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Interactive Mechanism Between β-Amyloid Process, α7 Nicotinic Receptor, Glial Cell Activation and Oxidative Stress in Alzheimer's Disease

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To my family with love

ABSTRACT

Alzheimer's disease (AD) is the most common form of dementia with pathological features including the accumulation of neuritic plaques (NPs) and neurofibrillary tangles (NFTs). These degenerative processes are also accompanied by impaired cholinergic transmission, oxidative stress, activated glia and inflammation reaction. Amyloid β (Aβ) is the major constituent of NPs and plays a causative role in the pathogenesis of AD. Increasing evidence suggests that the interactions between Aβ process and nicotinic receptors (nAChRs) may play an important role in the pathogenesis of AD.

The main purposes of this thesis were to investigate the potential neuropathological effects of Aβ on the cholinergic transmission, especially on neuronal nAChRs and the possible interactive mechanisms among Aβ process, nAChRs, and activated glia in the pathogenesis of AD.

The distribution changes of different nAChRs on astrocytes and neurons were investigated in the brains of subjects carrying the Swedish APP 670/671 mutation (APPswe) and sporadic AD. Significant increases of astrocytes expressing the α7 nAChR subunits, along with significant decreases in the levels of α7 and α4 nAChR subunits on neurons, were detected in both APPswe and sporadic AD brains. The increased expression of α7 nAChRs on astrocytes and the decreased expressions of α7, α4 nAChR subunits on neurons in the brain of APPswe were more pronounced in comparison to the sporadic AD brain. While the increased expression of astrocytic α7 nAChRs were positively correlated with the extent of neuropathological alternations. Furthermore, the astrocytic α7 nAChRs were morphological associated with amyloid plaques. The elevated expression of astrocytic α7 nAChR may participate in the Aβ cascade and the formation of NPs.

Aβ-induced oxidative stress was investigated as a possible mechanism for the deficits of neuronal nAChRs in both AD brains and the PC12 cells. It was found that lipid peroxidation induced directly by Aβ may be involved in the deficits in nAChRs in the PC12 cells. Similarly, a correlation between increased lipid peroxidation and the loss of α4 nAChR subunit was detected in AD brain. These findings suggested that lipid peroxidation stimulated by Aβ might be a mechanism for the loss of neuronal nAChRs in AD brain.

The possible interaction s among Aβ, acetylcholinesterase (AChE), activated glia and nAChRs were investigated in the brains of the double transgenic mice carrying the APPswe mutation and overexpressing the human AChE (hAChE-Tg//APPswe mice). Results showed that Aβ deposition occurred more early in the brain of the double transgenic mice compared to the single APPswe mice. These results confirmed that AChE might promote the deposition of Aβ *in vivo*. The increased expression of the α7 nAChRs and astrocytes in the brain of these mice probably participate in the Aβ cascade and the formation of amyloid plaques.

The developmental expression of different nAChRs were investigated in the brains of the α7 deficient (α7 -/-), α7 heterozygous null (α7 +/-) and α7 wild-type (α7 +/+) mice during the postnatal developmental period. Significant increases of the α4 and α3 nAChR subunits in binding, protein and mRNA levels were detected in the brains of α7 -/- and α7 +/- mice compared to α7 +/+ mice. The increased number of α4, α3-containing nAChRs, co-assembled with the α5 nAChR subunit, may compensate for the lack of or decrease in α7 nAChR and contribute to the normal brain development of α7 -/- and α7 +/- mice in brain.

In conclusion, these studies show that selective changes of nAChR subunits were found between neuron and astrocytes in AD brain. The decreased neuronal nAChRs might reflect the neurodegenerative condition of neurons in AD brain. The Aβ-induced lipid peroxidation was suggested as a possible mechanism of the deficits of neuronal nAChRs in AD brain. The selective increase of astrocytic α7 nAChRs and its morphological association with amyloid plaques might suggest that the astrocytic α7 nAChRs are involved in the Aβ cascade and the formation of NPs in AD brain. Based on the possible interaction between Aβ process and nAChRs, especially the α7 nAChRs, the α7 nAChRs might be a promising target for neuroprotective therapy in AD.

LIST OF PUBLICATIONS

This thesis is based upon the following publications, which are referred to in the text by their Roman numerals I-V:

I. **Yu WF***, Guan ZZ*, Bogdanovic N, Nordberg A. (2005) High selective expression of alpha7 nicotinic receptors on astrocytes in the brains of patients with sporadic Alzheimer's disease and patients carrying Swedish APP 670/671 mutation: a possible association with neuritic plaques. *Experimental Neurology 192: 215-225.*

These authors contributed equally to this work

II. Guan ZZ, **Yu WF**, Shan KR, Nordman T, Olsson J, Nordberg A. (2003) Loss of nicotinic receptors induced by beta-amyloid peptides in PC12 cells: possible mechanism involving lipid peroxidation. *Journal of Neuroscience Research 71: 397-406.*

III. **Yu WF**, Nordberg A, Ravid R, Guan ZZ. (2003) Correlation of oxidative stress and the loss of the nicotinic receptor alpha 4 subunit in the temporal cortex of patients with Alzheimer's disease. *Neuroscience Letters 338: 13-16.*

IV. Svedberg MM, Unger C, **Yu WF**, Mousavi M, Guan ZZ, Nordberg A. Consequences of increased soluble and insoluble β-amyloid levels at different ages in the brain of transgenic mice overexpressing both human acetylcholinestrase and the APPswe mutation. *Submitted.*

V. **Yu WF**, Guan ZZ, Nordberg A. Postnatal upregulation of α4 and α3 nicotinic receptor subunits in the brain of α7 nicotinic receptor-deficient mice. *Submitted.*

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CONTENTS

LIST OF ABBREVIATIONS

4-HNE	4-hydroxy-2-nonenal
5-HT	Serotonin
Aβ	β-Amyloid
ACh	Acetylcholine
AChE	Acetylcholinesterase
AD	Alzheimer's disease
AICD	APP intracellular domain
APOE	Apolipoprotein E
APP	Amyloid precursor protein
BACE	β-site cleaving enzyme
BuChE	Butyrylcholinesterase
CNS	Central nervous system
FAD	Familial Alzheimer's disease
GABA	γ-amino butyric acid
GFAP	Glial fibrillary acidic protein
Glu	Glutamine
mAChR	Muscarinic acetylcholine receptor
MDA	Malondialdehyde
mRNA	Messenger RNA
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5, diphenyl tetrazolium bromi
nAChRs	Nicotinic acetylcholine receptors
NFTs	Neurofibrillary tangles
NGF	Nerve growth factor
NMDA	N-metyl-D-aspartate
NPs	Neuritic plaques
PET	Positron emission tomography
PS	Presenilin
RT-PCR	Reverse transcription polymerase chain reaction
SAP	Serum amyloid P
TBARS	Thiobarbituric acid reactive substances

INTRODUCTION

ALZHEIMER'S DISEASE

Alzheimer's disease (AD) is a progressive neurodegenerative disorder clinically characterized by progressive memory loss and cognitive impairment. This neurodegenerative disease was first described by the German psychiatrist Alois Alzheimer nearly a century ago (Alzheimer et al. 1995). Today, AD is the most common form of dementia, representing 40-70% of all dementia cases and affecting approximately 5-10% of the population over 65 years of age and more than 20% over age 80 (Fratiglioni et al. 1999; Fratiglioni et al. 2000; Wimo et al. 2003). The common symptoms of this disease are short-term memory impairment and a general cognitive decline. The duration of this disease is typically 5-15 years. Clinical diagnosis of AD is based on a combination of several facotrs, including evaluation of the patient' s history, neurological and psychiatrical investigations, neuroimaging, and cognitive testing. Definitive AD can only be diagnosed after a postmortem neuropathological examination.

Risk factors

While the etiology of sporadic AD is still unknown, a number of risk factors for the development of AD have been identified. Increasing age is the greatest definite risk factor for the development of AD. Other risk factors suggested by epidemiological studies are genetics, female gender, head trauma, and hypertension (Launer et al. 1999; Munoz and Feldman 2000). In addition to the most common sporadic form, AD can appear in early-onset familial form (familial Alzheimer's disease, 'FAD'), suggesting that genetics play an important role in the etiology of this disease (Tanzi 1999).

Genetics in AD

The early-onset FAD has been showed to be associated with the gene mutations residing in the amyloid precursor protein (APP), presenilin 1 and 2 (PS1 and PS2) genes on chromosome 21, 14 and 1, respectively (Hardy 1997; Blacker and Tanzi 1998; Van Gassen and Annaert 2003). Meanwhile, the late-onset sporadic AD is associated with gene coding for apolipoprotein E (APOE) on chromosome 19 (Corder et al. 1993; Weiner et al. 1999). FAD mutations account only 1-5% of all AD cases and mutations in PS1 are the most frequent cause of FAD (Rogaeva et al. 2001).

APP

So far there are sixteen known mutations within the vicinity of the β-amyloid (Aβ) region on the APP gene (http://www.molgen.ua.ac.be/ADMutations/). These mutations include the APP 670/671 double Swedish mutation (Mullan et al. 1992), the APP692 Flemish mutation (Hendriks et al. 1992), the APP717 London mutation (Chartier-Harlin et al. 1991; Goate et al. 1991) and the APP693 Arctic mutation (Nilsberth et al. 2001). Due to their strategic localisation at the enzymatic cleavage sites of the β and γ-secretases, all known APP mutations alter APP metabolism and subsequently lead to an accumulation of

Aβ, which forms the major component of AD amyloid plaque deposits (Hardy 1996; Selkoe 2001).

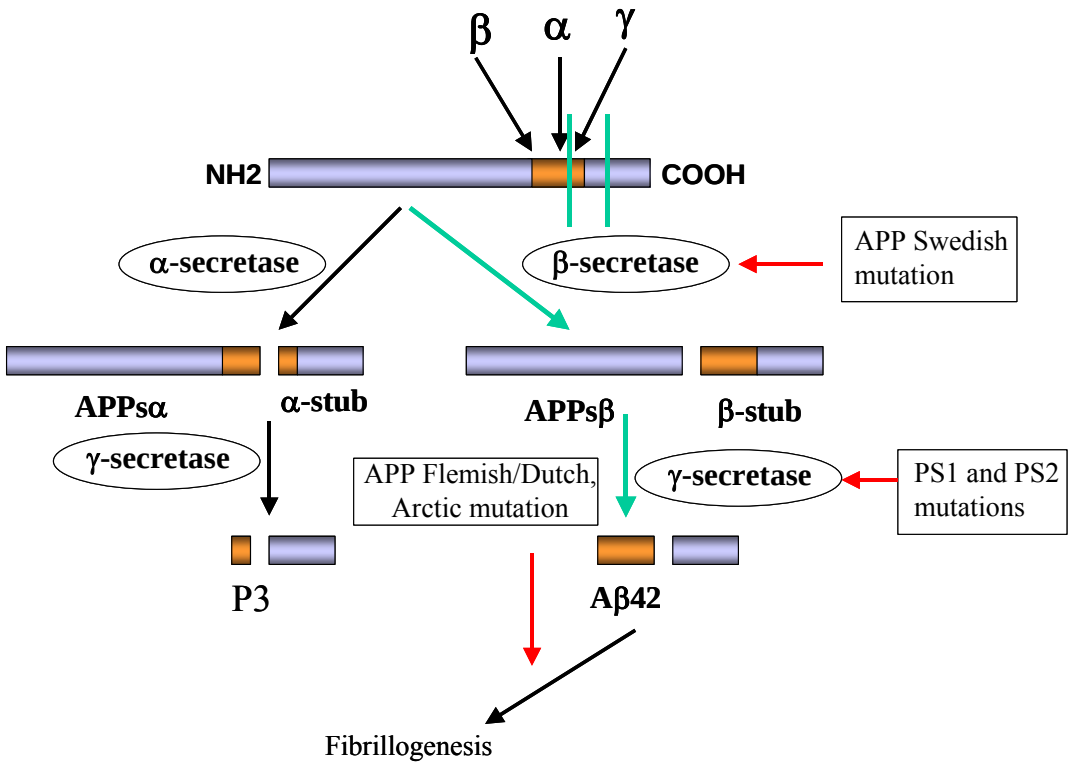


Figure 1. Position of several APP mutations and proteolytic cleavage of APP via amyloidogenic or non-amyloidogenic pathway.

Presenilin 1 and 2

The vast majority of all known familiar AD mutations have been found in the gene encoding PS1 (Schellenberg et al. 1992; Sherrington et al. 1995). To date, 146 mutations in PS1 on chromosome 14 have been reported to cause autosomal dominant FAD in several hundred families worldwide (http://www.molgen.ua.ac.be/ADMutations/). In contrast to PS1, very few mutations leading to AD have been found in the PS2 gene, with a later age of onset than PS1 mutation carriers (Renbaum and Levy-Lahad 1998). Mutations in both of these genes lead to the increased production of the more fibrillogenic Aβ1-42 by selectively increasing γ-secretase cleavage of APP (Lemere et al. 1996; Scheuner et al. 1996). PS1 mutations cause the earliest and most aggressive forms of AD, commonly leading to a clinical onset of AD before the age of 50 (Lippa et al. 2000).

APOE

APOE is a lipoprotein that plays a central role in lipoprotein metabolism and cholesterol homeostasis (Poirier 1994). APOE has three different isoforms, E2, E3 and E4 encoded by the three alleles of varying frequency: APOE ε2 7-8%, ε3 77-78% and ε4 14-16%. APOE ε4 is identified as a susceptibility gene for late-onset AD (Corder et al. 1993). The frequency of the APOE ε4 allele is increased in AD patients and a dose dependent risk for developing AD is observed with one or two copies of the ε4 allele (Corder et al. 1993; Strittmatter and Roses 1995). The mechanism(s) by which APOE ε4 allele contributes to AD pathogenesis is somewhat unclear. Several studies from knockout and transgenic mice

suggest that APOE ε4 facilitates the aggregation and deposition of Aβ (Bales et al. 1997; Holtzman et al. 2000).

Neuropathology

The pathological hallmarks of AD are extracellular neuritic plaques (NPs), intercellular neurofibrillary tangles (NFTs), neuronal cell loss and synaptic pathology (Braak and Braak 1997). NPs and NFTs are considered to be critical for the diagnosis of AD. The plaques are formed by fibrils of Aβ and can be divided into two forms: NPs and diffuse plaques in AD brain (Braak et al. 1998; Small and McLean 1999). NPs, the mature and classical plaques in AD brain, contain a compact extracellular deposit of amyloid fibrils and are surrounded by the dystrophic neuritis, reactive astrocytes and activated microglia (Itagaki et al. 1989; Stalder et al. 1999; Vehmas et al. 2003). Compared to the classical NPs, which represent only a minority of plaques, diffuse plaques are more common in the AD brain. Diffuse plaques have a more amorphous form of Aβ deposits and few or no reactive astrocytes and activated microglia can be seen surrounding these plaques. Diffuse plaques may be the early forms of plaques and the NPs are thought to evolve from them (Rozemuller et al. 1989). Evidence has shown that the formation of amyloid plaques is a primary event in the pathogenesis of AD (Braak et al. 1999; Morris and Price 2001). Both reactive astrocytes and activated microglia directly participate in the formation and morphological evaluation of amyloid plaques (Koenigsknecht and Landreth 2004; Nagele et al. 2004). Recently, direct imaging of amyloid plaques in AD brain by positron emission tomography (PET) has provided a new way to further understand the role of Aβ in AD brain (Klunk et al. 2004; Nordberg 2004).

NFTs are composed of hyperphosphorylated microtubule-associated tau protein (Grundke-Iqbal and Iqbal 1999). The abnormally hyperphosphorylated tau accumulates in neurons in the form of paired helical filament, twisted ribbons, and straight filaments (Iqbal et al. 2005). It has been demonstrated that the density of NFTs correlates well with the loss of cells and synapses which are also characteristic traits in the AD brain (Masters and Beyreuther 1995; Braak et al. 1999). The number of NFTs shows a consistent and strong correlation with dementia severity (Braak and Braak 1991; Arriagada et al. 1992; Bierer et al. 1995).

Aβ plays a causal role in the pathogenesis of AD

Generation of Aβ from APP requires two proteolytic processes by several different proteases (Seubert et al. 1993). Two major proteolytic processings of APP are outlined in Figure 1. The amyloidogenic pathway results in production of intact Aβ, whereas the non-amyloidogenic pathway precludes intact Aβ-formation. In the amyloidogenic pathway, APP is first cleaved at the β-secretase site by β-site cleaving enzyme (BACE), generating a soluble extracellular fragment of APP (sAPPβ) and a membrane-bound 99 amino acid residue C-terminal fragment (C99) (Hussain et al. 1999; Vassar et al. 1999). In the non-amyloidogenic pathway, the cleavage of APP by α-secretase leads to the generation of a soluble extracellular fragment of APP (sAPPα) and a membrane-bound 83 amino acid residue fragment (C83). C99 and C83 are further processed by cleavage at the γ-secretase site, located within the APP transmembrane domain, to generate Aβ and a short protein fragment referred as p3, respectively. γ -secretase cleavage of C83 leads to generation of Aβ-peptides of different lengths. Of the two main variants formed, Aβ1-40 is the most abundant

whereas Aβ1-42 is produced to a lesser extent. In addition to Aβ and p3, a soluble cytosolic fragment, referred to as APP intracellular domain (AICD), is generated. All AICD fragments analyzed to date have been shown to start at a position a few residues distal to the sites for γ-secretase cleavage (Sastre et al. 2001; Yu et al. 2001; Weidemann et al. 2002). This indicates that there is an additional cleavage site in APP, which has been termed the ε-cleavage site. The γ- and ε-secretase cleavages are closely linked to each other and may be performed by the same enzymatic activity (Chen et al. 2002; Schroeter et al. 2003). Pathogenic mutations in APP either affect the N-terminal (β-secretase) cleavage site of the Aβ domain in APP, leading to elevated levels of Aβ1-40 and Aβ1-42 (43), or affect the C-terminal (γ-secretyase) cleavage site, leading to elevated levels of Aβ1-42 (43) specifically (Goate et al. 1991; Mullan et al. 1992; Cai et al. 1993; Suzuki et al. 1994). Mutations in PS1 lead to the elevation of Aβ1-42 (43) only, by selectively increasing γ-secretase cleavage of APP (Lemere et al. 1996; Scheuner et al. 1996).

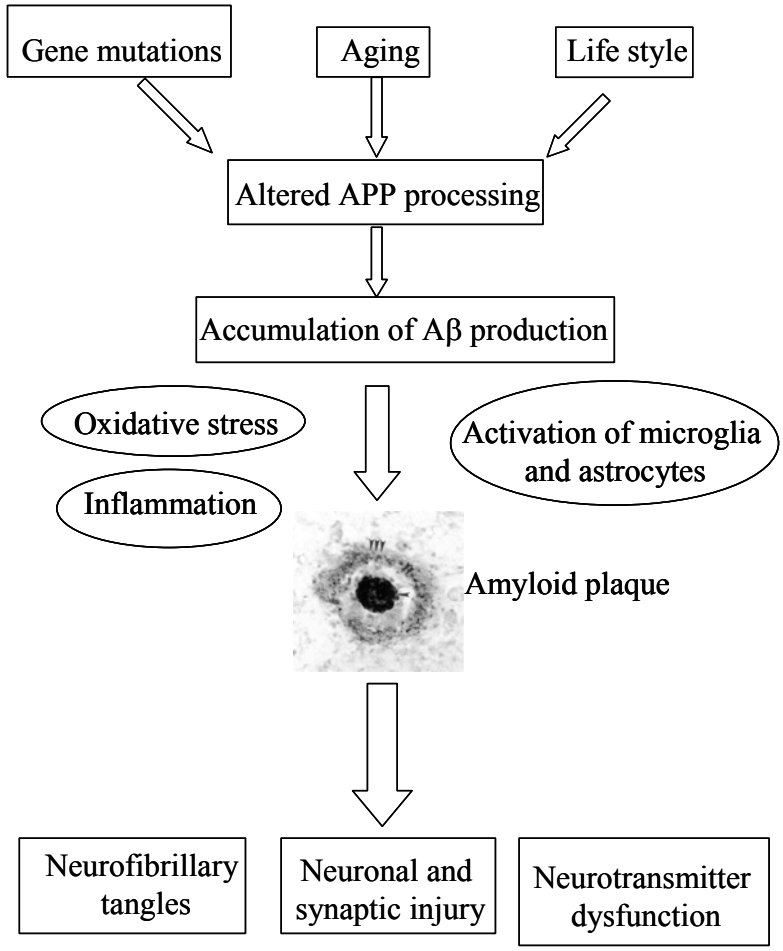


Figure 2. The amyloid cascade hypothesis of AD.

The amyloid cascade hypothesis has been one of the leading theories in the pathogenic events causing AD (Selkoe 1991; Hardy and Higgins 1992). This hypothesis postulates a causative role for Aβ deposition in the pathogenesis of AD, and the NFTs, inflammatory response, cell loss, vascular damage and dementia follow as a direct result of this deposition (Hardy and Selkoe 2002). Evidence that Aβ play a causal role in the development of AD came from the following studies. First, mutations in APP, PS1 and PS2 genes that are linked to the early onset forms of FAD increase the production of total Aβ (Citron et al. 1992; Cai et al. 1993) or specifically increase the relative amount of Aβ1-42 (Duff et al. 1996; Citron et

al. 1997; Tomita et al. 1997). Second, individuals with Down's syndrome overproduce Aβ and develop AD-like dementia and neuropathology (Olson and Shaw 1969). Third, the levels of deposited Aβ correlate with cognitive decline and severity of the disease in both AD patients and in transgenic mice (Hsiao et al. 1996; Naslund et al. 2000; Gordon et al. 2001). Fourth, fibrillar Aβ has been shown to be neurotoxic in vitro and is able to mediate neurotoxic effects, inflammatory responses and abnormal tau phosphorylation (Yankner et al. 1990; Akiyama et al. 2000; Lewis et al. 2001). Moreover, several reports suggest a causal link between Aβ and impaired neuronal function and cognitive decline (Chen et al. 2000; Janus et al. 2000b; Morgan et al. 2000). Recently, attention has been focused on which form (monomers, oligmers, protofibrils or fibrils) of amyloid species has the most deleterious effects. Several studies have suggested that the oligomeric and protofibrillar forms of amyloid as the most toxic (Lambert et al. 1998; Dahlgren et al. 2002; Walsh et al. 2002; Cleary et al. 2005).

Even if the amyloid cascade hypopthesis is convincing, it does not fully explain the role of tangles and/or inflammatory response (McGeer and McGeer 1998; Lee 2001). One argument against the amyloid cascade hypothesis is that the APP/PS1 double transgenic mice only develop plaques pathology, but not the NFTs in the brain (Janus et al. 2000a). Recently, amyloid deposition was shown to precede tangle formation in a triple transgenic mouse model overexpressing mutant APP and mutant tau on a PS1 mutation knock-in background (Oddo et al. 2003). Furthermore, the absence of plaque pathology in patients with tau mutations, causing frontotemporal lobe dementia, and the presence of both plaques and tangles in patients with APP mutations, causing AD, suggests that amyloid toxicity precedes tau in the pathological development of AD.

Oxidative stress in AD brain

The central nervous system (CNS) is especially vulnerable to free radical damage as a result of the brain's high oxygen consumption rate, its abundant lipid content and the relative paucity of antioxidant enzymes compared with other tissues (Coyle and Puttfarcken 1993). AD brain is under extensive oxidative stress as manifested by lipid peroxidation, protein oxidation, and DNA oxidation (Christen 2000; Butterfield et al. 2001; Butterfield and Lauderback 2002). Aβ might be the central to the pathogenesis of AD. Aβ has been shown to induce protein oxidation and lipid peroxidation in vitro and in vivo (Butterfield and Lauderback 2002). Many studies have indicated that Aβ-induced oxidative stress is involved in the pathogenesis of AD (Butterfield 1997; Butterfield et al. 2001).

Lipid peroxidation is an important mechanism of neurodegeneration in AD brain. Many studies have shown increased lipid peroxidation in several regions of AD brain, where the histopathologic alterations are very noticeable (Lovell et al. 1995; Marcus et al. 1998). It has been shown that there is a strong regional correlation between the thiobarbituric acid reactive substances (TBARS), one indicator of lipid peroxidation, antioxidant enzymes, the presence of NPs and NFT in AD brain (Lovell et al. 1995). Aβ is widely reported to cause lipid peroxidation in brain cell membranes in a manner that is inhibited by free radical antioxidants (Gridley et al. 1997; Bruce-Keller et al. 1998; Daniels et al. 1998; Mark et al. 1999). Aβ leads to an increased level of 4-hydroxy-2-nonenal (4-HNE), one of the major products of lipid peroxidation, in hippocampal and cortical neuronal cells (Mark et al. 1997). Increased 4-HNE was found in AD brain and it was proven to be toxic to hippocampal neuronal cells (Mark et al. 1997). 4-HNE can also increase the vulnerability of cultured

hippocampal neurons to excitotoxicity, as well as an alteration in multiple cellular functions including glucose or glutamate transport (Keller et al. 1997; Mark et al. 1997).

Protein oxidation is an important factor in ageing and age-related neurodegenerative disorders (Stadtman 1992; Berlett and Stadtman 1997; Butterfield and Boyd-Kimball 2004). Oxidative modification of proteins can lead to diminished specific protein functions, which may ultimately result in cell death (Stadtman 1992; Hensley et al. 1995; Dean et al. 1997). Protein oxidation is most often indexed by the presence of protein carbonyls (Stadtman 1992). Protein carbonyls arise from a direct free radical attack on vulnerable amino acid side chains or the protein backbone or from the products of glycation, glycoxidation, and lipid peroxidation reactions with proteins (e.g., 4-HNE and acrolein). In AD frontal lobe containing copious amounts of NPs and NFT, protein oxidation is significantly elevated compared to normal aged-matched controls (Smith et al. 1991). A significant increase in protein carbonyls was reported in the parietal lobe, and a trend towards elevated protein carbonyls was found in the frontal, temporal and occipital lobes and hippocampus in AD (Lyras et al. 1997). Increased protein carbonyl immunoreactivity in the hippocampus and superior middle temporal gyrus in AD brain were also reported (Aksenov et al. 2001).

Reactive astrocytes and activated microglia in AD brain

Several lines of evidence have proposed that inflammation may play a significant role in the pathogenesis of AD (McGeer and McGeer 1998; McGeer and McGeer 2001). According to the "inflammation hypothesis", the deposition of amyloid in AD brain brings about activation of microglia and astrocytes, initiating a proinflammatory cascade that results in the release of potentially neurotoxic substances, cytokines, and other related compounds, bringing about degenerative changes in neurons (Akiyama et al. 2000; Morgan et al. 2005). Reactive astrocytes and activated microglia are often found in and around amyloid plaques in the brains of AD patients and APP transgenic mice (McGeer and McGeer 1995; Frautschy et al. 1998). Activated astrocytes and microglia display dynamic complexity in the pathogenesis of AD (Minagar et al. 2002; Morgan et al. 2005). They contribute significantly to the inflammatory response in AD brain by secreting a range of immunological proteins such as cytokines, chemokines and complement pathway components (Benveniste 1997; Dong and Benveniste 2001). These inflammatory molecules contribute to the inflammatory processes and cause the degeneration of neurons (Johnstone et al. 1999; Aloisi 2001).

Furthermore, activated astrocytes and microglial may be involved in the formation, evolution of amyloid deposits (Koenigsknecht and Landreth 2004; Nagele et al. 2004). In response to local neurodegeneration in AD brain, reactive astrocytes are capable of accumulating substantial amounts of neuron-derived, Aβ1-42-positive material and other neuron-specific proteins. Immunohistochemical analyses have suggested that astrocytes overburdened with these internalized materials can eventually undergo lysis, and radial dispersal of their cytoplasmic contents, including Aβ1-42, can lead to the deposition of a persistent residue in the form of small, glial fibrillary acidic protein (GFAP)-rich, astrocytic amyloid plaques (Akiyama et al. 1999; Thal et al. 2000; Nagele et al. 2003). Microglia, most of which appear to be derived from blood monocytes and recruited from local blood vessels, rapidly migrate into and congregate within neuritic and dense-core plaques, but not diffuse plaques. Instead of internalizing and removing Aβ from plaques, microglia appear to contribute to their morphological and chemical evolution by facilitating the conversion of existing soluble and oligomeric Aβ within plaques to the fibrillar form.

The cholinergic neurotransmitter system in AD

The cholinergic neurotransmitter system is the most important nervous pathways in the brain and is involved in many higher cognitive functions, such as learning and memory (Paterson and Nordberg 2000; Changeux and Edelstein 2001; Levin 2002). There are several pathways providing cholinergic projection to the frontal, parietal and occipital cortices, hippocampus, thalamus and olfactory bulb from the origin of the nuclei where the cholinergic fibers arise (Mesulam et al. 1983; Gotti and Clementi 2004). Cholinergic neurotransmission is mediated by the key endogenous molecule acetylcholine (ACh) interacting with two different types of receptors: muscarinic (mAChRs) and nicotinic (nAChRs) acetylcholine receptors. The mAChRs are metabotropic receptors, belonging to the G-protein coupled receptor family. Activation of these receptors stimulates second messenger systems and is responsible for mediation of slower cellular events (Caulfield 1993). The nAChRs are transmitter/ligand-gated ion channels belonging to a gene family of homologous receptors including N-metyl-D-aspartate (NMDA), γ-amino butyric acid (GABA), and serotonin receptor 3 (5-HT3) (Paterson and Nordberg 2000; Karlin 2002). nAChRs mediate fast synaptic events when activated by ACh or other ligand transmitters.

Structure, distribution and functional properties of nAChRs

nAChRs have a pentameric structure and are composed of five different α and β subunits or α subunits alone, which are arranged around a central pore. To date, nine different α subunits (α2-α10) and three β subunits (β2-4) of nAChRs have been cloned and sequenced in human brain (Sargent 1993; Elliott et al. 1996). Each subunit has the ability to combine with different subunits and form heterogenous or homomeric receptors, giving rise to various subtypes with individual pharmacological and physiological profiles and distinct anatomical distributions in the brain (Clementi et al. 2000; Pereira et al. 2002). The most abundant nAChR subtype in the brain, which accounts for most of the high affinity nicotine binding sites, is made up of α4 and β2 subunits and has high affinity for cytisine, epibatidine, and nicotine, and low affinity for α-bungarotoxin (Adem et al. 1988; Hall et al. 1993). The other main nAChR subtype, which accounts for most of the high affinity of α-bungarotoxin binding sites, is made up of the α7 subunits and has low affinity for nicotine, ACh, and cytisine (Dani 2001; Karlin 2002).

nAChRs are widely distribution in the human brain. They are located pre- and postsynaptically, and also peri- and extrasynaptic sites where they may modulate neuronal function by a variety of actions (Gotti et al. 1997; Court et al. 2000; Paterson and Nordberg 2000). The distribution of nAChRs in the human brain has been mapped by radioligand binding and autoradiography studies with nicotinic ligands. The α4β2 nAChR subtype, measured by [^{3}H] nicotine and [^{3}H] epibatidine in human post mortem brain tissue, is high in the thalamus, caudate nucleus, substantia nigra, moderate in some regions of cerebral cortex, cerebellum and is low in the hippocampus, amygdala and pons (Adem et al. 1988; Nordberg et al. 1988; Adem et al. 1989; Marutle et al. 1998). The levels of the α7 nAChR subtype, measured by [^{125}I] α-bungarotoxin in human post mortem brain tissue, are high in the hippocampus and substantia nigra and low density in the cerebral cortex (Court and Clementi 1995; Breese et al. 1997).

nAChRs play an important role during the brain development in differentiation, guidance of axons and synapse formation (Lipton and Kater 1989; Kinney et al. 1993; Role and Berg 1996). Functional nAChRs are present in fetal mouse cerebral cortex as early as embryonic day 10 and the α3, α4, α7 nAChR subunits proteins are detectable

immunocytochemically in cortical cells from embryonic day 10 to birth (Atluri et al. 2001). The levels of [^{3}H] nicotine binding, [^{125}I] α-bungarotoxin binding as well as mRNA for nAChR α4, α7 and β2 subunits increase during early developmental periods in the neocortex and hippocampus of mouse and rat brains compared to the adult (Fiedler et al. 1990; Shacka and Robinson 1998). Furthermore, the developmental profiles of nAChRs correlate well with the structural rearrangement and progressive neurochemical maturation in many brain regions (Huang 1989; Kostovic and Rakic 1990; Gould et al. 1991; Naeff et al. 1992). The high expression of nAChRs during the early developmental period of mouse brain suggests an important role of nicotinic receptor signalling in the maturation of brain structure during this period. α7 nAChRs with their high Ca^{2+} permeability might be of special importance during brain development, a period of intense calcium-dependent plasticity (Ghosh and Greenberg 1995; Wong and Ghosh 2002).

nAChRs are also involved in many brain functions, especially in various complex cognitive functions, such as attention, learning, memory consolidation and sensory perception (Cordero-Erausquin et al. 2000; Levin 2002). Due to the wide presynaptic and preterminal distributions of the nAChRs in neocortical, hippocampal and cerebella regions in the brain, the nAChRs are involved in the modulation of a number of neurotransmitter releases processes including ACh, dopamine (DA), γ-amino-butyric acid (GABA), serotonin (5-HT) and glutamine (Glu) (Gray et al. 1996; Albuquerque et al. 1997; Alkondon et al. 1997; Radcliffe et al. 1999). The involvement of nAChRs in different neurotransmitter system in the brain suggests an important role for them in a number of important physiological and pathological processes in the brain.

nAChRs in AD

A consistent and significant loss of nAChRs has been observed in the cortical regions of AD patients compared to age-matched healthy controls in early studies (Nordberg and Winblad 1986; Nordberg 1992; Quirion 1993). When the distributions of [^{3}H] nicotine, [^{3}H] epibatidine and [^{3}H] cytisine were measured in AD neocortical regions, marked reduction were observed compared to control brains (Hellström-Lindahl et al. 1999; Sihver et al. 1999). A significant decreased binding of [^{125}I] α-bungarotoxin has also been reported in the hippocampus of AD patients as compared with controls (Hellström-Lindahl et al. 1999). Significant reductions in the numbers of [^{3}H] epibatidine and [^{3}H] nicotine binding sites were observed in various cortical regions of the Swedish 670/671 APP mutation family subjects (Marutle et al. 1999). In vivo studies with PET have demonstrated a decrease in nAChR binding in association with a decline of cognitive function in AD patients (Nordberg et al. 1995; Nordberg 1999). In the protein levels, reduced expressions of α4, α7, and α3 nAChR subunits have been demonstrated in AD brains by several groups (Martin-Ruiz et al. 1999; Wevers et al. 1999; Guan et al. 2000). The regional expression of nAChR α4 and α3 subunit mRNA species showed no difference between AD and control tissues in any region analyzed (Hellström-Lindahl et al. 1999). The level of the nAChR α7 mRNA was significantly higher in the hippocampus of AD brains compared to controls, suggesting that the loss in nAChRs can not be attributed to alterations at the transcriptional level (Hellström-Lindahl et al. 1999).

The mechanisms for the changes of nAChRs seen in AD are still somewhat unclear. Possible factors such as amyloid peptide accumulation, hyperphosphorylation of tau protein, oxidative stress, and modification of cell membranes may be related to nAChR deficts (Loo

et al. 1993; Alonso et al. 1994; Farooqui et al. 1995; Edelberg and Wei 1996; Kar and Quirion 2004).

The α7 nAChRs are functional homomeric ligand-gated ion channels that are widely expressed throughout the mammalian brain, and have been implicated in cognitive function and neuroprotection (Seo et al. 2001). α7 nAChR induced neuroprotection has been demonstrated in apoptotic, glutamate-induced excitotoxicity, and Aβ-induced neurotoxicity (Shimohama et al. 1998; Jonnala and Buccafusco 2001; Kihara et al. 2001). α7 nAChRs were also proposed to be relevant to the pathogenesis of AD by interactng with Aβ (Dineley et al. 2001; Auld et al. 2002). α7 nAChRs and Aβ1-42 are co-localized in NPs and neurons, and they can form a stable complex with high affinity (Wang et al. 2000b; Wang et al. 2000a). Increased Aβ loads as well as increased α7 nAChRs were detected in the brains of APP/PS1 transgenic mice (Dineley et al. 2001). The formation of Aβ1-42/α7 nAChR complexes may be an important step for the accumulation of Aβ1-42 in neurons (Nagele et al. 2002). The accumulation of Aβ1-42 in neurons will eventually result in the lysis of neurons, followed by their selective loss and the dispersal of their cytoplasmic contents, including the accumulated Aβ1-42, into the surrounding extracellular space to form amyloidogenic plaques (Cuello 2005).

Cholinesterase in AD

The cholinesterase enzyme exists in two different forms in humans, AChE and butyrylcholinesterase (BuChE) (Taylor and Radic 1994; Greig et al. 2002). AChE is the main cholinesterase in the CNS, while BuChE, originated from glial cells, is more common in serum (Darvesh et al. 2003). Decreased AChE activity and stable or increased BuChE activity were detected in the brains of AD (Arendt et al. 1992). Most of the neocortical AChE activity in AD brain was found associated with NPs, in which it colocalized with Aβ deposits including both the diffuse amyloid deposits and the mature NPs (Geula and Mesulam 1989; Carson et al. 1991; Moran et al. 1993). Further studies showed that AChE promoted the aggregation of Aβ peptides and accelerated the formation of amyloid plaque, suggesting that AChE may play a pathogenic role in AD by influencing Aβ processing (Inestrosa et al. 1996; Rees et al. 2003). It has also been shown that Aβ which aggregates with AChE is more toxic to cells compared to aggregates of Aβ alone (Reyes et al. 2004).

Treatment strategies in AD

Different treatment strategies have been explored to prevent or slow the progression of AD. Pharmacological treatment of AD is based on the use of acetylcholinesterase (AChE) inhibitors and the NMDA antagonist, memantine, which have beneficial effects on cognitive, functional, and behavioural symptoms of the disease. Other new therapeutic approaches include memantine, cholinergic receptor agonists, Aβ peptide vaccination, secretase inhibitors, metal chelators and anti-inflammatory drugs.

AChE inhibitors

The deficiency in cholinergic neurotransmission in AD has led to the development of AChE inhibitors as the first-line treatment for mild to severe AD. The common mechanism of action underlying this class of agents is an increase in available ACh through inhibition of the AChE. Treatment with various AChE inhibitors including donezepil, rivastigmine and galanthamine have been shown in clinical studies to improve cognitive, functional and behavioural symptoms of AD (Rogers et al. 1998; Cummings 2000; Tariot et al. 2000).

Tacrine, donepezil, and galantamine selectively inhibit AChE. Galantamine also improves cholinergic neurotransmission by acting as an allosteric ligand at nAChRs to increase presynaptic ACh release and postsynaptic neurotransmission (Scott and Goa 2000). PET studies performed in AD patients treated with AChEI have shown improvement in cerebral blood flow, glucose metabolism and increased number of nAChRs (Nordberg 1999).

Memantine

Memantine, a non-competitive NMDA receptor antagonist, is approved in several countries for symptomatic treatment of moderate to severe AD. Clinical studies have shown that AD patients receiving memantine have significantly less deterioration in cognitive and functional measurements (Reisberg et al. 2003; Tariot et al. 2004). Moreover, AD patients receiving long-term memantine treatment experienced a significant benefit in all main efficacy assessments including functional, global, and cognitive assessments (Reisberg et al. 2006). By blocking the NMDA receptor, memantine can inhibit the NMDA receptor-dependent excitotoxicity, which has been hypothesized to play a role in the progressive neuronal loss. In addition, pre-clinical evidences shows that memantine acts as an open channel blocker for several ligand-gated ion channels (Buisson and Bertrand 1998; Oliver et al. 2001; Maskell et al. 2003).

Cholinergic receptor agonists

Selective M1 mAChR activation protect neurons from Aβ-induced damage via activation of the Wnt signalling pathway (Farias et al. 2004). Administration of AF267B, a selective M1 mAChR agonist, has been shown to rescue the cognitive deficits and reduce both the Aβ and tau pathological changes in the brains of a transgenic model of AD (3×Tg-AD) that progressively manifests several disease-relevant features, including plaques, tangles, cholinergic dysfunction, and cognitive impairments (Oddo et al. 2003; Caccamo et al. 2006). Selective nAChR agonists are also candidates for symptomatic and neuroprotective AD therapy (Nordberg 2003). The potential therapeutic benefit of nAChR agonists in AD is based upon the observation that nicotine improves memory in animals, healthy subjects, and AD patients (Rusted and Warburton 1992; Newhouse et al. 1997). Experimental data in cultured neurons suggest that the activation of α4β2 nAChRs provides protection against Aβ induced neurotoxicity (Kihara et al. 1997). Both nicotine and AChEI are believed to attenuate the toxicity of Aβ mediated through the activation of the α7 nAChRs (Zamani et al. 1997; Svensson and Nordberg 1998).

Anti-amyloid therapies

Since Aβ plays a causal role in the pathogenesis of AD, blocking the production of Aβ has been suggested as one potential therapeutic approach in AD treatment (Citron 2004; Mattson 2004). Those proteases that generate Aβ, β- and γ-secretase, are the potential targets for therapeutic development (Wolfe 2001). Inhibition the β- or γ-secretase activities or stimulation α-secretase activity has been shown to reduce Aβ levels (Citron 2000; Wolfe 2002). Blocking the assembly of Aβ into oligomers and fibrils and enhancing the clearance of Aβ are other potential strategies for preventing the toxic effects of this peptide. Serum Amyloid P component (SAP) inhibitors, nicotine-like substances and cholesterol-lowering drugs have been suggested as potential therapeutic means to prevent the assembly of Aβ into oligomers and fibrils or enhancing the clearance of Aβ (Botto et al. 1997; Refolo et al. 2001; Nordberg et al. 2002; Hellström-Lindahl et al. 2004; Unger et al. 2005a).

Another therapeutic strategy for both preventing Aβ build-up and enhancing the clearance of this peptide is immunization, either actively with the Aβ peptide itself or passively with anti-Aβ antibodies. Active immunization with Aβ in APP transgenic mice resulted in a decrease in amyloid plaques and an improvement of cognitive behavioural deficits (Schenk et al. 1999; Bard et al. 2000; Morgan et al. 2000). Based on the successful clearance of Aβ, active immunization trials were performed in AD patients. Due to the development of an aseptic, vasocentric meningoencephalitis in a subset of patients, the trial was terminated (Orgogozo et al. 2003). Neuropathological investigations of post mortem brain tissue from AD patients who had participate in the study showed a decreased number of Aβ plaques compared to controls, but an increase in the number of NFT was also evident (Nicoll et al. 2003; Ferrer et al. 2004). Passive immunization with anti-Aβ antibody has been suggested to be a potentially safer alternative therapy. However, a recent study showed meningoencephalitis subsequent to peripheral and intercerebral passive immunization of APPswe transgenic mice (Lee et al. 2005).

Other therapies

Other tentative therapeutic candidates include treatment with nerve growth factor (NGF) (Eriksdotter Jonhagen et al. 1998), anti-inflammatory therapy (McGeer et al. 1996), antioxidants, e.g., vitamin E and C (Sano et al. 1997) and the cholesterol-lowering drugs, statins (Jick et al. 2000).

TRANSGENIC MICE MODELS

During the last decade, various transgenic mouse models that express one or several mutant forms of human proteins associated with FAD, including APP, PS and tau, have been developed. Transgenic mice models have become important research tools in AD since they can provide insight into the pathological and biochemical processes involved in the pathogenesis of this disease. These transgenic mice displayed increased Aβ production and deposition in the brain and cognitive deficits between 3 and 15 months, depending on the mutation of APP, gene promoter, as well as the mouse's genetic background (Higgins and Jacobsen 2003). Transgenic mice that overexpress the APP695 isoform containing a K670M/N671L mutation (Tg2576) have learning and memory impairments and show amyloid plaques in the brain at 9 months of age (Hsiao et al. 1996). Even though these transgenic mice do not display all of the neuropathology changes of AD brains, they may provide a useful tool to investigate some mechanisms of the pathological abnormalities in these brains. Double transgenic mice that overexpress the APP695 isoform containing a K670M/N671L mutation and overexpress the human AChE (hAChE-Tg//APPswe) show amyliod plaques in the brain at 6 months of age (Rees et al. 2003). A triple-transgenic model (3×Tg-AD) harboring $PS1_{M146V}$, APP_{SWE}, and tau_{P301L} transgenes was generated recently (Oddo et al. 2003). The 3×Tg-AD mice progressively developed amyloid plaques, tangles and show cholinergic dysfunction and cognitive impairments, providing a valuable transgenic model of AD for evaluating putative AD therapies (Oddo et al. 2004; Oddo et al. 2005). The main characteristics and neuropathological features of several transgenic mouse models are summarized in Table 1.

α7 KNOCKOUT MICE

Knockout mice, in which one or more genes of interest are silenced, provide unique opportunities to analyse diverse aspects of gene function *in vivo*. Since the first targeted disruption in 1995 of the gene encoding β2 nAChRs, all but a few of the mammalian

nAChR subunits have been disrupted (i.e. α7, α4, α3, α9, β4 and β3). The development of knockout mice has extended understanding of nicotinic receptors, revealing that some subunits are necessary for viability, whereas others mediate modulatory effects on learning and memory, anxiety, dopaminergic neurotransmission, seizure threshold, development of the visual system and autonomic function.

α7 knockout mice have been generated and used to demonstrate the functions of individual nAChR subunits (Orr-Urtreger et al. 1997). In the α7 null mice, the neuropathological examination revealed normal structure and cell layering in the brain. The result of autoradiography with [^{3}H] nicotine revealed no detectable abnormalities of high-affinity nicotine binding sites, but there was an absence of high-affinity [^{125}I] α-bungarotoxin binding sites. Null mice also lack rapidly desensitizing, methyllycaconitine-sensitive and nicotinic currents that are present in hippocampal neurons. Studies have demonstrated that mice lacking the α7 subunit are anatomically and behaviorally normal, suggesting that whereas α7 subunit may play a developmental role that is not essential for normal development or neurological function. It is still unclear whether there are compensatory changes in other nAChR subunits, such as α3, α4, β2 and β4 subunits, during the early stages of brain development in the α7 knockout mice. It therefore would be is interesting to investigate whether the other nAChR subunits can take place the function of α7 subunit partly during the early stages of brain development in this animal model.

Table 1: Characteristics and neuropathological features of transgenic models

Line	Transgene	Aβ deposits	NFT	Neuron loss	nAChRs	Reference
Tg2576	APP695 (K670N/M671L)	9 mo	No	No	nAChRs ↑	(Hsiao et al. 1996)
APP23	APP751 (K670N/M671L)	6 mo	No	Yes	Not reported	(Sturchler-Pierrat et al. 1997)
APP/LD	APP695 (V642I)	12-15 mo	No	No	Not reported	(Moechars et al. 1999)
TgCRND8	APP695 (K670N/M671L + V717F)	3 mo	Not reported	Not reported	Not reported	(Chishti et al. 2001)
PDAPP	APP695, 715 and 770 (V717F)	6-9 mo	No	No	Not reported	(Games et al. 1995)
PSAPP	APP695 (K670N/M671L) + PS1 (M146V)	3 mo	Not reported	No	α7 nAChRs ↑	(Holcomb et al. 1998)
APP/PS1	APP695 (K595N/M596L) +PS1 (A246E)	9-10 mo	Not reported	Not reported	No change	(Borchelt et al. 1997; Marutle et al. 2002)
TAPP	APP695 (K670N/M671L) + tau (P301L)	6 mo	9-10 mo	Yes	Not reported	(Lewis et al. 2001)
JNPL3	tau (P301L)	No	9-10 mo	Yes	Not reported	(Lewis et al. 2000)
3 × Tg-AD	APP695 (K670N/M671L) + PS1 (M146V) + tau (P301L)	6 mo	15 mo	Not reported	Not reported	(Oddo et al. 2003)
hAChE-Tg	Overexpress human AChE	No	No	Attenuated dendritic branch	α4 nAChRs ↑	(Beeri et al. 1995; Svedberg et al. 2002)
hAChE-Tg//APPswe	Overexpress human AChE + APP695 (K670N/M671L)	6 mo	Not reported	Not reported	Not reported	(Rees et al. 2003)

AIMS OF THE THESIS

Aβ accumulation, NFTs and loss of neurons are consistent features of AD. These pathological processes are accompanied by oxidative stress, activated glia, inflammation and impaired cholinergic transmission. The main purpose of this thesis was to provide a further understanding of how these pathological processes, influence cholinergic transmission, especially that mediated by nAChRs, and the possible interactive mechanisms among Aβ processing, nAChRs, and activated glia in the pathogenesis of AD in both AD patients and transgenic mouse models. Since Aβ is thought to play a causal role in the pathogenesis of AD, a special focus has been on the interaction between Aβ and nAChRs. Figure 3 illustrates the main aim of this thesis and those models that were used to investigate their interactions.

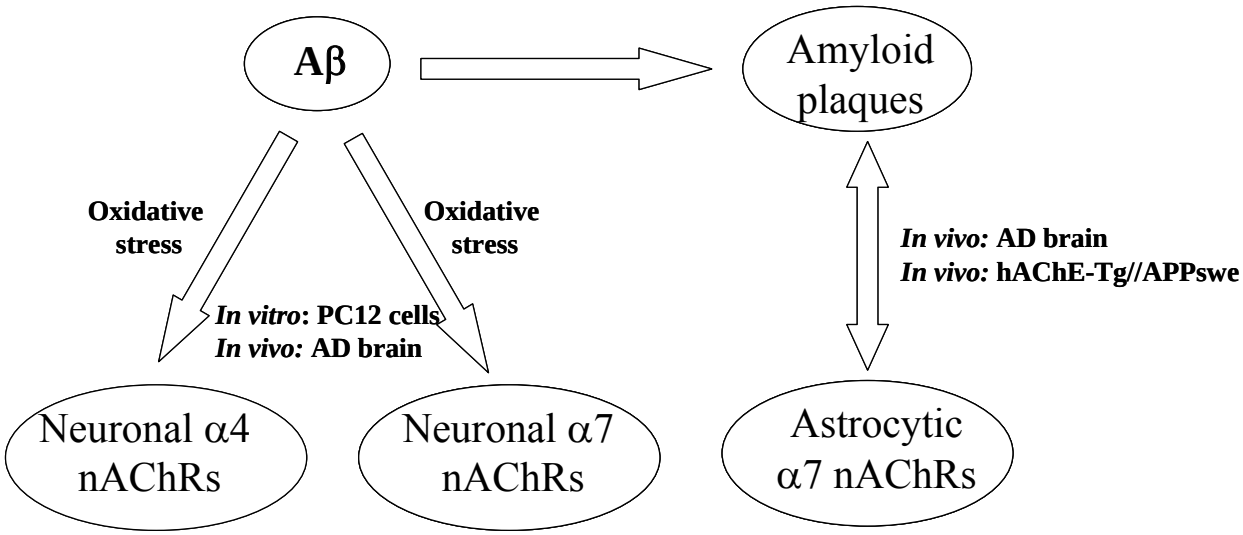


Figure 3. General focus of this thesis

The special aims are:

- To characterize the distribution changes of different nAChR subunits in neurons and activated glia in the brains of both sporadic AD and familiar AD carrying the Swedish APP670/671 mutation (APPswe) and their possible association with the pathological changes of both sporadic AD and APPswe *(Paper I)*.

- To study the effect of Aβ on the nAChRs and its possible mechanism in PC12 cells and to investigate whether oxidative stress damages is related to deficits in nAChRs in AD brain *(Paper II and III)*.

- To investigate the possible interaction among Aβ, AChE, activated glia and nAChRs in the brains of different ages of transgenic mice both overexpressing human AChE and carrying the Swedish APP670/671 mutation (hAChE-Tg//APPswe) *(paper IV)*.

- To characterize the developmental changes of different nAChR subtypes in the brains of α7 knockout mice during their postnatal development *(Paper V)*.

MATERIALS AND METHODS

HUMAN POSTMORTEM TISSUE (Paper I, III)

Human brain tissues were obtained from the Huddinge brain Bank at Karolinska University Hospitl, Huddinge (coordinator Dr. Nenad Bogdanovic) (Paper I) and the Netherlands Brain Bank (coordinator Dr. Rivka Ravid) (Paper III). Subjects were matched according to age, postmortem delay, tissue storage time, smoking history and previous drug treatment. All experimental procedures were approved by the ethical committee in Karolinska institutet.

Paper I

Autopsy samples from the hippocampus and medial gyrus of the temporal cortex were obtained from four AD patients carrying the Swedish APP 670/671 mutation (mean age: 63.3 ± 5.3 years, range 56–68 years), four younger control subjects (mean age: 63.2 ± 2.8 years, range 59–66 years), six sporadic AD patients (mean age: 80.5 ± 8.2 years, range 66–89 years), and six older control subjects (mean age: 81.8 ± 8.8 years, range 71–94 years). All AD patients were clinical and histopathologically verified according to NINCDS-ADRDA and CERAD criteria. The control subjects had no clinical history of psychiatric or neurological disorders, nor did their brains exhibit any neuropathological characteristics of dementia. All of these subjects were non-smokers, except for one patient whose history of smoking was unclear in the AD patients carrying the Swedish APP 670/671 mutation. Mean postmortem intervals for the AD patients carrying the Swedish APP 670/671 mutation, the younger control subjects, the sporadic AD patients, and the old control subjects were 21.7 ± 1.3 h, 22.5 ± 2.6 h, 21.3 ± 1.9 h, and 23.2 ± 2.3 h, respectively.

Paper III

Brain tissues were obtained from ten sporadic AD patients (mean age: 78.8 ± 11.7 years) and twelve age-matched control subjects (mean age: 79.3 ± 9.9 years). All AD patients were clinically and histopathologically verified according to NINCDS-ADRDA and CERAD criteria. The control subjects had no known history or symptoms of neurological or psychiatric disorders. Both the AD patients and control subjects have non-smoking histories. The mean postmortem intervals of the AD patients and controls were 4.15 ± 0.65 and 7.5 ± 3.7 h, respectively.

CELL CULTURE (PAPER II)

PC12 cells (the rat pheochromocytoma cell line), obtained from the German Collection of Microorganisms and Cell Cultures (Germany) were grown in RPMI 1640 medium, supplemented with 10% heat inactivated horse serum, 5% heat-inactivated fetal bovine serum and 25 u/ml penicillin-streptomycin (Gibco BRL Life Technologies AB, Sweden) on plates precoated with collagen. The cultures were maintained in a humidified atmosphere containing 5% CO2 at 37°C.

Aβ peptides and treatment (Paper II)

Aβ1-40, Aβ25-35 and Aβ35-25 were obtained from the Department of Medical Biochemistry and Microbiology, Uppsala University, Sweden. For aggregation, Aβ1–40 was

dissolved in autoclaved distilled water and incubated at 37°C for 48 h before use. Aβ25–35 and Aβ35-25 were not pre-aged before treatment because they aggregate within a few hours under cell culture conditions (Forloni et al. 1993).

TRANSGENIC AND KNOCKOUT MOUSE MODELS (PAPER IV, V)

All animal experimental protocols were approved by the local ethics committee and carried out in accordance with the guidelines of the Swedish National Board for Laboratory Animals (CFN). All mice were born and bred in our own colony and housed under the same conditions with controlled temperature, humidity, and enriched environment. The mice had access to food and water ad libitum and were maintained on a 12 h light dark cycle. All mice used in these studies were sacrificed by cervical dislocation during the light cycle.

Paper IV

The transgenic mice overexpressing APP695 and containing a KM670/671NL mutation driven by a hamster prion protein gene promoter in a C57BL/6J x SJL F1 hybrid mice and were kindly provided as a gift from Dr. Karen Hsiao-Ashe (Hsiao et al. 1996). The transgenic mice carrying human AChE cDNA under the control of 586 bp of the authentic human AChE promoter were kindly provided as gifts from Dr. Hermona Soreq (Beeri et al. 1995).

Male APPswe transgenic mice and female hAChE-Tg transgenic mice were used to breed a colony of double transgenic (hAChE-Tg//APPswe) transgenic mice. Offspring from C57BL6J crossed with FVBN mice (Bomice & Mollegaard Breeding Laboratories, Ejby, Danmark) served as control animals. Offspring of the matings were genotyped by PCR analysis of the tail tip DNA. Analysis of the APP and AChE transgenic used previously described primers (Beeri et al. 1995; Hsiao et al. 1996).

Paper V

The α7 heterozygous null mice (α7 +/-) (C57BL/6J background) were purchased from the Jackson Laboratory (Bar Harber, ME, USA) and were used to breed a colony of experimental animals, including the α7 deficient (α7 -/-), α7 +/- and α7 wild-type (α7 +/+) mice. Genotyping was done according to the protocol described on the Jackson Laboratory website (www.jax.org). Experiments were performed on 7-day to 3-month-old α7 -/-, α7 +/- and α7 +/+ mice. Six mice in each group at each time point were used. Mice were sacrificed by decapitation at the ages of 7, 10, 14, 21, 28 days and 3 months. The date of birth is considered to be postnatal day 0 (P0). The mouse brains were taken out and the neocortex, hippocampus and cerebellum were dissected and stored at –80 °C until use.

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

nAChR, GFAP immunohistochemical staining (Paper I)

Immunohistochemical staining was performed on formalin-fixed paraffin-embedded human brain sections. Prior to staining, sections were deparaffinized, rehydrated, and rinsed in Tris–NaCl buffer (0.05 M Tris, 0.15 M NaCl, pH 7.6) at room temperature (RT) for 10 min, followed by treatment with 1% hydrogen peroxide in order to quench endogenous peroxidase activity. For immunostaining of nAChR subunits or GFAP, the sections were treated in a microwave oven 10 min in 0.05 M citrate-buffered saline (pH 6.0) for antigen retrieval. Subsequently, the sections were incubated in Blocking Buffer (DAKO) for 30 min

at RT to block non-specific reactions and thereafter incubated with anti-α7, α4, α3, and β2 nAChR antibodies (diluted 1:1000 in Tris–NaCl buffer) or rabbit anti-cow GFAP antibody (diluted 1:200 in Tris–NaCl buffer) overnight at 4°C. Following a thorough rinse in Tris–NaCl buffer, the sections were incubated with the secondary antibodies, i.e., biotinylated goat anti-mouse IgG or biotinylated goat anti-rat IgG, biotinylated horse anti-goat IgG or biotinylated goat anti-rabbit IgG (diluted 1:200 in Tris–NaCl buffer) for 60 min at RT, respectively. For visualization of immunoreactivity, sections were incubated subsequently with avidin-biotinylated enzyme complex and DAB and thereafter dehydrated with increasing concentrations of ethanols, cleared with xylene, and mounted in Permount.

Negative control for the immunohistochemical procedure was performed by incubating sections with non-immune serum instead of the primary antibodies, which resulted in no detectable labeling.

Double-labeling nAChR, GFAP and Aβ (Paper I, IV)

In order to examine the colocalization of different nAChR subunits in relationship to astrocytes and amyloid plaques, double staining was performed employing antibodies against individual nAChR subunits and astrocytes or against the α7 nAChR subunit and Aβ, respectively. For colocalization of nAChR subunits and astrocytes, the sections were first incubated with rabbit anti-cow GFAP antibody (diluted 1:200 in Tris–NaCl buffer) overnight at 4°C and then visualized for immunoreactivity by incubating with chromogen DAB as described above. Subsequently, the sections were rinsed in Tris–NaCl buffer and treated with 1% hydrogen peroxide in distilled water. Finally, the sections were incubated with antibodies against α7, α4, α3, and β2 nAChR subunits at 4°C overnight and then the immunoreactivity was visualized with the second chromogen Vector SG substrate kit.

In the case of colocalization of α7 nAChR subunit and Aβ, after first being treated with 1% hydrogen peroxide (30 min, RT) and incubated in 100% formic acid (3 min, RT), the sections were incubated with the antibody against Aβ (diluted 1:500 in Tris–NaCl buffer) overnight at 4°C. Following a thorough rinse in Tris–NaCl buffer, sections were incubated with the secondary antibodies, biotinylated goat anti-mouse IgG (diluted 1:200 in Tris–NaCl buffer) for 60 min at RT. To achieve visualization of the immunoreactivity, sections were incubated with avidin-biotinylated enzyme complex and DAB, followed by rinsing in Tris–NaCl buffer and treatment with 1% hydrogen peroxide in distilled water. Subsequently, the sections were incubated with antibody against the α7 nAChR subunit at 4°C overnight and thereafter the immunoreactivity was visualized with the second chromogen Vector SG substrate kit.

Negative controls for this double-labeled immunohistochemical staining were performed by omission one of the primary antibodies, which resulted in no detectable staining.

Membrane preparation (Paper I, II, IV, V)

Homogenates of PC12 cells were prepared with binding buffer and were centrifuged at 40,000 × *g* for 15 min at 4°C. The resulting pellets were washed twice in binding buffer. The final pellets were re-suspended in the binding buffer ready for receptor binding assay.

Crude membrane preparations (P2) were prepared from the temporal cortex of human brain and the cortex and hippocampus of transgenic mouse brain. The brain tissues were homogenized in 0.32 M sucrose and then centrifuged at 1000×*g* for 10 min at 4°C. The

resulting supernatant was centrifuged at 17,000×*g* for 30 min at 4°C. The final pellet was re-suspended in either 10 volumes of a phosphate buffer ([^{125}I] α-bungarotoxin binding assay) or fresh 0.32 M sucrose solution ([^{3}H] epibatidine and [^{3}H] cytisine binding assays). Membrane preparations were stored at –20°C until receptor binding assays were performed. The protein content in all preparations was measured according to the method of Lowryusing bovine serum albumin as the standard (Lowry et al. 1951).

Receptor binding assays (Paper I, II, IV, V)

[^{125}I] α-bungarotoxin binding assay

Membrane preparations (0.2 mg protein) were pre-incubated in the binding buffer (10mM Na-phosphate buffer, pH 7.4, containing 50mM NaCl and 0.1% BSA) with 1 μM (final concentration) of cold α-bungarotoxin (for non-specific binding) or without (for total binding) at 37°C for 30 min. The membrane preparations were then incubated with 3.5 nM (for human brain) or 2 nM (for PC12 cells and mouse brain) [^{125}I] α-bungarotoxin (specific radioactivity 260 Ci/mmol; DuPont NEN USA) for an additional 30 min in a final binding volume of 200 μl. The reaction was terminated by adding 1 ml cold binding buffer and the samples were centrifuged at 17, 000 × *g* for 10 min at 4°C. The pellet was washed twice with binding buffer by centrifugation at 17, 000 × *g* for 10 min at 4°C. The bottom of the microtube containing the pellet was cut and the radioactivity was determined in a γ-counter (Packard, USA).

[^{3}H] epibatidine binding assay

Membrane preparations (0.2 mg protein) were incubated with 2.5 nM (for PC12 cells) or 0.1 nM (for mouse brain) [^{3}H] epibatidine (specific radioactivity 54.6 Ci/mmol; DuPont NEN, USA) in 50 mM Tris-HCl buffer (PH 7.4), final volume 1 ml, at 25°C for 3 h. The samples were then filtered through Whatman GF/C glass filters presoaked with 0.3% polyethylenimine solution for 3-4 h and washed three times with assay buffer. Radioactivity trapped on the filters was counted in a scintillation counter. Non-specific binding was determined in the presence of 0.1 mM (-)-nicotine.

[^{3}H] cytisine binding assay

Membrane preparations (0.2 mg protein) were incubated with 2 nM [^{3}H] cytisine (specific radioactivity 35.2 Ci/mmol; DuPont NEN, USA) in 50 mM Tris-HCl buffer (pH 7.4), final volume 1 ml, at 4°C for 1.5 h. The samples were then filtered through Whatman GF/C glass filters presoaked with 0.3% polyethylenimine solution for 3-4 h and washed three times with assay buffer. Radioactivity trapped on the filters was counted in a scintillation counter. Non-specific binding was determined in the presence of 0.1 mM (-)-nicotine.

Western blot assay (Paper II, III, V)

PC12 cells, human brain tissues and mouse brain tissues were homogenized in ice-cold buffer (50 mM sodium phosphate, 50 mM NaCl, 2 mM EDTA, 2 mM EGTA, and 1 mM phenylmethylsulfonyl fluoride, pH 7.4). Total membranes were pelleted by centrifugation at 60, 000 × g for 60 min at 4°C. Membranes were then resuspended in ice-cold buffer containing 2% Triton X-100, protease inhibitors, as well as 1 mM phenylmethylsulfonylfluoride. The suspension was mixed for 2 h at 4°C and then

centrifugated at 100,000 × g for 60 min at 4°C. The supernatant containing detergent-solubilized membrane protein was used for western blot analysis. Protein concentration was determined by the DC Protein Assay kit (Bio-Rad, USA).

The solubilized membrane protein was separated on 10% SDS-polyacrylamide gel followed by transfer to a PVDF membrane (Amersham, USA). Polyclonal goat antibodies against nAChR α3, α4, α7, α5 and β2 subunits (1:1000, Santa Cruz Biotechnology Inc, CA, USA) were incubated with the membranes overnight at 4°C. After washing, the membranes were incubated with horseradish peroxidase-conjugated anti-goat IgG antibodies (1:3000, Santa Cruz Biotechnology Inc, CA, USA) for 60 min at room temperature. Finally, enhanced Chemiluminescence (ECL; Amersham, USA) was used for exposure. The signal intensity of protein bands was quantitated with progress Scion Image (National Institutes of Health, MD, USA).

Immunoprecipitation (Paper V)

The membrane protein fractions were prepared from the mouse brain as described in the western blotting procedure. 500 μg membrane protein from each sample was pre-cleared by incubation with 0.25 μg of control IgG and 20 μl agarose conjugate protein A (Santa Cruz Biotechnology) for 30 min at 4 °C. Agarose was spun down by centrifugation at 1,000 × g for 30 seconds at 4°C and the supernatant was transferred to a new tube. The supernatant was incubated with 1 μg rabbit anti-nAChR α5, or 1 μg goat anti-nAChR α3 and α4 antibodies (Santa Cruz Biotechnology) for 2 h at 4 °C with rocking, respectively. Agarose conjugated rabbit IgG and goat IgG were used as negative controls. Then, 20 μl agarose conjugate protein A were added and incubated for 3 h at 4 °C with rocking. The pellet was collected by centrifugation at 1,000 × g for 30 seconds at 4° C and the supernatant was carefully aspirated and discarded. The pellet was washed three times with RIPA buffer (Santa Cruz Biotechnology), each time repeating the centrifugation at 1,000 × g for 30 seconds at 4° C. After the final wash, the pellet was resuspended in electrophoresis sample buffer, boiled and loaded onto gels for western blotting. The proteins were transferred onto membranes and detected for α3, α4 and α5 nAChR subunits, as described above in the western blotting procedure.

RNA isolation and RT-PCR (Paper IV, V)

Total RNA was isolated from the neocortex and hippocampus by using a TOTALLY RNA isolation kit (Invitrogen life technologies, Grohingen, the Netherlands), which includes treatment with RNase-free DNase in order to remove traces of contaminating genomic DNA (Mousavi et al. 2001; Mousavi et al. 2004). The RT and PCR reactions were performed as previously described (Mousavi et al. 2001). Detection of mRNA was performed by PCR. PCR oligonucleotide primers for different nAChR subunits were both rat specific and newly designed mouse specific primers created with the Cybergene primer designing program (Mousavi et al. 2001; Mousavi et al. 2004).

PCR was carried out in a reaction volume of 50 μl using a master mix containing 2 mM $MgCl_2$, 1 × PCR buffer, 0.2 mM dNTPs, 0.5 μM each of the forward and reverse primers (1 μM each primer for cyclophilin), 0.5 μl Taq DNA polymerase (Promega), and 5 μl of RT reaction mixture. After an initial denaturation of 94°C for 3 min, 30 cycles were performed consisting of denaturation at 94°C for 30 s, annealing for 30 s, extension at 72°C for 30 s followed by a final extension at 72°C for 10 min. Oligonucleotide primers of mouse cyclophilin were used as an internal standard. The PCR products were analysed on a 1.5%

agarose gel containing 0.5 μg/ml ethidium bromide (Sigma-Aldrich, Stockholm, Sweden) and visualized under ultraviolet light. The densitometric analysis was performed using the TINA 2.09G software (raytest Isotopenmeβgeräte GmbH βezestraβe 4). The results are expressed as the ratio of the signals of nAChR subunits mRNA to the corresponding cyclophilin signal.

QUANTIFICATION OF AD NEUROPATHOLOGICAL FEATURES (PAPER I)

Two-dimensional quantitation of the NPs and NFT in the sections was performed according to the Bielschowsky procedure. NP and NFT were counted in 1 mm^2 throughout the cortical layers and the hippocampus. Two consecutive measurements were performed in the superficial and deeper layers of the brain regions, using three randomly chosen areas in which the cytoarchitectonic characteristics of the region were well preserved. Only NP and NFT completely inside or crossing only two free, clearly defined edges of the area under observation were counted. The numbers of NPs and NFT were corrected for the shrinkage factor, which reflected the shrinkage of the brain tissue in the period from cutting the slabs to final mounting of sections, and reported as a numerical density in the particular brain area.

3-(4,5-DIMETHYLTHIAZOL-2-YL)-2,5,DIPHENYL TETRAZOLIUM BROMIDE REDUCTION ASSAY (PAPER II)

For 3-(4,5-dimethylthiazol-2-yl)-2,5,diphenyl tetrazolium bromide (MTT) analysis, exponentially growing PC12 cells were plated at a density of 5,000 cells in 100 μl medium containing 10% dialyzed FBS per well in 96-well plates precoated with collagen. The day after plating, the culture medium was replaced by the same medium but containing 5 μM of $A\beta_{25-35}$, $A\beta_{1-40}$ or $A\beta_{35-25}$. The cultures were incubated for 48 h and 10 μl of 5 mg/ml MTT stock were added to each well, after which the incubation continued for another 4 h. After incubation, 100 μl of a solution containing 20% SDS and 50% dimethylformamide (pH 4.8) were added to each well, and the cultures incubated overnight. Absorption values at 570 nm were determined with a spectrophotometer.

DETECTION OF LIPID PEROXIDATION AND PROTEIN OXIDATION (PAPER II, III)

The levels of MDA (malondialdehyde), one product of lipid peroxidation, were detected in PC12 cells and AD brains by the thiobarbituric acid-reactive substance (TBARS) assay (Subbarao et al. 1990). At the terminating stage, the treatment was stopped by adding 10 mM butylated hydroxytoluene and 10 μM EDTA. After the cells and brain tissues were harvested and centrifuged, the supernatant was collected and mixed with trichloroacetic acid to yield a 4.5% trichloroacetic acid mixture. Two volumes of TBARS reagent (containing 0.45% thiobarbituric acid and 12% acetic acid) were then added. The mixture was incubated at 90°C for 60 min. TBARS in the supernatants was detected by a spectrophotometer at wavelength of 532 nm.

Levels of conjugated dienes in the cells treated with Aβ were measured employing the method of Recknagel and Glende (Recknagel and Glende 1984). The cells were scraped from culture plates and extracted in chloroform: methanol: water (2:1:1 v/v). Chloroform layers from two extractions were combined and dried under nitrogen. Samples were resuspended in a known volume of acetonitrile and absorbance was determined at 235 nm.

Protein carbonyls were quantified by reaction with DNPH (Levine et al. 1994). The PC12 cells or human brain tissues were homogenized in 50 mM phosphate buffer (pH 7.4) containing protease inhibitor and then centrifuged at 11,000 × *g* for 10 min. The supernatant was incubated with 10 mM DNPH in 2 M HCl. The blank control was incubated with 2 M HCl. The mixture was allowed to stand at RT for 1 h, then vortexed at 5-min intervals in the dark. Hydrazone derivatives were extracted with 20% (v/v) trichloroacetic acid (final concentration 10%) and the tubes were left in ice for 10 min and centrifuged at 40,000 × *g* for 5 min to collect the protein precipitates. Another wash was carried out, using 10% trichloroacetic acid, and protein pellets were broken mechanically. The protein pellet was washed three times with ethanol/ethyl acetate (1:1, v/v). After the last wash, the precipitated protein was dissolved in 6 M guanidine hydrochloride in 20 mM sodium phosphate buffer (PH 6.5) by incubation at 37°C for 15 min with gentle vortex mixing. Absorbance was measured at the optimal wavelength (355-390 nm) against the complementary blank.

Aβ1-40 AND Aβ1-42 LEVELS (PAPER IV)

Preparation of tissue and measurements for tris-soluble and guanidinium-soluble Aβ1-40 and Aβ1-42 was described earlier (Hellström-Lindahl et al., 2004). In brief, cortical mice brain tissue was homogenised in 20 mM Tris HCl at pH 8.5, containing protease inhibitor. This was followed by centrifugation at 100,000 × g for 1 h at 4°C. Supernatant was used for analysis of tris-soluble (soluble) Aβ. The pellet was extracted in 5 M guanidium HCl in 20 mM Tris HCl, at pH 8.0 at room temperature and then diluted with phosphate buffered saline including 0.5% bovine serum albumin, 0.05% Tween 20 and protease inhibitors followed by centrifugation at 13,100 × g for 20 min at 4°C. The supernatant was used for measurements of guanidinium-soluble (insoluble) Aβ1-40 and Aβ1-42. The level of Aβ1-40 and Aβ1-42 peptides were analyzed with the Signal Select™ Human Aβ1-40 and Aβ1-42 colorimetric sandwich ELISA kits (BioSource International, Inc. USA) according to the instructions of the manufacturer. The values were calculated by comparison with a standard curve of synthetic human Aβ1-40 and Aβ1-42. All samples were analyzed in the linear range of the ELISA.

Aβ AND GFAP IMMUNOHISTOCHEMICAL STAINING (PAPER IV)

Frozen hAChE-Tg//APPswe, APPswe and control mice brains were sectioned sagittally on a cryostat at –20°C to 10μm thickness. Sections were thaw-mounted onto poly-L-lysine coated slides (Histolab, Sweden), and directly re-frozen in the cryostat. The slides were stored in –80°C until use.

For immunofluorescence labelling of Aβ or GFAP, the sections were pre-treated with concentrated formic acid (for Aβ) for 5 min and incubated for 1 h in 5% normal goat serum in tris buffered saline (TBS) at pH 7.6. Brain sections were stained with 6E10, a mouse monoclonal antibody that is directed against Aβ 1-17 (MAB1560, Chemicon, USA) to determine Aβ deposits or a polyclonal rabbit anti GFAP (Z0334, DakoCytomation, Denmark) to label astrocytes overnight at 4°C. Sections were then incubated with Cy2 (cyanine 2-OSu) conjugated goat anti mouse or Cy3 (cyanine 3-OSu) conjugated goat anti rabbit secondary antibodies (Chemicon, USA). For control staining, the primary serum was omitted and resulted in no detectable labelling.

Double immunofluorescence labelling of Aβ and GFAP was performed by pretreatment of sections with concentrated formic acid for 5 min, followed by incubating the sections in 5% normal goat serum in TBS at pH 7.6. Sections were incubated with a

cocktail of primary antibodies (anti-6E10/anti-GFAP) overnight at 4°C, followed by a secondary antibody cocktail consisting of Cy2-conjucated goat anti mouse and Cy3-conjucated goat anti rabbit antibodies (Chemicon, USA) as indicated for single fluorescent immunostaining. All images were generated by using an Axiophot microscope (Carl Zeiss AG, Göttingen, Germany) equipped with a Hamamatsu digital camera (Hamamatsu Photonics K.K., Hamamatsu City, Japan) and a computerized imaging system (Openlab, version 2.1 for Mac OS, Improbvision, Coventry, England). Post-processing was performed using Adobe PhotoShop (version 7.0) software for PC.

GFAP AND INTERLEUKIN 1β LEVELS (PAPER IV)

A polyclonal GFAP antibody (DakoCytomation, Denmark) was coated in the wells of microtiter plates. After blocking non-specific binding with non-fat dry milk, aliquot of the SDS homogenates were diluted and added to the wells of the plate. Following appropriate blocking and washing steps, a monoclonal GFAP antibody (VWR, Sweden) was added to the "sandwich". An alkaline-phosphatase-linked IgG antibody was then added and a coloured reaction product was obtained by subsequent addition of enzyme substrate. The spectophotometric absorption at 405 nm was measured and expressed as GFAP immunoreactivity/mg total protein.

Preparation of cortical mice brain tissue was preformed as for tris-soluble Aβ1-40 and Aβ1-42, as previously described. The level of Il-1β peptides was analyzed by using the Signal Select™ Mouse Interleukin 1β colorimetric sandwich ELISA kits, (BioSource International, Inc. USA) according to the instructions of the manufacturer. The values were calculated by comparison with a standard curve of synthetic mouse Il-1β. All samples were analyzed in the linear range of the ELISA.

SYNAPTOPHYSIN LEVEL (PAPER V)

The protein level of synaptophysin was quantitated by western blotting as described above. Briefly, the solubilized membrane protein was separated on 10% SDS-polyacrylamide gel followed by transfer to a PVDF membrane (Amersham, USA). The membrane was blocked in blocking buffer to reduce non-specific binding. The blot was then incubated with rabbit antibody against synaptophysin (1:3000, Dakocytomation, Glostrup, Denmark) overnight at 4°C. After washing, the membranes were incubated with horseradish peroxidase-conjugated anti-rabbit IgG antibody (1:3000, Santa Cruz Biotechnology Inc, CA, USA) for 60 min at room temperature. Finally, enhanced chemiluminescence (ECL; Amersham, USA) was used for exposure. The signal intensity of protein bands was quantitated with progress Scion Image (National Institutes of Health, MD, USA).

STATISTICAL ANALYSIS

Paper I

Group differences were analyzed for statistical significance by using ANOVA followed by post hoc test. The correlation of the percentage of astrocytes expressing the α7 nAChR subunit and the number of NP and NFT tangles was performed employing the correlation *Z* test.

Paper II

The two-tailed Student's *t*-test or ANOVA followed by Student-Newman-Keuls test were used to analyze the effect of treatment of Aβ25-35, Aβ1-40 and Aβ35-25 with or

without vitamin E on the protein levels and receptor binding sites of different nAChRs, cytotoxicity, lipid peroxidation and protein carbonyl level in PC12 cells.

Paper III

The two-tailed Student's *t*-test was used to compare the lipid peroxidation, the protein carbonyl level, and the protein level of α4 nAChR subunit, between AD patients and age-matched controls. A simple regression was used to evaluate the possible correlation between the increased level of lipid peroxidation and the decreased level of α4 nAChR subunit protein in AD patients.

Paper IV

Statistical analysis was carried out by using one factor ANOVA followed by post hoc test to calculate the significance between transgenic and control data within the same age group or different age groups. A simple regression was used to evaluate the possible correlations.

Paper V

The one-way ANOVAs followed by Newamn-Keuls post-hoc comparisons were used to analyze the differences of α7 +/+, α7 +/- and α7 -/- mice data within the same age group.

RESULTS AND DISCUSSION

The distribution changes of nAChRs in sporadic AD brain

The distribution changes of the α7 and the α4 nAChR subunits were detected in sporadic AD brains by immunohistochemical staining. Our results revealed that the α7 nAChR subunit was strongly expressed on both astrocytes and neurons, whereas the α4 nAChR subunit was only expressed on neurons (Fig. 6). A significant increase in the percentage of astrocytes expressing the α7 nAChR subunit was detected in the CA1, CA2 and CA3 regions of hippocampus and temporal cortex of sporadic AD brain compared to the aged-match controls (Fig. 4). In contrast, the expressions of the α7 and α4 nAChR subunits on neurons were significantly lower in all regions of the hippocampus and in all layers of the temporal cortex of sporadic AD brain in comparison to age-matched controls (Fig. 4).

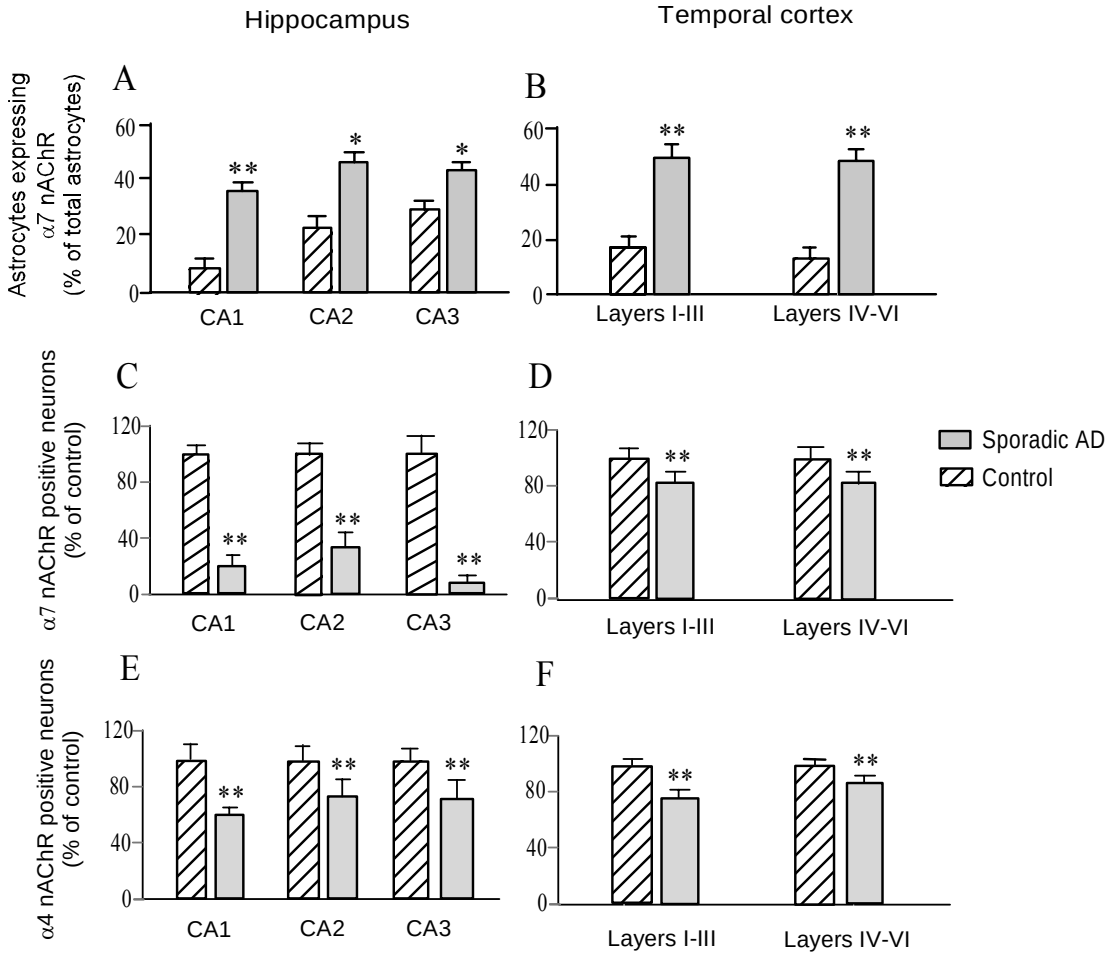


Figure 4. Increased percentages of α7 nAChR-positive astrocytes (A, B), decreased neuronal α7 nAChR subunit (C, D) and α4 nAChR subunit (E, F) in the hippocampus and temporal cortex regions of sporadic AD brain. Data are expressed as a percentage of the control group. *P < 0.05, **P < 0.01 compared to the age-matched control groups, as analyzed by the Student's unpaired t-test.

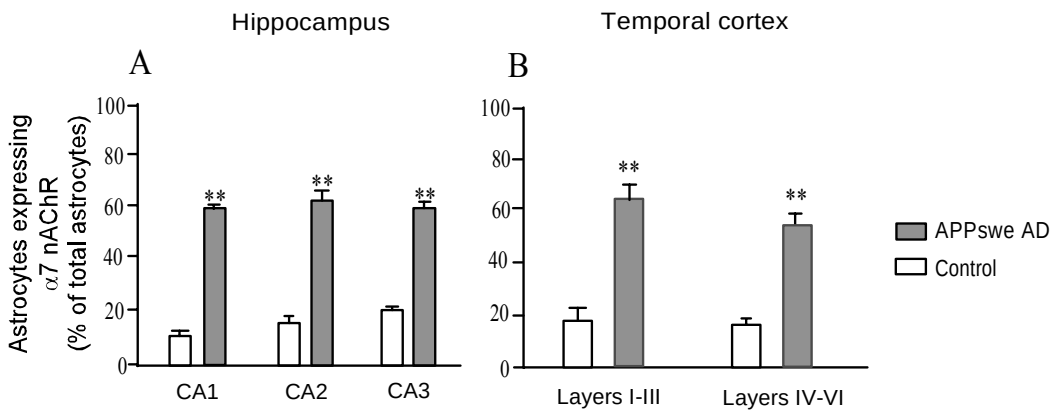
Our result is in agreement with previous studies that showed a significant decrease of the protein levels of neuronal α4 and α7 nAChR subunits and a significant increase of the astrocytic α7 nAChR subunits in AD brain (Wevers et al. 1999; Teaktong et al. 2003). Consistent and pronounced loss of the α4 nAChR subunit is well documented in several

brain regions of the sporadic AD brain (Warpman and Nordberg 1995; Martin-Ruiz et al. 1999; Guan et al. 2000; Wevers et al. 2000). However, conflicting results have also been reported in the level of α7 nAChRs in the AD brain (Martin-Ruiz et al. 1999; Burghaus et al. 2000; Guan et al. 2000). Since the levels of α7 nAChRs detected by ligand binding assays and Western blotting only provide information concerning the total binding capacity or total level of this protein, respectively, they do not explain the possible differential expression of the receptor protein on astrocytes and neurons. The values obtained by these methods will reflect the balance between the increased expression of the α7 nAChRs in astrocytes and a decreased expression of the α7 nAChRs in neurons. Our results might provide an explanation for the conflicting results obtained upon analyzing the levels of α7 nAChRs by binding assays or Western blotting in different studies on sporadic AD brain.

The distribution changes of nAChRs in AD carrying the Swedish APP670/671 mutation

A double mutation in codons 670 and 671 of the APP gene on chromosome 21 has been detected in a Swedish family with early-onset familial AD (APPswe) (Mullan et al. 1992) and shown to alter APP metabolism resulting in an over-expression of Aβ and consequently extensive formation of amyloid plaques (Citron et al. 1992). APPswe brains show more severely neuropathological changes, e.g., larger number of NPs and intracellular NFTs than are observed in sporadic AD brains (Bogdanovic et al. 2002).

We investigated the distribution changes of the α7 and the α4 nAChR subunits in APPswe brain by immunohistochemical staining. An increased of the percentage of astrocytes expressing the α7 nAChR subunit was detected in the CA1, CA2 and CA3 regions of hippocampus and temporal cortex of APPswe brain compared to the aged-match controls (Fig. 5). The expression of the α7 and α4 nAChR subunits on neurons were significantly lower in all regions of the hippocampus and in all layers of the temporal cortex of APPswe brain compared to age-matched controls (Fig. 5). The increased expression of α7 nAChRs on astrocytes and the decreased expressions of α7, α4 nAChR subunits in neurons were more pronounced in comparison to the sporadic AD brain. The presence of α7 nAChR on both astrocytes and neurons together with the elevated level of expression of α7 nAChR on astrocytes in the AD brain, especially in APPswe individuals, suggests that α7 nAChR may play a role in connection with Aβ pathology that differs from that played by the α4 nAChR receptor subunit that presents only neuronally. These results also confirm our earlier studies on α4 containing receptor binding in APPswe brains (Marutle et al. 1999).



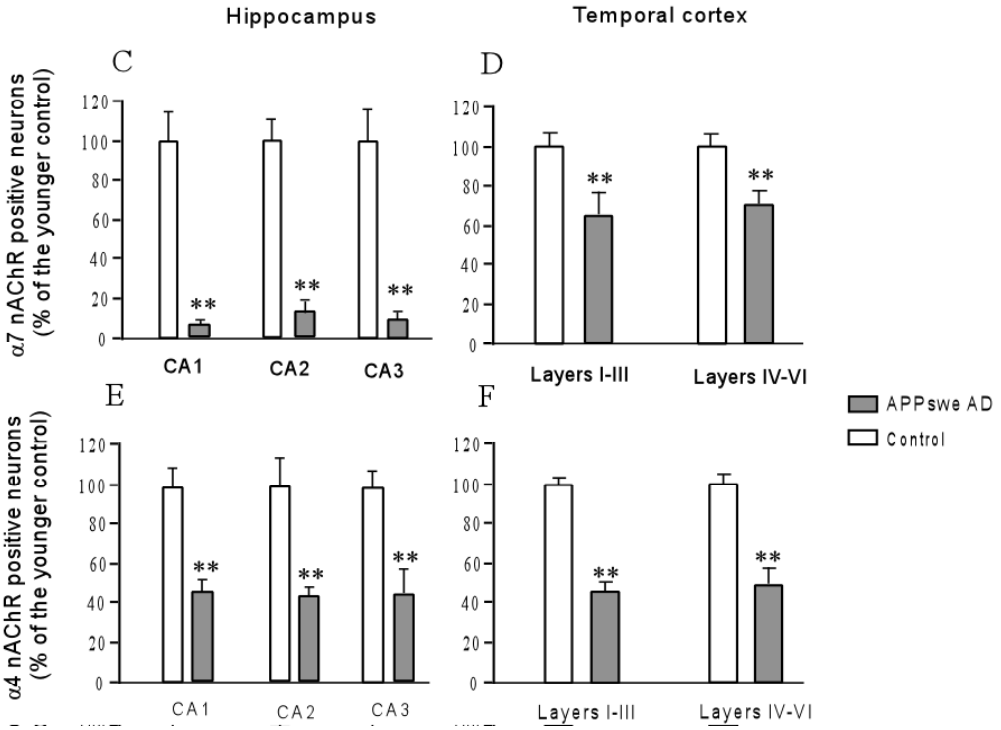


Figure 5. Increased percentages of α7 nAChR-positive astrocytes (A, B), decreased neuronal α7 nAChR subunit (C, D) and α4 nAChR subunit (E, F) in the hippocampus and temporal cortex regions of APPswe brain. Data are expressed as a percentage of the control group. **P < 0.01 compared to the age-matched control groups, as analyzed by the Student's unpaired t-test.

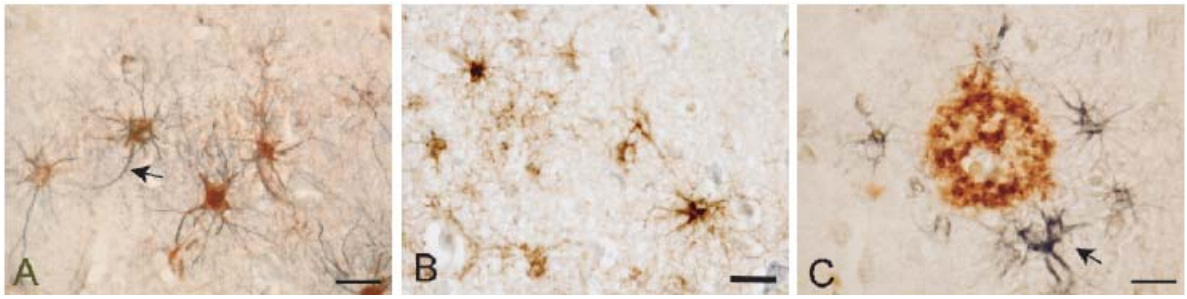


Figure 6. Expression of the nAChR subunits on astrocytes in human brain. Double immunolabeling for GFAP (brown) and α7 nAChR (blue) (A) or α4 nAChR (blue) (B) demonstrated that the α7 subunit was expressed on astrocytes. Double immunolabeling for Aβ (brown) and α7 nAChRs (blue) (C) showed the association of astrocytic α7 nAChR with NPs. The arrows indicate α7-positive astrocytes. Scale bar = 20 μm. (For colour figures see page 39)

Astrocytic α7 nAChR and neuropathological features in AD brains

In sporadic AD brain, the amyloid plaques consisted of both neuritic and diffuse forms. In the case of the APPswe brain, most of the plaques observed were neuritic forms, with a dense core and ring or with a condensing core and dispersed Aβ, and only a few amyloid plaques were in the diffuse form. In both the sporadic AD and APPswe brains, double staining for the nAChR α7 subunit and Aβ confirmed that α7 nAChR-positive astrocytes were associated with Aβ-immunoreactive plaques (Fig. 6). The α7 nAChR-positive astrocytes were often observed to surround or lie in the vicinity of the NPs and only seldom

around the diffuse plaques (Fig.7). Furthermore, a significant positive correlation was detected between the percentage of the astrocytes expressing α7 nAChR subunit and the number of NPs in both the hippocampus and temporal cortex of AD brains. A positive correlation was observed between the percentage of α7 nAChR-positive astrocytes and the density of NFTs in the CA1 region of the hippocampus of AD patients. Our results provide clear evidence that α7 nAChR-positive astrocytes are associated with NPs in the AD brain. A recent study suggested that astrocytes might provide a major contribution to amyloid plaques by enhancing Aβ42 accumulation and leading to selective lysis of Aβ42-burdened neurons (Terai et al. 2001; Nagele et al. 2003). The increased expression of α7 nAChRs on astrocytes in the AD brain, especially in APPswe cases, suggests a possible role of α7 nAChRs in these pathological processes.

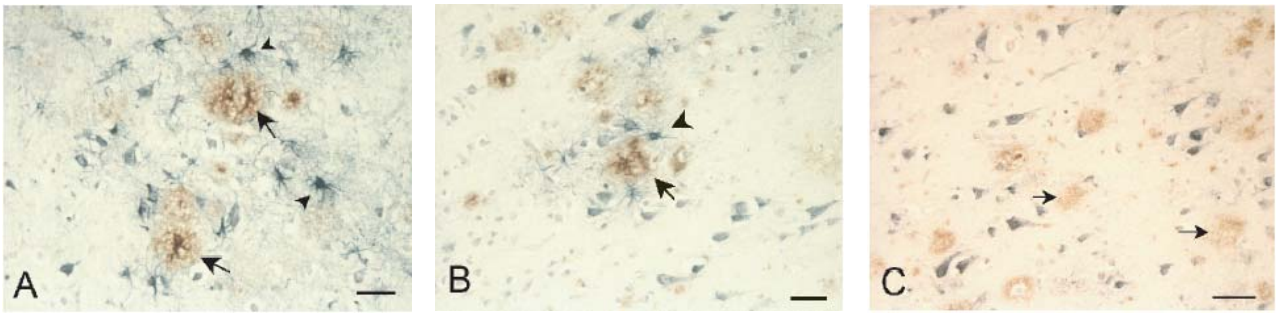


Figure 7. Double immunolabeling of Aβ (brown) and α7 nAChR (blue) in AD brains. The astrocytes expressing α7 nAChR surrounded or lie in the vicinity of neuritic plaques (large arrows) in both APPswe (A) and sporadic AD (B) brains; with only a few positive astrocytes being seen near the diffuse plaques (small arrows) in sporadic AD (C). Scale bar = 40 μm. (For colour figures see page 39)

Aβ-induced lipid peroxidation - a mechanism of the loss of nAChRs

Loss of neuronal nAChRs has been shown in AD brain, with decreased receptor ligand-binding sites and subunit protein levels in the cortical regions (Nordberg and Winblad 1986; Martin-Ruiz et al. 1999; Wevers et al. 1999; Guan et al. 2000; Wevers et al. 2000). Aβ, which plays a causal role in the pathogenesis of AD, has been shown to induce the decrease of nAChRs in PC12 cells (Guan et al. 2001a). Several studies have also shown that Aβ are involved in the generation of oxidative stress, including the lipid peroxidation and protein oxidation (Mark et al. 1997; Huang et al. 1999; Yatin et al. 1999). To prove that the Aβ-induced oxidative stress might be a possible mechanism of the loss of nAChRs, PC12 cells were treated by Aβs alone or together with the prior addition of antioxidant. The levels of lipid peroxidation, protein oxidation and the levels of nAChRs were measured.

Exposure of PC12 cells to 5 μM $A\beta_{25-35}$ for 48 h resulted in significant increases of MDA and conjugated dienes, two metabolic products of lipid peroxidation. In contrast, no significant increase of the level of carbonyl groups, an indicator of protein oxidation, was detected (Fig. 8).

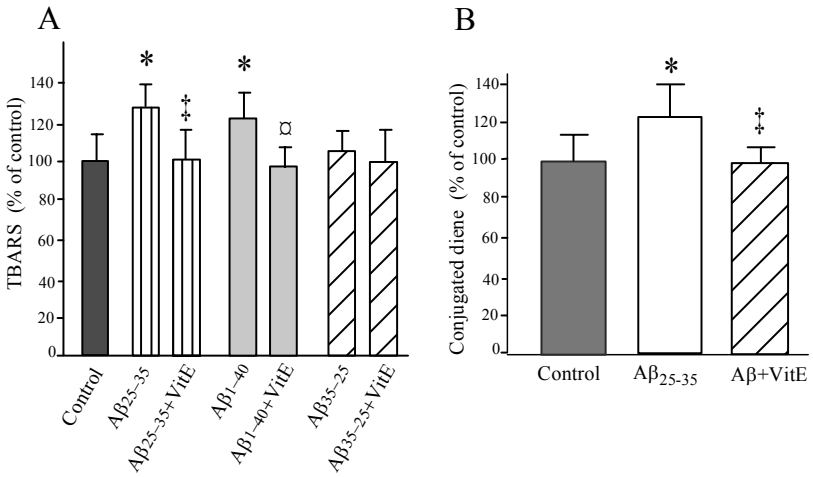


Figure 8. Lipid peroxidation products in PC12 cells treated by Aβs and Vitamin E (VitE). *$P < 0.05$ compared to control; $P < 0.05$ compared to the cells treated with $A\beta_{25\text{-}35}$; ¤$P < 0.05$ compared to the cells treated with $A\beta_{1\text{-}40}$. Two-paired Student's *t*-test.

A significant reductions of the numbers of [^{3}H] epibatidine, [^{125}I] α-bungarotoxin binding sites, and the protein levels of the nAChR α3 and α7 subunits were detected in the PC12 cells after exposure to 5 μM $A\beta_{25\text{-}35}$ and $A\beta_{1\text{-}40}$ for 48 h (Fig. 9). Interestingly, the increased lipid peroxidation and the decrease in nAChRs induced by Aβs in PC12 cells were completely prevented with addition of the antioxidant, vitamin E, before the treatment with Aβs (Fig. 8 & 9).

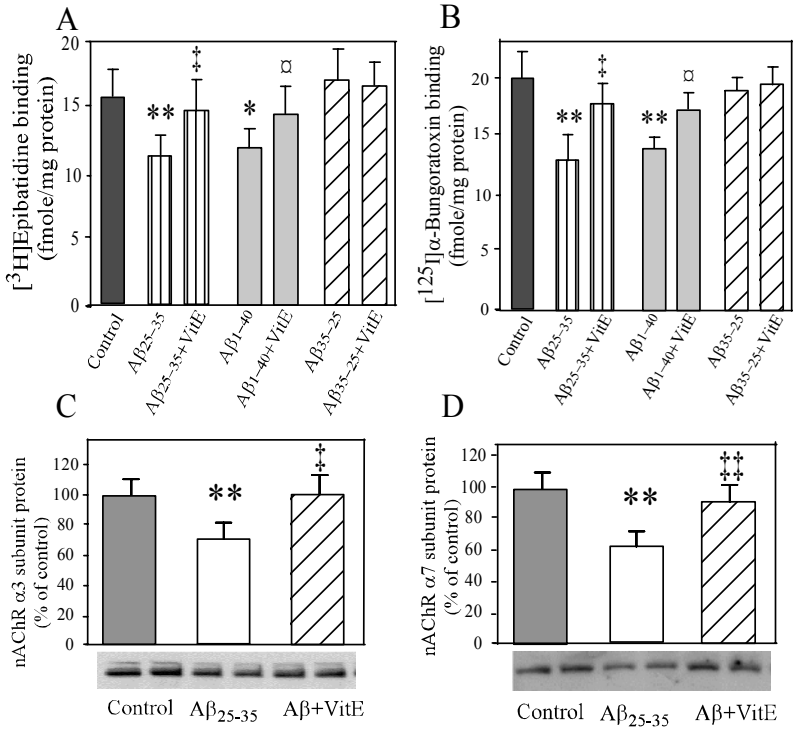


Figure 9. [^{3}H]Epibatidine, [^{125}I] α-bungarotoxin binding sites, nAChR α3 and α7 subunits at protein levels in PC12 cells treated by Aβs and vitamin E (VitE). *$P < 0.05$ compared to control; $P < 0.05$ compared to the cells treated with $A\beta_{25\text{-}35}$; ¤$P < 0.05$ compared to the cells treated with $A\beta_{1\text{-}40}$. Paired Student's *t*-test.

Recent studies have provided strong evidences for Aβ-associated oxidative stress damage to neurons as a fundamental process in AD (Miranda et al. 2000). Free radicals have been shown to impair the function of some membrane neurotransmitter receptors, such as muscarinic and serotonergic receptors (Abe et al. 1985; Hirata et al. 1995). Our results provided evidence that Aβ-induced lipid peroxidation may be involved in the loss of nAChRs. It has been postulated that peroxidation of membrane lipids may lead to a disturbance in membrane integrity and dysfunction of cells (Curtis et al. 1984; Richter 1987). In addition, 4-HNE, an aldehyde product of lipid peroxidation, that is neurotoxic in tissue culture and in vivo studies, is elevated in intracerebral ventricular fluid in AD (Markesbery and Lovell 1998). Aβ can increase the levels of free and protein-bound 4-HNE in cultured cells, and HNE mediates Aβ-induced oxidative damage of neuronal membrane proteins, which may be associated with Aβ-induced loss of nAChRs.

Lipid peroxidation and the loss of α4 nAChRs in AD brain

Oxidative stress has been implicated in the pathogenesis of AD (Smith et al. 2000). Several studies have shown that oxidative stress induced damage, including lipid peroxidation and protein oxidation, are increased in AD brain (Markesbery and Lovell 1998; Montine et al. 1998; Guan et al. 1999). Our earlier work suggested that a decrease in nAChRs may be result from lipid peroxidation in vitro (Guan et al. 2001b). We proposed that the increase level of oxidative stress may be a possible cause for the nAChR deficits in AD brain. In this study, we investigated whether the nAChRs are influenced by membrane lipid peroxidation or by a direct oxidation of the receptor proteins in AD brain. Lipid peroxidation, and protein oxidation in whole brain tissues and in cellular membrane protein fractions, as well as the protein levels of α4 nAChR subunits were measured in the temporal cortex AD brain.

The levels of MDA, one product of lipid peroxidation, were significantly increased in AD brains, whereas no significant changes in protein oxidation either in whole brain tissues or the cellular membrane protein fractions were detected in AD brains (Fig. 10). The protein levels of α4 nAChR subunit were significantly decreased in AD brains compared to the controls. Furthermore, there was a correlation between the increased levels of lipid peroxidation and the decreased levels of α4 subunit protein in AD brains (Fig. 10).

Our results demonstrate a significant increase in lipid peroxidation and a trend of increasing protein oxidation in the temporal cortex in AD patients, indicating an elevated situation of oxidative stress in AD brain (Smith et al. 2000). In addition, a significant decrease of the α4 subunit protein was detected in the temporal cortex of AD, which is consistent with the previous findings (Martin-Ruiz et al. 1999; Guan et al. 2000). The changed levels of nAChRs in cellular membranes may be thought toresult from to lipid peroxidation or protein oxidation damage due to an attack by free radicals in the AD brain. We therefore evaluated the oxidative damage to membrane receptor proteins in the temporal cortex of AD by employing Western blots. No difference was found between AD brains and age-matched controls in the oxidation of the membrane protein that corresponded to the molecular mass of the α4 subunit protein, indicating that there is no direct oxidation of the α4 subunit protein in AD brain. Interestingly, increased lipid peroxidation correlated well with the decreased levels of the α4 subunit in the temporal cortex of AD, which suggests that lipid peroxidation may demonstrably influence the expression of nAChRs in cellular membranes in these brains.

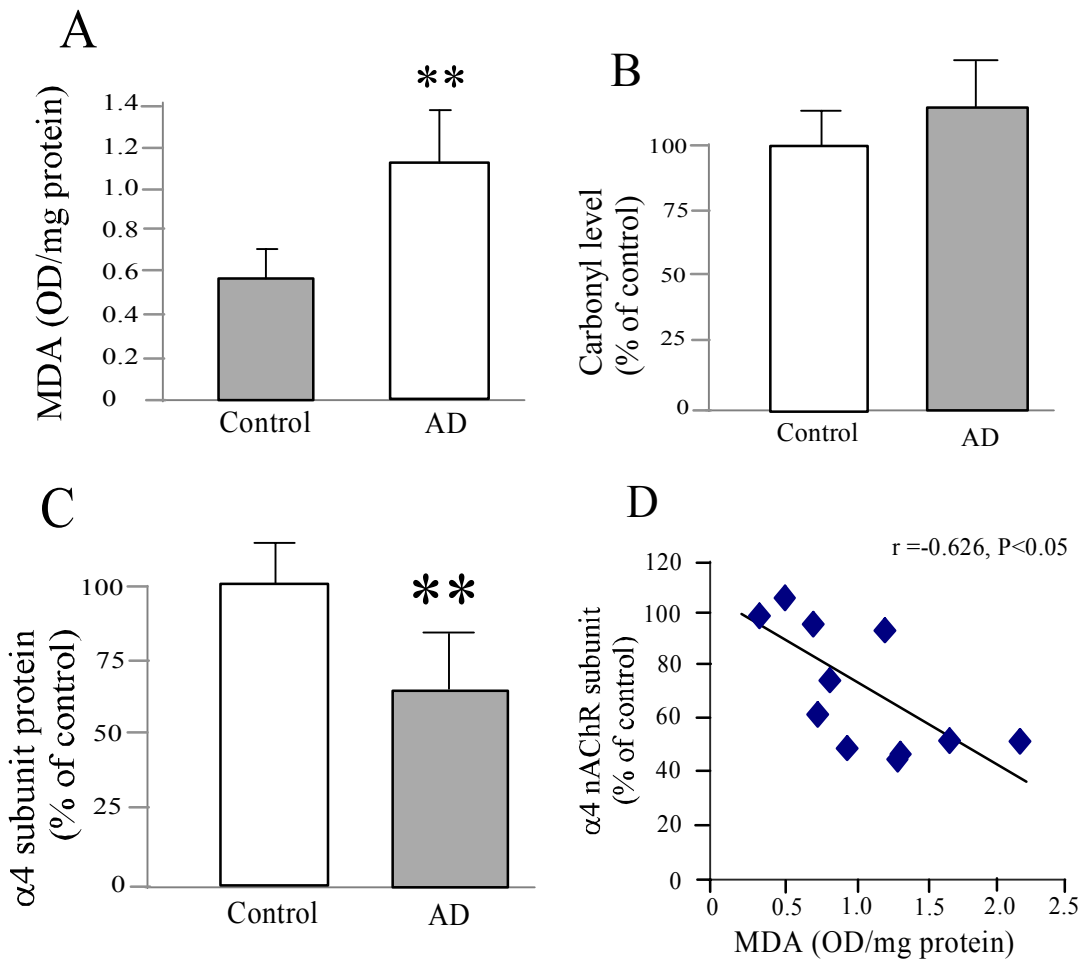


Figure 10. Lipid peroxidation (A), protein oxidation (B), rotein levels of α4 nAChR subunit (C) and the correlation between lipid peroxidation and the levels of the α4 nAChR subunit (D) in the temporal cortex of AD brain.

Aβ pathology and Aβ levels in hAChE-Tg//APPswe transgenic mice

Despite a decrease in AChE activity in the brain of AD patients, an increased activity of AChE is observed around the amyloid plaques early in the process of Aβ deposit (Inestrosa and Alarcon 1998). Studies have demonstrated that the increase in AChE may have a direct effect on the plaque formation in the brain (Talesa 2001; Rees et al. 2003). To investigate the possible interaction between Aβ, AChE, activated glia and nAChRs *in vivo*, the hAChE-Tg//APPswe transgenic mice are generated by crossing hAChE-Tg mice with APPswe transgenic mice.

The regional Aβ plaque formation in the brain of hAChE-Tg//APPswe mice was assayed by immunofluorescence labelling with the mouse antibody 6E10 directly against $A\beta_{1\text{-}17}$ (Fig. 11). Our study detected Aβ plaques in the neocortex and hippocampus of 7 months old transgenic mice (Fig. 11A, 10D). By 10 months of age small numbers of deposits were consistently detected in transgenic mice in both cortex and hippocampus (Fig. 11B, 10E). These Aβ plaques in 10 months old hAChE-Tg//APPswe mice were larger than those in age-matched APPswe mice (Fig. 11C, 10F).

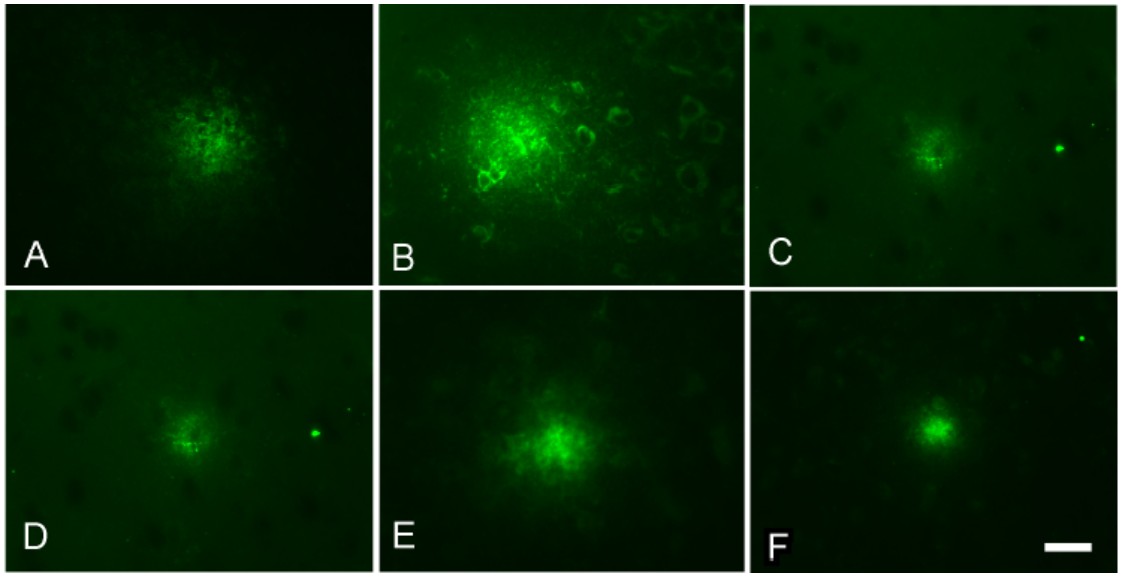


Figure 11. Aβ plaques in hAChE-Tg//APPswe and APPswe transgenic mice brain. Aβ plaques in the neocortex **(A)** and hippocampus **D)** of 7 -months -old hAChETg// APPswe mice. Aβ plaques in the neocortex **(B)** and hippocampus **(E)** of 10 -months -old hAChE-Tg//APPswe mice. Aβ plaques in the neocortex **(C)** and hippocampus **(F)** of 10-months-old APPswe mice. Scale bar = 30 μm.

This finding is in agreement with previous study employing this particular mouse model, which showed that the Aβ deposition occurred earlier and denser in these double transgenic mice, at ages of 6-12 month, in the cerebral cortex than age-matched APPswe transgenic mice (Rees et al. 2003). It has been shown that AChE directly modulates Aβ fibrillogenesis *in vitro* (Inestrosa et al. 1996). Our studies provide *in vivo* evidences for AChE promoting Aβ plaques, by comparing Aβ deposition between the hAChE-Tg//APPswe and APPswe transgenic mice.

An increase of the level of soluble Aβ1-40 was found at 10 months in comparison to 1 month old hAChE-Tg//APPswe transgenic mice (Fig. 12A). No significant change in insoluble Aβ1-40 level was detected (Fig. 12B). A significant decrease in insoluble Aβ1-42 level was found at 7 and 10 months compared with 1-month-old hAChE-Tg//APPswe transgenic mice (Fig. 12C). A qualitative comparison of the levels of the soluble and insoluble Aβ in the neocortex of 3 and 10 months old hAChE-Tg//APPswe and APPswe transgenic mice shows that the levels of both insoluble Aβ1-40 and Aβ1-42 were higher in hAChE-Tg//APPswe than in the APPswe transgenic mice. Conversely, the level of soluble Aβ1-40 was higher in APPswe than in hAChE-Tg//APPswe (Unger et al. 2005b; Unger et al. 2005a).

The result that the soluble level of Aβ1-40 was lower and the insoluble levels of Aβ1-40 and Aβ1-42 were higher in hAChE-Tg//APPswe mice, compared to single APPswe mice, suggests that AChE may promote Aβ formation *in vivo*. Complexes with AChE and Aβ peptide are more toxic to the cells than Aβ aggregated alone (Alvarez et al. 1998; Reyes et al. 2004). In addition, it has also been shown that AChE directly modulates Aβ fibrillogenesis suggesting that AChE may play a role in the development of the plaques by accelerating Aβ deposition (Inestrosa and Alarcon 1998; Bartolini et al. 2003).

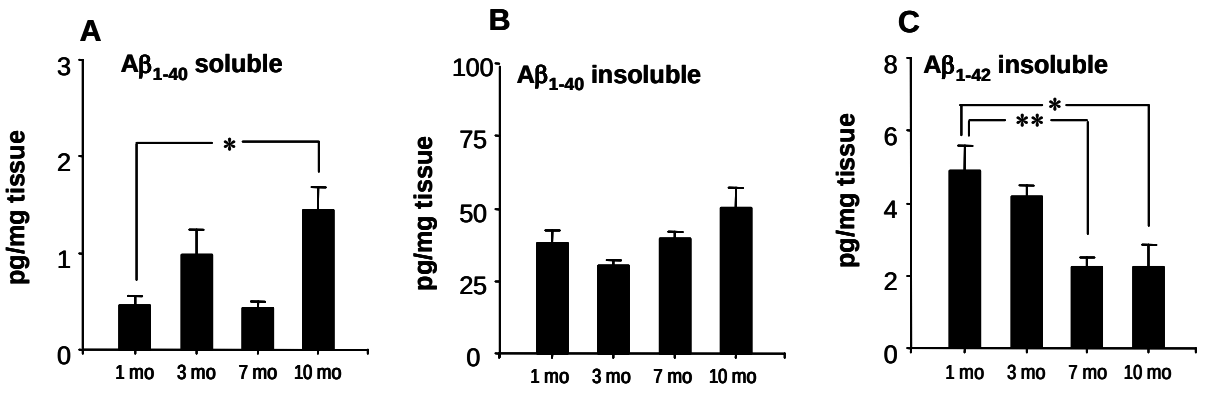


Figure 12. Aβ1-40 and insoluble Aβ1-40 and Aβ1-42 in the neocortex of 1, 3, 7, 10-month-old hAChE-Tg//APPswe mice. Results are expressed as mean ± SEM. Mo: months. One factor ANOVA. *$P < 0.05$, ** $P < 0.01$, compared to 1 month of age.

Activated astrocytes and α7 nAChRs in hAChE-Tg//APPswe transgenic mice

Immunostaining was performed in order to visualize reactive astrocytes in the brain of hAChE-Tg//APPswe mice. Clusters of GFAP immunoreactive astrocytes were seen closely associated with Aβ plaques in the neocortex and hippocampus in 10 months old hAChE-Tg//APPswe mice (Fig. 13). A significant increase in immunoreactivity for GFAP was detected by sandwich ELISA in the hippocampus at 1 month of age in the hAChE-Tg//APPswe transgenic mice compared to control mice. Interestingly, a significant increase of interleukin 1β, a pro-inflammatory marker, was detected in the neocortex of hAChE-Tg//APPswe mice at 7 months of age compared to non-transgenic mice.

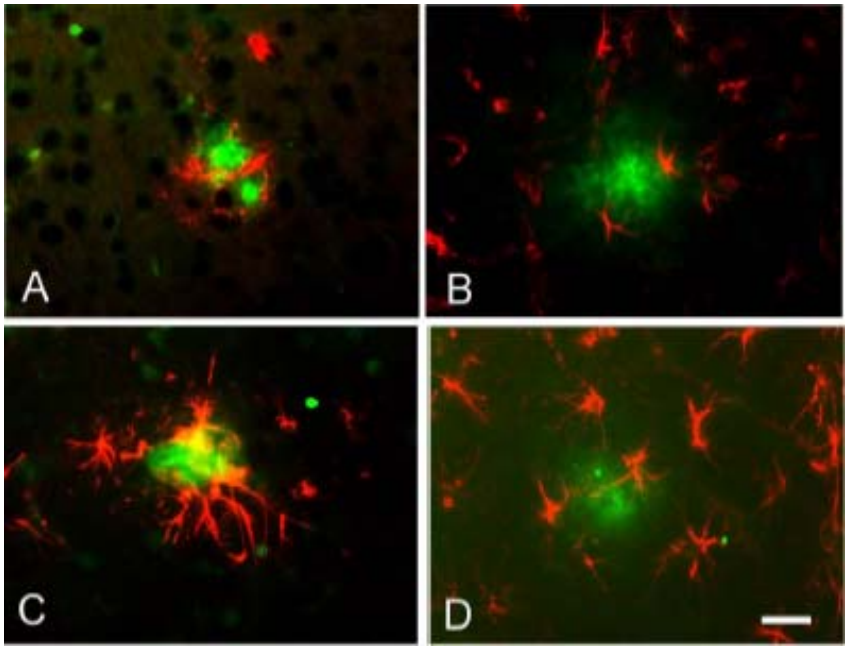


Figure 13. Double labelling of Aβ plaques and GFAP in hAChE-Tg//APPswe mice brain. Aβ deposits associated with GFAP immunoreactive astrocytes in the cortex **(A)** and hippocapmus **(B)** of 7-months-old transgenic mice. Of 10 months old transgenic mice, a similar association of Aβ deposition with GFAP immunoreactive astrocytes was observed in the neocortex **(C)** and hippocampus **(D)** in the transgenic mice. Scale bar = 30 μm. (For colour figures see page 39)

A significant increase in mRNA levels of the α7 nAChR subunit, measured by RT-PCR, was detected in the neocortex of hAChE-Tg//APPswe transgenic mice at 3 and 7 months of age compared to controls (Fig. 14). Similarly, a significant increase in [^{125}I] α bungarotoxin binding was found in the corticles of hAChETg//APPswe mice at 7 months of age compared to age-matched non-transgenic controls (Fig. 14).

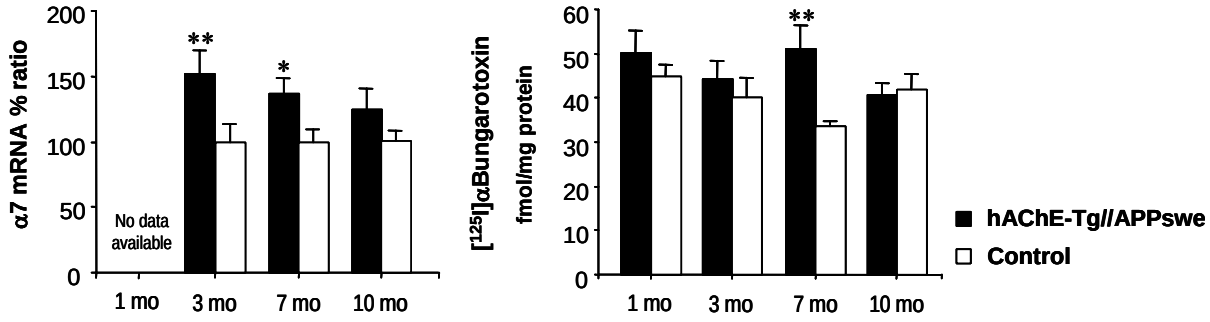


Figure 14. mRNA levels of the α7 nAChR subtypes and specific binding of [^{125}I] α-bungarotoxin in the cortex of hAChETg//APPswe and control mice. Results are expressed in mean ± SEM. *P < 0.05, **P < 0.01, significance level between transgenic and control mice by one factor ANOVA.

The presence of reactive astrocytes in association with Aβ deposition has been proposed as a pathogenic mechanism of the neuronal degeneration in AD brain (Dickson 1999). These reactive astrocytes contribute significantly to the inflammatory response in AD brain (McGeer and McGeer 1998; Akiyama et al. 2000). We observed an increased expression of GFAP in the hippocampus in the hAChE–Tg//APPswe transgenic mice at 1 month of age compared to control mice. Furthermore, there was increased expression of GFAP in the hippocampus in the hAChE-Tg//APPswe mice compared to that of APPswe transgenic mice at 10 months of age. Elevated levels of the pro-inflammatory cytokine IL-1β have been found associated with Aβ and reactive astrocytes in the brains of AD patients and APPswe transgenic mice (Dickson 1999; Mehlhorn et al. 2000). We observed an increase in the pro-inflammatory cytokine IL-1β in the neocortex of hAChETg//APPswe transgenic mice compared to controls at 7 months of age. Similarly, Aβ depositions have been associated with activated microglia, reactive astrocytes, and increased pro-inflammatory cytokines (Dickson 1999; Apelt and Schliebs 2001; Streit 2004).

The α7 nAChRs have been proposed to play an important role in the pathogenesis of AD by interaction with Aβ and regulation of the accumulation of Aβ (Wang et al. 2000b; Wang et al. 2000a). The binding of Aβ with neuronal α7 nAChR might play a key role in facilitating intraneuronal accumulation of Aβ (Nagele et al. 2002). The accumulation of Aβ in neurons will eventually result in the lysis of neurons, followed by a selective loss of neurons and form amyloidogenic plaques (Cuello 2005). Interestingly, the α7 nAChRs were shown distributed on both neurons and astrocytes (Graham et al. 2003; Teaktong et al. 2003). Our study in AD brain has showed that the elevated expressin of astrocytic α7 nAChRs might participate in the Aβ cascade and formation of NPs. In this study, the α7 nAChRs were increased in the cortex of the hAChE-Tg//APPswe transgenic mice compared to controls. The increased levels of the α7 nAChRs have also been reported in the brains of APPswe and APP/PS1 transgenic mice (Dineley et al. 2001; Bednar et al. 2002). The increased α7 nAChRs on the hAChE-Tg//APPswe transgenic mice probably participate in the Aβ cascade and formation of amyloid plaques in the brain of this transgenic mice.

Postnatal developmental changes of nAChRs in the brains of α7 -/- mice

nAChRs, especially α7 nAChR, have been found to be involved in neuronal differentiation and migration, as well as synapse formation during the brain development (Lipton and Kater 1989; Role and Berg 1996; Coronas et al. 2000). α7 nAChR are highly expressed in rat brain from early embryonic through postnatal stages, and in the postnatal human brain (Barrantes et al. 1995; Broide et al. 1995; Falk et al. 2002). Due to their high Ca^{2+} permeability, α7 nAChR may impact many developmental processes of neuron via Ca^{2+} -dependent processes, such as activation of second messenger systems and induction of immediate-early gene expression (Role and Berg 1996; Albuquerque et al. 1997; Broide and Leslie 1999). Additionally, α7 nAChR may contribute to synaptic strengthening during the early brain development (Jones et al. 1999). In attempt to understand the functional role of α7 nAChR in the brain, a mouse model with a null mutation in the α7 gene (Acra7) was developed (Orr-Urtreger et al. 1997). The α7-deficient mice are viable and anatomically normal, except for some cognitive abnormalities (Young et al. 2004). Little is known about the changes of nAChRs during the early developmental period of α7 -/- mouse brain. In this study, we characterized the developmental changes of different nAChRs in the brains of α7 -/-, α7 +/- and α7 +/+ mice during the postnatal developmental period.

Our results show that nAChRs display different developmental patterns in different brain regions during the postnatal developmental period of mouse brain. The [^{3}H] epibatidine and [^{3}H] cytisine binding sites start to increase from postnatal day 7 (P7), reach peak levels at P21, and then decline to adult levels at P28 in the mouse neocortex and hippocampus. In contrast, the number of [^{125}I] α-bungarotoxin binding sites increases in the cortex and hippocampus of α7 +/+ and α7 +/- mice at P7, reached its peak at P10 and thereafter decreases gradually to adult levels at P84. Significant increases in the number of [^{3}H] epibatidine and [^{3}H] cytisine binding sites were detected in the cortex and hippocampus of α7 -/- and α7 +/- mice during the postnatal developmental period compared to α7 +/+ mice (Fig. 15). A 40% decrease in the number of [^{125}I] α-bungarotoxin binding sites was detected in the α7 +/- mice compared to the α7 +/+ mice. Interestingly, small number of [^{125}I] α-bungarotoxin binding sites was detected in the cortex and the hippocampus of α7 -/- mice. Similarly, significant increases of the protein and mRNA levels of α4 and α3 nAChR subunits were detected in the brains of α7 -/- and α7 +/- mice during this developmental period compared to the α7 +/+ mice.

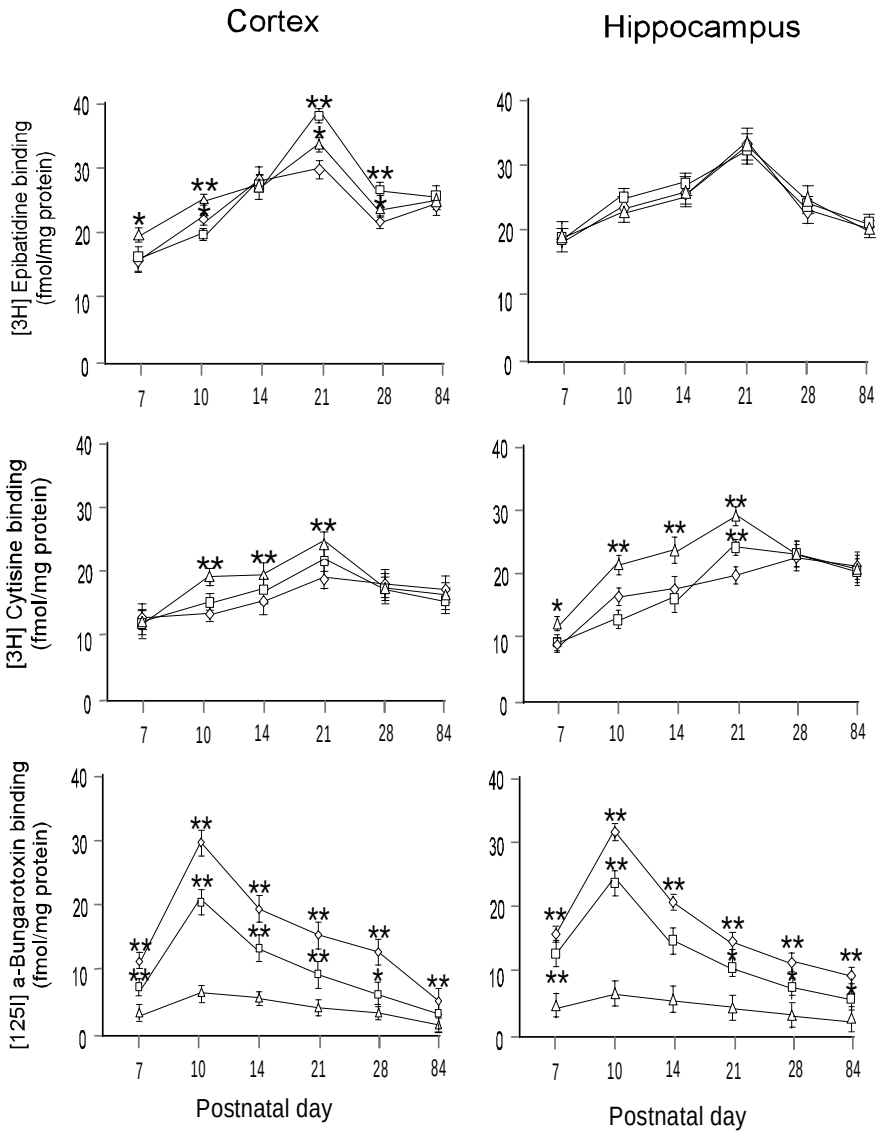


Figure 15. Developmental changes in [3H] epibatidine, [3H] cytisine and [125I] α-bungarotoxin binding sites in different brain regions of α7+/+, α7 +/-, α7 -/- mice brain. Data are means ± SEM of six separate determinations. *P< 0.05, **P< 0.01 compared to α7+/+ mouse, one-way ANOVA with Newamn-Keuls post-hoc comparisons.

In agreement with our results, several previous studies have shown that the number of [^{3}H] nicotine and [^{125}I] α-bungarotoxin binding sites, as well as the mRNA levels of nAChR α4, α7 and β2 subunits, were increased during early developmental periods in the neocortex and hippocampus of mouse and rat brains compared to the adult (Shacka and Robinson 1998). Furthermore, the developmental profiles of nAChRs correlate well with the structural rearrangement and progressive neurochemical maturation in many brain regions (Gould et al. 1991; Naeff et al. 1992). The high expression of nAChRs during the early developmental period of mouse brain suggests an important role of nAChR signaling in the maturation of brain structures during this period. Interestingly, our study show significant increases in the numbers of [^{3}H] cytisine (α4 nAChR) and [^{3}H] epibatidine (α3 and α4 nAChR) binding sites, and increases in protein and mRNA levels of α4 and α3 nAChR subunits, in the brains of α7 -/- and α7 +/- mice during the postnatal developmental period. We propose that the increases in α4 and α3 nAChRs may replace the functional role of α7 nAChRs and

compensate for the lack of or decrease in α7 nAChRs during the brain developmental period of α7 -/- and α7 +/- mice.

Possible co-assemble of the α4, α3 with α5 nAChR subunit in mouse brain

Immunoprecipitation was performed to investigate whether α4 and α3 nAChR subunits could combine with α5 nAChR subunit in the brain of α7 -/- mice. Immunoprecipitation assays with polyclonal antibody against α5 nAChR followed by immunoblotting for α4 or α3, demonstrated the association between α5 and α4 or α3 nAChR subunits (Fig. 16A). Similarly, reciprocal immunoblotting assays with antibody against α5 nAChR after immunoprecipitation with α4 or α3 nAChR subunit antibodies, respectively, showed the α4 and α3 nAChR were associated with α5 nAChR subunit (Fig. 16B).

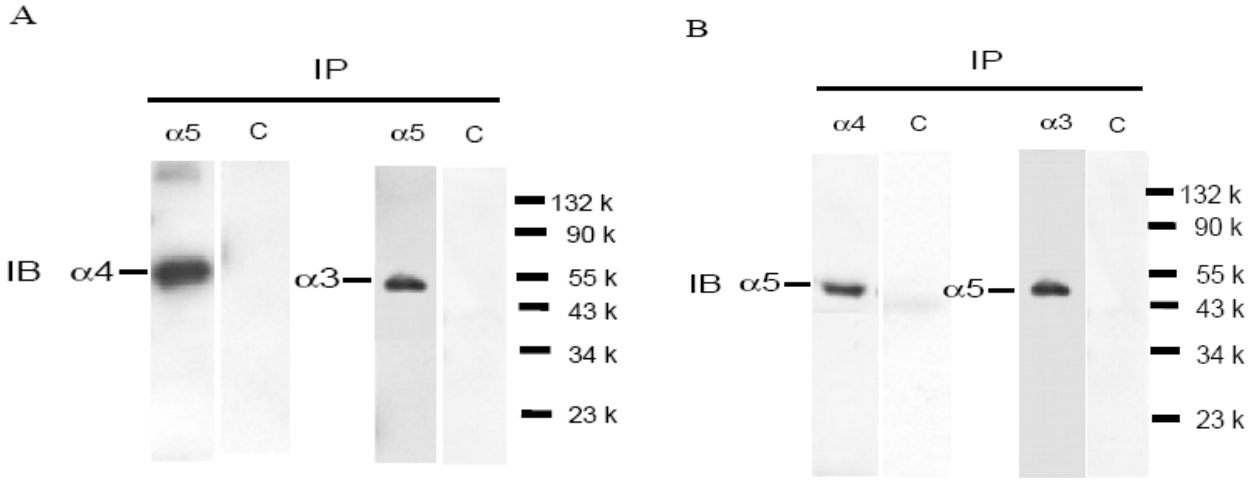


Figure 16. α4 and α3 nAChR subunits are co-assembled with the α5 nAChR subunit in the brains of α7 -/- mice. (A). Immunoprecipitation from the brain extracts of 3-weeks old α7 -/- mice with anti-α5 nAChR subunit antibody and then subjected to SDS-PAGE, electroblotted, and probed with either α4 or α3 nAChR subunit antibody. (B). Immunoprecipitation from the brain extracts of 3-weeks old α7 -/- mice with α4 and α3 nAChR subunit antibodies and then subjected to SDS-PAGE, electroblotted, and probed with α5 nAChR subunit antibody .The results showed that the α4 and α3 nAChR subunits are co-assembled with the α5 nAChR subunit in the brain of α7 -/- mice. Similar results were obtained in three experiments. IP: immunoprecipitate IB: immunoblotting C: negative control.

The cholinergic neurotransmitter system has been proposed to serve trophic functions and influence brain development (Lipton and Kater 1989; Lauder 1993; Role and Berg 1996). The nAChRs use Ca^{2+} signaling as a second messenger system. The α7 nAChR and the α4, α3-containing nAChRs can generate and mediate Ca^{2+} signals in different ways during the early developmental period of mouse brain (Role and Berg 1996; Albuquerque et al. 1997; Atluri et al. 2001). Ca^{2+} signals in turn can impact many developmental processes in neural cells, including cell proliferation, differentiation, apoptosis, survival, and neurite extension (Lipton and Kater 1989; Lauder 1993; Pugh and Berg 1994; Role and Berg 1996). Compared to the α4- and α3-containing nAChRs, the α7 nAChR show higher permeability to Ca^{2+} and this high degree of Ca^{2+} permeability is likely to underlie many of its functions (Role and Berg 1996; Messi et al. 1997). Interestingly, combinations of the α5 nAChR subunit with the α4- and α3-containing nAChRs can significantly increase their Ca^{2+} permeability to a similar level as observed for NMDA receptors and α7 nAChR (Ramirez-Latorre et al. 1996; Gerzanich et al. 1998). The possible combination of increased α4- and

α3-containing nAChRs with the α5 nAChR subunit in mouse brain may have the potential to compensate for the lack or decrease of α7 nAChR during the brain development of α7 -/- and α7 +/- mice. To test this hypothesis, we immunoprecipitated mouse α5 nAChR subunit and probed immunoblots with antibodies directed against α4 and α3 nAChR subunits. The results showed that the α4- and α3-containing nAChRs may combine with the α5 nAChR subunit in the brains of these mice. In the case of lack of or decrease in α7 nAChR density, it is plausible that an increase in α4- and α3-containing nAChRs, with high Ca^{2+} permeability incorporated through the α5 nAChR subunit, may replace the function of α7 nAChRs and contribute to the brain development of α7 -/- and α7 +/- mice.

CONCLUDING REMARKS

AD is the most common form of dementia. One characterized pathological feature of AD is the accumulation of Aβ. This process is also accompanied by oxidative stress reactions, glia cell activation, inflammatory reactions and the disturbance of the cholinergic system. nAChRs are ligand-gated ion channels that are widely distributed in human brain. Numerous subtypes of nAChRs exist, with α4β2 and α7 the main subtypes in human brain. Consistent loss of α4β2 nAChRs has been observed in AD brain while Aβ is the major constituent of neuritic plaques and has been shown to be causative for the pathogenesis of AD. Aβ-induced neurotoxicity might be related to nAChR deficits in AD brain. The α7 nAChRs have been proposed to play a role in the pathogenesis of AD by interaction with Aβ. α7 nAChRs and Aβ co-localize in NPs and individual cortical neurons. These two proteins bind together with high affinity and form a stable complex. Since the interaction between the Aβ and nAChRs might play an important role in the pathogenesis of the AD brain, one aim of this thesis is to characterize the changes nAChRs in AD brain and their possible interactions between Aβ. Transgenic mice that overexpress the mutant forms of APP, alone or together with other proteins, including AChE, have an overproduction of Aβ plaques in the brain. It is a significant advantage to further understand the underline mechanisms, such as Aβ deposition and the relation between Aβ, glia activations and cholinergic neurotransmission in these transgenic mice.

The distribution changes of different nAChRs were investigated in the brains of the APPswe and sporadic AD by using immunohistochemical staining. Significant increases in the total numbers of astrocytes and of astrocytes expressing the α7 nAChR subunit, along with significant decreases in the levels of α7 and α4 nAChR subunits on neurons, were observed in the brains of both APPswe and sporadic AD. The increased expression of α7 nAChRs on astrocytes and the decreased expressions of α7, α4 nAChR subunits on neurons in the brain of APPswe were more pronounced in comparison to the sporadic AD brain. The decreased neuronal nAChRs might reflect the neurodegenerative condition of neurons in AD brain. The increased level of astrocytic α7 nAChR was positively correlated with the extent of neuropathological alternations in both APPswe and sporadic AD brains. Furthermore, the astrocytic α7 nAChR was morphologically associated with amyloid plaques in AD brain. The elevated expression of α7 nAChR on astrocytes might participate in the Aβ cascade and formation of NPs, thereby playing an important role in the pathogenesis of AD.

Significant reductions in different nAChR subunits on neurons has been reported (Martin-Ruiz et al. 1999; Wevers et al. 1999; Guan et al. 2000) in AD brain. Whether Aβ-induced oxidative stress and damage is a possible mechanism to account for the deficits in neuronal nAChRs in AD brains was investigated in both AD brain and cell culture. The results from cell culture showed that lipid peroxidation induced directly by Aβ might be involved in the deficits of nAChRs. Similarly, increased lipid peroxidation, loss of α4 nAChR subunits and the correlation between them were detected in AD brain. These findings suggested that lipid peroxidation stimulated by Aβ might be a mechanism for the loss of nAChRs associated with the pathogenesis of AD.

Despite a decrease in AChE activity in the brain of AD patients, increased activity of AChE is observed around the amyloid plaques early in the process of Aβ deposition (Inestrosa and Alarcon 1998). Studies have demonstrated that the increase in AChE may have a direct effect on plaque formation in the brain (Talesa 2001; Rees et al. 2003). The possible interactions among Aβ, AChE, activated glia and nAChRs in the brain of hAChE-

Tg//APPswe mice were investigated in this thesis. Results showed that the soluble level of Aβ1-40 was lower and the insoluble levels of Aβ1-40 and Aβ1-42 were higher in hAChE-Tg//APPswe mice, compared to single APPswe mice, confirming that AChE may promote Aβ formation *in vivo*. Significant increases of GFAP, as well as the mRNA and the binding sites of α7 nAChRs, have also been detected in hAChE-Tg//APPswe mice compared to control mice. Furthermore, immunostainnig showed astrocytes were associated with Aβ deposition in the brain of hAChE-Tg//APPswe mice. These findings suggested that the hAChE-Tg//APPswe transgenic mice may be a suitable model to study the impact of accelerated Aβ accumulation in the brain, which may lead to novel therapies in AD.

The α7 nAChR plays an important role during the development of the brain. The developmental expressions of different nAChRs in the brains of α7 -/-, α7 +/- and α7 +/+ mice during the postnatal developmental period were investigated in this thesis. The results showed increased numbers of [^{3}H] cytisine (α4 nAChR) and [^{3}H] epibatidine (α3 and α4 nAChR) binding sites, and increased protein and mRNA levels of α4 and α3 nAChR subunits in the brains of α7 -/- and α7 +/- mice compared to α7 +/+ mice during this developmental period. Furthermore, the α4 and α3 nAChR subunits appeared to co-assemble with α5 nAChR subunit in the brains of these mice. Our results suggest that an increased number of α4, α3-containing nAChRs, with high degree of Ca^{2+} permeability co-assembled with α5 nAChR subunit, may have a compensatory effect on the lack or decrease of α7 nAChR during the development of α7 -/- and α7 +/- mice in brain.

In conclusion, these studies show that selective changes of different nAChR subunits between neurons and astrocytes in AD brain. The decreased neuronal α4 and α7 nAChRs might reflect the neurodegenetative condition of neurons in AD brain. The Aβ-induced lipid peroxidation was suggested as a possible mechanism of the deficits of neuronal nAChRs in AD brain. The selective increase of astrocytic α7 nAChRs and its morphological association with amyloid plaques might suggest that the astrocytic α7 nAChRs are involved in the Aβ cascade and the formation of NPs in AD brain. Based on the possible interaction between Aβ process and nAChRs, especially the α7 nAChRs, the α7 nAChRs might be a promising target for neuroprotective therapy in AD.

Figure 6

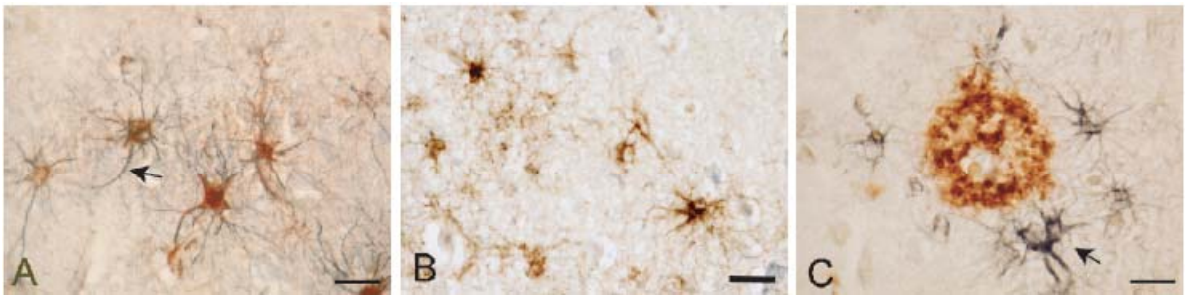


Figure 7

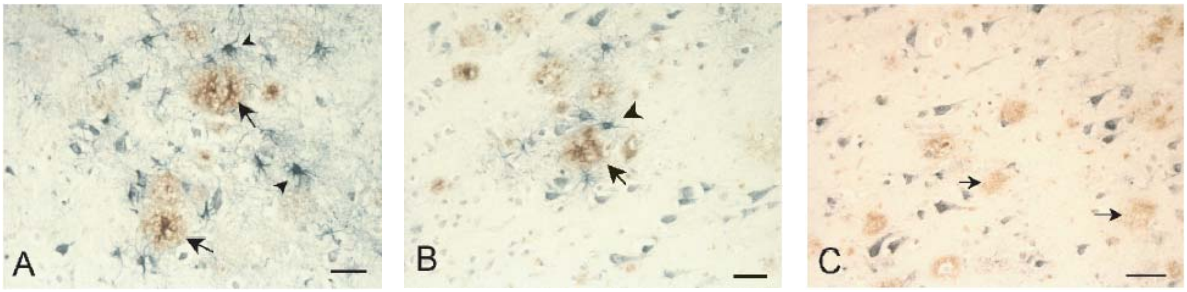
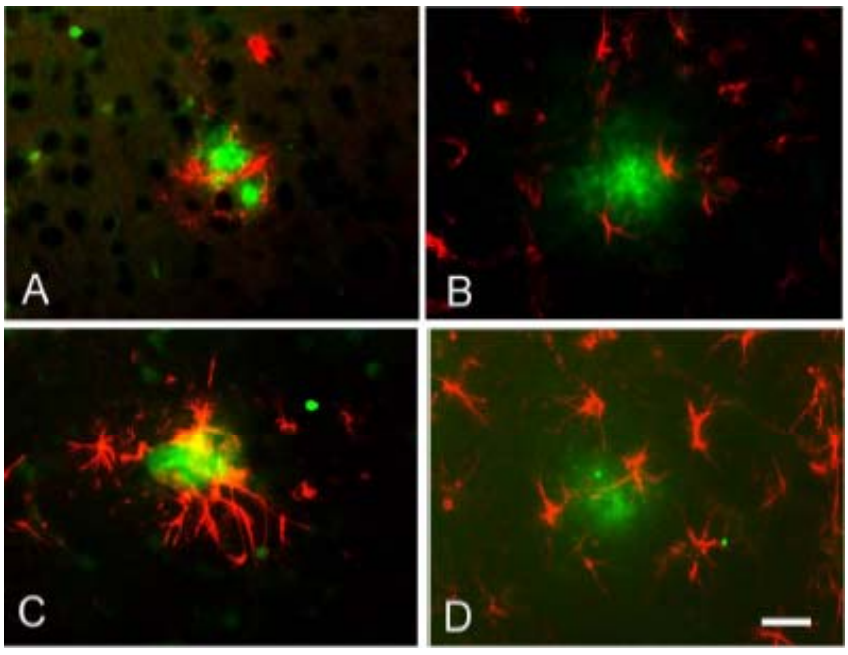


Figure 13



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