Cerebral amyloidosis in a transgenic mouse model of Alzheimer's disease: Impact and therapy

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Abbreviations

ACh	acetylcholine
AChE	acetylcholinesterase
AD	Alzheimer's disease
APP	amyloid precursor protein
Aβ	β-amyloid
CAA	cerebral amyloid angiopathy
ChAT	choline acetyltransferase
mAChR	muscarinic acetylcholine receptor
nAChR	nicotinic acetylcholine receptor
NBM	nucleus basalis of Meynert

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Summary

Senile dementia is a diagnostic category that includes all types of cognitive and memory impairments that occur in the elderly. Alzheimer's disease (AD) is the most common form of dementia, which is characterized by progressive impairments in memory, cognition, praxis, language, and behavior. Neuropathologically, major features of AD include extracellular accumulation of amyloid β (Aβ) peptide in form of plaques, intracellular tangles composed of hyperphosphorylated tau protein, selective neurodegeneration, and synapse loss. More specifically, the cholinergic system is compromised in AD. Cholinergic disruption in the neocortex and the hippocampus is exemplified by diminished density of cholinergic terminals and fibers, reduction in cholinergic receptors, and decreased choline acetyltransferase (ChAT) and acetylcholinesterase (AChE) enzyme activities. In addition, loss of cholinergic neurons in the basal forebrain, the major input to cortex and hippocampus, has been reported.

The principal risk factors for AD include aging, mutations in the genes of amyloid precursor protein (APP), presenilin (PS) 1 and PS2, and the presence of the ε4 allele of the apolipoprotein E (apoE) gene. Mutations in APP, PS1 and PS2 genes are inherited in an autosomal dominant fashion and are the cause of early-onset familial AD (FAD). FAD accounts only for a minor percentage of all AD cases. It is, however, clinically and pathologically indistinguishable from the much more common sporadic AD found in the elderly. FAD mutations result in alteration of APP processing, leading to overproduction of Aβ and thus formation of plaques. Based on the knowledge on the genetic factors leading to AD, transgenic mice carrying FAD mutations were generated and used to elucidate the role of Aβ in AD pathogenesis. The studies presented herein make use of the well-established APP23 mouse model of cerebral amyloidosis. APP23 mice bear the FAD Swedish mutation on the APP gene, which results in increased production of amyloidogenic Aβ and develop amyloid deposits progressively with age. Moreover, linked to extensive amyloid deposition, these mice show additional AD-like characteristics such as cerebral amyloid angiopathy, microglial activation, selective neuron loss, and cognitive impairment.

The purpose of the first part of the present research was to study the interaction of amyloidosis and the cholinergic system. Therefore, we elucidated the extent of the cholinergic changes in APP23 mice to deduce the contribution of amyloidosis to the cholinergic deficit seen in AD. Stereological quantification of cortical cholinergic fibers and measurement of ChAT and AChE enzyme activities implied a cholinergic disruption in the neocortex. To establish whether this deficit was due to a loss of cholinergic source neurons in the basal forebrain, ChAT-positive neurons in different nuclei such as the medial septum and the nucleus basalis of Meynert (NBM) were quantified with stereological methods. No decrease in cholinergic neuron number could be found, suggesting that cortical cholinergic deficit is a local phenomenon and is solely attributable to plaque formation. To further study the interaction between cholinergic system and amyloid plaque formation and to test the hypothesis that cholinergic depletion has an effect on amyloid plaque formation, the major cholinergic source to the neocortex, the NBM, was experimentally lesioned. This lesion induced a cortical loss of cholinergic fibers and enzyme activities, and subsequent plaque formation was monitored. Results revealed a decreased plaque load in denervated areas. The outcome of this experiment suggested that amyloid deposition and the cholinergic system are somehow linked but the hypothesis that loss of cholinergic input does promote plaque formation could not be confirmed.

The aim of the second study was to clarify the impact of Aβ on neocortical synapses. Although amyloid load has been shown to correlate with loss of synapses in AD, this finding is complicated by the presence of neurofibrillary tangles and the loss of subcortical input. Therefore, we followed neocortical synaptic changes throughout the development of plaques. The presynaptic vesicular protein synaptophysin was analyzed in different age groups of APP23 and wild-type mice, from 3 months of age with no plaques, to 24 months with severe plaque load. Densitometric analysis of Western blots did not reveal any differences between APP23 mice and wild-type controls. This finding was further supported by stereological synapse counting, which showed that no synapses are lost with aging in either genotype group. Moreover, APP23 mice did not bear decreased synapse number compared with wild-types. Our results suggest that Aβ

deposition is not sufficient to account for the synapse loss seen in AD. Alternatively, a possible trophic effect of APP may prevent or delay a loss of synapses in our mouse model.

In the last part of the work presented here, we studied the effect of passive immunization with antibodies against Aβ in APP23 mice. Vaccination holds great potential in the fight against AD, and clinical trials with human patients have been undertaken. However, we have shown that although very efficient in removing amyloid deposits, immunization may bear the danger of leading to cerebral hemorrhages. Although this finding might appear discouraging, it contributes to the understanding of the mechanisms involved in plaque formation and thus eventually leads to new therapeutical approaches.

In summary, through the use of transgenic mice, the presented studies have brought improved understanding of the pathogenesis of AD. We have shown that cerebral amyloidosis is the cause for cortical cholinergic fiber and enzyme activity loss. Interestingly, this fiber loss does not lead to retrograde degeneration of cholinergic basal forebrain neuron cell bodies. Furthermore, overall synapse number was not changed in the neocortex even with high plaque load. Together, these findings exemplify the complexity of the impact of amyloid. The question remains open, whether the toxicity of amyloid plaques has a direct effect or plays a more regulatory role in the complex cascade leading to neurodegeneration. Clearance of amyloid by passive immunization concomitantly induced microhemorrhages, further demonstrating the complex effects of amyloid. Together, these studies are part of a better understanding of the pathophysiological mechanisms leading to AD and are fundamental for new therapies based on the causes of AD.

1. Introduction

1.1 Alzheimer's disease

In 1907, Dr. Alois Alzheimer, a German neurologist and psychiatrist, described the neuropathological features found in the brain of Auguste Deter, a 56-year-old woman who had suffered from progressive dementia (Alzheimer, 1907). Dementia is a syndrome characterized by deterioration of previously acquired intellectual abilities. Today, Alzheimer's disease (AD) is known as a progressive neurodegenerative disorder that occurs predominantly in later life. Following symptom onset, the course of the disease varies considerably from a few years to over 20 years, with a mean survival time of approximately 8 years (Forstl and Kurz, 1999). AD represents the most common form of dementia, with a prevalence of 10% in 65-year-olds and up to 40% for 90-year-olds (Ebly et al., 1994; Lautenschlager et al., 1996; Lobo et al., 2000). The number of individuals affected by this devastating condition is thought to increase as the clinical management of other life-threatening conditions reduces death by other causes, thus resulting in a growing population of the elderly. Causing tremendous costs for institutional care for AD patients, AD represents a major public health problem.

1.1.1 Clinical presentation

There is a great variation in the clinical presentation of the neuropsychological and cognitive abnormalities in AD. Nonetheless, initial presentation typically involves memory and learning impairment, and word-finding problems. As the disease progresses, the symptoms become increasingly distressing, as language problems, spatial disorientation, dysfunction of the motor system, disturbed circadian rhythm, and restlessness cause major problems. In latter stages the patient is completely debilitated and requires constant supervision (for review see Morris et al., 1989; Forstl and Kurz, 1999).

1.1.2 Neuropathological hallmarks

A variety of neuropathological hallmarks are found in the AD brain. Characteristic features include extracellular deposits of β-amyloid (Aβ), intracellular neurofibrillary tangles, neuronal and synaptic loss, and depletion of the cholinergic system.

Aβ can be deposited in form of spherical neuritic plaques of 10-120 µm diameter which consist of extracellular masses of Aβ filaments. These can be associated with dystrophic dendrites and axons, activated microglia, and reactive astrocytes (Selkoe, 1991; Dickson, 1997; Selkoe, 1999, 2001). More frequently, Aβ is deposited in form of amorphous diffuse plaques which are not associated with dystrophic processes (Selkoe, 1991; Dickson, 1997; Selkoe, 1999, 2001). Aβ can also be deposited in leptomeningeal and cortical vessels, a feature called cerebral amyloid angiopathy (CAA) (Vinters, 1987; Yamada et al., 1987; Jellinger, 2002). CAA compromises the integrity of the vessel wall and can lead to cerebral hemorrhage. Cerebral amyloidosis occurs predominantly in the neocortex and the hippocampus, but diffuse amyloid deposits are also found in cerebellum, striatum, and thalamus (Selkoe, 1991, 2001). It has been shown that Aβ in the form of peptides, oligomers, and plaques are neurotoxic and can inhibit cholinergic signaling (Yankner, 1996; Auld et al., 1998; Hartley et al., 1999; Pettit et al., 2001; Urbanc et al., 2002).

Neurofibrillary tangles are intraneuronal cytoplasmic structures composed of paired helical filaments of the hyperphosphorylated microtubule-associated protein tau (Goedert, 1993; Lee et al., 2001). As consequence of the hyperphosphorylation, tau is unable to bind to microtubules and is believed to self-assemble into paired helical filaments. Tangle-bearing neurons lose their ability to function and eventually die. The first regions affected by tangles are the entorhinal cortex, amygdala, hippocampus, and nucleus basalis of Meynert (NBM). As the disease progresses, also neocortical areas are affected (Goedert, 1993; Braak et al., 1999).

Additional features in the AD brain are decrease in neocortical thickness and enlargement of the third and lateral ventricles (Bundgaard et al., 2001). While no global neocortical loss occurs in AD, selective neuronal loss occurs in the entorhinal cortex, the CA1 region of the hippocampus, the hilus and the subiculum (Regeur et al., 1994; West et al., 1994; Gomez-Isla et al., 1996). It has been established that synapses are lost in AD. Several studies have shown a decreased amount of synaptic markers in the hippocampus (Masliah et al., 1994; Scheff and Price, 1998; Sze et al., 2000; Bertoni-Freddari et al., 2002), and in the neocortex (DeKosky and Scheff, 1990; Scheff et al., 1990; Terry et al., 1991; Sze et al., 2000; Tiraboschi et al., 2000; Masliah et al., 2001; Bigio et al., 2002). Moreover, this synaptic loss has been demonstrated to correlate with cognitive impairment (DeKosky and Scheff, 1990; Terry et al., 1991). However, whether synapse loss is an early event in AD remains contradictory. It is possible that at least in some regions such as the frontal cortex, synapse loss might be an early event in the progression of the disease (Masliah et al., 2001). On the other hand, several studies have found that loss of synaptic markers occurred only in late stages of AD, and failed to detect synaptic deficit in mild cases of AD (Masliah et al., 1994; Mukaetova-Ladinska et al., 2000; Tiraboschi et al., 2000; Minger et al., 2001).

1.2 The cholinergic system and Alzheimer's disease

A special emphasis falls to the cholinergic system. Originally, the observation that healthy people treated with anticholinergic agents show cognitive deficits and loss of memory lead to the postulation of the cholinergic hypothesis saying that cognitive deficit is due to cholinergic dysfunction (Bartus et al., 1982). Acetylcholine (ACh) has been one of the best-studied transmitters and is still subject of intensive research. The vast knowledge on this neurotransmitter system and its role in AD (see 1.2.2) resulted in enhanced drug research and therapeutical approaches involving the cholinergic system (see 1.4.1).

1.2.1 Synthesis and release of acetylcholine

ACh is synthesized in the neuronal cytoplasm from choline and acetyl coenzyme A by the soluble enzyme choline acetyltransferase (ChAT). Following synthesis, ACh is transported from the cytoplasm into synaptic vesicles by a vesicular acetylcholine transporter (VAChT) protein, where the transmitter is stored until release. After release from the presynaptic nerve terminal and binding to postsynaptic muscarinic (mAChR) and nicotinic (nAChR) receptors, ACh is hydrolyzed by extracellular acetylcholinesterase (AChE) to yield choline and acetate (Soreq and Seidman, 2001; Giacobini, 2003). Choline is taken up by the high-affinity choline transporter (CHT) located at the presynaptic plasma membrane, and may be used as a precursor in the synthesis of new neurotransmitter by ChAT (Fig. 1) (Soreq and Seidman, 2001). The process of choline uptake in presynaptic terminals by the high-affinity CHT is thought to be essential for efficient ACh recycling and is the rate-limiting step in ACh synthesis (Tucek, 1985). The high-affinity choline transport system is distinct from the multiple low-affinity choline transport pathways that exist to provide choline for metabolic needs and membrane phospholipid synthesis (Okuda and Haga, 2003).

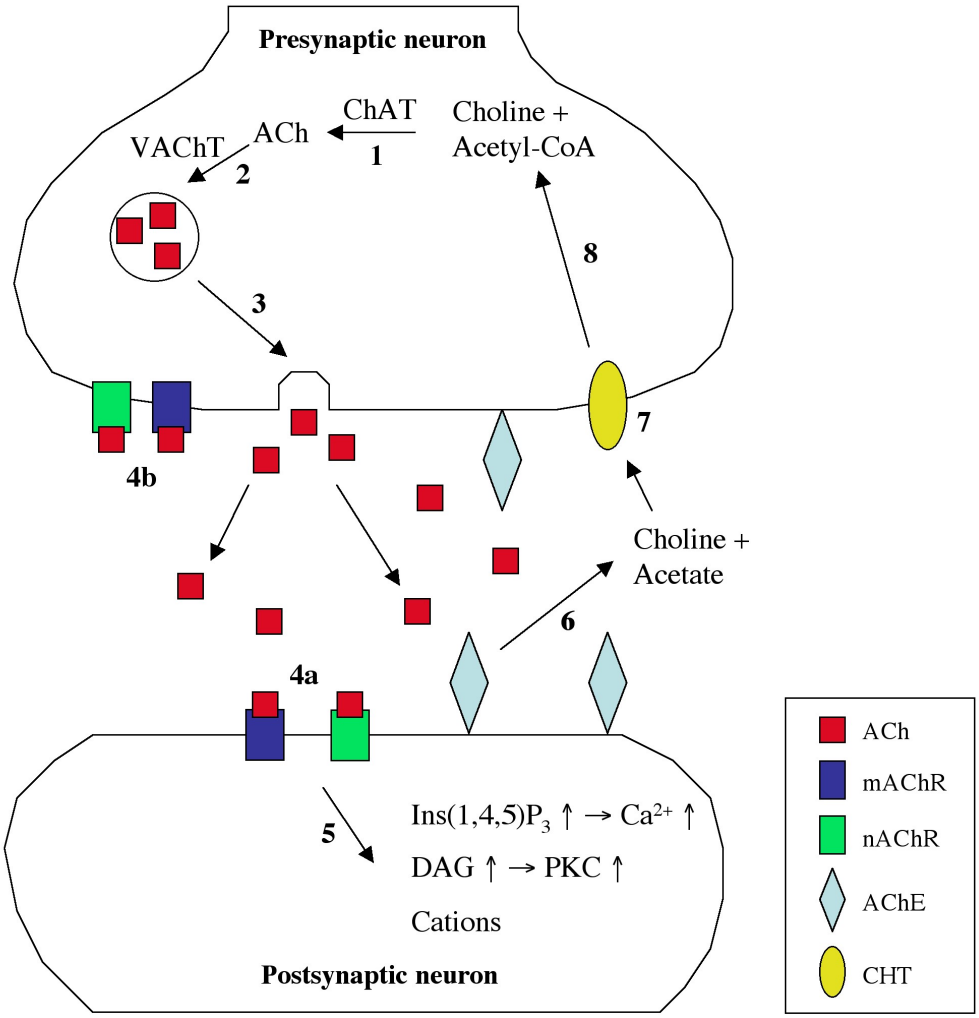

Figure 1. The cholinergic synapse

1 In the presynaptic neuron, ChAT catalyzes the synthesis of ACh from choline and acetyl coenzyme A.
2 ACh is packaged in synaptic vesicles via VAChT.
3 Action potentials trigger the release of ACh into the synaptic cleft.
4a ACh binds to mAChR and nAChR receptors located on the postsynaptic membrane.
4b ACh binding to presynaptic receptors regulate ACh release via feedback response.
5 mAChR receptors transduce signals through a pathway involving diacylglycerol (DAG), inositol-1,4,5-trisphosphate (Ins(1,4,5)P_3) and Ca^{2+}-dependent protein kinase (PKC). ACh binding to nAChR modulates permeability for cations.
6 ACh is hydrolyzed in the synaptic cleft by AChE which terminates cholinergic synaptic transmission.
7 Choline is taken up by CHT at the presynaptic plasma membrane.
8 Choline is recycled to produce ACh.

(Soreq et al., 2001; modified)

1.2.2 Cholinergic changes in Alzheimer's disease

ACh plays an important role in learning and memory (Winkler et al., 1995; Muir, 1997). Deficits in cholinergic neurotransmission are correlated with the severity of dementia (Wilcock et al., 1982; Bierer et al., 1995). Early in AD, many aspects of the cholinergic neurotransmitter system undergo profound changes (Bartus et al., 1982). In the neocortex and the hippocampus, cholinergic fiber loss, significant reduction of ChAT and AChE enzyme activities, decrease of cholinergic receptors, and diminished density of cholinergic terminals have been reported (Shimohama et al., 1986; Perry et al., 1992; Ransmayr et al., 1992; Svensson et al., 1997; Geula et al., 1998; Davis et al., 1999). In addition, cholinergic neurons in the basal forebrain, the major input to cortex and hippocampus, are lost (Whitehouse et al., 1981; Whitehouse et al., 1982; Vogels et al., 1990; Jope et al., 1997; Cullen and Halliday, 1998). The cortical cholinergic deficit is likely linked to the loss of cholinergic neurons in the basal forebrain. This loss could be caused by a disruption of their cortical terminal field followed by retrograde degeneration, as Aβ has been shown to be neurotoxic (Yankner, 1996; Hartley et al., 1999; Urbanc et al., 2002). Alternatively, death of cholinergic basal forebrain neurons could be explained by the formation of intracellular tangles in these neurons (Cullen and Halliday, 1998). A link between cerebral amyloidosis and the cholinergic system is suggested by the observation that AChE is accumulated by neurites associated with Aβ plaques in AD brain (Moran et al., 1993).

Whether the cholinergic system influences the deposition of amyloid or whether Aβ has an effect on the cholinergic system remains unclear due to contradictory findings. On the one hand, it has been suggested that AChE accelerates the assembly of amyloid peptide into insoluble β-amyloid fibrils thus enhancing plaque formation (Inestrosa et al., 1996; Rees et al., 2003). Different hypotheses could explain the underlying mechanism: (i) AChE could modulate amyloid formation by inducing a conformational change in Aβ, (ii) AChE could act as a nucleator, thus increasing the rate of fibrillogenesis, (iii) AChE could bind to and stabilize the growing of amyloid fibrils, (iv) AChE could act together with other plaque-associated proteins and promote fibrillization. On the other hand, Aβ has been shown to increase AChE levels by

elevating AChE expression and release from astrocytes, thus providing an explanation for the accumulation of AChE seen around plaques in AD brain (Saez-Valero et al., 2003). Furthermore, in vitro studies have shown that activation of muscarinic M1 and M3 receptors by cholinergic agonists results in increased DAG formation and PKC activation and stimulates the nonamyloidogenic α-secretory pathway of amyloid precursor protein (APP), and thus decreases the generation of amyloidogenic APP fragments (Nitsch et al., 1992; Lin et al., 1999). In agreement with these findings, in vivo studies showed that treatment with M1 and M3 muscarinic receptor agonists results in decreased levels of full-length APP, suggesting a stimulation of α-secretase pathway (Lin et al., 1999). Further support for an amyloid-lowering effect by stimulation of cholinergic receptors comes from Nordberg et al. who show that treatment with nicotine decreases both insoluble $A\beta_{1-40}$ and $A\beta_{1-42}$ in cortex and hippocampus of transgenic mice (Nordberg et al., 2002). The mechanism of induced attenuation of β-amyloidosis by nicotine administration may involve an effect of nicotine on the β-pleated sheet of Aβ. Nicotine might therefore act on Aβ aggregation, thus leading to a reduction in the accumulation of Aβ. In contrast, other studies suggest that cholinergic deprivation leads to Aβ deposition (Beach et al., 2000). It might be possible that cholinergic deafferentiation leads to increased release of Aβ by deafferented neurons. Taken together, this data imply a link between the cholinergic system and Aβ formation.

1.3 Genetics of Alzheimer's disease

The majority of AD cases are sporadic, whereas only a small percentage of cases are hereditary (Selkoe, 1999). Familial AD (FAD) is clinically and pathologically indistinguishable from sporadic AD, except for the early age of onset before age 50 (Lippa et al., 1996). Autosomal dominant mutations have been discovered in the following genes: (i) APP gene on chromosome 21 (Goate et al., 1991), (ii) presenilin (PS) 1 gene on chromosome 14 (Sherrington et al., 1995), and (iii) PS 2 gene on chromosome 1 (Rogaev et al., 1995). FAD mutations result in overproduction of total

Aβ (Citron et al., 1992; Cai et al., 1993) and/or increase of the more fibrillogenic form of Aβ, $A\beta_{1-42}$ (Suzuki et al., 1994; Tamaoka et al., 1994) (see section 1.3.1).

Besides the autosomal dominant mutations causing early-onset AD, several susceptibility genes have been discovered. These genes increase the risk of developing AD. One of these genes is the apolipoprotein E (apoE) on chromosome 19 (Roses 1996; Laws et al., 2003). Carriers with the ε4 allele have an increased risk of developing AD and lowers the age of dementia onset in a dose-dependent fashion, whereas the ε2 allele may protect against AD, or at least delay its onset (Strittmatter and Roses, 1995). The major function of apoE is to mediate the clearance of lipoproteins. It has been proposed that apoE2 and apoE3 isoforms complex with Aβ and lead to Aβ clearance. The observation that the formation of apoE-Aβ complexes is promoted by apoE2 and apoE3 but not apoE4, together with the increased plaque burden seen in AD patients expressing apoE4 leads to the assumption that apoE4 might result in impaired Aβ clearance (Laws et al., 2003). Another genetic risk factor is the insulin-degrading enzyme (IDE) gene on chromosome 10. IDE cleaves small proteins such as Aβ, insulin and glucagon. As consequence, IDE hypofunction leads to accumulation of cerebral Aβ (Bertram et al., 2000; Ertekin-Taner et al., 2000; Myers et al., 2000; Farris et al., 2003). Moreover, homozygosity for haplotype B of the cystatine C gene on chromosome 20 has been shown to be a further risk factor (Finckh et al., 2000). Another gene involved in late-onset AD has been suggested to be α-2 macroglobulin (Pericak-Vance et al., 1997), although these results have not been consistently replicated (Gibson et al., 2000; Poduslo et al., 2002). Other putative genes contributing to late-onset AD could be located on chromosomes 4, 6, 9, 12, and 20 (Pericak-Vance et al., 1997; Pericak-Vance et al., 2000; Poduslo and Yin, 2001).

1.3.1 APP and Aβ

APP is an ubiquitously expressed type 1 transmembrane glycoprotein encoded by a gene located on chromosome 21 (Masters et al., 1985). The exact function of APP is not yet clear, but it has been suggested to play a role in cell adhesion, cell growth, neuron

viability, neuroprotection, axonogenesis, arborization, and regulation of haemostasis (Perez et al., 1997; Storey and Cappai, 1999). Alternative splicing of the APP gene gives rise to at least three transcripts that encode proteins of 695, 751, and 770 amino acids (Hardy, 1997; Hartmann, 1999; Selkoe, 1999). All of the APP isoforms contain the 39-43 amino acids long Aβ domain. APP can undergo different proteolytic cleavage pathways. Cleavage by α–secretase occurs inside of the Aβ domain at amino acid 17 of Aβ (cleavage between residues 687 and 688 of APP) and therefore does not result in Aβ production but in release of soluble sAPPα and an 83-residue C-terminal peptide (C83) (Fig. 2). Cleavage by β-secretase at amino acid 1 of Aβ (cleavage between residues 671 and 672 of APP) produces sAPPβ and a 99-residue C-terminal (Fig. 2). Both C83 and C99 are substrates for γ-secretase which cuts at amino acid 39-43 of Aβ (cleavage at residues 710-714 of APP) and yields a 3-kD peptide called p3 from cleavage of C83, and the 4-kD Aβ from cleavage of C99 which is highly amyloidogenic and is deposited in form of plaques (Fig. 2) (Hardy, 1997; Hartmann, 1999; Selkoe, 1999). Several mutations in the APP gene, especially near the cleavage sites, have been found to be the cause of a shifted secretase activity resulting in increased Aβ production. An overview is given in Table 1.

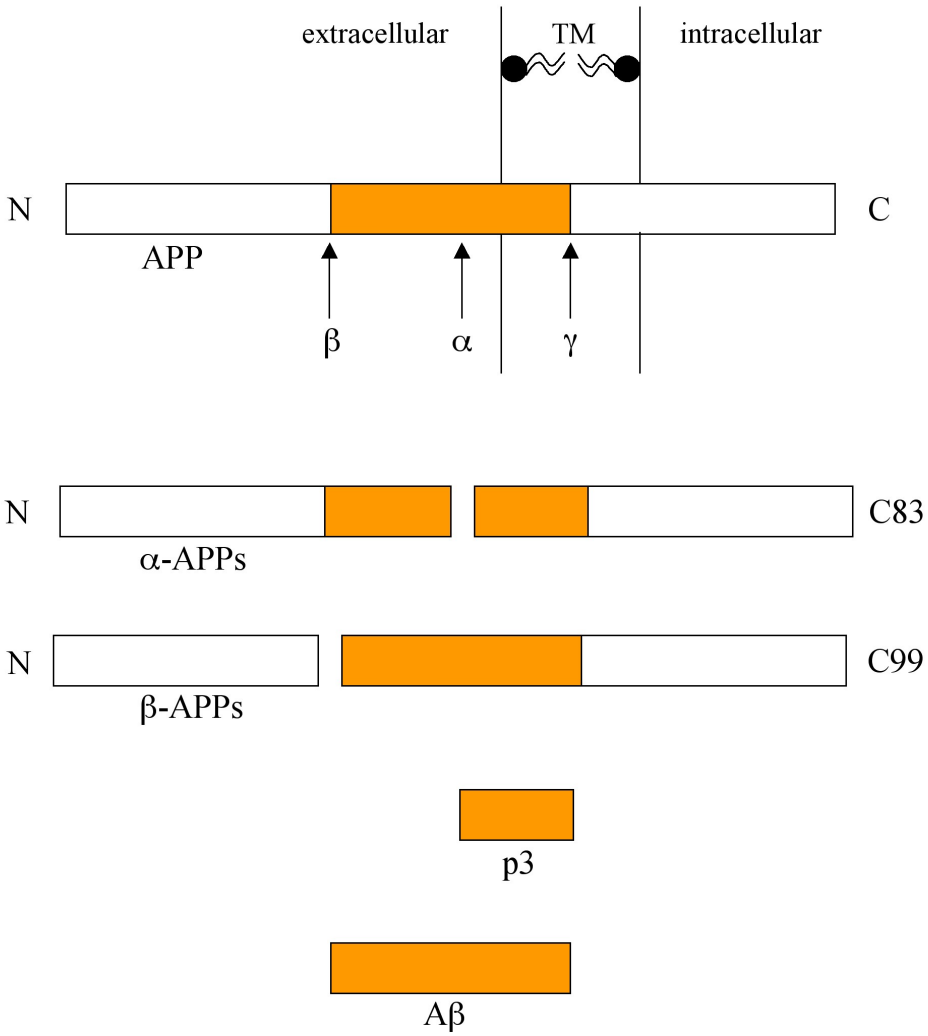

Fig. 2. APP processing. α-secretase cuts within the Aβ region to produce α-APPs and an 83-residue C-terminal fragment (C83). β-secretase generates β-APPs and a 99-residue C-terminal fragment (C99). Both C83 and C99 are substrates for γ-secretase, which performs proteolysis in the middle of the transmembrane (TM) domain to produce a p3 peptide from C83 and Aβ from C99.

Table 1. Examples of APP mutations related to increased Aβ production (Hardy, 1997; De Jonghe et al., 1998; Ancolio et al., 1999; Kumar-Singh et al., 2000b; Kwok et al., 2000; De Jonghe et al., 2001; Grabowski et al., 2001; Nilsberth et al., 2001; Pasalar et al., 2002).

Mutation (amino acid substitution)	APP codon	Effect
Swedish (Lys→Asn/Met→Leu)	670/671	Increase of $A\beta_{40}$ and $A\beta_{42}$
Flemish (Ala→Gly)	692	Increase of $A\beta_{40}$ and $A\beta_{42}$
Arctic (Glu→Gly)	693	Increased protofibril formation
Iranian (Thr→Ala)	714	Increase of $A\beta_{42}$
Austrian (Thr→Ile)	714	Increase of $A\beta_{42}$
German (Val→Ala)	715	Increase of $A\beta_{42}$
French (Val→Met)	715	Increase of $A\beta_{42}$
Florida (Ile→Val)	716	Increase of $A\beta_{42}$
London (Val→Phe/Gly/Ile)	717	Increase of $A\beta_{42}$
Australian (Leu→Pro)	723	Increase of $A\beta_{42}$

Several candidates have been suggested to represent the secretases: the adamalasyn proteinase (ADAM10) and the tumor necrosis factor-α (TNF-α) converting enzyme (TACE or ADAM17) are putative α–secretases (Marcinkiewicz and Seidah, 2000; Esler and Wolfe, 2001); β-secretase is an aspartic protease (beta-site APP-cleaving enzyme, BACE, memapsin 2 or Asp 2) (Sinha et al., 1999; Vassar et al., 1999; Yan et al., 1999; Lin et al., 2000; Marcinkiewicz and Seidah, 2000; Cai et al., 2001; Esler and Wolfe, 2001); and γ-secretase consists of a complex of proteins including presenilin, the glycoprotein nicastrin, and the transmembrane proteins aph-1 and pen-2 (Wolfe et al., 1999; Esler et al., 2000; Yu et al., 2000; Esler and Wolfe, 2001; Francis et al., 2002; Edbauer et al., 2003).

1.4 Therapeutic strategies for Alzheimer's disease

Different strategies have been undertaken to treat AD. To this date, the most widespread approaches include symptomatic treatments such as AChE inhibitors that ameliorate cholinergic deficits (see 1.4.1) and drugs that act against neuroinflammation (see 1.4.2). More recently, approaches aimed to act on the causes of the disease have been developed. Strategies to prevent amyloid deposition include immunization and modulation of the secretases (see 1.4.3 and 1.4.4).

1.4.1 Modulation of the cholinergic system

The most consistent neurotransmitter disruption involves the cholinergic system, and therefore intensive pharmacological research and therapy is based around rectifying this transmitter deficit. Pharmacological enhancement of cholinergic activity involves presynaptic agents such as choline, lecithin, and piracetam that increase ACh synthesis and release (Winkler et al., 1998). In addition, postsynaptic agents that directly stimulate ACh receptors such as muscarinic agonists (e.g. oxotremorine) and nicotinic agonists (e.g. galanthamine, nicotine) are also known to play an important role (Albuquerque et al., 1997). Moreover, involvement of synaptic agents that increase the availability of ACh by limiting its breakdown, namely AChE inhibitors, have been described (Bores et al., 1996; Cutler et al., 1998; Winkler et al., 1998; Grutzendler and Morris, 2001). Therapy with cholinergic precursors has been proven to be ineffective, whereas agonists appear to be promising although the principal beneficial effect of nicotinic agonists and cholinomimetics compounds may not be to enhance memory but rather to improve attention (Winkler et al., 1998; Whitehouse, 1999). Nicotine treatment has been shown to induce upregulation of nAChR sites, and the mechanism by which this happens is thought to involve reduced turnover of cell surface receptors as a result of posttranscriptional mechanisms (Marks et al., 1992). Activation of muscarinic M1- and M3-receptors leads to activation of protein kinase C, which has been shown to stimulate α-secretase-mediated processing of APP, leading to decreased Aβ production (Nitsch et al., 1992; Hung et al., 1993; Lin et al., 1999). Potential problems with this

strategy might be undesirable side effects as a consequence of the chronic activation of muscarinic receptors. AChE inhibitors have shown success in enhancing cognitive function and improve participation in activities of daily living, and have become the main approach to symptomatic treatment. The most commonly used AChE inhibitors will be listed here. The aminoacridine **tacrine** (Cognex®) was among the first drugs approved for the treatment of AD. However, its short half-life (2 to 3 hours) and the therefore high rate of administration, together with severe side effects such as hepatoxicity, transaminase elevation, nausea, vomiting, anorexia, and diarrhoea limited its success. Similarly, the tertiary amine **physostigmine** has an unfavorable ratio: too high incidence of adverse effects such as gastrointestinal distress for the modest efficacy. Also the clinical utility of the carbamate derivative of physostigmine, **eptastigmine**, may be limited due to its potential adverse haematological effect without a benefit superior to other AChE inhibitors. Originally used by millions of patients in Africa in the treatment of schistosomiasis, the organophosphate **metrifonate** represents a well-tolerated AChE inhibitor with side effects only at higher doses. More promising is the phenanthrene alkaloid **galanthamine** (Reminyl®). In addition to its function as AChE inhibitor, it acts as nicotinic agonist, a property that may provide an additional therapeutic mechanism. Galanthamine was originally used for the treatment of myasthenia gravis and has a low rate of adverse effects. Particularly successful in preserving the patient's independence in activities of daily living and improvement of cognition has been shown by treatment with the carbamate **rivastigmine** (ENA-713; Exelon®). Exelon has the property of not only inhibiting AChE, but also the other enzyme involved in the breakdown of ACh, butyrylcholinesterase. Its efficacy and the low incidence of its side effects, mainly gastrointestinal problems, lead to its broad use. The other similar successful AChE inhibitor, the piperidine-based **donepezil** (Aricept®), is distinguished by its long half-life (70 hours) and therefore easy administration (once daily), its high efficacy, and its great tolerance with no severe side effects. However, neurotransmitter approaches, though potentially useful in providing short-term relief and delaying intensive care, do not solve the fundamental problem of neuronal degeneration and death. It should be emphasized that regaining function is unlikely to occur, at best a stable cognitive and functional state of the patient can be achieved.

1.4.2 Anti-inflammatory drugs

A chronic inflammatory response, exemplified by activated microglia, reactive astrocytes, complement factors, and increased inflammatory cytokine expression, is associated with Aβ deposits in AD brain (Akiyama et al., 2000; Emmerling et al., 2000). Nonsteroidal anti-inflammatory drugs (NSAIDs) can have side effects such as gastric irritation and ulceration due to the function of prostaglandins in the maintenance of the gastric mucosa. However, their use is still preferable to steroid anti-inflammatory agents which can cause immunosuppression, endocrine and psychological dysfunction, infertility, and neurodegeneration (Akiyama et al., 2000). Anti-inflammatory drugs such as used in patients with arthritis have been suggested to delay the onset and slow the progression of AD by inhibiting the cyclooxygenase and therefore prostaglandin synthesis, resulting in reduced inflammatory response (McGeer et al., 1996; Launer, 2003). Besides an anti-inflammatory action, some NSAIDs have been shown to decrease the release of $A\beta_{1-42}$ from different types of cultured cells overexpressing APP. The lowering of $A\beta_{1-42}$ concentration was associated with an increase of $A\beta_{1-38}$ concentration, suggesting the property to change the processing of APP by γ-secretase to produce the non-amyloidogenic $A\beta_{1-38}$ (Weggen et al., 2001). However, although epidemiological studies suggest that NSAIDs reduce the risk of developing AD, clinical trials in patients with mild to moderate AD have been negative (Aisen et al., 2003; Launer, 2003). Further clinical trials are needed to establish the efficacy of NSAIDs in slowing down the progression of, or preventing, AD and to make recommendations on the dose and the period of time of NSAID treatment.

1.4.3 Immunization

A therapeutic approach that is not simply based on the relief of AD symptoms but on the prevention of amyloid deposition lead to the search for a vaccine. Passive immunization with antibodies against Aβ (Bard et al., 2000; DeMattos et al., 2001), or active immunization with Aβ (Schenk et al., 1999; Janus et al., 2000; Morgan et al.,

2000) have been proven to significantly lower amyloid burden and reverse cognitive deficits in mice. However, we have recently shown an association of cerebral hemorrhages with amyloid-laden vessels in APP23 mice after passive immunization (see section 4), which could also occur in humans, as over 80% of AD cases exhibit CAA (Vinters, 1987; Jellinger, 2002; Pfeifer et al., 2002). Although immunization represents a promising strategy in the fight against AD, before further trials are undertaken, patients may be screened for the presence and severity of CAA. A problem encountered in human trials is the development of symptoms of central nervous system inflammation. Roughly 6% of the patients in the phase II clinical trial developed meningoencephalitis and therefore the trial had to be suspended (Munch and Robinson, 2002; Senior, 2002; Nicoll et al., 2003; Orgogozo et al., 2003). The mechanism of this inflammatory reaction is unknown. It is suggested that T-cells enter into the brain due to a breakdown of the blood-brain-barrier and mediate an immune reaction (Weiner and Selkoe, 2002). However, preliminary analysis suggests that immunization might work. Blood serum analysis of immunized patients showed that patients who generated high levels of antibodies against Aβ remained cognitively stable whereas patients who did not generate such antibodies showed worsened cognition (Hock et al., 2003). Analysis of the first autopsy case of the human clinical trial revealed low levels of Aβ plaques, of dystrophic neurites and of astrocyte clusters. The low Aβ immunoreactivity was associated with microglia, which might be linked to clearing mechanisms. However, other AD-associated pathologies such as neurofibrillary tangles and CAA remained unchanged (Nicoll et al., 2003). A promising approach which prevents inflammatory response by directly activating lymphocytes might be mucosal (intranasal) application of Aβ (Lemere et al., 2002). Alternatively, N-terminal Aβ fragments lacking the T-cell-activating epitope but which still reduce aggregation of Aβ could be administered (McLaurin et al., 2002).

However, repeated antibody administration can lead to an antibody response, and the resulting serum immune complexes can deposit in blood vessels leading to vasculitis and/or glomerulonephritis (Sigurdsson et al., 2002). Since the process of amyloid deposition occurs over decades, a passive immunization approach would require multiple applications, increasing the probability of adverse effects. On the other hand,

passive immunization offers the great advantage that treatment can be discontinued if needed. This approach may be used in individuals who have a diminished immune response to the antigen, a phenomenon common in the elderly who are the target population for AD therapy.

Treatments that circumvent the problems of immunotherapy include the treatment with agents which can bind Aβ. Peripheral administration of the secretory protein gelsolin or the ganglioside GM1, which show high affinity for Aβ, can reduce Aβ levels in transgenic mice without any antibody or immune response (Matsuoka et al., 2003). Moreover, application of fragments of Aβ antibodies lacking the Fc region leads to significant clearance of Aβ simply by acting as an Aβ binding agent without Fc-mediated phagocytosis (Bacskai et al., 2002). Successful reduction of amyloid deposits by immunization of Fc-Receptor knock-out mice further supports that another clearance mechanism besides Fc-mediated phagocytosis exists (Das et al., 2003).

Based on this data, three main possible mechanisms are suggested to be responsible for Aβ clearance:

I) Aβ antibodies enter the CNS, attach to plaques and induce Fc-mediated phagocytosis of amyloid plaques (Bard et al., 2000).

II) Aβ antibodies act as a “peripheral sink” which modifies the dynamic equilibrium of Aβ between CNS and plasma, facilitating Aβ efflux from the brain to the plasma (DeMattos et al., 2001).

III) Antibodies to the N-terminus of Aβ have been shown to have anti-aggregation properties and dissolve existing fibrils (Solomon et al., 1996; 1997).

Based on the promising findings that immunization against Aβ results in decreased amyloid burden and behavioral stabilization, future research holds great potential for treating or even preventing AD.

1.4.4 Secretase modulators

With the identification of the secretases (see section 1.3.1), new approaches to treat AD have become possible, although no drug is available yet. Inhibition of β- and γ-secretases or enhancement of α-secretase should result in decreased Aβ production. However, this approach might not be as simple as it seems. Stimulation of α-secretase can be accomplished by phorbol esters or by muscarinic M1-and M3-receptor agonists (Nitsch et al., 1992; Hung et al., 1993; Lin et al., 1999) (see 1.4.1). However, activation of α-secretase will affect not only APP but also unrelated proteins that are natural substrates of ADAM10 and ADAM17 such as notch, prion protein precursor, L1 adhesion molecule, transforming growth factors, and p75 tumor necrosis factor receptor (Dewachter and Van Leuven, 2002). β-secretase is thought to be an ideal therapeutic target as it catalyzes the first step of Aβ production. The major challenge represents the large active site of BACE, thus, there is a high threshold on the size of the compound that could act as inhibitor (Hong et al., 2000). β-secretase knock-out mice have no phenotype except for a dramatic reduction in Aβ levels, but β-secretase substrates other than APP and the lately discovered sialyltransferase are likely to exist and have still to be identified (Esler and Wolfe, 2001; Citron, 2002a, b; Dewachter and Van Leuven, 2002). This is indeed the case for γ-secretase; notch, a molecule important for a variety of cell fate decisions, is also a substrate of γ-secretase, and its inhibition is lethal (Esler et al., 2000; Yu et al., 2000; Esler and Wolfe, 2001; Francis et al., 2002). Interestingly, the recent finding that lithium, a glycogen synthase kinase inhibitor, interferes with γ-secretase inhibiting Aβ generation without affecting notch processing, raises the hope that specifically acting compounds may be valuable in the treatment of AD (Phiel et al., 2003).

1.5 Mouse models of Alzheimer's disease

Because of the extensive knowledge of their genome, the simplicity of their maintenance and breeding, their high fertility, and their modest body size, mice represent a valuable tool as transgenic animal models. With the development of

transgenic mice it became possible to clarify the role of genetic factors, study pathogenic mechanisms, and test the efficacy of compounds as well as novel therapeutical approaches such as immunization. Several transgenic mouse models have been generated through expression of mutated human APP (Games et al., 1995; Hsiao et al., 1996; Sturchler-Pierrat et al., 1997; Moechars et al., 1999; Bronfman et al., 2000; Kumar-Singh et al., 2000a; Van Dorpe et al., 2000). These mice develop cerebral amyloidosis progressively with age. Concomitantly, they show additional AD-like pathologies such as memory deficit, gliosis, and dystrophic neurites. The APP23 mouse, first described by Sturchler-Pierrat (1997), represents a model for the Swedish double mutation and shows a broad spectrum of AD-like hallmarks. These mice not only develop remarkable amounts of extracellular compact and diffuse Aβ deposits, but also CAA which eventually can lead to hemorrhagic strokes (Calhoun et al., 1999; Winkler et al., 2001). Amyloidosis was associated with microglial activation (Stalder et al., 1999), region-specific neuron loss (Calhoun et al., 1998; Bondolfi et al., 2002), aberrant sprouting with ectopic synapse terminal formation (Phinney et al., 1999), cognitive impairment (Kelly et al., 2003), and disruption in the cholinergic system (Boncristiano et al., 2002) (see section 2).

In addition to mice with mutations in the APP gene, mouse models bearing PS mutations have been generated (Duff et al., 1996; Citron et al., 1997). Except for increased production of $A\beta_{42/43}$, no abnormal pathology could be observed. Interestingly, co-expression of both PS and APP mutations lead to increased plaque deposition (Borchelt et al., 1997; Holcomb et al., 1998; Lamb et al., 1999; Gordon et al., 2002). To mimic the other main hallmark of AD, namely tangles, transgenic mice expressing mutant human tau have been created (Lewis et al., 2000). These mice exhibit neurofibrillary tangles similar to those seen in human tauopathies. To generate mice with simultaneous both hallmarks, tau mutant mice were crossed with APP mutant mice (Lewis et al., 2001). Interestingly, besides amyloid plaques, double mutant mice exhibited substantially enhanced neurofibrillary tangle pathology in limbic areas as compared to single tau mutant mice. These observations suggest that a high Aβ environment or APP dysfunction could be responsible for the modulation and enhancement of the tau phenotype in double transgenic mice. In general, it can be said

that such double-transgenic mice offer the possibility to study the interaction between different mechanisms and provide a better model for the complex features of AD. Although no mouse model reproduces all the hallmarks of AD in one, transgenic mice provide a valuable tool to study molecular mechanisms and to develop therapies against AD.

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Cholinergic changes in the APP23 transgenic mouse model of cerebral amyloidosis

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ABSTRACT

Alzheimer's Disease (AD) is a neurodegenerative disorder that is characterized by extracellular deposits of amyloid-β peptide (Aβ) and a severe depletion of the cholinergic system, although the relationship between these two events is poorly understood. In the neocortex, there is a loss of cholinergic fibers and receptors and a decrease of both choline acetyltransferase (ChAT) and acetylcholinesterase enzyme activities. The nucleus basalis of Meynert (NBM), which provides the major cholinergic input to the neocortex, undergoes profound neuron loss in AD. In the present study, we have examined the cholinergic alterations in amyloid precursor protein transgenic mice (APP23), a mouse model of cerebral β-amyloidosis. In aged APP23 mice, our results reveal modest decreases in cortical cholinergic enzyme activity compared with age-matched wild-type mice. Total cholinergic fiber length was more severely affected, with 29% and 35% decreases in the neocortex of aged APP23 mice compared with age-matched wild-type mice and young transgenic mice, respectively. However, there was no loss of cholinergic basal forebrain neurons in these aged APP23 mice, suggesting that the cortical cholinergic deficit in APP23 mice is locally induced by the deposition of amyloid and is not caused by a loss of cholinergic basal forebrain neurons. To study the impact of cholinergic basal forebrain degeneration on cortical amyloid deposition, we performed unilateral NBM lesions in adult APP23 mice. Three to 8 months after lesioning, a 38% reduction in ChAT activity and significant cholinergic fiber loss were observed in the ipsilateral frontal cortex. There was a 19% decrease in Ab levels of the ipsilateral compared with contralateral frontal cortex with no change in the ratio of Aβ40 to Aβ42. We conclude that the severe cholinergic deficit in AD is caused by both the loss of cholinergic basal forebrain neurons and locally by cerebral amyloidosis in the neocortex. Moreover, our results suggest that disruption of the basal cholinergic forebrain system does not promote cerebral amyloidosis in APP23 transgenic mice.

Key words: Alzheimer’s disease; APP; amyloid; basal forebrain; cholinergic system; ChAT; AChE; neurodegeneration; nucleus basalis of Meynert; neocortex; lesion; mouse; stereology; hippocampus; aging

Alzheimer's disease (AD) is a progressive neurodegenerative disorder that affects a large proportion of elderly people. Although genetic factors seem to strongly contribute to disease susceptibility, only a small number of cases are caused by dominant mutations (Selkoe, 1999). To date, all such mutations alter processing of the amyloid precursor protein (APP), leading to changes in the production or fibrillization of amyloid-β (Aβ), the major constituent of amyloid plaques found in AD brain (Hardy, 1997; Selkoe, 1997; Haass and Steiner, 2001)

Besides the extracellular deposition of Aβ, the AD brain is characterized by intracellular neurofibrillary tangles and profound changes in the cholinergic system (Bartus et al., 1982; Coyle et al., 1983; Goedert, 1993). In both neocortex and hippocampus of AD brain, a loss of cholinergic fibers and terminals, decreases in cholinergic receptors and/or signal transduction, and significant reductions in choline acetyltransferase (ChAT) and acetylcholinesterase (AChE) enzyme activities have been reported (Coyle et al., 1983; Perry et al., 1992; Ransmayr et al., 1992; Bierer et al., 1995; Jope et al., 1997; Geula et al., 1998; Ladner and Lee, 1998).

The major cholinergic innervation to the cerebral cortex originates from the nucleus basalis of Meynert (NBM), together with the horizontal limb of the diagonal band of Broca, the ventral pallidum, the magnocellular preoptic area, the substantia innominata, and the nucleus of the ansa lenticularis (hereafter referred to as the NBM complex). Cholinergic innervation to the hippocampus is mainly provided by the medial septum (MS) and the vertical limb of the diagonal band of Broca (VDB) (McKinney et al., 1983; Gaykema et al., 1990; Kitt et al., 1994). In AD brain, a profound loss of these cholinergic basal forebrain neurons has been reported (Whitehouse et al., 1982; Vogels et al., 1990; Jope et al., 1997; Cullen and Halliday, 1998). This neuron loss may be caused secondarily as a result of Aβ neurotoxicity to the cholinergic terminals followed by retrograde degeneration. Alternatively, degeneration of cholinergic basal forebrain neurons may be the primary lesion with subsequent loss of cortical cholinergic innervation.

The relationship between cerebral amyloidosis and cholinergic depletion in AD remains poorly understood (Roberson and Harrell, 1997; Auld et al., 1998; Geula et al., 1998).

It has been demonstrated that stimulation of muscarinic acetylcholine receptor subtypes increases non-amyloidogenic APP processing (Nitsch et al., 1992). It has also been reported that AChE accelerates the assembly of Aβ into insoluble amyloid fibrils (Inestrosa et al., 1996). Accordingly, dysfunction of the cholinergic system may influence cerebral amyloidosis. Vice versa, it has been demonstrated that Aβ is neurotoxic to cholinergic neurons and that low concentrations of Aβ can directly inhibit cholinergic signalling (Auld et al., 1998; Pettit et al., 2001). Thus, increased Aβ levels may contribute physiologically and/or pathologically to the cholinergic changes in AD brain.

Several APP transgenic mouse models have been generated that exhibit age-related Aβ deposition in plaques and vessels predominantly in the neocortex and hippocampus (Games et al., 1995; Hsiao et al., 1996; Sturchler-Pierrat et al., 1997). The amyloid deposits display major characteristics of human AD plaques and human cerebral amyloid angiopathy including congophilic Aβ cores, amyloid-associated cell death, dystrophic neurites, activated microglia, and reactive astrocytes (Masliah et al., 1996; Frautschy et al., 1998; Calhoun et al., 1999; Phinney et al., 1999; Stalder et al., 1999; Van Dorpe et al., 2000; Winkler et al., 2001; Bondolfi et al., 2002). These mouse models offer the opportunity to study cholinergic alterations that result from, or lead to, cerebral amyloidosis. To this end we have used biochemical and morphological techniques to assess cholinergic changes in neocortex and basal forebrain of APP23 transgenic mice. Moreover, to test the hypothesis that cortical cholinergic depletion has an effect on amyloid plaque formation we have lesioned the NBM in APP23 mice.

MATERIALS AND METHODS

Animals

The generation of the B6,D2-TgN(Thy1-APP_{Swe}) transgenic mouse line (APP23) is described elsewhere (Sturchler-Pierrat et al., 1997). In brief, APP_{751} cDNA with the Swedish mutation (K670N-M671L) was inserted into an expression cassette comprising

a murine Thy-1.2 gene construct, and mice were generated by pronuclear injection. The founder mice were then back-crossed with C57BL/6 mice. APP23 mice of generations F5-F10 and corresponding wild-type mice were used. The wild-type control mice were either littermates or nontransgenic age-matched mice from another litter of the same generation of backcrossing.

ChAT and AChE enzyme activities

Mice were killed by decapitation, and the frontal cortex was dissected on ice. The tissue was weighed, snap-frozen on dry ice, and stored at –80°C until analysis. Tissue was homogenized 1:50 (w/v) in 10 mM EDTA containing 0.5% Triton X-100 at pH 7.4. ChAT enzyme activity was measured by a slight modification (Kelly and Moore, 1978) of the method of Fonnum (1975). In brief, tissue homogenates were incubated in a waterbath at 37°C in an incubation mixture containing (in mM): 300 NaCl, 8 choline iodide, 20 EDTA, 0.1 eserine hemisulfate, and 0.2 acetyl-[1-^{14}C]-coenzyme A in 50 phosphate buffer, pH 7.4 (final concentrations). After stopping the production of radioactively labeled acetylcholine with ice-cold 10 mM sodium dihydrogen phosphate, pH 7.4, the solutions were transferred into scintillation vials, and sodium tetraphenylboron (0.5% in acetonitrile; Merck, Darmstadt, Germany) and the scintillant (0.05% PPO in toluene) were added. Labeled acetylcholine was determined by liquid scintillation counting in the biphasic aqueous, toluene scintillation solution mixture. ChAT enzyme activity was expressed in µmol per 100 mg protein per hour. Protein concentration was measured using the BIO-RAD (Munich, Germany) protein assay.

For AChE activity, samples of the same homogenates were sonicated**,** and aliquots were assayed for AChE activity by colorimetric determination by the method of Ellman (Ellman et al., 1961; Cutler et al., 1998). In short, after centrifugation for 15 min (15,000 rpm, 4°C), aliquots from the clear supernatant were used as enzyme source. Twenty µl of supernatant (quadruplicates) were placed in a 96-well flat-bottom micro-test plate. The reaction was started by adding the following substrate-reagent mixture, using an automatic plate dispenser (Titertek Autodrop): 0.5 mM acetylthiocholine iodide (Fluka, Buchs, Switzerland), 1 mM tetraisopropylpyrophosphoramide (iso-OMPA; Sigma,

Buchs, Switzerland), and 0.25 mM 5,5'-dithiobis-2-nitrobenzoate (Fluka) in 0.1 M phosphate buffer, pH 7.4. The plate was then placed in the automatic Micro-Reader (Molecular Devices, Palo Alto, CA; UVmax) which recorded the occurrence of the yellow reaction product at 405 nm. The data were processed by a program (Softmax; Molecular Devices) controlled by the plate reader. The calculation of the enzymatic activity (performed by the computer program) is based on a change in optical density in the linear range over time using the molar extinction coefficient of the reaction product (13.3 cm^2/mmole). AChE enzyme activity was expressed in nmol per mg of protein per min.

Immunohistochemistry

Perfusion- or immersion-fixed brains were paraffin embedded according to a previously published procedure (Calhoun et al., 1998a) that involved post-fixation in increasing alcohol concentrations and clearing with Cedarwood oil and methyl salicylate (Aldrich, Buchs, Switzerland). Serial coronal sections (25 μm) were cut with a microtome, and immunohistochemistry was performed using the avidin-biotin-peroxidase method (Calhoun et al., 1998a). In brief, paraffin sections were deparaffinized in xylene, then placed in 100% ethanol for 10 min followed by 30 min in methanol with 0.3% H_2O_2. Sections were rinsed in PBS and incubated for 1 hr in 5% goat or horse serum (Vector Laboratories, Burlingame, CA) in a humid chamber. Sections were then incubated overnight with a primary antibody in PBS with 3% serum. After rinsing, sections were incubated for 1 hr with biotinylated secondary antibody (Vector Laboratories) diluted 1:200 in PBS. Sections were rinsed again and incubated with the avidin-biotin-peroxidase complex (1:50; ABC Elite Kit; Vector Laboratories) in PBS. Finally, sections were reacted with 3′,3-diaminobenzidine-dihydrochloride (DAB; Sigma; 0.08%) and 0.03% hydrogen peroxide in PBS, rinsed, dehydrated, cleared in xylene, and coverslipped. On NBM-lesioned tissue (see below) immunohistochemistry was done on free-floating, fixed-frozen sections according to a previously published protocol (Phinney et al., 1999), similar to the procedure described above. The following antibodies were used: polyclonal anti-Aβ (NT12; 1:500 and 1:2000 for paraffin and

frozen sections, respectively) (Schrader-Fischer and Paganetti, 1996) and polyclonal anti-ChAT (AB144P; 1:500 for paraffin sections; Chemicon, Temecula, CA).

AChE histochemistry

Mice were deeply anesthetized with an intraperitoneal injection of an overdose of pentobarbital (50 mg/ml Nembutal) and transcardially perfused with 4% paraformaldehyde in PBS. Brains were removed, post-fixed for 24 hr in 4% paraformaldehyde, cryoprotected in 30% sucrose in PBS, frozen in isopentane at –25°C, and serially sectioned at 40 µm on a freezing-sliding microtome. AChE immunohistochemistry was performed according to a previously published method (Hedreen et al., 1985). Some sections were also immunostained with a polyclonal antibody to AChE (Marsh et al., 1984) (gift of J. Massoulie, Paris, France). No qualitative difference was noted between the histochemical and immunohistochemical reaction confirming previous findings in the rat (Jucker et al., 1996). Histochemical AChE staining was however more reliable and more distinct and was taken for quantitative analysis of cholinergic fiber length (see below).

To study the localization of AChE in more detail, some of the tissue was fixed with paraformaldehyde plus 1% glutaraldehyde, cut on a vibratome (60 µm), stained for AChE, and plastic-embedded according to a previously published protocol (Stalder et al., 1999). Semithin (0.5 µm) and ultrathin (80 nm) sections were cut on a ultramicrotome. Sections were collected on formvar-coated nickel grids and viewed under high-power light microscopy or electron microscopy.

Stereological assessment of cholinergic fiber length

Stereological techniques were used to estimate the total length of AChE-positive fibers in the neocortex (Table 1). To this end, every 16th section throughout the neocortex was histochemically stained for AChE according to the above described protocol. The volume of the neocortex was estimated by superimposing a point-grid on each section and counting the points over neocortex, according to the Cavalieri principle (West et al.,

1991; Calhoun et al., 1998b). AChE-positive fiber density was then estimated by superimposing a system of test lines, and intersections of test lines with fibers were counted within the volume of disectors systematically spaced throughout the neocortex using the 100x objective (2756x final magnification) with a numerical aperture of 1.3 (Stocks et al., 1996). This number of intersections between fibers and test lines of a known length produces a result directly related to the length of the fibers themselves within the disector volume (Howard et al., 1992). Because length measurement was performed on coronal sections in all cases, it is assumed that no overall directional orientation with respect to the plane of section exists for cholinergic fibers as they innervate the cortex. AChE-positive fiber length was calculated by multiplying the region volume by the fiber length density (West, 1993). Stereological analysis was performed with the aid of Stereologer software and a motorized x-y-z stage coupled to a videomicroscopy system (Systems Planning and Analysis, Alexandria, VA). Post-processing section thickness was measured using a focus drive accurate to ± 0.1 µm (Applied Scientific Instrumentation, Eugene, OR). Coefficient of error was calculated according to West et al. (1991) and was well below biological variability. Region definitions of neocortex were based on a recent mouse brain atlas (Franklin and Paxinos, 1997).

Stereological assessment of amyloid load

Amyloid load was assessed on every 20th paraffin section throughout the neocortex immunostained with NT12. The percentage of neocortical volume occupied by amyloid (amyloid load) was determined by sampling through the entire neocortex with a 20x objective (numerical aperture: 0.45) and counting the percentage of points from a superimposed point grid that hit amyloid (Table 1) (Calhoun et al., 1998b). Stereological analysis was performed with the Stereologer software described above.

Number and volume of ChAT-positive basal forebrain neurons

For estimation of the number (West et al., 1991) and volume (Jensen and Gundersen, 1993) of ChAT-positive cells in the basal forebrain, systematic random series of ChAT-

stained paraffin sections were analyzed using a fractionator sampling scheme. For quantitative analysis ChAT-positive neurons in the NBM complex that provide the major cholinergic innervation to the neocortex were combined. Similarly cholinergic neurons in the MS and VDB, that provide the cholinergic innervation to the hippocampus were combined for analysis. Every eighth section throughout the NBM complex and every fourth section throughout the MS-VDB were collected. On each section a point grid was placed randomly over the region to determine the systematic-random placement of the optical disectors. The total number of ChAT-positive neurons within the three-dimensional optical disectors throughout the region were then counted. Objects were counted by focusing through the counting frame using a 63x objective with a numerical aperture of 1.25. The nuclei of ChAT-positive cells were the selected objects for counting (Table 1). Additionally, the volume of each counted cell was estimated. For practical reasons, the tissue could not be randomly rotated during sectioning, and it was thus assumed that there is no change in preferred orientation of the cells in the basal forebrain (Gundersen et al., 1988). A vertical line with three perpendicular grid lines were superimposed, and intersections of grid lines with the cell soma were identified. The mean length of these lines is proportional to neuron volume (Jensen and Gundersen, 1993), although in this case the value is orientation-dependent. Stereological analysis was performed with the Stereologer software described above.

NBM Lesions

Animals were deeply anesthetized using a combination of ketamine (10 mg/kg body weight; Ketalar; Parke-Davis, Ann Arbor, MI) and xylazine (20 mg/kg body weight; Rompun, Bayer, Germany) in saline administered intraperitoneally. Using stereotaxic surgery, the scalp was cut, a hole was drilled, and an electrode was lowered into the NBM [anteroposterior (bregma), -0.2 mm; lateral, 1.5 mm; dorsoventral (dura), -4.5 mm]. A current flow of 0.5 mA was passed for 15 sec. Half of the mice received an additional sham lesion on the contralateral side, in which the electrode was lowered, but no current was passed through. The lesion side was alternated evenly over the groups.

Mice were killed 3 - 8 months later by decapitation. Brains were removed, and a small piece of the ipsilateral and contralateral frontal neocortex was dissected and combined

with a small piece from the ipsilateral and contralateral motor-somatosensory cortex as previously described (Kelly and Moore, 1978). The dissected tissue was weighed, snap-frozen on dry ice, and assayed for ChAT enzyme activity (described above) and APP/Ab (see below). The rest of the brain was immersion-fixed in 4% paraformaldehyde, cryoprotected in 30% sucrose and frozen. Serial coronal 40 µm sections were cut throughout the frontal cortex and alternate sections were stained for Ab and AChE or double stained for both. Amyloid load was then determined stereologically on the Aβ stained sections (see above), discarding the sections missing the dissected pieces. This yielded a total of 5-15 Aβ-stained sections through the frontal cortex per mouse. Percentage of amyloid load was determined for both hemispheres separately.

Aβ load and APP processing using Western blotting

It has previously been demonstrated that amyloid cores in APP23 mice are completely soluble in SDS (Kuo et al., 2001). Thus, the tissue samples used to determine ChAT activities (see above) were diluted 1:3 (weight/volume) in 1.5x SDS-sample buffer (containing 1% SDS) and separated using a 10% SDS-polyacrylamide gel containing 8 M urea (Klafki et al., 1996). For amyloid quantification 2 µl was loaded from the unlesioned and lesioned side. After separation, proteins were transferred to Immoblin-P (Millipore, Bedford, MA) with a Biorad semidry transfer apparatus for 1 hr at 25V, according to Wiltfang (Wiltfang et al., 1997). Membranes were blocked in 5% non-fat milk in PBS with 0.1% Tween-20 (PBS-T) and reacted overnight at 4 °C with Aβ antibody 6E10 (Signet, Dedham, MA) at 1:2000 in PBS-T followed by a goat anti-mouse peroxidase conjugate (Chemicon, Temecula, CA) at 1:2500 in PBS-T for 30 minutes at room temperature. Bands were visualized using Supersignal (Pierce, Rockford, Il) and developed onto Kodak X-OMAT AR film (Rochester, NY). Different exposures of the film were digitized and band density measurements for Aβ40 and Aβ42 were made using NIH Image Version 1.61 (NIH, Bethesda, MD). Each sample pair was run at least three times, and the mean was taken. Only bands within the linear range of the film were analyzed.

To analyze APP processing in more detail, the β-secretase cut, the N terminal secreted fragment (sAPPβ), and the corresponding C terminal fragment C99 were additionally analyzed. For the analysis of sAPPβ, proteins were separated on a 8% SDS-polyacrylamide gel and transferred to Immobilon-P membranes as described previously (Andra et al., 1996). The blot was reacted with rabbit neoepitope antiserum 852 against the C terminus of sAPPβ (P. Paganetti and M. Staufenbiel, unpublished observations). As an internal control, an antibody to tubulin was used.

Statistics

All statistical analysis was done using StatView 5.0.1. The mean ± SEM is indicated. Significance level was set at $p<0.05$.

RESULTS

Disruption and loss of cholinergic fibers in neocortex of aged APP23 mice

To study the impact of cerebral amyloidosis on the cholinergic system, we have analyzed AChE-positive fiber length and ChAT-positive boutons in neocortex of APP23 mice. Three age groups with similar numbers of male and female APP23 mice were examined: young (6 months; n=6), adult (15 months; n=5), and aged (24 months; n=7). Corresponding controls were young (6 months; n=6), adult (15 months; n=8), and aged (24 months; n=5) wild-type mice. Quantitative analysis of the amyloid load revealed no amyloid deposition in the young APP23 mice, 4.0±1.8% in adult APP23 mice, and 28.6±1.4% in aged APP23 mice (Fig. 1A,B). Consistent with previous reports, the majority of the amyloid deposition in APP23 mice was compact in nature and congophilic (Calhoun et al., 1998b; Stalder et al., 1999). Amyloid deposits were also found in vessels and as extracellular diffuse amyloid (Fig. 1B). Neocortical volume did not differ between the groups at any age (mean volume for all mice=17.4 mm^3; one hemisphere).

Histochemical staining for AChE revealed a dense laminated network of cholinergic fibers throughout the neocortex of wild-type mice and young APP23 mice. In adult and aged APP23 mice, however, there was a loss and often dramatic disruption of the cholinergic fiber network (Fig. 1C,D). In particular in the aged APP23 mice, a loss of AChE-positive fibers throughout the entire neocortex was evident with intense staining of dystrophic structures at the plaque periphery and diffuse staining of the amyloid cores (Fig. 2A). High-power microscopic analysis identified these dystrophic structures as fibers that often formed loops ending in dystrophic boutons (Fig. 2A). Semithin and ultrathin sectioning through the diffusely stained AChE-positive amyloid cores demonstrated that AChE–immunoreactivity was associated with amyloid fibrils (Fig. 2B).

ChAT immunohistochemistry revealed punctate staining of cholinergic boutons throughout the neocortex (Fig. 2C,D). Punctate staining was often very dense revealing the shape ofindividual cholinergic fibers with their immunostained boutons. No qualitative difference in staining was noted between wild-type and young APP23 mice. In contrast, in adult and aged APP23 mice a reduction of ChAT-positive boutons was observed in neocortical areas with a high amyloid burden, such as entorhinal and frontal cortex (Fig. 2D). Large dystrophic ChAT-positive structures were present at the periphery of the amyloid plaques and resembled the AChE-positive dystrophic fibers described above.

For quantification of cholinergic fiber length, AChE-histochemistry was chosen because of robust staining and significant penetration of the stain into the tissue sections, both prerequisites for stereological analysis. Results for total length of AChE-positive fibers in neocortex revealed the astonishing length of 600 m per hemisphere and was similar for all three groups of wild-type mice (Fig. 3). In contrast to this lack of age effect in wild-type mice, there was a clear reduction in fiber length with aging in the APP23 mice that reached 35% compared with young APP23 mice, and 25% compared with adult APP23 mice. In comparison with age-matched wild-type mice there was a 29% reduction. Consistently, ANOVA revealed a significant age effect ($F_{(2,31)}=6.40$; $p<0.01$), a significant transgene effect ($F_{(1,31)}=4.59$; $p<0.05$), and a significant age x transgene interaction ($F_{(2,31)}=4.30$; $p<0.05$). Newman-Keuls *post hoc* analysis indicated that the fiber loss in aged APP23 mice was statistically significant compared with aged wild-

type mice ($p<0.01$) and with young ($p<0.01$) and adult ($p<0.05$) APP23 mice (Fig. 3A). A significant negative correlation between AChE-positive fiber length and amyloid load was apparent when adult and aged APP23 mice were combined ($R^2=0.46$; $p<0.05$) (Fig. 3B). Males and females were combined for AChE-fiber length analysis because previous ANOVA analysis did not reveal any significance for, or interaction with, gender.

ChAT and AChE activities in the frontal cortex of APP23 mice

ChAT and AChE enzyme activities were measured in the frontal cortex (Table 2). Three age groups consisting of similar numbers of both sexes of APP23 mice (6 months, n=8; 19 months, n=11; 24 months, n=6) and age-matched wild-type control mice (6 months, n=8; 19 months, n=9; 24 months , n=7) were used. Because of logistical issues, the young, adult, and aged mice were analyzed separately, thus preventing comparison between age groups. Because no difference between males and females was apparent, males and females werecombined. Results revealed a modest but significant decrease of ChAT activity in the frontal cortex of aged APP23 mice as compared with age-matched controls ($t_{(11)}=2.27$; $p<0.05$) (Table 2). Differences in AChE activities did not reach significance, although there was a trend towards decreased activities in transgenic mice in all three groups (Table 2).

Number and volume of ChAT-positive neurons in the basal forebrain

Two age groups of APP23 mice (young: 8 months, n=8; aged: 27 months, n=8) and corresponding wild-type mice (young: 8 months, n=7; aged: 27 months, n=6) were used with sex equally balanced within groups and treated as one group. ANOVAs for ChAT-positive neuron number were calculated separately for the NBM complex and the MS-VDB (Fig. 4). Results did not reveal a significant effect of age or transgene on neuron number ($p>0.05$) (Fig. 5A,B). There was also no change in volume of ChAT-positive cells in the NBM complex (Fig. 5C). Rather unexpectedly, a significant transgene effect ($F_{(1,25)}=8.76$; $p<0.05$) with a nonsignificant age x transgene interaction was found for the volume of ChAT-positive cells in the MS-VDB, indicating that both young and aged APP23 mice have smaller cholinergic neurons in the MS-VDB (Fig. 5D).

Lesions of the nucleus basalis of Meynert reduce neocortical Aβ load

Groups of male and female mice were lesioned at 5, 7, 10, and 13 months of age and were killed at 9, 15, 13, and 20 months of age, respectively. Only mice with a complete lesion of the NBM complex and a >20% ChAT decrease in the ipsilateral compared with contralateral frontal cortex were included in the analysis (n=11; mean ChAT decrease: 38 ± 4%). Histologically, in these mice there was a considerable loss of AChE-positive fibers in the ipsilateral frontal cortex (Fig. 6).

Stereological analysis of the amyloid load on Aβ-immunostained sections revealed a mean decrease in the amyloid load of 22 ± 8% in the ipsilateral versus contralateral frontal cortex (Fig. 6A,B). The individual changes ranged from +14% to -63% with a decrease in 8 out of 11 mice. This difference did not reach statistical significance (p=0.11, Wilcoxon signed rank test). In contrast, a significant decrease was observed when Aβ40 was analyzed using densitometry of Western blots. The mean decrease was 19 ± 9% with a range from +30% to–61% (Fig. 6C). Eight out of 11 mice revealed a decrease (p=0.04, Wilcoxon signed rank test). The ratio between Aβ42 and Aβ40 was not significantly different between the ipsilateral (0.29) and contralateral side (0.26).

To further study APP processing we have analyzed sAPPβ and C99, the two cleavage products of b-secretase. No significant difference was found for sAPPβ between ipsilateral and contralateral side (-7%, p=0.15, paired t-test) and this difference was identical to the one found for tubulin. Consistently, no apparent difference for C99 between ipsilateral versus contralateral was found (results not shown). These results indicate that cholinergic denervation does not lead to a globally detectable shift of APP metabolism to the amyloidogenic pathway in APP23 mice.

DISCUSSION

Cerebral amyloidosis is a hallmark lesion of AD, and genetic analysis has demonstrated that Aβ is central to AD pathogenesis (Selkoe, 1999). Similarly, depletion of the cholinergic system is a robust finding in AD and correlates with cognitive impairment

(Collerton, 1986; DeKosky et al., 1992; Bierer et al., 1995). Yet, the link between cerebral amyloidosis and the cholinergic deficit remains poorly understood.

The present study was undertaken to investigate alterations in the cholinergic system in the APP23 mouse model of cerebral amyloidosis. The mice develop amyloid plaques and cerebrovascular amyloid throughout the neocortex and hippocampus with only modest amyloid deposition in the basal forebrain (Sturchler-Pierrat et al., 1997; Calhoun et al., 1998b). Individual amyloid deposits in APP23 mice are also morphologically similar to those in AD brain and include congophilic amyloid cores, amyloid-associated dystrophic neurites, astrocytosis and microgliosis (Jucker et al., 2001). A difference between amyloid plaques in mouse compared with AD brain is the lack of paired helical filaments in plaque-associated dystrophic neurites (Sturchler-Pierrat et al., 1997). Moreover, there is a difference in solubility and chemical composition of the amyloid (Kuo et al., 2001).

Results of the present study reveal a robust 30% decrease in cholinergic fiber length with distorted and dystrophic cholinergic fibers surrounding the amyloid very similar to that in AD brain (Geula et al., 1998). Because fiber loss in the mice correlated with cortical Aβ load and because we did not find a loss or shrinkage of cholinergic neurons in the NBM, our results suggest that the cortical cholinergic deficit in APP23 mice is locally induced by Aβ deposition. These results are also consistent with earlier observations that retrograde degeneration in the NBM only occurs after more severe cortical tissue damage (Sofroniew et al., 1983; Liberini et al., 1994). In other transgenic mouse models, a lack of neuron loss in the basal forebrain has also been reported. However, in these mouse models, no significant cortical cholinergic deficits have been observed possibly because of the age of the mice and/or the lower neocortical amyloid burden (Wong et al., 1999; Bronfman et al., 2000; Hernandez et al., 2001; Jaffar et al., 2001), although in one plaque-burdened mouse line a decrease in vesicular acetylcholine transporter-positive bouton area and density in frontal cortex was reported (Wong et al. 1999).

Rather unexpectedly, our results reveal that cholinergic neurons in the MS-VDB of APP23 mice are significantly smaller compared with wild-type mice. Amyloid

deposition is unlikely to account for this observation, because a decrease in neuron volume was also found in the 8-month-old APP23 mice that exhibited negligible amyloid deposition in hippocampus and no deposition in the MS-VDB. It has been reported that in SN56 cells (N18TG2 neuroblastoma cells fused with mouse primary septal neurons), low concentrations of Aβ can induce long-lasting downregulation of the cholinergic activities without evidence of neurotoxicity (Pedersen et al., 1996; Auld et al., 1998). Thus, it is possible that Aβ levels in the 8-month-old APP23 mice are high enough to induce cholinergic hypoactivity and shrinkage of MS-VDB neurons, although it is unclear why this region would be preferentially affected. Interestingly, in another APP transgenic mouse model, a selective decrease in size of MS cholinergic neurons, but not NBM cholinergic neurons, has also been reported (Bronfman et al., 2000). In this model however, shrinkage was observed in aged transgenic mice but not in young transgenic mice without amyloid deposition. This difference may be explained by the much lower levels of APP expression/Ab in this latter model. In AD a shrinkage of basal forebrain cholinergic neurons has also been reported (Vogels et al., 1990).

Cholinergic fiber loss in AD neocortex displays considerable regional variability with reductions exceeding 50% in some cortical areas (Mesulam, 1996; Geula et al., 1998). In addition, there is a poor correlation between amyloid plaques and fiber loss, suggesting that amyloid deposition in neocortex cannot be the exclusive cause of the cholinergic loss. Thus, it is likely that in AD brain the significant loss of cholinergic basal forebrain neurons contributes to the cortical cholinergic deficit and accounts for the more pronounced fiber loss as compared with APP23 mice. NBM cholinergic neurons are among the first neuronal groups with neurofibrillary tangle formation and among the first neurons that are lost in AD (Mesulam, 1996; Cullen and Halliday, 1998; Sassin et al., 2000).

In contrast to the significant fiber loss in APP23 mice, our results revealed only a modest loss of ChAT and AChE enzyme activities. Correlative analysis of ChAT and AChE enzyme activities and amyloid load in AD brain have revealed conflicting findings. Some studies have found negative correlations (Perry et al., 1981; Mountjoy et al., 1984; Zubenko et al., 1989; Beach et al., 2000a), whereas others have found no

relation (Wilcock et al., 1982; DeKosky et al., 1992; Geula et al., 1998). In AD and in APP23 mice, a significant amount of ChAT and AChE staining is associated with dystrophic neurites surrounding amyloid plaques (Benzing et al., 1993; Moran et al., 1993). Moreover, in both AD and APP23 mice, AChE is a component of amyloid-containing plaques and it has been suggested that AChE accelerates amyloid fibril formation (Gomez-Ramos et al., 1992; Mesulam et al., 1992; Inestrosa et al., 1996). It is possible that this notable accumulation of ChAT and AChE within and around plaques in APP23 mice accounts for the only modest overall decrease in enzyme activities.

It has been hypothesized that cholinergic depletion in AD contributes to cerebral amyloidosis. This hypothesis is based on the observation that activation of protein kinase C through muscarinic receptor binding stimulates the nonamyloidgenic pathway of APP processing by increasing sAPPa production and reducing Aβ generation (Buxbaum et al., 1992; Nitsch et al., 1992; Hung et al., 1993). Thus, the loss of the cholinergic innervation may lead to increased production of Aβ and amyloid deposition. In *vivo* support for altered APP processing has been provided in both NBM-lesioned rats and in rats after muscarinic agonist treatment (Rossner et al., 1997; Lin et al., 1999). However, Aβ levels were not assessed in these studies. In contrast, Aβ levels were assessed six months after NBM lesions in rabbits and resulted in a 2.5- and 8-fold increase in neocortical Aβ40 and Aβ42, respectively, however no deposition of amyloid was found (Beach et al., 2000b). Based on these findings we initiated similar NBM lesions in APP23 mice that develop cerebral amyloidosis with aging.

ChAT reduction after NBM lesions in APP23 mice was 38% and is similar to the reduction reported in NBM-lesioned mice, rats, and rabbits (Smith, 1988; Beach et al., 2000b). However, we failed to see increased Aβ levels or deposition in the lesioned hemisphere in APP23 mice. Perhaps it is difficult to further shift the processing of transgenic APP toward the b-secretase pathway because the Swedish double mutation in APP23 mice already greatly favors this pathway (Citron et al., 1992).

The observation that NBM lesions in APP23 mice actually result in a decrease in neocortical amyloid deposition may be explained by the finding that AChE accelerates the assembly of amyloid peptide into amyloid fibrils (Inestrosa et al., 1996).

Accordingly, the cholinergic deficit in APP23 mice may result in reduced amyloid formation. However, the observations of amyloid plaque reduction after NBM lesions may also be the result of enhanced clearing mechanisms in a lesioned-deafferented brain tissue and not be specific to the cholinergic system. It has been reported that traumatic brain injury results in a 30% regression of amyloid burden in the hippocampus of PDAPP transgenic mice (Nakagawa et al., 2000). Moreover, entorhinal cortex lesions appear to inhibit amyloid plaque formation in the deafferented dentate gyrus (S. Sisodia, personal communication). The mechanism is not clear but may involve enhanced microglia clearance of amyloid in the denervated-lesioned brain areas.

In conclusion, our results suggest that the cerebral amyloidosis in neocortex of APP23 mice causes significant cholinergic fiber loss and severe disruption of the cholinergic fiber network. Because the mice do not lose basal cholinergic forebrain neurons, these results suggest that the cholinergic deficit in APP23 mice is locally induced by the amyloid in neocortex. In light of the role and importance of the neocortical cholinergic system for cognition (Winkler et al., 1995) the cholinergic changes in APP23 mice may contribute to the recently described cognitive impairment of these mice (Kelly et al., 2002). Importantly, our lesion results suggest that in the APP23 mouse model, loss of cholinergic forebrain neurons and a subsequent loss of cortical cholinergic activity does not promote amyloid deposition.

FIGURE CAPTIONS

Figure 1 Amyloid deposits and cholinergic disruption in neocortex of aged APP23 mice. (**A**) No amyloid is detected in 24-month-old wild-type mice. (**B**) Immunostaining for Aβ reveals numerous compact amyloid plaques, diffuse amyloid (arrowheads), and amyloid deposits in vessels (arrow) in neocortex of a 24-month-old APP23 mouse. (**C, D**) Histochemical staining for AChE reveals a disruption and a decrease in cholinergic fiber density in neocortex of 24-month-old APP23 mice (D) compared with 24-month-old control mice (C). The disruption is most evident around plaques but occurs throughout the neocortex. Scale bars, 120 µm.

Figure 2 Cholinergic disruption and dystrophy in APP23 neocortex. (**A**) AChE-positive fibers around an amyloid plaque with diffuse staining of the amyloid cores in the neocortex of an aged APP23 mouse (arrowhead). Insert: high-power analysis of the abnormal and often swollen AChE-positive fibers with terminal large boutons in vicinity of the amyloid plaque (arrowheads). Fibers frequently grow towards the amyloid but then form loops or sharply turn around to grow away from the amyloid to turn later again towards the amyloid (arrow). (**B**) High-power analysis of diffuse AChE-staining associated with the amyloid cores. Insert: AChE staining in semithin sections suggests an association of AChE with the amyloid core. Subsequent ultrastructural analysis confirms that AChE reactivity (arrow) is associated with amyloid fibers (a). (**C**) Immunostaining for ChAT reveals dense punctate staining of cholinergic boutons in the frontal cortex of an aged wild-type mouse. (**D**) In contrast, a loss of cholinergic boutons is apparent in aged APP23 mice. Dystrophic ChAT-positive boutons and neuritic structures are present around amyloid plaques (arrowheads). Scale bars: (A), 50 µm; (B), 1.6 µm; (C,D), 50 µm.

Figure 3 AChE-positive fiber length and amyloid load in neocortex of APP23 mice. (**A**) Total length of AChE-positive fibers in neocortex of young (6 months), adult (15 months), and aged (24 months) APP23 mice (black bars) and age-

matched wild-type mice (open bars). Aged transgenic mice had a significant loss of fiber length compared with aged wild-type mice (**p<0.01) and with young transgenic mice (**p<0.01). Indicated is the mean ± SEM for one hemisphere only. (**B**) A significant negative correlation between AChE-positive fiber length and amyloid load was apparent when adult (circles) and aged (squares) APP23 mice were combined.

Figure 4 ChAT-positive neurons in the basal forebrain of APP23 mice. **(A,B)** No apparent difference in neuron number was observed in the NBM of 27-month-old APP23 mice (A) compared to aged-matched wild-type mice (B). (**C,D**) Similarly, no apparent difference in ChAT-positive neuron number in the medial septum (MS) was noted between 27-month-old APP23 (C) mice and age-matched control mice (D). For quantification see Figure 5. Scale bar, 50 μm.

Figure 5 Number and volume of cholinergic neurons in the basal forebrain of APP23 mice. Number (**A)** and volume **(C)** of ChAT-positive neurons in the NBM complex in APP23 mice (black bars) and wild-type mice (open bars). No significant difference between transgenic and wild-type mice was noted. A similar analysis was done for the number (**B**) and volume (**D**) of ChAT-positive neurons in the MS-VDB in APP23 mice (black bars) and wild-type mice (open bars). For neuron volume, a significant reduction of 38% and 42% was found in the 8-month-old and 27-month-old APP23 mice, respectively (* p<0.05). Data are mean ± SEM. Indicated values are for one hemisphere only.

Figure 6 Amyloid load in the frontal cortex after NBM lesions. (**A, B**) Double labeling for AChE (brown) and Aβ (blue) in a 13-month-old APP23 mouse 3 months after a unilateral NBM lesion reveals a considerable loss of cholinergic fibers in the ipsilateral (B) compared with contralateral (A) frontal cortex. Stereological assessment revealed a 22% reduction of amyloid deposition in the ipsilateral compared with contralateral side. Scale bar: (A,B)

90 µm. (**C**) Western blotting of cortex homogenates. Samples were run in pairs, i.e. contralateral side (c) versus ipsilateral side (i). Shown are three mice. Results for all the mice revealed a 19% decrease of Aβ40 between the two sides. No difference in the ratio between Aβ40 and Aβ42 was found between contralateral and ipsilateral side.

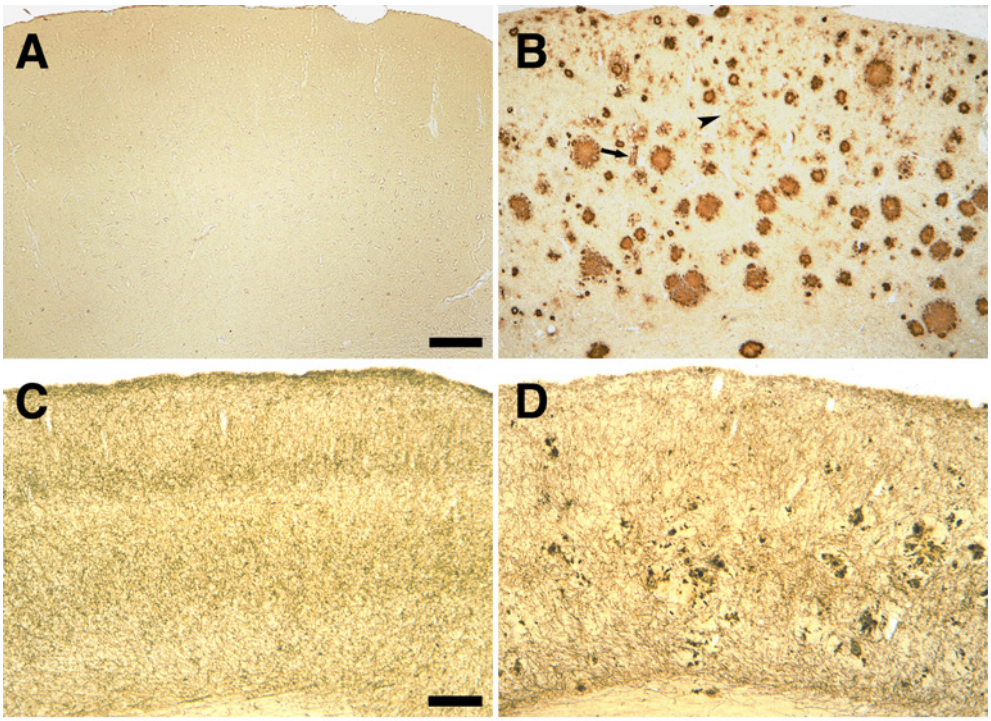

Figure 1.

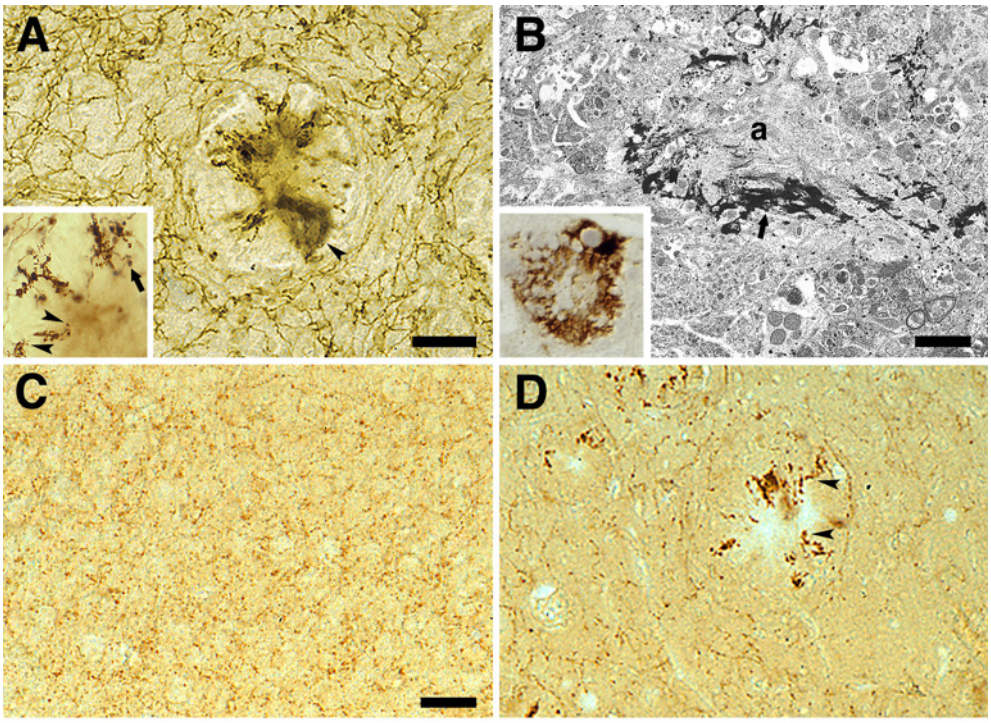

Figure 2.

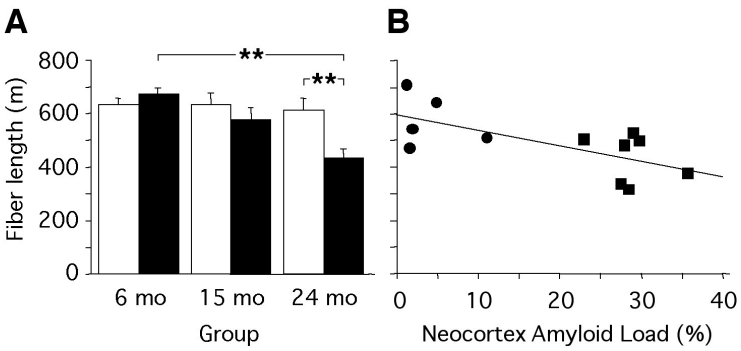
A
B
800
600
400
200
0
Fiber length (m)
**
**
6 mo
15 mo
24 mo
Group
0
10
20
30
40
Neocortex Amyloid Load (%)

Figure 3.

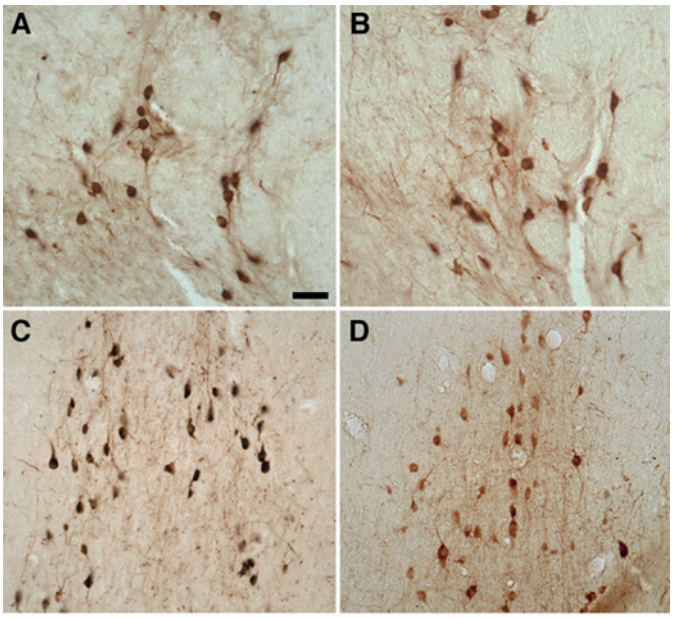

Figure 4.

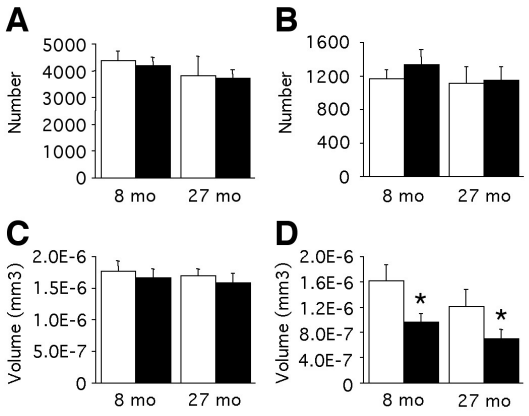
A
B
C
D
Number
Volume (mm3)
5000
4000
3000
2000
1000
0
1600
1200
800
400
2.0E-6
1.5E-6
1.0E-6
5.0E-7
1.6E-6
1.2E-6
8.0E-7
4.0E-7
8 mo
27 mo
*

Figure 5.

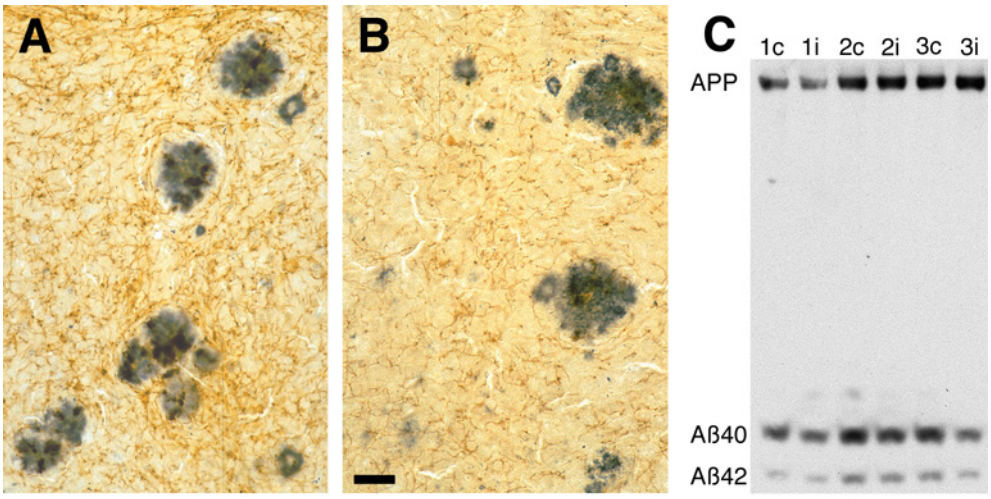

Figure 6.

Table 1. Stereological parameters for cholinergic fiber, amyloid load, and cholinergic basal forebrain neuron quantification. Values are mean ± SEM.

	Sections			Disector or Fractionator			Objects counted[b]	Results	
	Type	Thickness[a]	Sample	Area	Height	Spacing		Value[c]	CE
AChE fiber length in neocortex	40 µm frozen	15.6 ± 0.3 µm	1/16	1900 µm^2	8 µm	800 µm	525 ± 25	591.93 ± 19.2 m	0.047 ± 0.008
Amyloid load neocortex	25 µm paraffin	27.0 ± 0.3 µm	1/20	19000 µm^2	0 µm	800 µm	514 ± 86	18.4 ± 3.8%	0.038 ± 0.001
Cholinergic neurons in NBM complex	25 µm paraffin	23.2 ± 1.1 µm	1/8	8300 µm^2	18 µm	160 µm	120 ± 8	4015 ± 209; 1700 ± 72 µm^3	0.096 ± 0.003
Cholinergic neurons in MS/VDB	25 µm paraffin	23.6 ± 0.9 µm	1/4	8300 µm^2	14 µm	91 µm	154 ± 12	1196 ± 80; 1100 ± 120 µm^3	0.086 ± 0.003

[a]Section thickness measured to ± 0.1 µm after all processing / coverslipping was complete.
[b]Objects counted refers to the average number of intersections of fibers with test lines, number of points hitting amyloid, or number of cholinergic cells counted per subject.
[c]Value represents total length of AChE-positive fibers, amyloid load, or total number and volume of ChAT-positive neurons per hemisphere.

Table 2. ChAT and AChE enzyme activities in the frontal cortex. Aged transgenic mice showed a significant loss of ChAT activity as compared with age-matched wild-type controls (*$p<0.05$). No significant decrease of AChE activity was detected. Because of logistical issues the young, the adult, and the aged mice were analyzed separately, thus preventing comparison among age groups.

	Wild-type	APP23	Decrease (%)
	Mean ChAT activity ± SEM (μmol/100 mg protein/hr)		
young (6 mo)	17.12 ± 0.6	16.57 ± 0.4	3.2
adult (19 mo)	13.79 ± 0.7	12.67 ± 0.5	8.1
aged (24 mo)	14.12 ± 0.3	12.5 ± 0.7	11.4 *
	Mean AChE activity ± SEM (nmol/mg protein/min)		
young (6 mo)	59.41 ± 2.5	56.20 ± 0.8	5.4
adult (19 mo)	62.48 ± 2.4	56.56 ± 2.5	9.5
aged (24 mo)	64.51 ± 1.5	59.75 ± 4.3	7.4

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Manuscript submitted

No synaptic loss despite robust amyloid deposition in APP transgenic mice

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ABSTRACT

Major pathological findings in Alzheimer's disease (AD) brain include the deposition of amyloid β (Aβ) and synapse loss. Amyloid load has been shown to correlate with loss of synapses in AD patients, but this finding is complicated by the presence of neurofibrillary tangles and other pathologies. With the use of the APP23 transgenic mouse model that overexpresses human APP with the Swedish double mutation, we investigated whether the development of cortical plaque deposition was accompanied by synaptic bouton loss. With stereological methods, we show that despite robust age-related cortical amyloid deposition with synaptic degeneration in the vicinity of the amyloid plaques, the total number of cortical synaptophysin-positive presynaptic terminals in 24-month-old animals compared with 3-, 8-, and 15-month-old APP23 mice is not changed. Wild-type mice also do not show an age-related loss of presynaptic boutons in the neocortex and are not significantly different from APP23 mice. Synaptophysin Western blotting revealed no significant difference between APP23 mice and wild-type controls at 3 and 25 months of age. Our results suggest that Aβ deposition is not sufficient to account for synapse loss in AD. Alternatively, a putative trophic effect of APP may prevent, compensate, or delay a loss of synapses in this mouse model.

Key words: Alzheimer; aging; APP; brain; CNS; neocortex; neurodegeneration; stereology; synapses; transgenic mouse

INTRODUCTION

Alzheimer's disease (AD) is a progressive neurodegenerative disorder which affects a large proportion of the elderly population. Mutations have been found in several genes which cause hereditary familial AD [35]. These mutations alter processing of the amyloid precursor protein (APP), leading to increased production of amyloid-β (Aβ), the major component of amyloid plaques found in AD brain [35]. A consistent finding in AD is a decrease in synaptic density and/or presynaptic markers in the hippocampus [21,33,39] and the neocortex [10,20,32,41,42]. Synaptic loss has also been shown to correlate with cognitive decline [10,39-41]. However, recent studies suggest that synapse loss might not be an early event in the progression of AD, as a decrease in synapses is only seen in later stages of the disease, where pathology is more widespread [21,23,27,42]. Studies in transgenic mice are conflicting, with some reporting a loss of synapses while others found no change [4,11,12,14,18,26]. A trophic effect of APP on synaptic boutons has also been reported [25,30,43], raising the question whether an interaction of these factors may explain the conflicting reports.

The APP23 mice, a transgenic mouse model which overexpresses mutant human APP carrying the Swedish double mutation, develops amyloid plaques progressively from six months of age and shows a broad spectrum of AD-like hallmarks [1,2,5,8,37,38]. In the present study, we seek to determine whether the development of plaques is accompanied by synaptic loss similar to that observed in AD.

MATERIALS AND METHODS

Animals

The generation of the B6,D2-TgN(Thy1-APP_{Swe}) transgenic mouse line (APP23) is described elsewhere [38]. In brief, APP_{751} cDNA with the Swedish double mutation (K670N-M671L) was inserted into an expression cassette comprising the murine Thy-1.2 gene, and mice were generated by pronuclear injection. The founder mice were then back-crossed with C57BL/6J mice for at least 8 generations. For morphologic studies,

four age groups of male hemizygous APP23 mice (3 months, n=6; 8 months, n=3; 15 months, n=5; 24 months, n=3) and male littermate wild-type control mice (3 months, n=6; 8 months, n=4; 15 months, n=8; 24 months, n=3) were used. For Western blotting, two age groups of male hemizygous APP23 mice (3 months, n=7; 25 months, n=7) and male littermate wild-type control mice (3 months, n=7; 24 months, n=7) were used.

Tissue Processing

For morphological studies, animals were deeply anesthetized and transcardially perfused with 4% paraformaldehyde in PBS. One hemisphere was processed for paraffin embedding. Standard paraffin processing was slightly modified to increase antibody penetration and preserve punctate presynaptic bouton staining, thus optimizing quantification of synaptophysin immunocytochemistry with stereological techniques [7,28]. In short, this involved post-fixation in increasing alcohol concentrations, and clearing with Cedarwood Oil and Methyl Salicylate (Aldrich, Buchs, Switzerland). Serial coronal paraffin sections (25µm microtome setting) were cut throughout the entire hemisphere.

Immunohistochemistry

Immunostaining was performed on paraffin sections with a polyclonal antibody to synaptophysin (1:1000; Dako; Glostrup, Denmark) or to Aβ (NT12; 1:500; gift of P. Paganetti, Basel) [1]. Immunostaining was done using the avidin-biotin-peroxidase system (Vector Laboratories Inc.; Burlingame, CA, USA), and reactions were visualized with 3',3-diaminobenzidine-dihydrochloride (Sigma; 0.08%) and 0.03% hydrogen peroxide in PBS [7].

Stereology

Stereological techniques were used to estimate plaque load and the total number of synaptophysin-immunoreactive presynaptic terminals in neocortex of transgenic and control mice. A systematic-random series of every 20^{th} paraffin section yielded 12-14 sections/stain/animal. Number estimation was done by first estimating the region volume according to the Cavalieri principle, and then multiplying by numerical density

estimates made with the optical disector [45,46]. Numerical density of synaptophysin-immunoreactive boutons was measured by focusing through optical disectors (using a 100x, 1.3 n.a. lens) placed within the stained sections. Distinctly stained SY-IR boutons were counted through a depth of the tissue (from 1.5-4 µm) where full antibody penetration (typically up to 8 µm) was guaranteed [6,24]. An average of 10-15 boutons were counted within the 23.56 μm^3 disector volume. Dystrophic synaptophysin-immunoreactive boutons (typically 5-10 µm in diameter) found within and surrounding amyloid plaques were excluded from the counts. Coefficient of error was calculated according to [46] and was well under the observed biological variability. Amyloid load (% of volume occupied by plaques) was assessed stereologically as described previously [1,8]. Anatomical regions were defined according to the Franklin & Paxinos atlas [13]. Stereological quantitation was undertaken with the assistance of Stereologer™ software and a motorized x-y-z stage coupled to a video microscopy system (Systems Planning & Analysis, Alexandria, Virginia, USA). All reported values are for one hemisphere only.

As the 3-month-old group was processed completely independent of all other groups, and synapse quantification resulted in a lower synapse number in both transgenic and wild-type mice as compared with the other age groups, their synapse number was adjusted with a correction factor derived from the ratio between 8-month versus 3-month wild-type mice. This seemed legitimate because an additional experiment, in which groups of 3- and 24-month-old control and APP23 mice were processed simultaneously, revealed that both 3-month-old wild-type and APP23 mice have an equivalent number of synaptophysin-positive presynaptic terminals as 24-month-old wild-type and APP23 mice.

Western Blotting

Mice were killed by decapitation, the entire neocortex was dissected on ice, weighed, and frozen on dry ice. Five µl of tissue samples (final dilution 1:880) were loaded on a 10-20% Tricine Novex pre-cast gel **(**Invitrogen, Carlsbad, CA**).** After separation, protein was transferred to a PVDF membrane (Invitrogen) using the Novex Xcell II Blot Module. Membranes were blocked in 5% nonfat milk in PBS with 0.05% Tween 20 (PBS-T) and reacted overnight at 4°C with monoclonal antibody to synaptophysin

(Boehringer Ingelheim, Ingelheim, Germany) at 1:2000 in PBS-T followed by a goat anti-mouse peroxidase conjugate (Chemicon, Temecula, CA) at 1:2500 in PBS-T for 30 min at room temperature. Bands were visualized using ECL (Amersham, Buckinghamshire) and developed onto Kodak X-OMAT AR film (Rochester, NY). Band density measurements were made using NIH Image version 1.62 (National Institutes of Health, Bethesda, MD). Each sample was run at least twice, and the mean for each animal was used in subsequent analyses.

Statistics

All statistical analysis was done using StatView 5.01. The mean ± SEM is indicated. Significance level was set at $p<0.05$.

RESULTS

No amyloid deposits could be detected in the neocortex of 3-month-old APP23 mice or any wild-type mouse (Fig. 1A). Eight-month-old APP23 mice had a small number of compact plaques in the neocortex but lacked diffuse amyloid (Fig. 1B; mean amyloid load 0.1% ± 0.03). Fifteen-month-old APP23 animals showed compact plaque deposition and some diffuse amyloid (Fig. 1C; mean amyloid load 4.0% ± 1.8). The 24-month-old APP23 group exhibited widespread compact and diffuse amyloid deposits in neocortex (Fig. 1D; mean amyloid load 25.2% ± 2.6).

Qualitative light microscopy analysis of synaptophysin-staining did not reveal any age-related differences in staining pattern or intensity for the wild-type mice. At high magnification, the staining revealed a densely packed, punctate pattern with a distribution characteristic of synaptic boutons (Fig. 2A,C). Synaptophysin-immunostaining in young APP23 mice appeared very similar to the wild-type mice. In 15 and 24-month-old APP23 mice synaptic degeneration at the periphery of compact amyloid plaques was clearly visible in the form of large, dystrophic, intensely-stained synaptophysin-positive boutons (Fig. 2B,D). In the plaque center there was a lack of synaptophysin-immunostaining. In plaque-free regions and in areas with diffuse

amyloid, however, synaptic morphology and density appeared similar to young APP23 mice and wild-type mice.

Quantitative stereological analysis of total synaptophysin-immunoreactive presynaptic boutons in neocortex of 3, 8, 15, and 24-month-old mice revealed that neither wild-type nor APP23 mice differed significantly in total bouton number with respect to aging (Fig. 3). ANOVA of synaptic bouton number revealed no significant effect for genotype ($F_{(1,30)}$=4.14; p>0.05), or age ($F_{(3,30)}$=0.81; p>0.05), nor a significant age × genotype interaction ($F_{(3,30)}$=1.43; p>0.05).

The absence of a change in synaptophysin-positive boutons between transgenic and wild-type mice was further investigated by Western blotting for synaptophysin (Fig. 4). Again, densitometry of Western blots did not reveal any significant differences in the amount of synaptophysin between wild-type mice and APP23 mice at 3 months of age (mean optical density: 15.1 ± 0.2 vs. 14.7 ± 0.4; $t_{(12)}$=-0.88; p>0.05) or at 25 months of age (14.6 ± 0.4 vs. 14.4 ± 0.3; $t_{(12)}$=-0.45; p>0.05).

DISCUSSION

The present study was undertaken to investigate alterations in neocortical synapses in APP23 mice, a mouse model which exhibits cerebral amyloidosis progressively with age. Four age groups of transgenic mice and age-matched wild-type control mice were used, allowing comparisons at various stages of amyloid plaque deposition. Despite an amyloid load as high as 25%, a level corresponding to severely-affected AD patients, no overall reduction in synaptic bouton number in neocortex was observed in any group.

Previous studies have documented a robust decline of synaptic density and/or presynaptic markers in AD neocortex and hippocampus and this loss correlates with cognitive impairment [17,34]. However, despite robust synaptic dystrophy and degeneration at the periphery of compact amyloid plaques in aged APP23 mice [29] the present study did not reveal a decrease of total synaptophysin-positive presynaptic terminals in neocortex of aged APP23 mice in comparison to wild-type control mice. Wild-type B6 mice also did not undergo synaptic bouton loss with aging, as previously

reported for the hippocampus using similar stereological techniques [7]. These observations are also in line with our semiquantitative Western blot analysis in APP23 mice and wild-type controls at 3 and 25 months of age that also did not reveal any differences in synaptophysin between the groups.

A part of the synapse loss seen in AD neocortex, but not APP23 mice, could be attributed to a loss of basal forebrain and other subcortical neurons that project to the neocortex. In AD, basal forebrain neurons are lost, most likely because of the formation of intracellular tangles [9,16,44,47]. However, we have previously reported that in APP23 mice no basal forebrain neurons are lost, an observation consistent with other mouse models [1,15].

Recent investigations suggest that neocortical synapse loss in AD might be a late event, when pathology and cognitive state of the patients reaches advanced levels [21,23,42]. In at least one study, a biphasic synaptic response was reported with an increase in synaptic markers at early stages of AD, and a decrease at more advanced stages when tangles and a severe cognitive deficit were apparent [27]. This latter stage is not reproduced in the APP23 mouse model, as it does not develop tangles, and neuron loss in the mice does not reach the extent observed in AD [2,8]. The observation of an increase of synaptic markers in early stages of AD may suggest that compensatory mechanisms are active in an initial phase of AD pathogenesis. Thus, the lack of neocortical synapse loss in APP23 mice may also be attributable to compensatory mechanisms such as sprouting. Indeed, we have previously shown that cerebral amyloidosis in the hippocampus of APP23 mice induces aberrant sprouting of entorhinal fibers with ectopic terminal formation [29].

Studies with amyloid-depositing APP transgenic mice consistently report synapse degeneration at the periphery of compact amyloid plaques [3,14,22,29]. In contrast, controversial results have been reported regarding the loss of total number of synapses in any given brain area with amyloid. Some studies report a loss in the hippocampus or neocortex [4,14,18] whereas others find no change in these regions [11,19]. In APP transgenic mice that do not develop amyloid, synaptic stability or even an increase has been reported [25,31], an observation consistent with a trophic effect of APP overexpression [25,30,43]. At least one study demonstrates region-specificity of

synaptic changes: while in certain regions of the cortex synapses appear to be increased, in the hippocampus they can be decreased [12].

In addition to region-specific differences, and a balance between APP overexpression and amyloid plaque formation, methodological differences could account for the different findings in transgenic mice. Here, we have used a stereological technique which provides an accurate estimate of a total quantity whereas other studies have used optical or numerical density estimates of synaptic boutons which may be subject to shrinkage artifact or other artifactual and biological factors [6]. It is also possible that synapses are lost in specific cortical regions like the entorhinal cortex as it occurs in AD, but this loss might escape detection when the entire neocortex is measured. Unfortunately, separate quantification of the entorhinal cortex could not be performed in this study because in mice with a substantial plaque load, the boundaries of this region can no longer accurately be identified.

In conclusion, the APP23 mouse offers a model to study the effect of progressive amyloid deposition on behavioral, cellular, and molecular levels. Although it bears multiple AD-like hallmarks, the APP23 mouse does not show overall neocortical synaptic loss despite robust amyloidosis. These results are consistent with the hypothesis that amyloid deposition is not sufficient to account for synapse loss in AD. Alternatively, a putative trophic effect of APP or injury-related compensatory sprouting may prevent, compensate, or delay a loss of synapses in this mouse model. In any case, the presence of synaptic terminals does not necessarily predict synaptic function. In fact, recent studies have shown that synaptic dysfunction caused by diffusible oligomers of Aβ occurs in an early phase of AD pathogenesis in which structural changes of synapses have not yet occurred [36]. Further assay of synaptic function is necessary in these mice to examine the effect of increased levels of Aβ on plasticity and cognition.

Figure Captions

Figure 1 Amyloid staining in the neocortex of APP23 mice. (**A**) Three-month-old APP23 mice do not show any amyloid deposition. (**B**) At eight months, single compact plaques appear in the neocortex. The amyloid load in the mouse shown is 0.2%. (**C**) At 15 months, compact and some diffuse amyloid deposits are visible. Shown is the mouse with the highest amyloid load in its age group (10.9%). (**D**) Twenty-four-month-old APP23 mice show robust diffuse and compact cortical amyloid plaques. Shown is a mouse with a 27.8% amyloid load. Calibration bar in (A) is 100 μm for all panels.

Figure 2 Synaptophysin-immunostaining in neocortex of aged wild-type and APP23 transgenic mice. (**A**) Low-power overview of synaptophysin-immunostaining of a 24-month-old wild-type mouse and (**B**) an age-matched APP23 mouse. In plaque-free areas synaptic morphology and density appeared similar between wild-type and APP23 mice. In contrast, there was a lack of synaptophysin-immunostaing in the center of the amyloid plaques with dystrophic, intensely-stained abnormal boutons in the plaque periphery. (**C**) High magnification of synaptophysin-staining in the wild-type mice reveals distinct and puncate synaptic structures. (**D**) High magnification of the synaptophysin-staining demonstrates the loss of synaptophysin-staining in the center (asterisk) and the dystrophic synaptic boutons around the plaques (arrows). Insert: High-power view of swollen, dystrophic boutons. Calibration bar in (A) is 100 μm for A and B; calibration bar in (C) is 12 μm for C and D.

Figure 3 Number of synaptophysin-positive presynaptic terminals in the neocortex of wild-type (open bars) and APP23 mice (black bars). Stereological analysis did not reveal any significant differences in presynaptic terminals between age groups or when APP23 mice were compared to wild-type mice. Indicated is the mean ± SEM for one hemisphere only.

Figure 4 Western blotting revealed no difference in synaptophysin intensity between APP23 and wild-type animals, neither at 3 months (**A**) nor at 25 months of age (**B**). Odd numbers represent wild-type animals, even numbers represent APP23 animals.

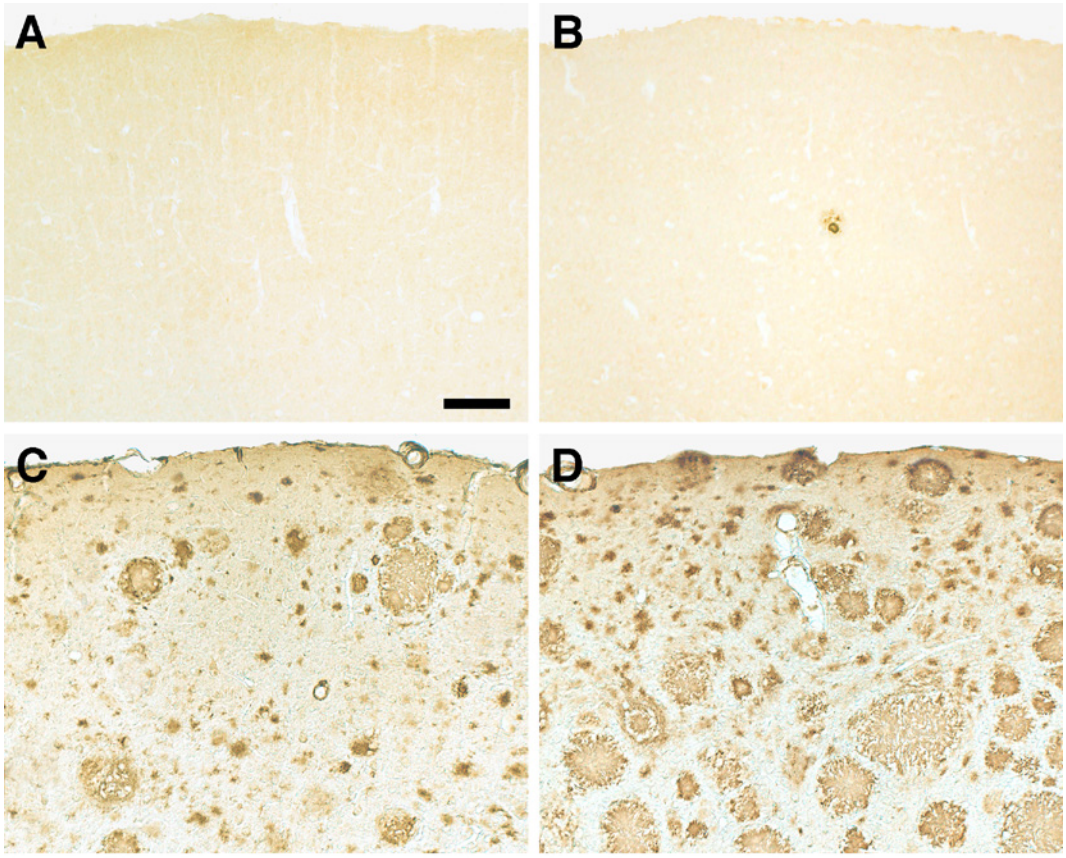

Figure 1

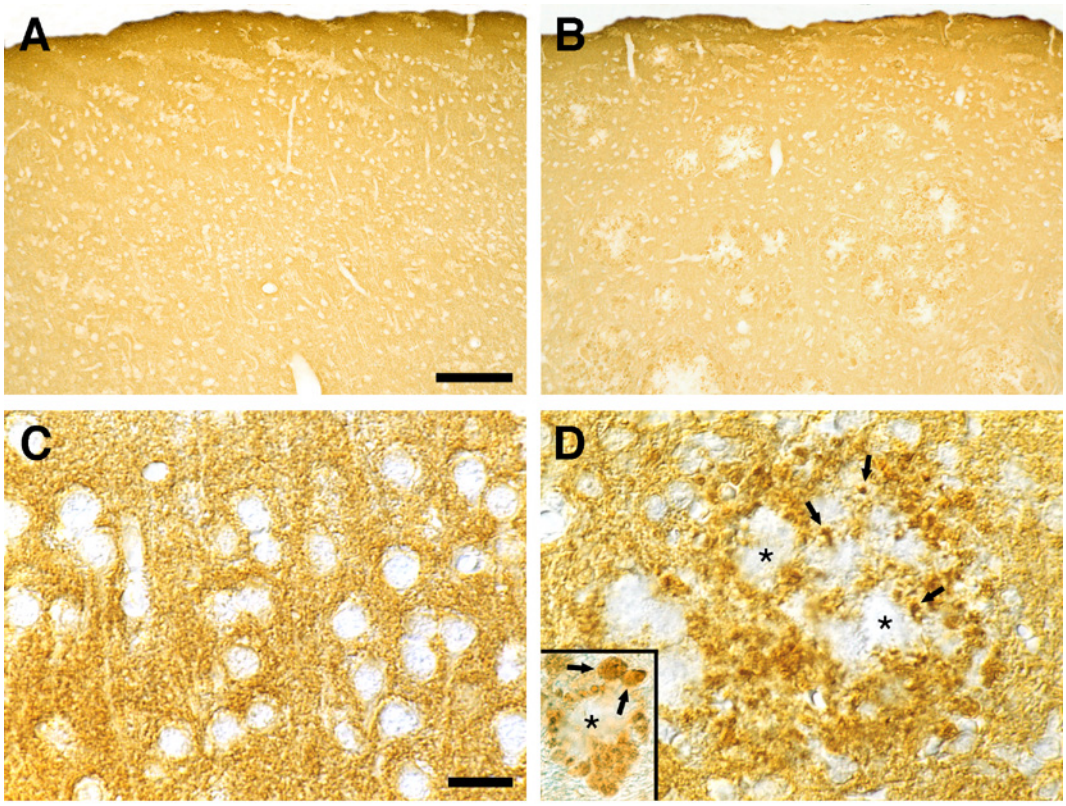

Figure 2

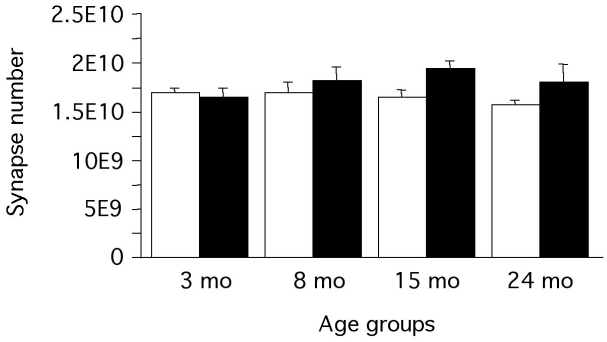
2.5E10
2E10
1.5E10
10E9
5E9
0
Synapse number
3 mo
8 mo
15 mo
24 mo
Age groups

Figure 3

Figure 4

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Cerebral hemorrhage following anti-Aβ immunotherapy

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Immunotherapy for Alzheimer's disease (AD) has been the subject of intense recent investigation. Both active and passive immunization against β-amyloid peptide (Aβ) in mouse models reduce levels of Aβ, prevent and clear amyloid plaques, and improve cognitive behavior (1). We studied passive immunization of APP23 transgenic mice, a model that exhibits the age-related development of amyloid plaques and neurodegeneration as well as cerebral amyloid angiopathy (CAA) similar to that observed in the human AD brain (2,3). Consistent with earlier reports, we found that passive Aβ-immunization results in a significant reduction of mainly diffuse amyloid. However, it also induces an increase in cerebral microhemorrhages associated with amyloid-laden vessels, suggesting a possible link to the neuroinflammatory complications of Aβ-immunization recently seen in a human trial (1).

Male 21-month-old APP23 mice (*n*=10) were passively immunized weekly by intraperitoneal injections of 0.5 mg of β1 mouse monoclonal immunoglobulin G_1 antibody that recognizes amino acids 3 to 6 of human Aβ (4). Age-matched APP23 control mice were injected with phosphate-buffered saline (*n*=11). After 5 months of treatment, stereological analysis of amyloid load (4) revealed a significant amyloid reduction (23%; *P*=0.0008) in neocortex of the immunized mice compared with controls (Fig. 1A,B). This reduction was largely accounted for by a reduction (33%; *P*=0.001) in diffuse amyloid. Enzyme-linked immunosorbent assay measurements of formic acid extracted brain samples (4) showed a significant reduction in Aβ42 (44.8 ± 2.7 and 34.7 ± 3.1 μg/g wet weight for control and immunized mice, respectively; *P*=0.03) but no significant reduction in Aβ40 (166.2 ± 11.3 compared with 152.7 ± 12.2 μg/g).

CAA frequency and severity (4) were not affected by immunization. However, immunized mice exhibited a more than twofold increase in the frequency of CAA-associated cerebral hemorrhage as well as a significant increase in hemorrhage severity over controls (Fig. 1 C to J). Most hemorrhages could clearly be attributed to amyloid-laden vessels and bleedings only occurred in brain areas affected with CAA. Moreover, we found six acute hematomas in immunized mice compared with only one hematoma in control mice. Similar immunization of 6-month-old APP23 mice (*n*=12), which

exhibited modest parenchymal amyloid but lacked significant CAA, revealed no hemorrhages.

Deposition of amyloid in cerebral blood vessels leads to a loss of smooth muscle cells and a weakening of the vessel wall in mice and humans (5,6). Our findings suggest that passive Aβ immunization increases the risk of cerebral hemorrhage by further weakening of the amyloidotic vessel wall. A potential mechanism is that antibody binding to vascular amyloid triggers a local inflammatory reaction, which might be sufficient to destabilize the already weakened vessel wall (6). A link between AD-type vascular pathology and inflammation has been suggested (7). Alternatively, antibody binding to soluble Aβ in blood may lead to increased vascular permeability with a concomitant invasion of plasma proteins and diapedesis that in turn may increase the risk of hemorrhage (6,8). We found no evidence for involvement of the extrinsic coagulation cascade, although we have previously reported that thrombolytic treatment enhances hemorrhagic diathesis in APP23 mice (4).

No adverse side effects have been reported in other mouse Aβ-immunization paradigms; however, the mouse models used in those studies do not develop significant CAA (1). As over 10% of people beyond 65 years of age and 80% of AD cases exhibit CAA (9,10), anti-Aβ immunotherapy protocols may be best developed in mouse models that show CAA in addition to brain parenchymal amyloid deposits. Although the anti-NH_2-terminal Aβ antibody used in this study resulted in an increase in CAA-associated microhemorrhages, further screening of antibodies that recognize other Aβ epitopes or conformations may identify antibodies that do not have this effect. Although difficult to diagnose pre-mortem (10), our results also suggest that the success of Aβ-immunotherapy may be improved by screening AD patients for the presence and severity of CAA before such therapies are undertaken.

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Supplementary Material

Animals and Immunization

APP23 mice (1) were passively immunized weekly for 5 months by intraperitoneal injections of 0.5 mg β1 mouse monoclonal IgG1 antibody, which recognizes amino acids 3-6 of human Aβ (2). Average serum titers were 1:20,000 and 1:4,000 at one and seven days after injections, respectively. Endogenous mouse IgG1 levels in blood were at least ten times higher than the injected amount of β1 antibody. At the end of the experiment mice were overdosed with pentobarbital and brains removed. One hemisphere was immersion fixed for morphological analysis. The other hemisphere was frozen and used for biochemistry.

Histology and Immunohistochemistry

Serial coronal 40 µm sections were cut from the formaldehyde-fixed hemispheres. Hematoxylin and eosin (H&E), and Congo red stainings were done according to standard protocols. The Berlin Blue method of Perls was used to visualize ferric iron in hemosiderin (3,4). Aβ-immunohistochemistry was done with polyclonal antibody NT12 and confirmed with two additional polyclonal antibodies to Aβ (1282, courtesy of D. Selkoe; 86/31, courtesy of C. Masters) according to previously published protocols (5,6). Selected sections were double stained for hemosiderin and Aβ (3,4).

Quantification of Amyloid Load and Cerebral Amyloid Angiopathy

Neocortical amyloid load was estimated on sets of every 20th systematically sampled Aβ-immunostained sections in two-dimensional disectors as previously described (6). Compact amyloid was defined as Aβ-positive/Congo red positive while diffuse amyloid was defined as Aβ-positive/Congo Red negative (6). CAA was quantified using a rating scale as used previously in mice (3,4) and very similar to that reported in humans (7).

Quantification of Cerebral Hemorrhage

Cerebral hemorrhage is followed by a delayed appearance of hemosiderin-positive microglia cells. Perls' Berlin Blue-stained clusters of hemosiderin-positive microglia were quantified on sets of every 10th systematically sampled sections throughout the entire neocortex as previously reported (3,4). *Hemorrhage Frequency* was obtained by counting the total number of perivascular clusters of hemosiderin positive microglial cells in all sections and multiplying the number with the section sampling fraction. *Hemorrhage Severity* was estimated by grading each hemorrhage counted: Grade 1: Cluster with 1-3 hemosiderinpositive perivascular microglia; Grade 2: Cluster with 4-10 hemosiderin-positive perivascular microglia; Grade 3: Cluster with more than 10 hemosiderin-positive perivascular microglia. The mean grade was taken as severity. *Hemorrhage Score* was obtained by multiplying hemorrhage frequency with severity providing a general estimate of the extent of microhemorrhage. Additional sets of every 5th section was stained for H&E and screened for remnants of acute intraparenchymal hematomas (3,4). All quantification was done by three observers (S.B., L.B., and A.S.) and yielded an inter-observer correlation of r=0.71-0.93. The mean was applied for statistical analysis.

ELISA

Human Aβx-40 and Aβx-42 levels were determined by sandwich ELISA from formic acid extracted brain homogenates as previously described (8,9). Control experiments, in which the β1 antibody was added to Aβ peptide standards, showed that the β1 antibody did not interfere with the ELISA detection of Aβ (unpublished observations).

Coagulation Screening

We have previously reported that thrombolytic treatment enhances hemorrhagic diathesis in APP transgenic mice with CAA (4). Accordingly, we tested the extrinsic coagulation cascade in the immunized mice by measuring prothrombin times (10). Briefly, 40 µl of platelet-poor, anticoagulated, mouse plasma (EDTA, ~12 mM) was mixed with 40 µl of a standard thromboplastin reagent provided by the manusfacturer

(recombinant human tissue factor, Innovin®, Dade) and with 40 µl of 50 mM $CaCl_2$. Results revealed that immunized mice had a trend towards shorter prothrombin times compared to PBS-injected control mice (15.4 ± 1.1s vs. 22.6 ± 4.0s; p=0.051). This observation does not support an involvement of the extrinsic coagulation cascade in the increased hemorrhages observed in APP23 mice after Aβ-immunotherapy.

Statistical Analysis

Statistical analysis was done with StatView 5.0.1. Indicated is the mean ± SEM. Significance level was set at $p<0.05$.

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FIGURE CAPTION

Fig. 1. Amyloid pathology in the neocortex of a control (**A**) and an age-matched immunized APP23 mouse (**B**). Hemosiderin staining reveals an increased number of microhemorrhages (arrowheads) in the immunized (**D**) compared with control mice (**C**). Hemorrhages in the immunized mice often reached considerable sizes (**E**). Double staining for hemosiderin (blue) and Aβ (brown) demonstrates that most bleedings were associated with amyloid-laden vessels (**F**). H&E staining reveals a fresh bleed in an immunized mouse (**G**). Quantification (4) of microhemorrhage frequency per unilateral neocortex (**H**) revealed a more than twofold increase in immunized (imm) compared with control (ctr) mice (**P*=0.02). Hemorrhage severity (**I**) and hemorrhage score (**J**) also showed significant increases (***P*=0.003 and **P*=0.01). Scale bars are 100 μm (A-D), 100 μm (E), 20 μm (F), and 200 μm (G).

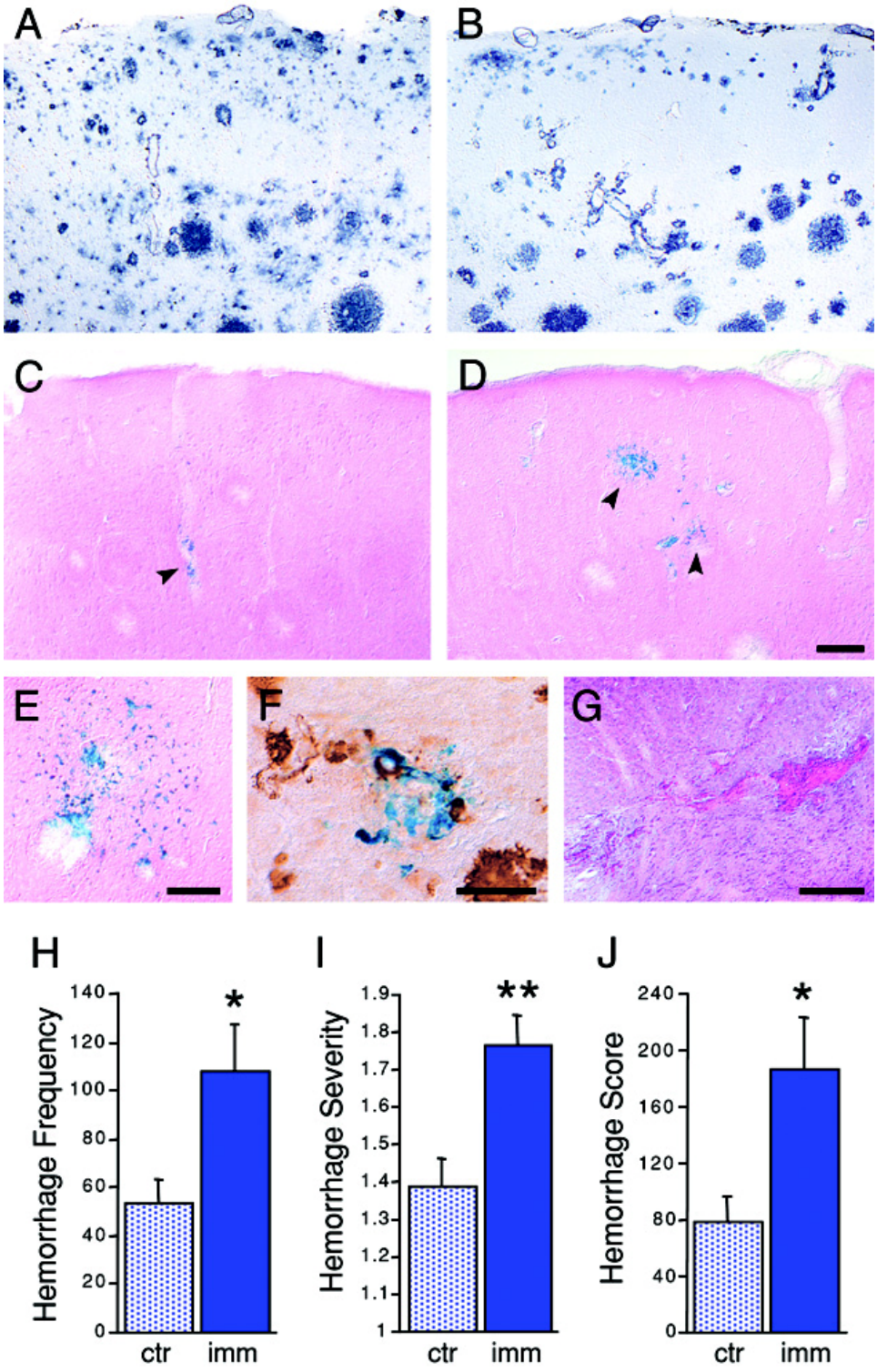
A
B
C
D
E
F
G
H
Hemorrhage Frequency
140
120
100
80
60
40
20
0
*
ctr
imm
I
Hemorrhage Severity
1.9
1.8
1.7
1.6
1.5
1.4
1.3
1.2
1.1
1
**
ctr
imm
J
Hemorrhage Score
240
200
160
120
80
40
0
*
ctr
imm

Figure 1

5. Conclusion

Although AD represents the most frequently encountered form of dementia in the elderly population, no definite cure for the disease is available yet despite intensive research. The development of genetically engineered mice proved to be a useful tool for studying different aspects of AD. The studies presented herein contribute to the understanding of the pathogenesis of amyloidosis and the impact of amyloid on neurons. Furthermore, we provided insights into the mechanisms of therapeutic immunization against Aβ.

It has long been recognized that ACh plays an important role in AD insofar as that the cholinergic system is severely disrupted. In the APP23 mouse we could confirm some of the features seen in AD, such as loss of cholinergic enzyme activities and substantial decrease of cholinergic fibers in the neocortex. In contrast to AD, we did not find a loss of cholinergic neurons in the basal forebrain. This finding answered several open questions, since the major cholinergic input to the neocortex comes from the basal forebrain. These results suggest that basal forebrain neurons in AD are not lost due to retrograde degeneration of cholinergic synapses and fibers in the neocortex, but most likely because of the formation of intracellular tangles. The cortical cholinergic deficit in mice is solely attributable to the presence of plaques. In AD, both loss of basal forebrain neurons and neurotoxicity of plaques contribute to the cortical cholinergic disruption, explaining the somewhat higher cholinergic deficit in AD compared with APP23 mice. Interestingly, our findings of a cortical cholinergic deficit with no change in basal forebrain neuron number were recently confirmed in the PDAPP mice (German et al., 2003). To further explore the interaction of ACh and Aβ, we lesioned the NBM in APP23 mice, knocking out an important cholinergic projection to the neocortex. The goal was to find out whether a cholinergic deficit would enhance amyloid production or rather delay it. The outcome of the experiment, a non-significantly reduced plaque load following cholinergic deficit, stands in contrast to other studies which show increased Aβ or APP levels after NBM lesion in rabbits and rats (Wallace et al., 1993; Wallace and Haroutunian, 1993; Beach et al., 2000). However, it should be mentioned that the

survival time after NBM lesion was substantially longer in our experiment, possibly shifting Aβ towards lower levels after an initial increase. Further evidence that cholinergic deafferentiation does not result in increased plaque formation comes from the observation that in human patients the presence of plaques precedes neocortical cholinergic deficit (Davis et al., 1999). Additionally, AChE has been shown to accelerate the assembly of Aβ peptides into fibrils in vitro, suggesting that AChE deficit should result in lowered plaque burden (Inestrosa et al., 1996). It can be argued that lesion experiments in animals do not reflect the proper situation in human AD, because loss of neurons occurs suddenly all at once, and not progressively with the course of the disease. However, lesion experiments can help to clarify mechanisms and links between systems.

Additionally to the disruption in the cholinergic system, it is well known that in AD neocortex and hippocampus, synapses are lost, and this loss has been correlated with cognitive decline (DeKosky and Scheff, 1990; Terry et al., 1991). However, whether synapse loss is an early event in AD remains contradictory. Recent studies suggest that synapse loss might be a late event in the course of the disease, when the pathology is most widespread, and is absent in mild AD (Masliah et al., 1994; Mukaetova-Ladinska et al., 2000; Tiraboschi et al., 2000; Minger et al., 2001). The outcome of our study, the quantification of cortical synapses in different age groups of APP23 and wild-type mice supports these findings. We failed to see any synaptic changes in APP23 mice at 3, 8, 15, and 24 months of age compared with wild-types. Several studies conducted in other transgenic mouse models are in agreement with our results (Dickey et al., 2003; Rutten et al., 2003). Other studies found even an increase in neocortical synaptic number in APP over-expressing mice or in rats infused with APP peptide (Mucke et al., 1994; Roch et al., 1994; Dodart et al., 2000). In contrast, investigations of synaptic numbers in the hippocampus suggest a decrease (Hsia et al., 1999; Dodart et al., 2000; Mucke et al., 2000; Buttini et al., 2002). Dodart et al. (2000) clearly show that at a given age, different regions of the brain undergo selective synapse changes; while the neocortex bears an increased number of synapses, the hippocampus can be affected by synapse loss. In a previous study, we have shown that also neurons undergo selective loss; while neuron loss is detected in the hippocampus of 15-months-old APP23 mice, no deficit is

seen in the neocortex at the same age (Calhoun et al., 1998). Region specificity may account for the differences regarding synapse number. Taken together, region specificity should be a factor to consider when studies are compared. Moreover, other studies have shown that that Aβ neurotoxicity is age- and species-specific (Geula et al., 1998). The synapses in our mouse model might therefore be less vulnerable to Aβ deposits than in human brain. We suggest that amyloid plaque deposition in the APP23 mouse model is not sufficient to account for the synapse loss seen in AD. Synapse loss in AD might be related to tangle formation or a loss of subcortical innervation.

Recently, a completely new way of AD treatment was approached. Vaccination against Aβ represents an innovative attempt to fight against one of the original causes of AD, and does not simply provide short-term relief of the symptoms. Since the first demonstration that immunization in mice results indeed in lowered plaque burden, it has been subject of intensive research. Although a lot of subsequent studies confirmed this effect and reported a reduction in cognitive dysfunction in mice, clinical studies in humans had to be halted because of serious side effects. Roughly 6% of the patients enrolled in the phase II trial developed meningoencephalitis. As phase I trial did not show any signs of brain inflammation, this phenomenon could be explained, at least in part, by modification of the protocol: in phase II patients received the highest dose of $A\beta_{42}$ and the lowest dose of adjuvant that were used in phase I (Senior, 2002). In addition, the low frequency of the reaction may explain why it became apparent only during the second trial, which was much larger than the first one. In fact, phase I was conducted with 20 and 64 patients which received single and multiple doses, respectively, whereas phase II was conducted with 372 patients. However, the failure of this first treatment attempt does not represent the end of the research pathway, but rather "the end of the beginning" (Schenk, 2002). In fact, a lot of new findings have been achieved, leading to valuable insights to the mechanism of immunotherapy. Alternative ways of immunization to the active one have been experimented: passive vaccination, intranasal application of the vaccine, immunization with Aβ fragments, and administration of agents which bind Aβ without inducing an immune response, all leading to successful decrease of amyloid burden. In our study, we made the important observation that in addition to the plaque-clearing effect of passive immunization, there

was an increase in number of cerebral bleedings associated with amyloid-laden vessels. This should be considered when future therapy trials are undertaken by first screening patients for the presence and severity of CAA. In short, despite the initial setback for the vaccine against AD, thanks to the work on immunization in transgenic mice, unpredicted discoveries have been made.

In conclusion it can be said that since the first discovery of AD nearly 100 years ago, significant progress in the understanding of the mechanisms of the disease has been made. As AD represents still a field of intensive research, and provides new insights almost every day, there is legitimate hope that AD will be defeated in the near future.

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6. Curriculum Vitae

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