSF and PET into the standardized clinical diagnosis of AD which might in the near future change the diagnostic guidelines[40-43].

Pathogenesis

The pathogenesis of AD is highly complex. Scientists have identified several factors that appear to play a role in the development of AD, but no definitive causes or mechanisms have been singled out. In 1906, Dr. Alois Alzheimer presented the first case of AD, a 51-year-old woman named Auguste Deter. In her autopsied brain, he described the extracellular accumulation of β amyloid (Aβ) and intracellular neurofibrillary tangles (NFTs) consisting of hyperphosphorylated tau under the microscope [44]. These two features still serve as the major pathological hallmarks of AD. The AD brain shows atrophy along with shrinkage of gyri, widening of sulci, and enlargement of ventricles. The cortical ribbon may be thinned, therefore, ventricular dilatation becomes obvious, especially in the temporal horn, due to atrophy of the amygdala and hippocampus[45]. The two pathological hallmarks of AD are found mainly in the brain parenchyma, especially in the cerebral cortex and hippocampus but also in the entorhinal cortex, neocortex [46]; furthermore, they are accompanied by other structural changes like granulovascular degeneration, dendritic atrophy, and synapse loss, especially cholinergic neurons being vulnerable[47]. In addition, the presence of inflammatory markers such as reactive microglia, astrocytes, and pro-inflammatory cytokines around the Aβ plaques reveal the existence of inflammation in AD brains[48-50].

Amyloid Precursor Protein (APP) processing

Amyloid- β precursor protein (APP) is a transmembrane glycoprotein containing a 47-residue cytoplasmic domain and one membrane-spanning domain[51]. The specific form of APP plays a key role in the modulation of neuronal plasticity, synapse stabilization, and memory consolidation[52]. Within the APP structure there are 40 and 42 amino acid sequences termed the Aβ region, which is located partly within the membrane (amino acids 29-42), and partially in the extracellular space (amino acids 1-28).

The exact biological function of APP and its homologues is still unknown. However, several *in vitro* and *in vivo* studies have provided strong evidence of the roles of APP on CNS such as: both in the developing and adult nervous system, in cell adhesion, neurite outgrowth, synaptogenesis, and modulation of synaptic plasticity, neuronal survival, vesicular transport, neuronal migration, and insulin as well as glucose homeostasis.

APP can be processed in two distinct reciprocal proteolytic pathways: a non-amyloidogenic (or α-secretase) and an amyloidogenic (or β-secretase) pathway (**Fig.**

1). Although APP processing has been studied extensively, there is not yet a complete picture of the processing events and the enzymes involved [53].

The non-amyloidogenic pathway is the major APP processing pathway in most cell types[54]. It is initiated by the cleavage at the α-secretase site by a member of the ADAM (adisintegrin and metalloprotease) family, of which ADAM10, ADAM9 [55,56], ADAM17 (TACE) [57] and ADAM19 have been identified as having α-secretase activity [58,59].

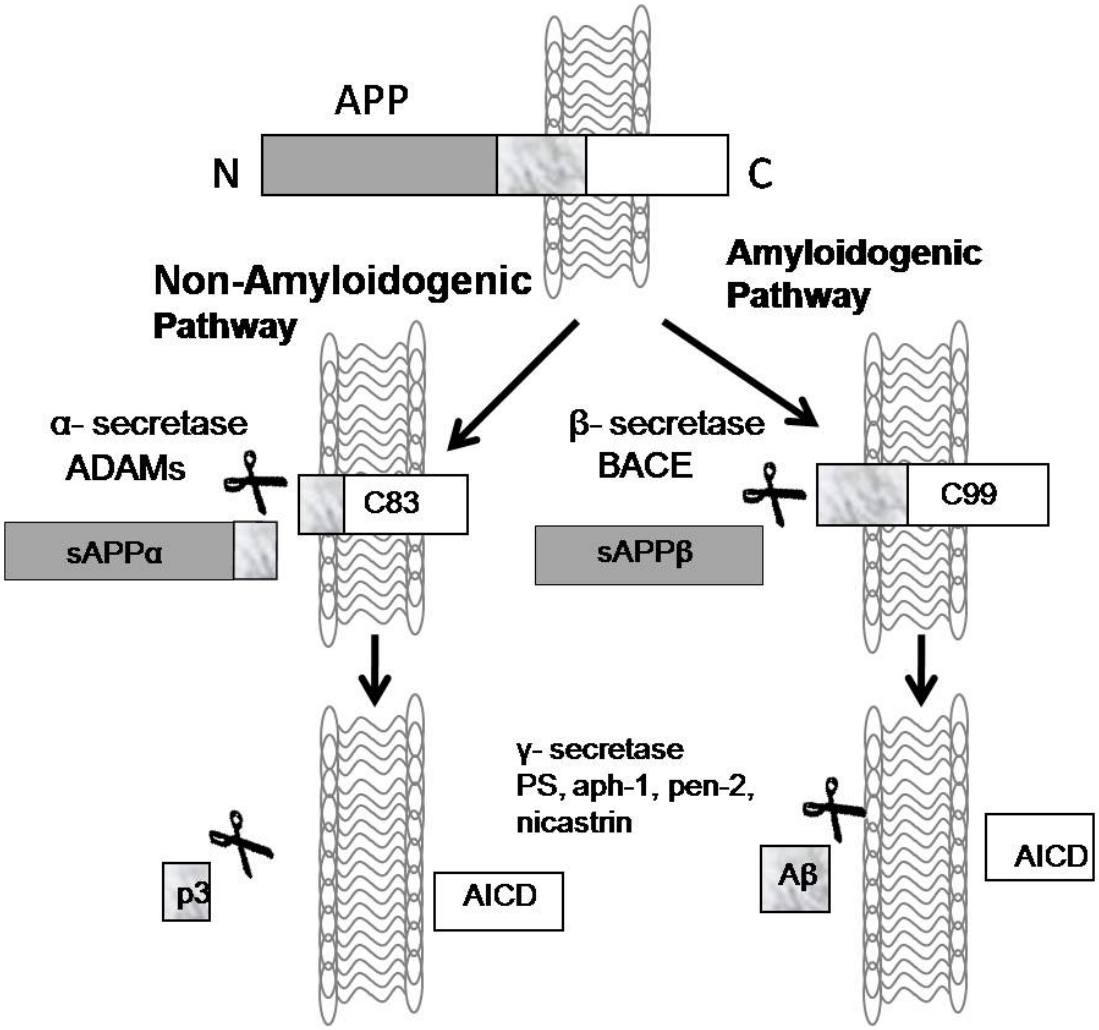

Figure 1. Schematic overview of the general processing of amyloid precursor protein. In non-amyloidogenic processing, APP is sequentially cleaved by α- and γ-secretase while in the amyloidogenic pathway APP is processed by β- and γ-secretase generating Aβ.

The cleavage occurs within the Aβ region (between residue 16 and 17), thereby preventing the formation of Aβ, generating a soluble extracellular fragment of APP (sAPPα). The sAPPα may have neuroprotective functions[60] and a membrane-associated

C-terminal fragment (CTFα) of 83 residues which is further processed by γ-secretase complex, generating a short p3 peptide, unable to form aggregates, and the APP intracellular domain (AICD).

In the amyloidogenic pathway, APP is processed by aberrant cleavage at the β-secretase site by the β-site cleaving enzyme (BACE), producing a large soluble N-terminal ectodomain fragment of APP (sAPPβ), and an Aβ-bearing membrane-associated C-terminal fragment (CTFβ) of 99 residues that are further processed within the transmembrane domain by γ-secretase complex, and generate $A\beta_{1-40}$ and $A\beta_{1-42}$ [61,62]. In addition, a soluble cytosolic fragment APP intracellular domain (AICD) is liberated. AICD can be translocated into the nucleus where it may function as a transcription factor[63]. The β-secretase has been shown to be the novel transmembrane aspartic protease, β-site APP-cleaving enzyme 1 (BACE1). BACE2, a protease homologous to BACE1, was also identified [64]. γ-secretase is a multiprotein complex consisting of presenilin-1 (PS1), nicastrin, Aph-1 (anterior pharynx-defective-1), and Pen-2 (PS-enhancer-2); all four proteins are necessary for full proteolytic activity[65].

Both α and β-secretase activity result in release of the large, extracellular sAPP domain. sAPPα has been reported to have neurotrophic properties *in vitro* [66,67]. Thus, any condition that favor Aβ production from APP, may lead to loss of neuroprotective sAPPα, along with the added neurotoxic effect of Aβ.

Aβ peptides can also be generated intracellularly at different subcellular regions whereas $A\beta_{1-40}$ is generated solely in the trans-Golgi network (TGN), while $A\beta_{1-42}$ is produced in the endoplasmic reticulum (ER) as well as in the Golgi compartments[68,69]. Aβ has also been found in mitochondria[70,71]. Although much of the intracellularly generated Aβ is enrolled to secretion, there is a significant pool of Aβ peptides that remain inside the cell. The pedigree of intraneuronal Aβ can be either by slow production of APP inside the neurons and/or taken up from the extracellular space. Several studies have described the internalization of Aβ from the extracellular pool[72-75]. In addition, stable Aβ oligomers are formed intracellularly in a variety of cell types, including primary human neurons[71].

Aβ pathology in Alzheimer's disease

In AD brain, amyloid exists in insoluble Aβ plaques (predominantly consisting of the 40 or 42 amino acid residue), but also as diffuse plaques and pre-amyloid deposits as

well as additional forms of intracellular and extracellular soluble Aβ. It has been suggested that fibrils, smaller peptide oligomers, water-soluble non-filamentous forms of Aβ might be toxic [76]. The fibrillization of Aβ is a multistep reaction where Aβ monomers assemble into oligomers. These oligomers further polymerize into protofibrils, which then mature into the Aβ fibrils that are found in the plaques [77]. With the advancement of the biomarker discoveries, it is now possible to measure fibrillar form Aβ *in vivo* using ^{11}C PIB tracer with positron emission tomography (PET) technique even at prodromal stage of AD [78].

Both *in vitro* and *in vivo* studies have shown that the purified Aβ oligomers but not monomers can disrupt cognitive function[79,80]. There are three types of Aβ oligomers that have been identified *in vitro*: **1)** very short oligomers ranging from dimer to hexamer size[81]; **2)** Aβ-derived diffusible ligands (ADDLs)- small oligomers ranging from 17 to 42 kDa[82]; and **3)** protofibrils, which can be seen in electron microscopy as short fibril intermediates[83]. However, the unstable and diversity nature of these Aβ arbitrates, makes it difficult to identify the specific species of Aβ oligomers that is responsible for the neurotoxic effects in AD.

Early cognitive impairment was manifested at 4-months of age in the triple transgenic AD mouse model, when NPs and NFTs are not present. This cognitive impairment correlated with the accumulation of intraneuronal Aβ in the hippocampus and amygdala [84]. In connection with intraneuronal Aβ, a number of studies have discussed the existence of intracellular Aβ accumulation in the brains of AD patients and animal models of AD and its impact on the pathogenesis of the disease such as synaptic impairment and neuronal loss[85-90]. This is in agreement with recent observation of a positive correlation between levels of isolated oligomers in autopsy AD brain and choline acetyltransferase (ChAT) activity and number of nicotinic receptors[91], supporting that the increased vulnerability of synaptic function caused by Aβ oligomers.

The amyloid cascade hypothesis

AD is considered to be a complex multifactorial disease and its definite disease mechanism is still under evaluation. Many theories have been proposed in the last century to explain the pathogenesis of AD. For explaining the disease process, one of the leading theories is the "amyloid cascade hypothesis." According to this hypothesis, the brain accumulation of Aβ is the primary influence that triggers the cascade of pathogenic events in AD, leading to the formation of NFT, inflammatory changes, and the loss of neurons and synapses in vulnerable regions that invariably accompany Aβ de-

position[92,93]. Yet, this hypothesis remains controversial mainly because the specific neurotoxic species of Aβ and its effects on neuronal function *in vivo* have not been defined. It has long been assumed that Aβ had to be assembled into extracellular amyloid fibrils and accumulation into amyloid plaques to exert its cytotoxic effects; however, clinical studies have been unable to confirm a relationship between amyloid plaque load and dementia severity, or loss of neurons and synapses[94,95]. Moreover, numerous amyloid plaques that match the brain regional pattern of distribution seen in AD patients may also be present in many non-demented individuals[96-98]. Another argument against the amyloid hypothesis has been the observation that amyloid plaques do not correlate as well with cognition scores as do neurofibrillary tangles[99-102].Therefore, a modified version of the amyloid hypothesis has been established which describes that it is soluble intraneuronal Aβ oligomers, not amyloid plaques that initiate the cascade leading to neuronal death and dysfunction[103-105]. Interestingly, recent discoveries are in agreement with the modified hypothesis, that the soluble Aβ oligomers rather than the actual plaques are more closely correlated to the clinical severity of disease and synapse loss[106,107]. An interesting observation is therefore, that APP arctic mutant AD patients show a reduction in $A\beta_{1-42}$ in CSF as well as reduced cerebral glucose metabolisms but no presence of fibrillar form of Aβ in the brain measured by PET indicating that high levels of oligomers, protofibrils are driving AD dysfunction[108].

An imbalance between production and clearance of the Aβ is a key momentum of the complex pathogenesis of AD. It is caused by overproduction of Aβ or by reduction of clearance from the brain. Clearance can be reduced by defective degradation, increased aggregation, disturbed transport across the blood-brain barrier or even inefficient peripheral removal of the peptide. Thus, entrapped Aβ in the brain undergoes accumulation and aggregation.

Tau protein

Tau proteins are a microtubule (MT) associated protein. The human tau is the product of alternative splicing from a single gene microtubule-associated protein tau (MAPT). It has six isoforms ranging from 352-441 residues with molecular weight of 45-65kDa[109]. They are abundant in the neurons of the CNS and are less common in non-neuronal cells, but are also expressed at very low levels in the CNS astrocytes and oligodendrocytes[110]. They are localized primarily in the distal portions of axons. In the normal brain, tau is necessary for stabilizing the MT network and regulation of

axonal transport[111]. Tau is a phosphoprotein, which has 79 potential Serine (Ser) and Threonine (Thr) phosphorylation sites on the longest tau isoform. Phosphorylation has been reported on approximately 30 of these sites in normal tau proteins. Tau can be post-transitionally modified by hyperphosphorylation, glycosylation, ubiquitination, glycation, oxidation, and proteolysis[112-114].

When tau is hyperphosphorylated, multi-steps of disorganization takes place such as: loss of the affinity of tau for the MTs[115], dissociation of tau from MTs and thereby MTs disassembly, abnormal accumulation of tau in the somatodendritic compartment, impaired axonal transport, loss of synapses and finally neuronal cell death[116,117]. The phosphorylation of tau is maintained by a mutual balance between kinases and phosphatases where kinases mostly cause phosphorylation of tau, and phosphatases do the opposite[118]. There are several kinases that are supposed to cause the tau hyperphosphorylation: the mitogen activated kinase (MAPK) family: extra cellular regulated kinase (ERK), P38 and c-Jun N-terminal kinase (JNK). However, the two major players for the hyperphosphorylation of tau are glycogen synthase kinase-3β (GSK3β) and cyclin dependent kinase-5 (CDK5)[119-121].

Hyperphosphorylation of tau and formation of neurofibrillary tangles in Alzheimer's disease

The hyperphosphorylation of tau is the most extensively studied in AD pathology. There are some tau phosphorylation sites like Thr212/Ser214 (AT100) that are thought to be AD specific [122]. Moreover, the severity of AD pathology has been correlated with some phosphorylating sites such as Ser202/Thr205 (AT8) and Ser396/Ser404 (PHF1), which showed strong signals in immunohistochemistry[123]. In the AD brain, tau is hyperphosphorylated and aggregated, forming paired helical filaments (PHFs), which is the main component of NFTs[124]. The impairment of neuronal function and cell death has been shown to be associated with the formation of NFTs. The number of NFTs seems to correlate more closely with the degree of neuronal loss and decline of cognitive function as well as the severity of AD[95,123,125]. It is quite plausible that NFTs might be responsible for a neurotoxic cascade. Tau mutations have been related to familial tauopathies other than AD, including frontotemporal dementia [126].

Interaction of Aβ and tau in AD

Even though the Aβ is believed to be the major player of the disease process, tau pathology is also strongly associated with the clinical expression and severity of AD[99,101]. In APP transgenic models, the manifestation of neurological deficits was evident before the deposition of significant amounts of $A\beta_{1-42}$, suggesting that the pathophysiology of AD may occur prior to amyloid deposition[98]. AD is also considered to be a tauopathy as the brain develops abnormal aggregation of the tau protein [127,128]. Despite the association of the earliest AD symptoms with plaque pathology[129], controversy has been generated by the association of clinical progression with tangles rather than plaques, and by the observation that tangles appear before Aβ deposition in the entorhinal cortex and the hippocampus[130,131]. Many studies agree that the amyloid cascade hypothesis mediates tau pathology as well as the association of Aβ to result in cognitive decline[132]. In addition, aggregated Aβ[133], Aβ fibrils[134], pre-aggregated Aβ[135], oligomers [106] as well as dimers[136] induced tau hyperphosphorylation, microtubule disassembly, and finally neuritic degeneration. On the other hand, reduced tau expression could block Aβ-induced cognitive impairments by reducing excitotoxicity in hAPP mice[137], suggesting that tau reduction uncouples Aβ from downstream pathological mechanisms. In primary neurons, tau was required for Aβ-induced microtubule disassembly[138]. A possible mechanism involving tau phosphorylation was suggested by a study stating that intracellular Aβ binds to soluble tau and promotes tau phosphorylation by GSK3β which was further supported by co expression of Aβ and tau within tangles in postmortem AD brain[139]. Here the authors hypothesize that Aβ-tau complex generates insoluble composites of both Aβ and tau within the neuron, and suggest that the blockade of the Aβ-tau binding may be a viable therapeutic target. Another observation showed that Aβ and p-tau pathology localize within the synaptic compartment in AD[140]. This also indicates the potential target of early Aβ/tau interactions in the synaptic terminals. Furthermore, tangles can be found in the absence of plaques, while the same amount of plaques could be found both in AD and in non-demented elderly.

NEUROTRANSMITTER SYSTEMS AFFECTED IN ALZHEIMER'S DISEASE

The cholinergic neurotransmitter system is believed to play a key role in AD pathology [141,142]. Other neurotransmitter systems are also affected in AD. Along with cholinergic deficits, AD patients demonstrated deficits in other neurotransmitter systems such as dopaminergic, noradrenergic, serotonergic, and glutarmatergic neurons as well as several peptide neurotransmitters.

THE CHOLINERGIC NEUROTRANSMITTER SYSTEM

The cholinergic projections originate from the nucleus basalis of Meynert (nbM) and reach several cortical areas[143]. There are two main cholinergic tracts, the medial and lateral pathways to the cerebral cortex and amygdala. The medial pathway supplies the parolfactory, cingulate, pericingulate, and retrosplenial cortices. The lateral pathway is subdivided into the capsular and perisylvian divisions, the later innervating frontoparietal operculum, insula, and superior temporal gyrus. The capsular division innervates the remaining parts of the frontal, parietal, and temporal neocortex.

A number of studies in humans indicate that the cholinergic pathways serve important functional roles in consciousness, awareness, attention, and working memory[144]. The cholinergic system in the brain is important for higher cognitive functions including memory and attention, both of which are impaired in AD. The dysfunction cholinergic neurotransmission is comprised of a reduction in ACh synthesis due to reduced ChAT and choline uptake, cholinergic neuronal and axonal abnormalities, and degeneration of cholinergic neurons[145]. In conjunction with AD, it is evident that cholinergic neurons in the hippocampus and nucleus basalis are greatly reduced[146]. The degeneration of cholinergic neurons eventually causes the dysfunction of this system, which is considered to be one of the seminal features of AD.

The cholinergic hypothesis

Extensive studies of the brains of AD have consistently found damage or impairments in the cholinergic projections that appeared to correlate well with the level of cognitive decline. As a result, the “cholinergic hypothesis” was developed, which essentially states that a loss of cholinergic function in the CNS contributes significantly to the cognitive decline associated with AD[141,142]. A recent study has shown an association of high levels of fibrillar Aβ with losses of neuronal nAChRs in autopsy human brain supporting the hypothesis that neuronal nAChRs may play a critical role in AD pathology[147]. Based upon the cholinergic hypothesis, currently available clinical treatment of the ChE inhibitors was formulated.

Cholinergic receptors

Cholinergic neurotransmission is mediated by the interaction of ACh with two different types of receptors: mAChRs and nAChRs. The mAChRs are metabotrophic receptors, belonging to the G-protein coupled receptor family whereas the neuronal nAChRs are

transmitter /ligand gated ion channels, permeable to Na^+, K^+, and Ca^+ ions and belong to the gene family of homologous receptors including NMDA, GABA, and 5-HT3 receptors.

The nAChRs have a pentameric structure and are composed of five membrane subunits consisting of α and β subunits[148]. Up to date, molecular biology studies have identified and cloned nine α (α2-α10) and three β subunits (β2-β4) of nAChRs in the rodent brain and eight nAChRs subunits (α3– α7, β2-β4) in the human brain Both α and β subunits preside over the pharmacological and functional properties of a defined subunit composition i.e., channel open times, ion selectivity, and rates of desensitization[149]. The nAChRs have a sparse but widespread distribution in the human brain. They are located pre- and post-synaptically, and also at peri and extrasynaptic sites where they may regulate neuronal function by a variety of actions[150-152]. The most abundant subtypes in the human brain are α4β2, α3β2, and α7 (homomeric) nAChRs.

Normal function of nicotinic acetylcholine receptors

The α7 nAChRs are thought to be involved in rapid synaptic transmission and play a role in learning[153] and sensory gating [154]. The α7 nAChRs subtype seems to play a role in the regulation of neuronal plasticity and differentiation during development[155] as well as neuroprotective mechanisms[156-158].

Nicotinic acetylcholine receptors in relation to Alzheimer's Disease

It is well known that nAChRs are closely related to cognition. Along with loss of cholinergic innervations, a severe loss of neuronal nAChRs, mainly in the cortical regions and the hippocampus has been reported [159-161]. Both molecular and neurochemical lines of evidence link a selective loss of different subtypes predominantly α4β2 nAChRs, but also α3 and α7 subtypes in AD brains[162-164] while α7 nAChRs are up-regulated in astrocytes[50]. This loss has been incorporated with *in vivo* PET studies indicating that deficits in nAChRs probably reflect an important step for the disease process of AD [152,165]. Moreover, it has been postulated that Aβ can bind to α7 nAChRs which might play a key role for the formation of Aβ plaque and degeneration of cholinergic neurons[166].

Cholinesterases and their role in Alzheimer's Disease

There are two different cholinesterase (ChE) enzymes present in the human brain: acetylcholinesterase (AChE) and butyrylcholinesterase (BuChE). AChE is present in the cholinergic nerve terminals -either intraneuronally, membrane bound or in the synaptic cleft (AChE-S), whereas BuChE is associated with glial cells or with neurons[167]. AChE comprises 90% of the total ChE in the temporal cortex of normal brain and mediates the inactivation of the most synaptic ACh[47].

A decreased activity of AChE, and a stable or increased activity of BuChE has been found in AD brain [47]. Studies confirmed that AChE activity is intact in aged normal cerebral cortex[168,169], but significantly reduced in the entire neocortex and the hippocampus of AD brains, even at the mild and moderate stages of the disease [168,170]. With the progression of disease, a continued loss of AChE activity was detected, and the lower cortical k3 values correlated significantly with the decline in memory and cognitive performances [170].

THE GLUTAMATERGIC NEUROTRANSMITTER SYSTEM IN ALZHEIMER'S DISEASE

L-glutamate is considered as the principal excitatory neurotransmitter in the mammalian CNS acting upon a variety of receptor types [171,172]. Glutamatergic neurons are widely distributed through the CNS, predominantly in the forebrain, where most of the cortical projections contain glutamate. There are two types of receptors that mediate the actions of glutamate: ionotropic receptors, which directly gate channels and metabotropic receptors, which involve signaling through messengers.

The N-methyl-D-aspartate receptors in relation to Alzheimer's disease

N-methyl-D-aspartate (NMDA) receptor is one of the major types of ionotropic glutamatergic receptors. The NMDA receptor is a heterogenic ligand-gated ion channel that interacts with multiple intracellular proteins by way of different subunits- namely NR1, NR2A-D, and subunits- namely NR1, NR2A-D and NR3A-B with different splice variants [173,174]. These receptors are involved in a variety of neural processes, including long-term potentiation (LTP) [175,176], long-term depression (LTD)[177,178], brain development, excitotoxicity[179], learning, and memory[180,181]. Prolonged activation of NMDA receptors results in excessive Ca^{2+} influx that can trigger excitotoxic signaling that propagates neuronal death, glutamate release, and subsequent apoptosis of neuron in hypoxia, ischemia, and neurodegenerative disorders such as AD [182,183].

Altered glutamate levels and loss of NMDA receptors are evident in post-mortem AD brain[183,184]. Recent studies revealed that NMDA receptors play a critical role in the Aβ induced neurotoxicity especially $A\beta_{1-42}$ [185,186], but also $A\beta_{1-40}$ [187]. It has been shown that stable Aβ oligomers decrease cell surface expression of NMDA receptors thereby inhibiting the induction of LTP, facilitate the LTD, alter dendritic spine density, and affect hippocampal synaptic plasticity[79,82,188-193]. Moreover, Aβ mediates and promotes NMDA receptor endocytosis possibly via the α7 nAChRs[193].

EPIGENETICS

Epigenetics refers to the heritable modifications in gene function or activity without changes in the DNA nucleotide sequence[194]. Epigenetic processes are genetic modifications that affect gene regulation by changing the DNA conformation. Therefore, gene function of any organisms is not only determined by the DNA code but also by the epigenetic phenomena. The epigenetic modifications can be carried out by histone modification or DNA methylation.

Histones

Within the chromosome, DNA is packaged by chromatin. This structural unit of chromatin is called nucleosome [195]. Nucleosomes are made up of 146 base pairs of double-stranded DNA wrapped around the core complex of 8 histones[196]. This core complex consists of two copies of each of the histone proteins H2A, H2B, H3, and H4, organized as a central $(H3\text{-}H4)_2$ tetramer flanked by two H2A-H2B dimers[197]. Histones are small basic proteins of 102-135 amino acids and their main function is to package the genomic DNA into nucleosome[198]. Each core of histone protein has two domains: a histone fold domain or globular domain, which is involved in histone-histone interactions as well as in packing DNA in nucleosomes; and a more flexible and charged amino-terminal 'tail' domain of 25-40 residues [199]. The tail lies on the outside of the nucleosome where it can interact with other regulatory proteins and with DNA. The basic N- terminal tails of the core histones act as signal integration platforms, whereby post-translational modifications are combined in a 'histone code' [200,201].

The histones $(H3\text{-}H4)_2$ tetramer are modified by acetylation (AC), methylation (Me), and phosphorylation (P); and H2A-H2B dimers are modified by acetylation, phosphorylation, ubiquitination (Ub), multiubiquitination, and ADP-ribosylation. The function of these modifications is important due to the possibility

that the nucleosome, with its modified tail domains, is not only a packer of DNA but also a carrier of epigenetic information. This indicates how genes are expressed as well as how their expression patterns are maintained from one cell generation to the next.

Histone acetylation

Acetylation of histone (Ac) is an enzymatic process by which inert chromatin is activated for RNA transcription[202]. A positive charge on lysine (Lys) residues of core histones is restored by histone deacetylation, permitting chromatin to change into a highly condensed, transcriptionally silent conformation or heterochromatin. Therefore, in most cases, histone acetylation permits transcription while histone deacetylation represses transcription. Nevertheless, in some cases transcriptional repression occurs as a result of histone acetylation. The balance between histone acetylation/deacetylation is controlled by the competitive activities of 2 superfamilies of enzymes: histone acetyltransferases (HATs) and histone deacetylases (HDACs)[203,204]. Acetylation of the histone tails might permit the access of transcription factors to DNA as well as assembly of nucleosome . H3 and H4 acetylation release a fraction of nucleosomal DNA from the packing of the nucleosome *in vitro*[205]. Therefore, different diseases can be the result of hyperacetylation of chromosomal regions that are generally silenced or deacetylation of chromosomal regions that are generally actively transcribed.

Histone methylation and ubiquitination

Similar to acetylation, methylation of histones also occurs on Lys residues, and is mediated by histone methyltransferases (HMTs)[206,207] while ubiquitylation occurs through the attachment of a ubiquitin to the Lys residues[208].

Histone phosphorylation

Histone H3 was the first histone whose phosphorylation was characterized in response to the activation of mitogenic signaling pathways[209]. Phosphorylation of serine 10 on H3 is mediated by ribosomal protein S6 kinase 2[210]. In addition, extra cellular signal-related kinase (ERK), mitogen activated protein kinase (MAPK), phosphatases PP1, and PP2A have been shown to regulate the phosphorylation of H3[211-213]. H3 acetylation and phosphorylation are thought to be partly interdependent.

Epigenetics in relation to memory formation and Alzheimer's disease

Memory is a process that is utilized by the brain for the long-term storage of information. Several studies have implicated that the formation of long-term memories is a complex process that can be mediated by many signaling pathways and the regulation of numerous genes[214,215]. Subsequent studies suggested both transcription and translation are important for the formation of long-term memories[216-218]. Acetylation of histone H3 was significantly increased after an animal underwent contextual fear conditioning- a hippocampus dependent learning model. This observation was the first indicator that epigenetic tagging of the genome occurs during consolidation of long-term memories[219]. The histone deacetylase inhibitors (HDACis), which cause histone acetylation, facilitated learning in rodents[220,221]. Another study also showed the involvement of H3 phosphorylation in memory storage[222]. These findings indicate that there might be a histone code for memory formation. The acetylation of H4, but not H3 was reduced in APPswe mice, a model of amyloid pathology [223]. Interestingly, administration of the pan-HDACi phenyl-butyrate was able to restore associative memory function and synaptic plasticity in APPswe mice[223,224] as well as in APP/PS1D9 mice, both of which are models for AD[225]. These findings suggest that histone acetylation is increasingly important as one of the key mechanisms to regulate gene-expression programs that are required for learning and memory. Consistent with mentioned findings, human autopsied brain studies indicate that the epigenetic marks at the DNA and histone level are modulated in relation to numerous insults associated with AD[226,227].

MOUSE MODELS RELATED TO ALZHEIMER'S DISEASE

The definite path of the disease mechanism of AD is still not fully understood. This is because all the molecular mechanisms are not always accessible for studying purposes in living humans. In this regard, the development of animal models has been a research priority to understand the pathogenesis and to test therapeutic strategies.

Table 1: Characteristics and neuropathological features of several transgenic mouse models for AD

Name	*Transgene*	*Aß dep.*	*NFT*	*Neuron loss*	*Gliosis*	*Reference*
APPswe	APP695 (K670N/M671L)	9 months	No (but AT8 IR)	No	Yes	[228]
hAChE-Tg	Overexpression of human AChE	No	No	Attenuated dendritic branching, reduced spine numbers	Not re-ported	[229]
hAChE-Tg //APPswe	Overexpression of human AChE + APP695 (K670N/M671L)	6 months	Not re-ported	Not reported	Yes	[230]
APP23	APP751 (K670N/M671L)	6 months	No (but AT8 IR)	Yes	Yes	[54]
PDAPP	APP695, 751 and 770 (V717F)	6-9 months	No (but AT8 IR)	No	Yes	[231]
TgAPParc	APP695 cDNA (E693Q)	4 months	Not re-ported	Not reported	Not re-ported	Ronnback, Zhu et al. 2011
TgArcSwe	APP695 cDNA (K670N/M671L, E693Q)	6 months	No (but AT8 IR)	Not reported	Not re-ported	[232]
APP/PS1KI	APP751 (K670N/M671L, V717I) + PS1 (M233T, L235P)	2.5 months	No	Yes	Yes	[233]
TAPP	APP695 (K670N/M671L) + tau (P301L)	6 months	9-10 months	Yes	Yes	[234]
3xTg-AD	APP695 (K670N/M671L) + PS1 (M146V) + tau (P301L)	6 months	15 months	Not reported	Yes	[84]
5xFAD	First transgene hAPP695 swe (K670N/M671L), Florida (I716V) and London (V717I); Second trans-gene: hPS1 (M146L and L286V) mutation	1.5-2 months		Yes	Yes	[235]

Among the animal models, mice offer several advantages in scientific research. They are small and easy to rear. Their progeny are abundant and they have very short gestation; furthermore, their size makes them manageable and, most notably, their whole genome has been mapped. The discovery of genes for FAD has allowed transgenic mouse models to be generated through the overexpression of the APP and/or presenilins harboring one or several mutations found in FAD. Although none of the transgenic mice reproduces the human condition exactly, they serve as important tools allowing for the study of similar pathological processes *in vivo,* which has provided valuable insights into disease mechanisms and opportunities to test therapeutic approaches. In addition, many of them develop amyloid pathology such as deposition of Aβ plaque and this feature depends on different factors such as single or multiple harboring mutations in APP or PS genes, promoter for driving over-expression of gene, and integration site and also the genetic background of the mice. In these mice, Aβ plaque appears ranging from 2.5 to 15 months of age. Conversely, studies on htau mice indicated that tau pathology might trigger an age-dependent learning impairment via disruption of synaptic function[236]. The main characteristics and neuropathological features of a number of transgenic mouse models are shown in Table 1. The transgenic mouse models of AD have also provided the key roles of soluble Aβ oligomers in the pathogenesis of disease as well as of the relationship between Aβ and tau pathologies. A significant number of studies of different AD mouse lines indicate that the onset and the severity of the Aβ deposits are directly linked to the level of soluble $A\beta_{1-42}$ peptide [84,96,237-240]. In addition, different studies of AD-related transgenic mice indicate that intraneuronal $A\beta_{1-42}$ triggers early neuronal loss as well as synaptic deficits[84,235] which is in agreement with the hypothesis that synaptic loss is one of the earliest events in AD pathogenesis[241,242]. Furthermore, evidence from AD transgenic mouse models supports the concept that Aβ may directly or indirectly interact with tau to accelerate NFT formation[234,243]. Altogether, AD transgenic models may allow to understand the molecular interactions of the disease pathogenesis *in vivo* as well as to evaluate potential therapeutic targets as well as to develop therapeutic strategies that might interfere or delay the disease progression.

THERAPEUTIC STRATEGIES OF ALZHEIMER'S DISEASE

The first clinically available and also present used treatment therapy in AD was based upon the cholinergic hypothesis . The advancement of understanding the complex molecular pathogenesis of AD, has resulted in a number of tentative therapeutic

interventions (Fig 2). However, AD is a complex multifactorial disease, therefore, the complete curative treatment is still a burning question.

Neurotransmitter therapy

The current available medications for AD include three different ChEIs and the NMDA receptor antagonist- memantine. Although they produce modest effect they also provide cognitive benefits to the patients.

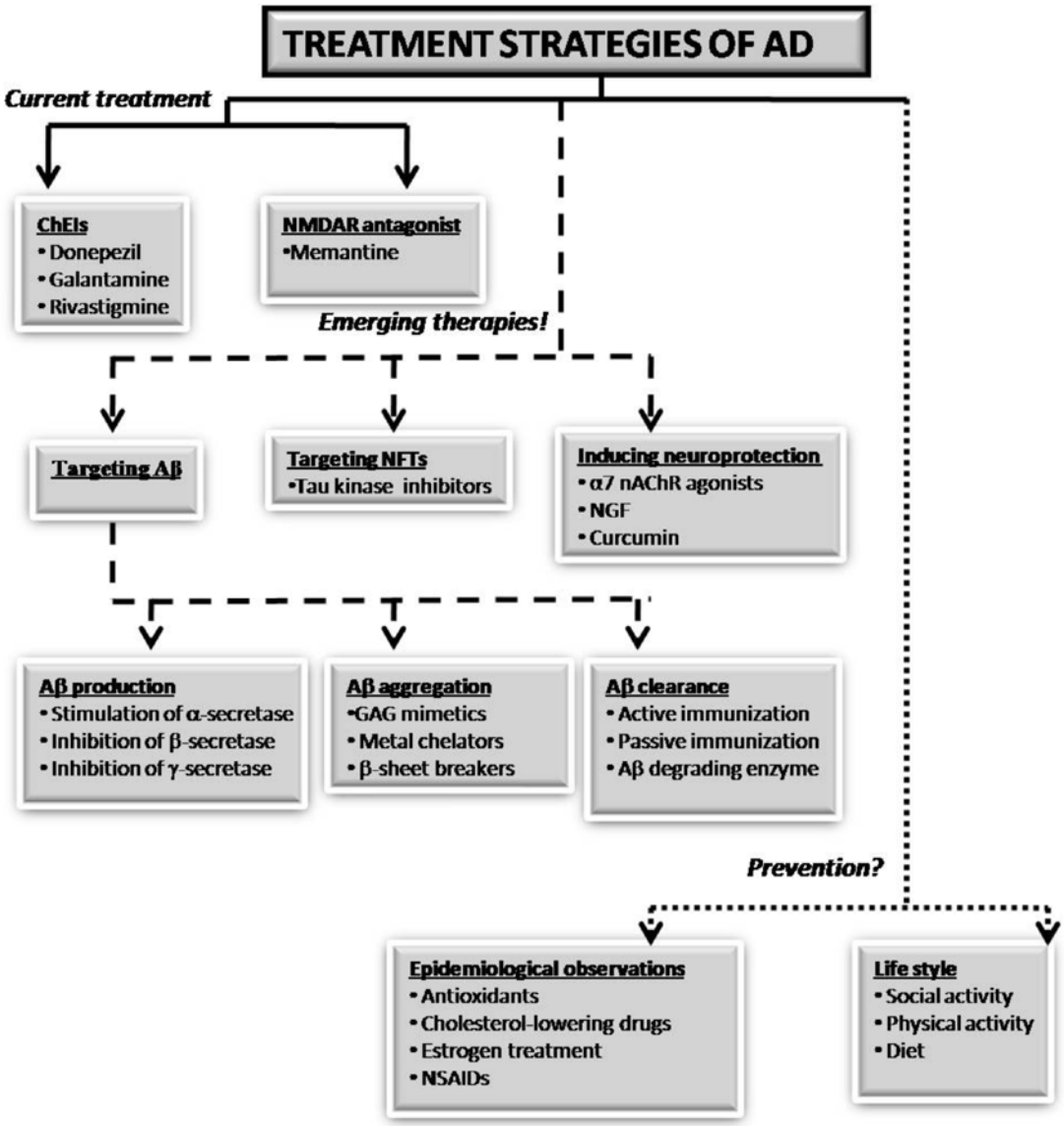

Figure 2. Schematic illustration of present and possible future therapies for AD

Cholinesterase inhibitors

Three ChEIs are available for treating mild to moderate AD patients (Fig 2). All these drugs inhibit AChE, a degrading enzyme of ACh, thereby increasing the concentration of ACh in the synaptic regions. This in turn, enhances and prolongs the actions of ACh to its receptors to improve cognitive function[155]. These drugs provide symptomatic improvement for several years but not prevent the disease progression. Several PET studies in patients treated with cholinesterase inhibitors have shown a significant correlation between cognition and cholinesterase activity, number of nicotinic receptors and cerebral glucose metabolisms[244-246].

N-methyl-D-aspartate receptor antagonist

Memantine is a specific non-competitive, moderate –affinity NMDA receptor antagonist[247,248]. According to different meta-analysis, memantine was shown to be effective in moderate to severe AD, slightly improving cognitive and global status but there was no improvement in function or behavior[249-251].

Targeting Aβ

The search of disease modifying interventions has focused largely on the compound targeting the Aβ pathway aiming to reduce Aβ load in the brain. There are several strategies targeting Aβ are under therapeutically evaluation. One such approach is to **inhibition of Aβ production** by inhibitors of β-secretase and γ-secretase. Pioglitazone[252] and rosiglitazone[253] drugs for diabetes mellitus could also down regulate β-secretase and APP. Semagacestat, a γ-secretase inhibitor has been studied extensively in AD trials [254]. However, these drugs have been discontinued after failure to demonstrate efficacy. Another approach to target **inhibition of Aβ aggregation** is by inhibiting Aβ assembly to Aβ oligomers and fibrils, which would prevent Aβ toxicity. Clioquinol (PBT-1)[255], PBT-2[256], glucosaminoglycans [tramiprosate (NC-531)][257], have all been suggested as possible therapies. **Removal of toxic aggregated Aβ deposits** from the brain thereby to attenuate the detrimental effects of AD is another therapeutic approach by using active or passive immunization. In 1999, Aβ immunization in PDAPP transgenic mice, prevented the plaque formation and astrogliosis as well as reduced the extent and progression of AD-like pathologies[258]. These preclinical observations led to the first clinical trial using active immunization with $A\beta_{1-42}$. However, the trial was halted due to serious adverse events of meningoencephalitis in

6% of treated patients[259]. Passive immunization with anti-Aβ monoclonal antibodies has also been tested in clinical trials. It is estimated that approximately 20 different antibodies for passive or active immunization are presently under clinical trials in AD patients. So far no clinical benefits on cognition have been reported although reduction in fibrillar Aβ plaques have been reported in brain by PET[260] as well as in post-mortem examinations[261].

Targeting tau

Inhibition of tau hyperphosphorylation and promotion of filament disassembly account two viable strategies for disease-modifying therapies [262]. In this regards, inhibitor of major tau phosphorylating kinase GSK3β and dissolving tau filaments by methylthioninium chloride are being considered. Neither lithium[263] nor valproate[264] was able to show significant improvements. This effort could be complicated due to the large number of tau phosphorylation sites, the unknown role of individual phosphorylation sites in disease pathogenesis and also the ability of multiple kinases to phosphorylate individual sites [265]. Recently an active immunization against the pathological phosphor-tau epitope phospho-Ser422 was performed in 3months old THY-Tau22 transgenic mouse model, which exhibit hippocampal NFT-like inclusions and display phosphorylation of tau on several AD-relevant tau epitopes. There was a decrease in insoluble tau species which correlated with a significant cognitive improvement in the Y-maze [266].

Other therapies

Different epidemiological studies of drugs have shown that the risk of AD is decreased by long-term treatment with cholesterol-lowering drugs [267], non-steroidal anti-inflammatory drugs (NSAIDs) [268] and estrogen[269]. However, none of these have drugs have been shown to treat AD patients. Different epidemiological studies of drugs have shown that the risk of AD is decreased by long-term treatment with cholesterol-lowering drugs [267], non-steroidal anti-inflammatory drugs (NSAIDs) [268] and estrogen[269]. However, none of these have drugs have been shown to treat AD patients. Several other epidemiological studies have also demonstrated that social activity, physically active people, and even consumption of Mediterranean diet have low risk to develop AD[270,271]. Therefore, in future there is possible to formulate new therapeutic preventive strategies.

Several treatment strategies focusing on neuroprotective mechanisms are of interest. As already discussed α7 nAChR may play a vital role in AD would be a potential therapeutic approach[272]. Regenerative mechanisms induced by drug or cell transplantation increasing neurogenesis might be tentative new treatment strategies in the future[273,274].

There are different other strategies for treating AD patients. Inducing neuroprotection would be one tentative approach. As discussed earlier that α7 nAChR play a vital role in AD and has been found within and around the plaque[50]. Experimental studies showed that α7nAChR agonists could reduce Aβ related toxicity thereby it would be a potential therapeutic approach.

Nerve growth factor (NGF) stimulates growth of cholinergic neurons and showed neuroprotection in different neurodegenerative disorders. NGF infusion[275] or NGF implantation[276] in nucleus basalis Meynert of mild AD patients improve might be a possible strategy to improve brain function.

AIM OF THE THESIS

The general aim of the present work was to study changes of Aβ isoforms in relation to different ages of transgenic mouse models and to gain further insight into how different Aβ isoforms influence the phosphorylation of tau, inflammatory processes and synaptic function how different cholinergic drugs could affect Aβ processing.

SPECIFIC GOALS:

- To elucidate different forms of Aβ and their effects on synapses, α7 nAChRs and NMDA receptors, as well as to evaluate whether these effects are mediated via the ERK/MAPK signaling pathway at different ages of APPswe mice (Paper I).

- To investigate the effects of Aβ, under the constant influence of AChE, on synapses, α7 nAChRs and glial cells in hAChE-Tg//APPswe double transgenic mice. (Paper II)

- To characterize the consequences of Aβ isoforms in relation to different ages of APPswe mice and to explore if these Aβ isoforms have any effect on phosphorylation of tau, GSK3β and CDK5 (Paper III)

- To examine the consequences of the excess of Aβ on epigenetic changes, especially the regulation of histones in APPswe mice, APPswe transfected SH-SY5Y cells and human post-mortem brain tissue from AD patients. (Paper IV)

- To study the effects of treatment with different cholinergic drugs on modulation of amyloid processing in SH-SY5Y cells transfected with APPswe mutation. (Paper V)

MATERIALS AND METHODS

Table 2: Schematic illustration of different materials and methods that have been used in the different papers.

Paper	Subject	Treatment	Parameter
I	• APPswe mice at ages 7, 21 and 90- days	NO	• Aβ ELISA • Synaptophysin (WB) • sAPPα (WB) • [^{125}I]α-bungarotoxin autoradiography
II	• hAChE-Tg/APPswe mice at ages 1, 3, 10-months used for baseline • 14- months of age used for treatment	• (+)-Nicotine • (-)-Nicotine	• Aβ and GFAP ELISA • GFAP (IH) • Synaptophysin (WB) • [^{125}I]α-bungarotoxin
III	• APPswe mice oat ages 7, 21, 90- day and 15-months	NO	• Aβ MALDI-TOF MS • Aβ oligomers (WB) • tTau & pTau (WB) • GSK3 and CDK5 (WB)
IV	• APPswe mice of 4-month-old • Postmortem brain from AD subjects • SH-SY5Y/APPswe cell line (treatment)	• DAPT, • NaB	• Aβ Mesoscale ELISA • TH3, AcH3, PH3, and 2MeH3 (WB) • AcH3 and NeuN (IH)
V	• SH-SY5Y/APPswe cell line • HEK293/APPswe cell line	• Nicotine • JN403 • Varenicline • Galantamine • (+)-Phenserine • (-)-Phenserine	• Aβ ELISA • sAPPα ELISA • sAPPβ ELISA

SUBJECTS

Animals (Paper I, II, III, IV)

All the housing and laboratory measures were followed according to the principles of Laboratory Animal Care (National Institutes of Health, publication 86-23, revised 1996). 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, and S43/07). All

mice that were bred, born in our own colony and 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 marked and weaned at 21 days and housed alone (male) or with 1–4 siblings of the same sex (female) 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 over-expressing 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 [228], 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). 7-day to 15- month old animals were used to carry out different experimental measures (see Table 2).

APPswe mice used in Paper IV were bred and studied at the University of Birmingham, AL, USA.

hAChE-Tg//APPswe mice (Paper II)

To breed a colony of double transgenic(hAChE-Tg//APPswe) mice, male APPswe transgenic mice and female FVB/N mice carrying human AChE cDNA [229](hAChE-Tg mice: two females were kindly provided by Prof. Hermona Soreq, were used to breed our own colony of hAChE-Tg mice) were used. The control for this transgenic line was used as offspring of C57B6 mice crossed with FVB/N mice (Bomice & Mollegaard Breeding Laboratories, Ejby, Denmark).

Cell culture (Paper IV and V)

Human neuroblastoma SH-SY5Y cells and human embryonic kidney HEK293 cells stably transfected with human APP Swedish KM670/671NL double mutations were kind gift from Dr. Erikur Benedikz[277,278]. The cells were cultured in RPMI 1640 + Glutamax I media supplemented with 10% fetal calf serum and 0.05% gentamycin. The cells were grown in 5% CO_2 humidified incubator at 37^{o}C.

Human postmortem brain tissue (Paper iV)

Brain tissues of both AD and control subjects were obtained from the Netherland Brain Bank (NBB) (Amsterdam, the Netherlands). The clinical diagnosis of dementia was performed according to NINCDS-ADRDA criteria [39] and its severity was estimated according to the Global Deterioration Scale [279]. For selecting the control subjects, all other neurological or psychiatric disorders were excluded.

DRUG TREATMENTS (PAPERS II, IV AND V)

The two enantiomers of nicotine (Paper II)

Fourteen-month-old hAChE-Tg//APPswe mice and non-transgenic control mice were included to the treatment with L(-)-nicotine or D(+)-nicotine twice daily (at 8:00–9:00 and at 16:00-17:00) (s.c.) 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.

LY-374973 and Sodium Butyrate (paper IV)

LY-374973, N-[N-(3,5-difluorophenacetyl)-L-alanyl]-sphenylglycinet-butyl ester (DAPT) was prepared in DMSO (vehicle) at a concentration of 100 μM and treated for 16 h. Sodium butyrate (NaB) was prepared in ultra-pure H2O (vehicle) at a concentration of 400 μM and treated for 4 h. Human neuroblastoma SH-SY5Y/APPswe and wild-type cells were used for both drug treatments.

Cholinergic substances (paper V)

HEK293/APPswe and SH-SY5Y/APPswe cells were used to treat with nicotine, galantamine, (-)-phenserine, (+)-phenserine, JN403 and varenicline for 72 h at serial concentrations ranging from 10^{-9} to 10^{-5}M. The drugs were prepared in ultra-pure H2O (vehicle) at a concentration of 10^{-3}M and further diluted in complete media for the treatment purpose.

EXPERIMENTAL METHODS

ELISAs

Levels of $A\beta_{1–40}$ and $A\beta_{1-42}$ (Papers I, II, III and IV)

Homogenates were prepared from the dissected cortices with ice-cold homogenate buffer in 1:7 volumes (w/v) of 20 mM Tris-HCl buffer (pH 8.5) containing protease

inhibitor cocktail (Roche, Basel, Switzerland) (Papers I and II). The brain 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 inhibitor cocktail (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.

Dissected brain cortices were homogenized in ice-cold homogenate buffer [containing 50 mM Tris, 1 mM EGTA and EDTA, protease inhibitor cocktail (Sigma), phosphatase inhibitor 1 and 2 (Sigma), sodium butyrate and PMSF] and centrifuged at 16,000 *g* at 4 ° C for 30 min. The supernatants were used as Tris-extracted Aβ fraction (Paper III).

The media from drug as well as vehicle-treated HEK293/APPswe and SH-SY5Y/APPswe cells were used for measuring the amount of releasing $A\beta_{1–40}$ and only SH-SY5Y/APPswe cells were used for measuring $A\beta_{1–42}$ (Paper V).

The levels of $A\beta_{1–40}$ and $A\beta_{1–42}$ 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 $A\beta_{1–40}$ and $A\beta_{1–42}$ were calculated by comparison with standard curves of synthetic human $A\beta_{1–40}$ and $A\beta_{1–42}$, respectively.

GFAP immunoreactivity (Paper II)

For measurement of GFAP immunoreactivity, hippocampal tissue from mouse brain was homogenized in 1% sodium dodecyl sulfate (SDS). A DC protein assay kit (Bio-Rad, 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 ab-

sorption at 405 nm was measured and expressed as GFAP immunoreactivity/mg total protein

sAPPα and sAPPβ release (Paper V)

Secreted forms of APPα and APPβ from the media of drug-treated SH-SY5Y/APPswe cells were measured using highly sensitive human sAPPα and human sAPPβ-swe colorimetric sandwich ELISA kits (Immuno-Biological Laboratories Co., Ltd. Fujioka, Gunma, Japan). The experiments were performed according to the manufacturer's protocol. Samples were diluted 4X with EIA buffer (included in the assay kit) and plated in duplicates. All the samples were analyzed in the linear range of the standard curve of the ELISA.

Mesoscale ELISA (Paper IV)

Three different species of Aβ: 1-38, 1-40 and 1-42 were measured in media using a MULTI-SPOT human (6E10) Aβ Triplex Assay kit (Mesoscale Discovery, Gaithersburg, Maryland, USA) according to the manufacturer's instruction.

Immunoprecipitation and Mass Spectrometry (Paper III)

The dissected brain cortices (30–80 mg) were homogenized in ice with 1:5 volumes (w/v) Tris-buffered saline (20 mM Tris, 137 mM NaCl, pH 7.6) containing complete protease inhibitor cocktail (Roche, Basel, Switzerland). Formic acid (FA) was added to the sample (final concentration 70%) followed by sonication (power: 15 amplitude microns; tune: 'middle') and centrifugation at 30,000 *g* for 1 h at 4 ° C. The FA-soluble Aβ extract was dried and dissolved in FA, and finally neutralized using 0.5 M Tris. Immunoprecipitation (IP) using the KingFisher magnetic particle processor (Thermo- Scientific, Waltman, Mass., USA) and mass spectrometric analysis using MALDI-TOF MS were performed. An aliquot (8 µg) of the anti-Aβ antibodies 6E10 and 4G8 (Signet Laboratories, Dedham, Mass., USA), which is reactive to amino acids 1–17 and 17–24, respectively, was separately added to 50 µl each of magnetic Dynabeads M-280 Sheep Anti-Mouse IgG (Invitrogen, Carlsbad, Calif., USA). The 6E10 and 4G8 antibody coated beads were mixed and added to 5–10 µl brain homogenates, and diluted to 1 ml in 0.025% Tween 20 in PBS (pH 7.4). After washing by using the KingFisher magnetic particle processor, the Aβ isoforms were eluted using 100 µl 0.5% FA. MALDI-TOF MS measurements were performed using an Autoflex-instrument (Bruker Daltonics, Bremen, Germany) operating in linear mode at 19 kV

acceleration voltages. Each spectrum represents an average of 1,500 shots acquired 75 at a time. The MALDI samples were prepared with the seed layer method using α-cyano-4-hydroxycinnamic acid as the matrix. Each isoform in the spectra were normalized to the sum of all isoforms in the spectra. This kind of data shows the relative changes of all the different isoforms in response to age.

Immunoblotting (Paper I, II, III and IV)

Tissue preparation for APP, Synaptophysin, ERK (Paper I)

Homogenates of brain cortical tissue were prepared in ice-cold buffer of 20 mM Tris-HCl (pH 8.5), containing protease inhibitor cocktail (Roche, Basel, Switzerland) (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 were 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.

Tissue preparation for tau and tau-related kinases (Paper III)

Dissected brain cortices were homogenized in ice-cold homogenate buffer [containing 50 mM Tris, 1 mM EGTA and EDTA, protease inhibitor cocktail (Sigma), phosphatase inhibitor 1 and 2 (Sigma), sodium butyrate and PMSF] and centrifuged at 16,000 *g* at 4 ° C for 30 min. The supernatants were used as cytosolic fraction (Paper III).

Tissue preparation for histone H3 and AcH3, PH3, and 2MeH3 (Paper IV)

All procedures were performed at 4° C unless otherwise indicated. Using Dounce homogenizers, brain tissue from APPswe and control mice as well as human or cells were homogenized in 50 mM Tris (pH 7.5) containing 250mM sucrose, 25mM KCl, 0.5mM phenylmethylsulfonyl fluoride, 0.9 mM NaB and protease and phosphatase inhibitors (Sigma). Subsequently, the combined nuclear and membrane fraction was pelleted by centrifugation at 7,700 x *g* for 1 min. The histone fraction was acid-extracted from the pellet by re-suspension and incubation in 0.4 N H_2SO4 for 30 min. The re-suspension was centrifugation at 14,000 *g* for 10 min and the supernatant collected. Histone proteins were precipitated from the supernatant after adding 0.004% deoxycho-

late in trichloroacetic acid for 30 min. Precipitated histones were pelleted by centrifugation at 14,000 x *g* for 30 min, then washed with acidified acetone (0.1% HCl) followed by 100% acetone in the same manner. The crude histone pellet was res-uspended in 50 mM Tris (pH 8.0) and total protein concentration was quantified using the Bradford assay.

Experimental procedure for immunoblotting

Standard procedure was carried out for the experimental purpose. Briefly, extracted proteins were size-fractionated by SDS-PAGE in the corresponding gel type (see Table 3). After electrophoresis, (1) PVDF membranes were processed and (2) blocked either in 3-5% serum albumin (BSA) or non-fat dried milk dissolved in TBS-T solution (3) incubated with primary antibody either for 1 h at RT or overnight at 4ºC (Table 3) (6) incubated with secondary antibody (4) exposed in ECL Plus reagents, (5) exposed to film (6) developed.

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 (Paper III and IV). As regards, phospho-ERK1/2, phospho tau, phosphor-GSK3β and CDK5 band intensity were first normalized to the band intensity detected with corresponding total protein, and then all samples were standardized to the pooled sample (paper I, II) or β-actin (paper III) allowing comparison between groups.

Table 3: Gel types and antibodies used for immunoblotting assay in different papers

Paper	Gel type	Antibody	Epitope(s)	Dilution	Source
I	12-well 4–20% gradient	22C11 6E10 Synaptophysin Total ERK Phospho ERK	Total sAPP Human sAPPα $Thr^{202/Tyr204}$	1:1000 1:500 1:2000 1:1000 1:3000	Chemicon Chemicon DakoCytomation BioSource Cell Signalling
II	12-well 10% Tris-HCl mini gel	Synaptophysin		1:2000	DakoCytomation
III	26-well 10–20% Tris-HCl Criterion gels	Tau-5 MmAb AT8 MmAb PS396 MmAb PS404 Poly PS422 Poly GSK3 MmAb P-GSK3β Poly T-216 Poly Cdk5 Poly P35/25 RmAb β-Actin 82E1 MmAb NU-4 MmAb	Total tau $P\text{-}Ser^{199/202}Thr^{205}$ $P\text{-}Ser^{396}$ $P\text{-}Ser^{404}$ $P\text{-}Ser^{422}$ GSK3α and GSK3β P-GSK3β Ser^{9} P-GSK3α PY^{279}/βPY^{216} Total Cdk5 Total p35 and p25 Total β –actin Aβ1–16 (N-terminal end specific) Aβ oligomers 12- to 24-mers (not monomers)	1:1000 1:1000 1:1000 1:1000 1:1000 1:500 1:1000 1:1000 1:1000 1:1000 1:2000 1:1000 1:1000	BioSource Pierce Invitrogen Invitrogen Invitrogen Invitrogen Sigma Aldrich Cell Signalling BioSource Cell Signalling Cell Signalling Abcam IBL Gift from Drs. Klein and Lacor
IV	12-wells 15% Tris-HCl gels	Histone H3 Poly Acetyl H3 Poly Phospho H3 Poly 2MeH3 Poly	Total H3 $Acetyl\text{-}lys^{14}$ H3 $P\text{-}Ser^{10}$ H3 $Dimethyl\text{-}lys^{9}$ H3	1:1000 1:1000 1:1000 1:1000	Millipore Millipore Millipore Millipore
P = Phosphorylated; Poly = rabbit polyclonal antibody; MmAb = mouse monoclonal antibody; RmAb = rabbit monoclonal antibody					

Receptor autoradiography

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

All the experimental procedure was performed in RT. Briefly, (1) frozen brain sections (10 µm) were thawed (2) pre-incubation with binding buffer (50 mM Tris-HCl, 1 mg BSA/ml, pH 7.4) (3) incubated in the same buffer containing 2 nM (Paper I), 1.55 nM (characterization: Paper II), 1.94 nM (treatment: Paper II) [^{125}I]α-bungarotoxin (4) washed with 50 mM Tris-HCl buffer (5) rinsed in distilled water (6) dried (7) placed together with [^{125}I]microstandards on [^{3}H]-Hyperfilm and incubated for 8–29 days.

[^{3}H]MK-801 autoradiography (Paper I)

All the experimental procedure was performed in RT. Briefly, (1) frozen brain sections (10 µm) were thawed (2) pre-incubated with binding buffer (3) incubated with the same

buffer, containing 10 nM [^{3}H]MK-801 with 100 mM glutamic acid and 100 mM glycine (4) washed with binding buffer (6) rinsed in distilled water (7) dried, and (8) placed together with [^{3}H]microstandards on ^{3}H-Hyperfilm for 8 weeks.

Post-processing and image analysis

Receptor autoradiography films were developed in D-19 developer followed by fixing with Kodak fixer solution by manually.

The autoradiograms were either analyzed with a video camera (CCD-72: Dage-MTI, Michigan City, IN, USA) coupled to a Macintosh computer public domain NIH Image software (written by Wayne Rasband at the NIH, US) (Papers I), or first scanned by using a high-resolution scanner and then analyzed by using with Image J (Paper II).

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. Specific binding was calculated by subtracting non-specific binding from total binding.

Immunohistochemical staining (Papers II and IV)

Immunofluorescence labeling of Aβ and GFAP (Paper II)

All procedures were performed at 25° C (unless otherwise indicated). Immunofluorescence labeling of Aβ and GFAP was performed by (1) pre-treating frozen brain sections with concentrated formic acid (for Aβ) for 5 min (2) 1 h incubation with 5% normal goat serum (3) incubated 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 (4) incubated with Cy2-conjugated goat anti-mouse or Cy3-conjugated goat anti-rabbit secondary antibodies (1:200 each: Chemicon, Temecula, CA, USA) (5) double immunofluorescence labeling of Aβ and GFAP was performed by incubating the sections with a cocktail of the primary antibodies 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 labeling.

Immunofluorescence labeling of Acetylated histone H3and NeuN (Paper IV)

Whole brains of mice were collected and were processed for paraffin embedding. To localize acetylated histones to neurons, sections were processed as follows in PBS-based solutions and rinses: (1) (permeabilization) 0.1% Triton-X-100, 10 min, (2)

(blocking) 5% normal goat serum, 30 min, (3) anti-AcH3 and –NeuN (both 1:200), 48 hr, (4) biotinylated anti-mouse IgG_1 (1:250), 30 min, (5) avidin-biotin complex (1:50, Universal ABC kit, Vector Laboratories), 30 min, (6) streptavidin-AlexaFluor488 (1:200) and anti-rabbit AlexaFluor594 (1:400), 1 hr. Slides were coverslipped and nuclei counterstained with DAPI inclusion in mounting media (Vector Laboratories).

Image analysis

Images were generated by using an Anxiophot microscope (Carl Zeiss AG, Göttingen, Germany) equipped with a digital camera and a computerized imaging system. In Paper IV, images of AcH3 (red), NeuN (green), and DAPI (blue) staining were taken. ImageJ software was used and the ColocalizeRGB plugin (written by S. Caballero, http://grove.ufl.edu/~ksamn2/plugins.html#COLOC) to colocalize pixels in the three separate channels: red, green, and blue. The ratio of red-green (AcH3+NeuN) and red-blue (AcH3+DAPT) was compared in neocortical tissue of APPswe and non-transgenic mice to indicate dual labeling of cells by anti-AcH3 and –NeuN; other channel ratios were used to validate staining specificity. Values are presented as the mean % co-localization = (red-green)/(red-blue) obtained from 5 sections (3 images/section) for APPswe or non-transgenic mice.

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 II, III and Paper V one-way ANOVA followed by the Bonferroni/Dunnett's *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, III). One or two-way ANOVA followed by Dunnett's *post-hoc* and Student's t-test were used for analyzing immunoblotting of histone modification in SH-SY5Y cells and Student's t-test was used for the analysis data from brain tissue (APPswe mice or human) (Paper IV). One-way ANOVA followed by Bonferroni *Post-hoc* test were used in the drug treated and control SH-SY5Y/APPswe cells (Paper V).

RESULTS AND DISCUSSIONS

AGE-RELATED Aβ PATTERN IN APPswe AND hAChE-Tg//APPswe TRANSGENIC MOUSE MODELS

It is well known that the pathological changes seen in AD patients are ongoing for decades before symptoms become manifest. To follow the human pathology from very early lifetime to the onset of AD is quite unrealistic. In this regard, the development of transgenic mice provides a good tool to study the time course of biochemical and pathological processes from very early ages, which is inaccessible in humans. Most widely used mouse models express mutations in human APP, providing aggravated brain Aβ production. Along with mutations in APP, some models also express mutations in other AD-related proteins such as AChE, giving the opportunity to study even earlier Aβ pathology.

PRESENCE OF Aβ-PEPTIDES STUDIED FROM 7 DAYS TO 15-MONTHS OF APPswe MICE

The proportion of different forms of Aβ varied with different ages of APPswe transgenic mice. Interestingly detection of both soluble (Tris extracted) and

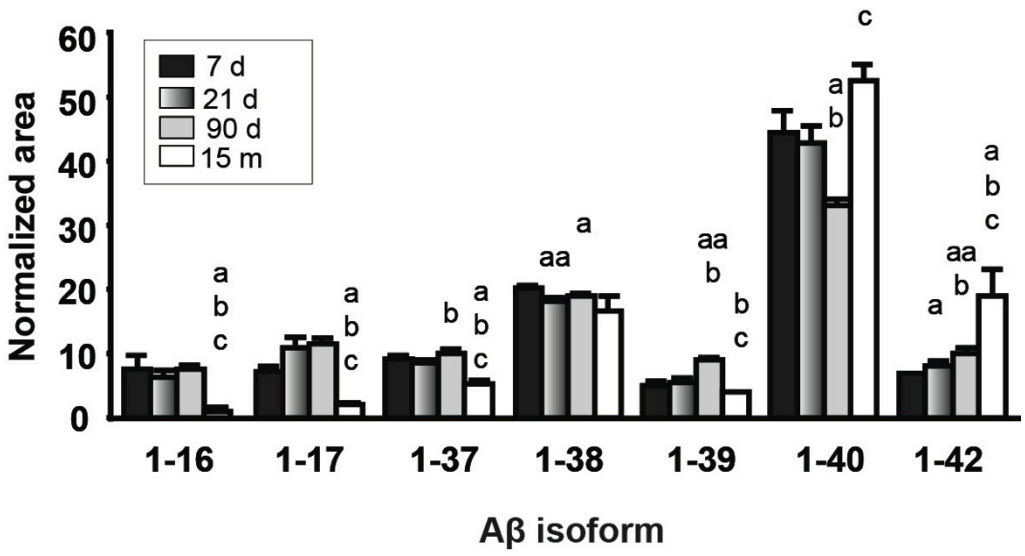

Figure 3. Levels of soluble Aβ isoforms in the cortices of APPswe mice at 7, 21, 90-days, and 15-months old age. Mass spectrometric signals for the detected Aβ isoforms are quantified in these groups of APPswe mice. [a] $P \leq 0.05$ and [a a] $P \leq 0.01$ compared with APPswe mice at 7-days of age; [b] $P \leq 0.05$ compared with APPswe mice at 21-days of age ; [c] $P \leq 0.05$ compared with APPswe mice at 90-days of age. Values are expressed as mean ±SEM.

insoluble (Guanidinium-extracted) Aβ isoforms at 7 days of APPswe mice, indicating that these mice produce Aβ very early life **(Paper I Table 1)**. APPswe mice younger than 15-months exhibited predominance of shorter peptides (**Fig. 3**). An age dependent increase of total levels of $A\beta_{1-42}$ was observed reflecting the plaques formation at older ages.

AGE-RELATED Aβ OLIGOMERS IN APPswe MICE

Studies have shown that the purified oligomers, but not the monomers play a critical role in the pathophysiology of AD[79] . In **Paper III**, oligomers were quantified starting from pentamers up to 26-mers as early as from 7-days-old APPswe mice where previous studies could detect dodecamer at 6 month-old APPswe mice[80,280]. This finding suggests that the dodecamer is present in this mouse model earlier than previously reported. The highest levels of Aβ oligomers were present at 90-days of age, which might reflect an age-dependent aggregation of Aβ into larger assemblies (**Fig. 4**). Furthermore, an interesting observation was that the levels of oligomers below 26-mers were lower in 15-month-old mice compared to 90-day-old mice, probably due to the increased fibrillization and aggregation in the older mice (**Fig. 4**).

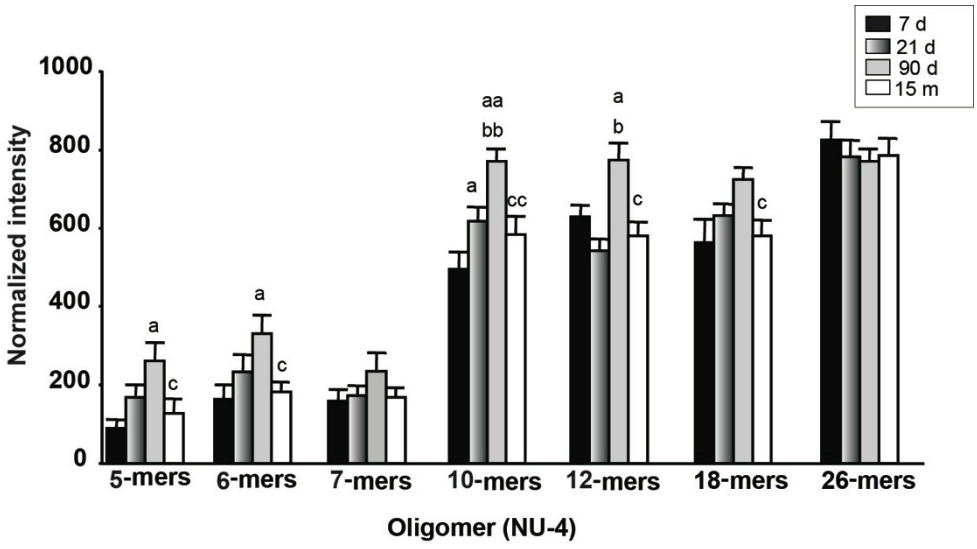

Figure 4: Levels of different oligomers (NU-4 antibody) in the cortices of 7, 21, 90-day, and 15-months-old APPswe mice. $^{a}P \leq 0.05$ and $^{aa}P \leq 0.01$ compared with APPswe mice at 7-days of age; $^{b}P \leq 0.05$ and $^{bb}P \leq 0.01$ compared with APPswe mice at 21-days of age ; $^{c}P \leq 0.05$ and $^{cc}P \leq 0.05$ compared with APPswe mice at 90-days of age. Values are expressed as mean ±SEM.

AGE RELATED EFFECT OF Aβ ON SYNAPTOPHYSIN IN APPswe MICE

Synapse loss is known as the best pathological correlate of cognitive impairment in AD [90,281]. Synaptophysin is a synaptic marker and its reduction indicates the loss of synapse. Both fibrillar and soluble Aβ oligomers cause synaptic loss[282,283]. Conversely, high APP and/or Aβ result increased synaptophysin levels [284-286]. On the contrary, synaptic activity has been shown to increase Aβ secretion *in vitro* and *in vivo*[189,287].

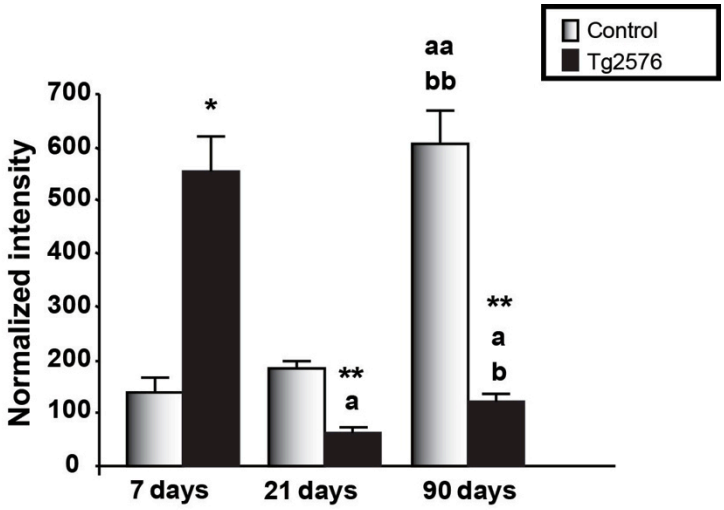

Figure 5. Levels of synaptophysin in the cortices of 7, 21 and 90-day old APPswe mice. * $P < 0.05$ and ** $P < 0.01$ compared with age-matched non-transgenic mice. [a] $P < 0.05$ and [aa] $P < 0.01$ compared with APPswe mice at 7-days of age. [b] $P < 0.05$ and [bb] $P < 0.01$ compared with APPswe mice at 21-days of age. Values are expressed as mean ± SEM.

In **paper I**, 7-day-old APPswe mice exhibited significantly higher levels of synaptophysin compared with non-transgenic controls (**Fig. 5**). A significant reduction in cortical synaptophysin levels were observed at 21 and 90- days compared with 7-days-old APPswe mice while there was an age dependent increase of synaptophysin levels in non-transgenic mice (**Fig. 5**). The findings of high levels of oligomers (**Fig 4**) and reduction of synaptophysin levels at 90-days of APPswe mice indicate that the oligomers are causing the reduction of synaptic function. As discussed earlier, 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. sAPPα is known to plays an important role in neurotrophic/neuroprotective functions and synaptogenesis[288,289]. The highest levels of total sAPP and human sAPPα were found at 90 days, and as expected not at 7 days of age, in the APPswe mice (**Paper I, Figs. 1 and 2, respectively**), there-

by supporting the hypothesis that soluble $A\beta_{1-40}$ contributed to the increased synaptophysin levels at 7-days of age.

EARLIER Aβ PLAQUE PATHOLOGY IN hACHE-TG//APPswe MICE

Most of the cortical AChE-S in AD brain was found associated with neuritic plaques[290], in which it co-localized with both diffuse and senile plaques[291,292]. When

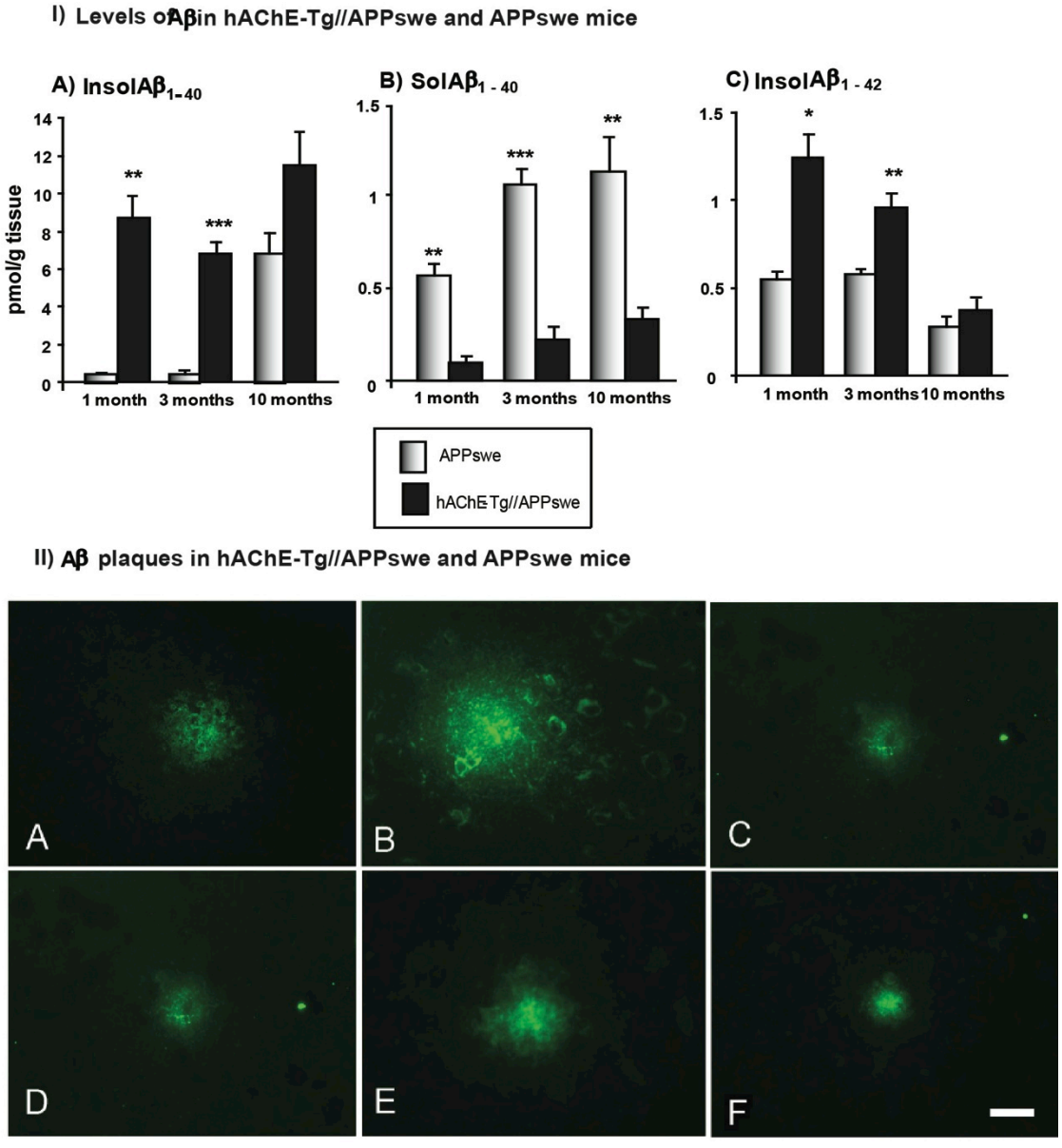

Figure 6. Aβ in the cortex of hAChE-Tg//APPswe and APPswe mice. I) Levels of Aβ in the cortices of 1, 3 and 10- month of hAChE-Tg//APPswe and APPswe mice. **A)** Insoluble $A\beta_{1-40}$ **B)** Soluble $A\beta_{1-40}$ **C)** Insoluble $A\beta_{1-42}$. *$P \leq 0.05$; ** $P \leq 0.01$; and *** $P \leq 0.001$*, compared with age-matched APPswe mice. Data are expressed as mean±SEM (pmol/g tissue). **II)** Aβ plaques in **(A)** cortex and **(D)** hippocampus of 7-month-old hAChE-Tg//APPswe mice. Plaques in the **(B)** cortex and hippocampus **(E)** of 10-month-old hAChE-Tg//APPswe mice. Plaques in the cortex **(C)** and hippocampus **(F)** of 10-month-old APPswe mice. Scale bar 30μm.

APPswe mice were crossed with the mice over-expressing human AChE-S (hAChE-Tg mice) (**Paper II**), the double transgenic hAChE-Tg//APPswe mice exhibited significantly increased levels of both insoluble $A\beta_{1–40}$ and $A\beta_{1–42}$ at 1 and 3-months of age while the levels of soluble $A\beta_{1–40}$ were decreased at 1, 3, and 10 months of age, compared with single transgenic APPswe mice **(Fig. 6I)**. In addition, Aβ deposition in the cortex and hippocampus of double transgenic hAChE-Tg//APPswe mice was evident already at 7- months of age (**Fig. 6II**), which is earlier than the onset of plaque pathology in APPswe mice, that starts at 9-months of age[293]. This finding is consistent with earlier studies that AChE-S may accelerate plaque formation[230]. Furthermore, the Aβ plaques appeared larger in 10-month-old hAChE-Tg//APPswe mice compared with the plaques in age-matched APPswe mice (**Fig. 6II)**, which also support this interpretation.

EFFECT OF Aβ ON α7 nAChRs AND NMDA RECEPTORS

Aβ aggregation and loss of nAChRs as well as NMDA receptors are well-known fea

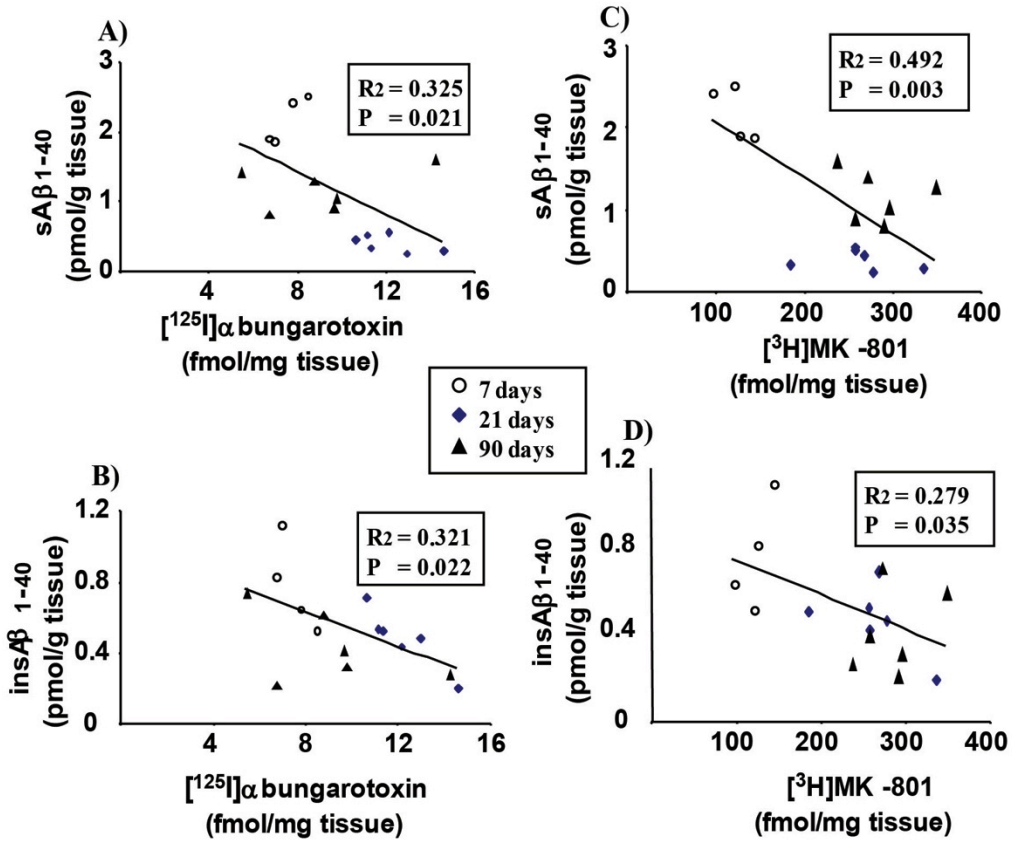

Figure 7. Correlation of $A\beta_{1-40}$ and [^{125}I]α-bungarotoxin or [^{3}H]MK-801 binding sites in the cortices of APPswe mice. Soluble $A\beta_{1-40}$ versus [^{125}I]α-bungarotoxin **(A)** and [^{3}H]MK-801**(C); i**nsoluble $A\beta_{1-40}$ versus [^{125}I]α-bungarotoxin **(B)** and [^{3}H]MK-801**(D)**. Each point represents data from one mouse**.**

tures of AD brain, and it is believed that these features are linked to each other. Studies suggested that α7 nAChRs play a pivotal role in Aβ pathology[166] while other studies showed that Aβ can reduce NMDA receptors [185]. Moreover, it has been reported that Aβ mediates and promotes NMDA receptor endocytosis possibly via the α7 nAChR [193]. Therefore, we investigated [^{125}I]α-bungarotoxin binding sites for α7 nAChR and [^{3}H]MK-801 for NMDA receptors at different ages of APPswe mice (**Paper I**). We found a significant negative correlation between Aβ and α7 nAChR as well as Aβ and NMDA receptors, suggesting that high levels of Aβ decrease the number of α7 nAChR as well as NMDA receptor binding sites (**Fig. 7**).

EFFECT OF β-AMYLOID ON TAU PHOSPHORYLATION AT DIFFERENT AGES OF APPswe MICE

Hyperphosphorylation of tau is one of the key features of AD. The interaction of Aβ and tau phosphorylation remains controversial. However different Aβ species have been shown to induce tau hyperphosphorylation[106,133-136]. Recent studies suggest that the functional impacts of tau phosphorylation depend on the specific phosphorylation sites and the extent of phosphorylation[118,294]. In **Paper III**, we used the APPswe transgenic mice of different ages ranging from 7-days to 15- months (**Table 2**) to study whether increased Aβ causes tau phosphorylation of six epitopes (**Paper III, Table 1**). Surprisingly, we did not observed an increased phosphorylation at any of the age groups compared with the non-transgenic control (**Paper III, Figs. 3 a–d**). Instead, we found a reduction of overall tau phosphorylation in adult brain. The reason for this finding could be due to differences between mouse and human tau or indicate that Aβ alone is not sufficient to induce phosphorylation of tau.

On the other hand, we observed that the smaller oligomers all showed a significant positive correlation with tau phosphorylation at AT8 and Ser[396] (**Fig. 8**). There might be different plausible explanations for the temporal correlation of these isoforms on tau phosphorylation such as: (i) they might indicate a first sign of AD pathology; (iii) certain pathological features in the human brain might be missing in the mouse brain, and therefore the pathology cannot proceed and develop into hyperphosphorylation of tau; and (iv) there could be a developmental effect in the brain of these mice not normally found.

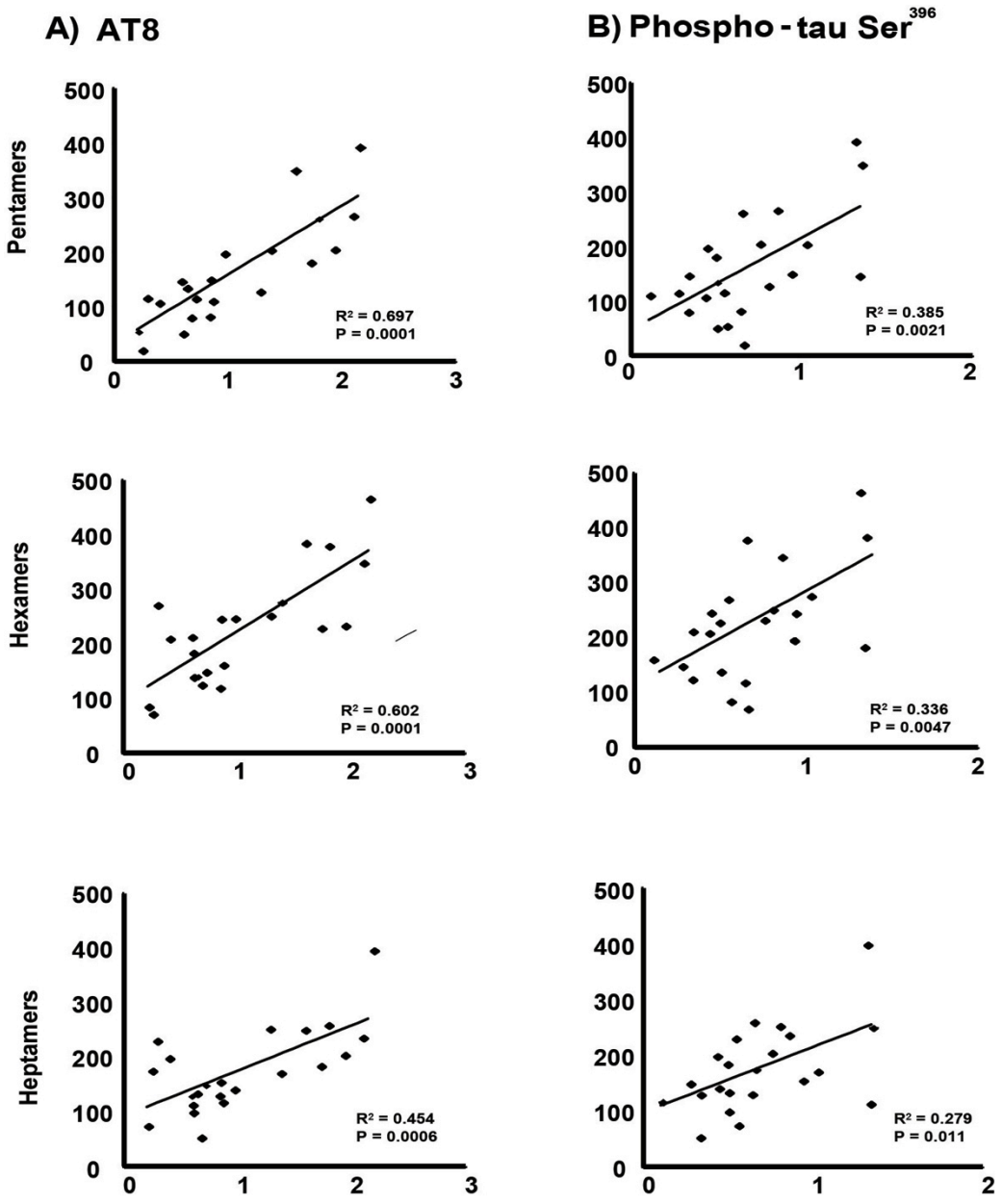

Figure 8. Correlation between small soluble Aβ oligomers and Tau phosphorylation in the cortices of APPswe mice at 7, 21, 90-days and 15-months of age. (**A**) AT8 and **(B)** Phospho-tau Ser 396 Simple regression analysis was used. Values are expressed as mean. Each point represents data from one mouse.

EFFECT OF β-AMYLOID ON DIFFERENT SIGNALING PATHWAYS

The signal transduction is important to relay the external signals to the cell interior. When a signaling pathway is activated, it leads to a cascade of cellular responses. The extra cellular regulated protein kinase/mitogen-activated protein kinase ERK/MAPK pathway plays an important role in differentiation and early embryonic differentiation

[295], synaptic plasticity[296], cell survival [297] as well as neuroprotection [298]. Therefore, we investigated ERK/MAPK signaling in APPswe mice due to the involvement in neuroprotection (**Paper I**). Opposite to what we expected, a significant decrease in ERK2/MAPK activity was observed at 7-days of age, which may indicate that the effects of Aβ in the brain are not mediated via ERK/MAPK pathway (**Paper I, Fig. 4**). In addition, a significant increase in activity was observed in 21 and 90-day-old APPswe mice, suggesting chronic activation of this signal transduction cascade as a consequence of the increased Aβ burden (**Paper I, Fig. 4**), which is in agreement with a previous study where chronic activation of ERK/MAPK signal transduction cascade was a consequence of the increased Aβ burden[299].

GSK3β [300], CDK5[301] are the major kinases that mediate tau hyperphosphorylation. Studies also suggest complex links between Aβ, tau phosphorylation as well as with GSK3β and CDK5[302,303]. In **Paper III,** we investigated GSK3β and CDK5 in relation to phosphorylation of tau in APPswe mice of different ages (**Table 2**). A significantly increased levels of phosphorylated GSK3β (P-Ser[9] and Y216) was observed in 7-day-old APPswe mice compared with non-transgenic controls (**Paper III, Fig. 4**). There were no differences at any other time points, indicating that Aβ does not activate GSK3β to a greater extent in this mouse model.

Hyperactivation of CDK5 due to the conversion of p35 to p25 by the calcium dependent protease calpain during neurotoxicity also contributes to the pathological state of neurons. Therefore, we investigated CDK5, p35 as well as p25 (**Paper III**). We found significantly reduced levels of CDK5 in 7-days-old APPswe (**Paper III Fig. 5a**) and also decreased levels of p35 in 90-days-old APPswe mice compared to age-matched non-transgenic controls (**Paper III Fig. 5c**) while there was a reduction in p25 levels in 7-days-old APPswe mice compared to non-transgenic controls, followed by an increase at 15-months of age (**Paper III Fig. 5b**) This result of high levels of p25 at older ages might be due to the prolonged Aβ exposure of the cells, which is in agreement with the studies where the conversion of p35 to p25 occurs in the presence of various neuronal insults, such as excitotoxic glutamate, hydrogen superoxide H_2O_2 and Aβ peptides [304].

EFFECT OF Aβ ON HISTONE MODIFICATION

The regulation of gene expression in response to environmental factors may play an important role in the progression of AD pathology. Without changes to the DNA sequence, gene expression can be modulated by epigenetic mechanisms such as histone modifications[305]. It is known that histone modification by methylation and phosphorylation are increased in AD brain[226,227]. In **Paper IV** we investigated changes in the histone levels associated with soluble Aβ exposure *in vitro* in SH-SY5Y/APPswe cells and primary cortico-hippocampal neuronal cultures, and *in vivo* in APPswe and human cerebral cortex.

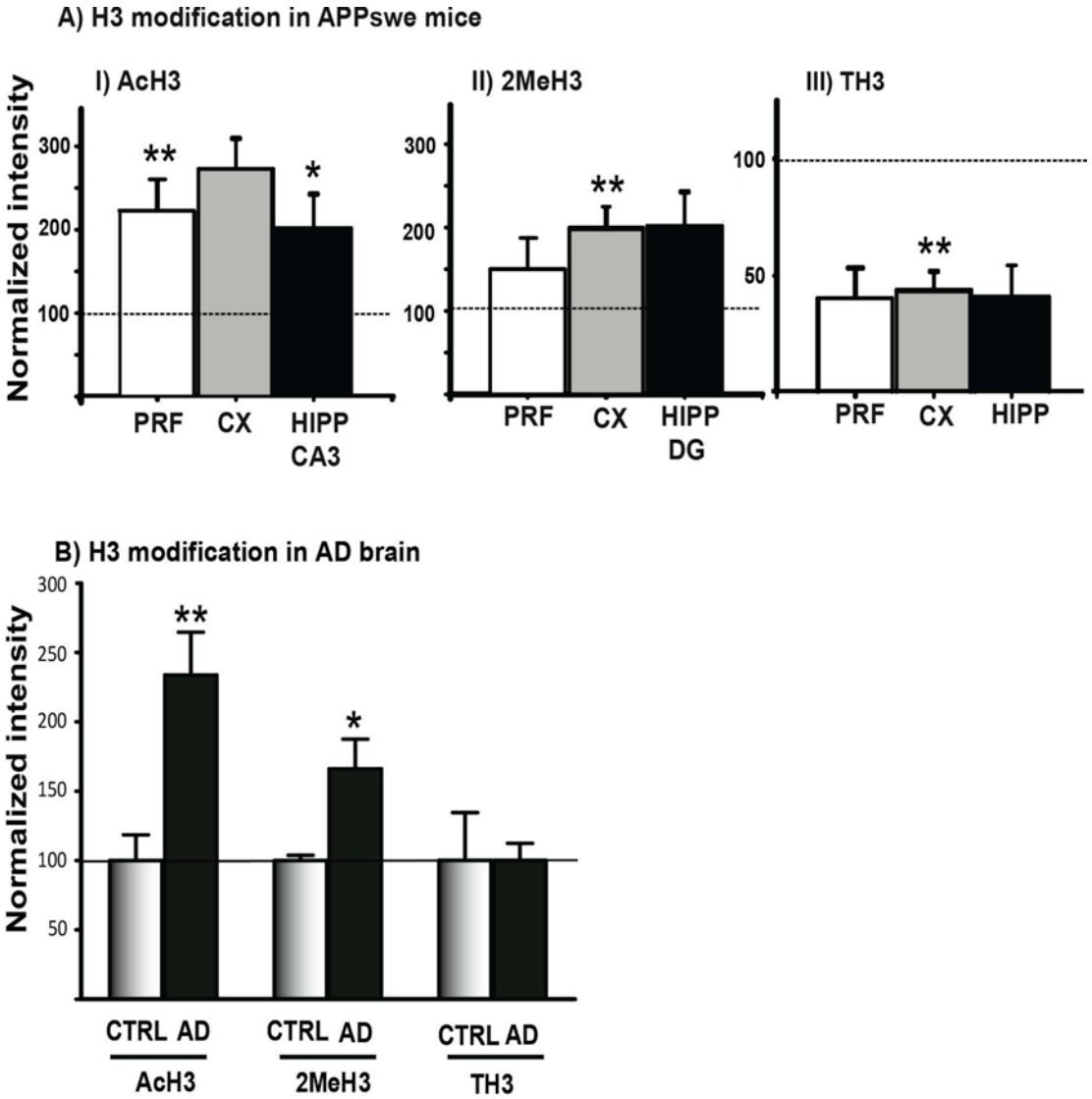

Figure 9. Modification of histone H3 in (A) 4-months-old APPswe at **(I)** Acetylate lysine 14 (ACH3) **(II)** Di-methylate lysine 9 H3, and (**III)** Total histone H3; and **(B)** AD brain. Values are expressed as mean ± SEM.

In APPswe mice, elevated levels of AcH3 and 2MeH3 were observed in the different brain regions compared to non-transgenic controls (**Fig. 9A I and II**) despite reduced TH3 (**Fig. 9A III)**. Consistent with these findings, AcH3 and 2MeH3 were significantly increased in the neocortical (occipital cortex) histone extracts in AD compared to non-demented, age-matched control subjects (**Fig. 9B**). As a conformation, DAPT treatment in SH-SY5Y/APPswe cells, a γ-secretase inhibitor showed a reduction of both $A\beta_{1\text{-}40}$ and AcH3. Besides, AcH3 (**Paper IV Fig 3C**) but not 2MeH3 (**Paper IV Fig. 3D**) were significantly increased with sodium-butyrate a histone de-acetylase inhibitor. These results suggest that acute and sustained Aβ exposure induced disrupted neocortical histone H3 homeostasis in cultures and APPswe brain, but may be sustained with chronic Aβ exposure as observed in end-stage AD neocortex.

EFFECTS OF CHOLINERGIC DRUG TREATMENT IN TRANSGENIC MOUSE MODEL AND SH-SY5Y/APPswe CELLS

Numerous studies suggested that cholinergic drugs might interact with the ongoing neuropathological processes beside their usual cholinergic effect. Disease modifying agent would be the therapy of choice for AD treatment. Inhibition of Aβ peptides or modulation of amyloid processing would be one of the major disease modifying targets for AD.

Effect of nicotine treatment in hAChE-Tg//APPswe mice

Studies have shown that both L(-) and D(+)-enantiomers of nicotine affect early stages of Aβ aggregation including delaying oligomerization and fibril formation, and thus reducing Aβ toxicity[306]. In addition, Aβ reduction was observed by chronic nicotine [L(-)-nicotine] treatment in 9-month-old APPswe mice (time of appearance of Aβ plaque)[155,307]. Opposite to these studies, in **Paper II** hAChE-Tg//APPswe mice of 14-months of age, treated with both L(-) and D(+)-nicotine for 10 days showed increased soluble $A\beta_{1\text{–}42}$ (**Fig. 10Ai**) and insoluble $A\beta_{1\text{–}40}$ (**Fig. 10Aii**) might be due to the over-expression of AChE in combination with Aβ pathology.

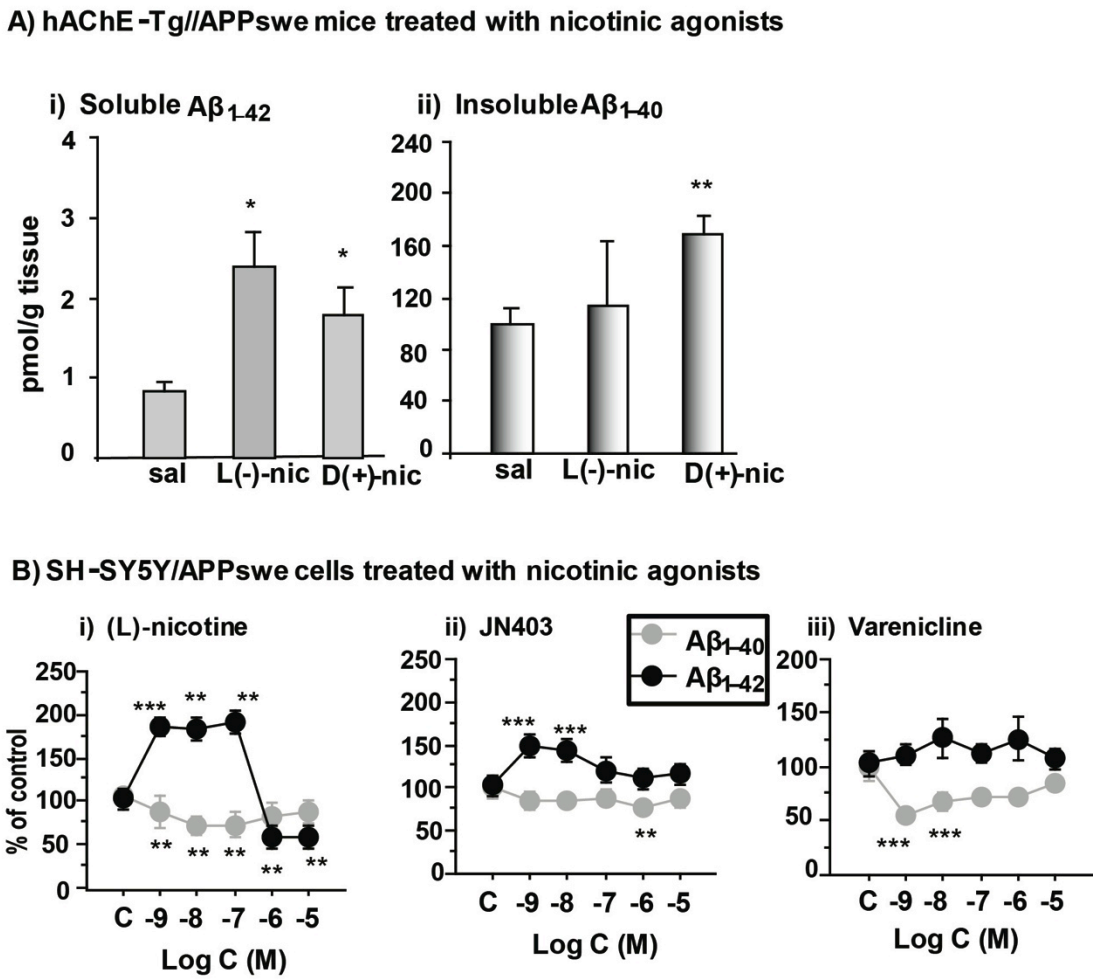

Figure 10. Levels of Aβ in (A) cortices of hAChE-Tg//APPswe mice treated nicotine: (i) Levels of soluble $A\beta_{1\text{-}40}$ (ii) Levels of soluble $A\beta_{1\text{-}42}$. **(B)** Release of soluble $A\beta_{1\text{-}40}$ and $A\beta_{1\text{-}42}$ from SH-SY5Y/APPswe cells treated with **(i)** Nicotine, **(ii)** JN403, and **(iii)** Varenicline. **P*≤0.05; ***P*≤0.01 and ****P*≤0.001, compared with untreated group. Values are expressed as mean ± SEM.

Effect of nicotinic agonists in SH-SY5Y/APPswe cells

A functional interaction of α7 nAChR–Aβ has been first described over a decade ago[166]; since then many studies have reported seemingly consequences of this function emphasizing a complex biology that underlies this interaction. Studies showed that α7 nAChR agonists provided neuroprotection against toxic effect of Aβ[308]. Therefore, it would be interesting to study α7 nAChR agonists on Aβ.

In **Paper V**, SH-SY5Y/APPswe cells were treated with nicotine as well as varenicline (a partial α7 nAChRs agonist) and JN403 (a pure α7 nAChRs agonist) for 72 hours. The release of $A\beta_{1\text{-}40}$ was significantly reduced by nicotine (F**ig. 10Bi**) and varenicline (F**ig. 10Biii)** while $A\beta_{1\text{-}42}$ were significantly increased by the treatment

with nicotine (F**ig. 10Bi**) and JN403 (F**ig. 10Bii**) compared to the control. The reduction of $A\beta_{1-40}$ release by nicotine and varenicline would be due to reducing the total $A\beta_{1-40}$ production or increase degradation while the result of increased release of $A\beta_{1-42}$ might be due to increase production or increase clearance of Aβ from the intracellular pool. However, complete mechanisms need to be understood to see the treatment effect in the intracellular aspect.

Effect of nicotine on APP

To investigate whether the selected nicotinic agonists could affect the amyloid processing in SH-SY5Y/APPswe cells, the amount of the release of sAPPα (non-amyloidogenic pathway) and sAPPβ (amyloidogenic pathway) were assessed. Varenicline reduced release of both sAPPα and sAPPβ compared to the untreated cells (**Paper V, Figs. 3C and 4C**). On the other hand, JN403 only reduced the sAPPβ levels (**Paper V Fig 4B**) while nicotine did not change either the sAPPα or sAPPβ (**Paper V, Figs. 3A and 4A**). Therefore, the results might indicate that different nicotinic agonists might have different mechanism of action.

Effect of nicotine on α7 nAChRs and astrocytes

It is well known that nicotinic agonists including nicotine , up-regulate the nAChRs, mainly the α4 nAChRs, while the α7 nAChRs are more resistant[155,293]. It has been suggested that there might be a functional interaction between α7 nAChR and Aβ [166]. Consistent with this hypothesis, we found an increased levels of α7 nAChR in the cortices and hippocampi of hAChE-Tg//APPswe transgenic mice treated with nicotine (**Paper II, Table 1**)- indicating a link to the elevated levels of Aβ in these mice. Furthermore, nicotine reduced the levels of GFAP- a marker for activated astrocytes in hAChE-Tg//APPswe mice, demonstrating the anti-inflammatory effect (**Fig. 11**). This is in agreement with the recent study where nicotine has been shown to reduce neuroinflammation [309] .

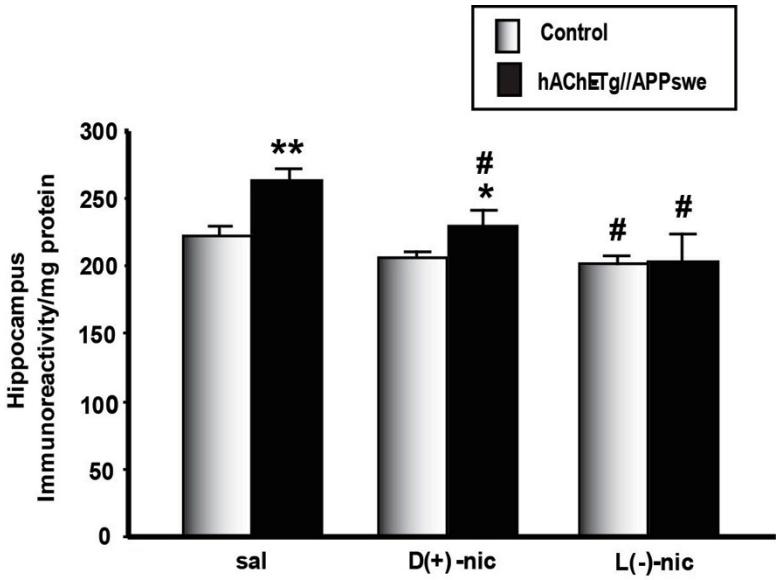

Figure 11. Expression of GFAP (in ELISA) in the hippocampus of hAChETg//APPswe and FVB/N//C57B6 control mice treated with saline, D(+)-nicotine, or L(-)-nicotine * $P \leq 0.05$; and ** $P \leq 0.01$, compared with control mice in the same treatment group. # $P \leq 0.05$, compared with saline-treated hAChE-Tg//APPswe mice or controls, respectively. Results are expressed as mean GFAP immunoreactivity/mg protein ±S.E.M.

Effect of ChEIs on amyloid processing

A number of studies have shown that ChEI treatment seems to be beneficial by having a neurotrophic effect[310], which also includes inducing neurogenesis in adult rodents[273,311] as well as long-term stabilization of neuronal functional activity in those brain regions where cognitive impairment is obvious in AD[246,312-314].

In **Paper V,** we treated SH-SY5Y/APPswe cells with galantamine as well as with both enantiomers (+) and (-)-phenserine. We observed that (-)-phenserine increased the release of $A\beta_{1-42}$ (**Paper V Fig. 2E**), but did not have any effect on sAPP. Whereas, (+)-phenserine, which is a weak ChE inhibitor and the anti-amyloid drug reduced $A\beta_{1-40}$ (**Paper V Fig. 1F**), as well as sAPPβ (**Paper V Fig. 4F**) indicating that the reduction of $A\beta_{1-40}$ was due to reduced levels of sAPPβ. These different findings of the two enantiomers of phenserine might have some other dissimilar properties, which include different regulatory mechanisms on amyloid processing. On the other hand, galantamine did not alter the release of Aβ levels while it reduced both sAPPα (**Paper V Fig. 3D**), and sAPPβ (**Paper V Fig. 4D**), indicating that galantamine interacts with APP processing.

CONCLUDING REMARKS

This work has been investigated the dynamic changes of different Aβ isoforms in relation to age in two types of AD-related transgenic mouse models and explored how these Aβ isoforms influence the synapses, cholinergic and glutamatergic receptors, phosphorylation of tau, inflammatory processes and how different cholinergic drugs affect Aβ and its processing. **Figure 12** contains a suggestion of the time course of Aβ and its influence on synapse in the AD-related transgenic mouse model.

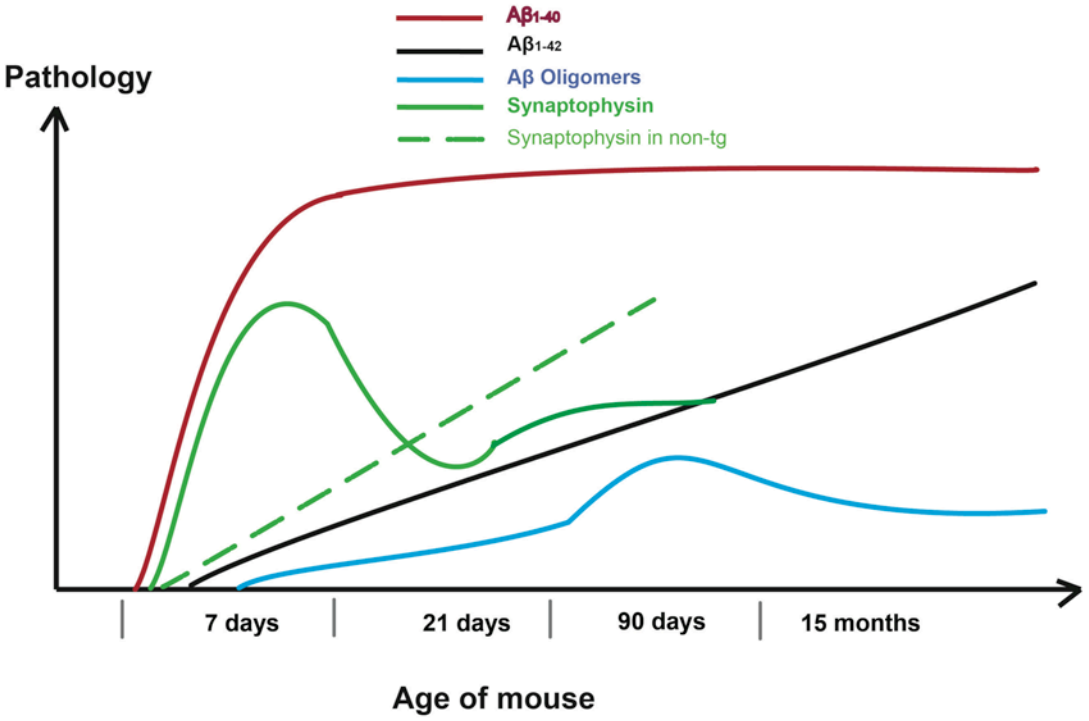

Figure 12: Illustration of the time course of different processes in APPswe transgenic mouse studied in this thesis.

From the main findings in this thesis the following conclusions can be drawn:

- The onset and consequences of Aβ pathology is exihited early in the brain of the different transgenic mouse models. The level of soluble $A\beta_{1–40}$ was the most abundant isoform in the cortices of APPswe mice. The levels remained fairly unchanged up to 15 months of age. There was an age-dependent shift in the Aβ pattern, where the brains of younger APPswe mice contained more of the shorter $A\beta_{1–16}$ and $A\beta_{1–17}$ peptide while the longer $A\beta_{1–42}$ (which is more prone to aggregate) increased with age.

- Oligomeric Aβ levels were highest at 90 days of age in the APPswe transgenic mouse model, making it a very interesting time point to study changes in other neuropathological hallmarks. The reduced levels of synaptophysin at that time point indicate that Aβ oligomers influence the number of synapses and thereby causing functional deficits in the brain.

- The findings of elevated acytylation and di-methylation of histone H3 in transgenic mice (as well as in postmortem AD brain) at the same age indicates that oligomeric Aβ causes changes in gene transcription in the cells of the brain. This might in turn lead to functional consequences and reduced cognitive performance.

- The different human Aβ isoforms did not directly increase tau phosphorylation in the APPswe mice. The reason for this finding could be due to the diffences in human and mouse tau or indicate that Aβ alone is not sufficient to induce phosphorylation of tau in this mouse model.

- Age-matched hAChE-Tg//APPswe mice displayed a different pattern of soluble and insoluble Aβ compared to the APPswe mice. This is probably due to the overexpression of AChE, leading to increased aggregation of Aβ and consequently earlier plaque formation. These also suggest that early intervention is needed to effectively reduce Aβ burden.

- The cholinergic drugs tested in this study all had effect on Aβ processing in SH-SH5Y/APPswe cells. Nicotine, the **α**7 nAChR agonist JN403 as well as (-)-phenserine showed increased release of Aβ probably due to a concurrent decrease of the intracellular Aβ pool. Moreover, reduction of sAPPβ by the anti-amyloid drug (+)-phenserine, the ChEI galantamine and JN403 as well as reduction of both sAPPα and sAPPβ by the nAChR agonist varenicline indicating that these substances modify the processing of amyloid.

In conclusion, to develop new therapeutic strategies it is important to understand the disease mechanisms. Therefore, this thesis has hopefully contributed valuble information in understanding the mechanisms of age-related Aβ pathology as well as provided suggestions of therapeutic targets.

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