Experimental approaches to study cerebral amyloidosis in a transgenic mouse model of Alzheimer's disease

Inauguraldissertation

zur

Erlangung der Würde eines Doktors der Philosophie

vorgelegt der

Philosophisch-Naturwissenschaftlichen Fakultät

der Universität Basel

von

Melanie Meyer-Lühmann

aus Deutschland

Basel, 2004

Genehmigt von der Philosophisch-Naturwissenschaftlichen Fakultät
auf Antrag von
Prof. Dr. Heinrich Reichert und Prof. Dr. Mathias Jucker

Basel, den 10. Februar 2004

Prof. Dr. Marcel Tanner
Dekan

Acknowledgements

First I would like to express special thanks to my supervisor Prof. Mathias Jucker for giving me the opportunity to carry out my PhD thesis and for supporting me throughout my doctoral work. In particular, I want to thank him for his enthusiasm towards my results and for supporting my participation at scientific international meetings. Additionally, I would also like to thank Prof. Heinrich Reichert and Prof. Konrad Beyreuther for their academical support and participation in this dissertation.

I am grateful to Prof. Michael Mihatsch for hosting my work at the Institute of Pathology. I would also like to acknowledge Prof. Alphonse Probst and Prof. Markus Tolnay for their advice as neuropathologists.

I am grateful to Martina Stalder, David Winkler, Luca Bondolfi, Sonia Boncristiano, Florian Ermini, Stephan Käser, Tristan Bolmont and Florence Clavaguera who were always a great help and a good companion during the daily lab life. I am especially indebted to Esther Kohler and Anne Stalder for their psychological support and Martin Herzig for his endless patience and help with my computer and other kinds of problems and for his friendship. Special thanks goes to Michelle Pfeifer, Janaky Coomaraswamy and Michelle Moore for proofreading the manuscript.

I would like to adress my special thank to the members of the photo group Thomas Schürch, Hans-Ruedi Zysset and Jan Schwegler for their help in preparing presentations and manuscripts.

I am grateful to Dr. Matthias Staufenbiel at Novartis in Basel and the members of his group for the general support of my studies and for scientific discussions.

I am indebted to Prof. Hans-Christoph Spatz at the University of Freiburg and Prof. John Wayne Aldridge at the University of Michigan at Ann Arbor who guided my first steps in science during my Diploma thesis and made my affinity for neuroscience grow.

I am grateful to my friends for bearing with me, as I worked my way to the end of this thesis.

Thanks are also due to my parents and Winfried Elis for encouraging me in this endeavor. Without their support of course, none of this would have been possible.

This work is dedicated to the memory of my grandmother Martha Huck.

Table of contents

Summary

Misfolding, aggregation and the accumulation of proteins in the brain are common characteristics of diverse age-related neurodegenerative diseases. Each of these neurodegenerative diseases is associated with abnormalities in the folding of a different protein leading to protein aggregation and finally to neuronal death. Alzheimer's disease (AD) is one of these protein conformational diseases characterized by two major neuropathological features: extracellular accumulation of amyloid-β (Aβ) peptide in the form of plaques and intracellular tangles consisting of hyperphosphorylated tau protein. Although the majority of AD cases are sporadic, three genes have been described whose mutations cause early-onset familial AD (FAD). The identification of mutations in these genes has provided new opportunities to explore pathogenic mechanisms using transgenic approaches. Based on the finding that mutations in these genes all lead to elevated levels of Aβ, new anti-amyloid therapies have been developed to either lower the production of Aβ or to clear the amyloid peptides.

In the past few years, several groups have generated transgenic mouse models of cerebral amyloidosis that exhibit age related Aβ deposition similar to AD patients through expression of mutated human amyloid precursor protein (APP). The studies presented herein were done using such a transgenic mouse model, the APP23 mouse, that overexpresses human APP with the Swedish mutation under the control of a neuron specific Thy-1 promotor. APP23 transgenic mice develop cerebral amyloidosis in an age- and region-dependent manner. Plaque formation starts early at 6 months of age and is associated with the typical AD-like pathology including cerebral amyloid angiopathy, neuron loss, glial activation and cognitive impairment.

The purpose of this thesis was to study the mechanism and initiation of amyloid formation as well as the spread of cerebral amyloidosis *in vivo*. The first series of studies were conducted to define the role and contribution of extracellular versus intracellular β-amyloid in plaque formation. To this end, we transplanted embryonic wildtype (wt) and APP23 transgenic (tg) brain tissue into the hippocampus and cortex of both APP23 and wt mice. We observed that APP23 grafts into wt hosts did not develop amyloid deposits up to 20 moths post-grafting. In contrast, both tg and wt grafts

into APP23 hosts developed amyloid plaques already 3 months post-grafting. The amyloid deposits in wt grafts were surrounded by neuritic changes and gliosis similar to the amyloid-associated pathology described in APP23 mice as well as in AD patients. These results suggest that the phenotype of the transplanted tissue is strongly influenced by the properties of the host. Moreover, these results provide evidence that diffusion of A in the extracellular space is important for the spread of A pathology, that amyloid formation starts extracellularly and that it is the extracellular amyloid that causes neurodegeneration.

The second set of experiments were performed to study the initiation of amyloid deposition and to clarify which factors are involved in the seeding process *in vivo*. Since seeded polymerization of A has already been demonstrated *in vitro* and *in vivo,* we replicated and advanced these findings by intracerebral injection of diluted brain extract from AD patients and brain extracts from aged APP23 transgenic mice into young pre-depositing APP23 mice. AD and APP23 brain homogenate induced a similar amount of seeded A deposits in the brain parenchyma and vessel walls four month post-infusion. This seeding was time- and concentration-dependent. In contrast, no seeding was observed when PBS was injected or when the same extract was injected into wt mice. To address whether A itself is the seeding agent we injected synthetic A into young APP23 mice. These synthetic A injections resulted in limited A deposition compared to that obtained with A-rich brain extract. Our findings suggest that A-containing human and mouse brain extracts can induce cerebral amyloidosis *in vivo*, and that A, in combination with additional factors, initiates amyloid formation.

The third part of the work presented here follows up on our previous finding that diffusion of A in the extracellular space plays an important role in the spread of cerebral amyloidosis. Therefore, we came up with the hypothesis that amyloid deposition and the accompanied pathophysiology could influence extracellular space (ECS) volume and interstitial fluid (ISF) diffusion properties. By using diffusion weighted magnetic resonance imaging (DWI), we determined the diffusion properties in the brains of young and aged APP23 transgenic mice and control littermates. Our results indicate that fibrillar amyloid formation and the associated gliosis are accompanied by a decrease in the apparent diffusion coefficient (ADC), suggesting that both build a

barrier for interstitial fluid diffusion. Thus, in elderly people, ADC measurements and the assessment of diffusion properties in the ECS could serve as a biomarker to detect pathological events in the brain of AD patients.

In summary, the studies presented herein have increased our understanding of the mechanisms leading to protein aggregation and finally to neurodegeneration in a transgenic mouse model. We have shown that factors other than local A production, such as diffusion in the extracellular space, are important in determining whether amyloid pathology will occur. Moreover, the results highlight the relevance of extracellular A to the pathogenesis of the disease. It still remains an open question whether A itself is sufficient to initiate plaque formation, and if so, what conformational form of A is required. Together, these studies provide insights into the mechanisms and disease pathways which may lead to AD.

1. Introduction

1.1 Protein misfolding and aggregation in neurodegenerative diseases

The misfolding and aggregation of proteins is one of the major threats for living organisms. The necessary information for a polypeptide chain to fold correctly into a three-dimensional structure is encoded in its primary amino acid sequence (Anfinsen, 1973). Normally, protein folding is assisted by chaperones that help correct folding and remove misfolded and malfunctioning proteins. However, under physiological conditions of high protein concentrations and cellular crowding (van den Berg et al., 1999; Minton, 2000) and especially at high temperatures, many proteins tend to form stable insoluble aggregates (Jaenicke, 1995). Aggregation occurs when folding/unfolding intermediates become trapped in partially misfolded states that successively associate, mainly through hydrophobic interactions, into an oligomeric continuum of increasingly larger, more stable, and less soluble complexes. In the aggregated state, inactive proteins are enriched in anti-parallel β-strands (Fink, 1998). They can appear as amorphous structures like inclusion bodies, or as ordered fibers (straight, unbranched, 10-nm wide fibrils) such as amyloid plaques and prion particles (Prusiner, 1998; McLaurin et al., 2000).

Age-related neurodegenerative diseases such as Alzheimer's disease (AD), Parkinson's Disease (PD), Huntington's Disease (HD), amyotrophic lateral sclerosis (ALS) and transmissible spongiform encephalopathies (TSE) form a pathologically diverse group that can affect thinking, skilled movements, emotion, cognition, memory and other abilities (Table 1). However, despite their differences in clinical symptoms and disease progression, these disorders do share some common features: most of them appear later in life and their pathology is characterized by neuronal loss and synaptic abnormalities (Martin, 1999). The hallmark feature of these conformational disorders is that proteins can fold into an alternative stable conformation, which results in its aggregation and accumulation as fibrillar deposits (Carrell and Lomas, 1997; Dobson, 1999). Insoluble aggregates of disease related proteins are deposited as inclusions or plaques, the characteristics of which are often disease specific. In AD, amyloid plaques are

deposited in the brain parenchyma and vessel walls, composed mainly of the amyloid-β protein (Glenner et al., 1984). In patients with PD, aggregates called Lewy bodies deposit in the substantia nigra (Forno, 1996), with the major constituent being α-synuclein (Spillantini et al., 1997). Intranuclear deposits of a polyglutamine-rich version of huntingtin protein are typical for the brains of HD patients (DiFiglia et al., 1997). In ALS, patients have aggregates that are mainly composed of superoxid-dismutase in the cell bodies and axons of motor neurons (Bruijn et al., 1998). Finally, the brains of humans that have been affected by TSE are characterized by aggregates of the prion protein (PrP) (Bolton et al., 1982).

Disease	Clinical features	Protein deposits	Toxic protein	Affected brain regions	Disease gene
Alzheimer's Disease	Progressive dementia	Extracellular plaques Intracellular tangles	Amyloid-β and Tau	Hippocampus, cerebral cortex	APP PS 1 PS 2
Parkinson's Disease	Movement disorder	Lewy bodies	α-Synuclein	Substantia nigra, hypothalamus	α-Synuclein Parkin UCHL1
Huntington's Disease	Dementia, motor and psychiatric problems	Nuclear and cytoplasmic inclusions	Huntingtin	Striatum, cerebral cortex	IT-15
Amyotrophic lateral sclerosis	Movement disorder	Bunina bodies	Superoxide dismutase	Motor cortex, brainstem	SOD1
Transmissible spongiform encephalopathies	Dementia, ataxia, psychiatric problems	Prion plaque	Prion protein	Various regions depending on disease	PRNP

Table 1. Clinical and pathological features of neurodegenerative disorders characterized by aggregation and deposition of abnormal protein (adapted from Soto, 2003).

Although these cerebral aggregates have been recognized as a typical feature of neurodegenerative diseases for many years, neuropathological studies have been unable to determine whether they are directly involved in the pathogenesis of the disease. Analysis of post-mortem tissue shows only a poor correlation between the amyloid deposit load and the severity of clinical symptoms (Terry et al., 1991; Gutekunst et al.,

1999). Moreover, the appearance of protein deposits in clinically normal, healthy, elderly people (Katzman et al., 1988) question the role of protein aggregates in neurodegeneration, even though protein aggregates accumulate mainly in the brain areas that show degeneration. Support for a causal role of protein misfolding in neurodegenerative diseases has come more recently from genetic studies since mutations in the respective fibrillar proteins have been found in AD, PD, HD, ALS and TSE (Hsiao et al., 1990; Goate et al., 1991; Group, 1993; Rosen et al., 1993; Polymeropoulos et al., 1997). Furthermore, the generation of transgenic animal models, which express mutant proteins, has provided good evidence for the contribution of protein misfolding to the disease pathogenesis. Several pathological and clinical features have been observed in transgenic models in which protein aggregates were successfully produced (Hsiao et al., 1990; Gurney et al., 1994; Games et al., 1995; Mangiarini et al., 1996; Davies et al., 1997; Masliah et al., 2000). AD may therefore be seen as one example of many neurodegenerative diseases in which an abnormally folded molecule accumulates in the brain and causes neuronal damage.

1.2 Alzheimer's disease

It is almost a hundred years ago since the German psychiatrist Alois Alzheimer (1864-1915) first described the clinical and pathological symptoms of the disease that now bears his name. At a meeting in 1906, he first presented the case of a 54 year old women who suffered from progressive dementia. Although his presentation of the typical clinical and morphological features of AD was not considered to be worthy of discussion at this time, it was published one year later (Alzheimer, 1907). The clinical manifestations of the disease consist of progressive memory loss, disordered cognitive function as well as altered behavior including paranoia, delusions, impairments of attention, perception, reasoning and comportment, and a progressive decline in language function (Price et al., 1993; Morris, 1996). Motor functions remain initially intact but deteriorate in the final phase of the disease leading to a picture resembling motor disorders such as parkinsonism (Selkoe, 2001). Mental functions and activities of daily living become progressively impaired (Forstl and Kurz, 1999). A state of dementia becomes identified when these deficits undermine the capacity for independent living. The onset of the dementia in AD is preceeded by a transitional preclinical period of

many years during which relatively isolated memory difficulties exceed those expected on the basis of normal aging, without however, becoming severe enough to interfere with daily living activities (Linn et al., 1995; Fox et al., 1998). Even normal aging does not necessarily imply that memory function has remained intact (Small et al., 1999).

AD is the most common form of dementia in the elderly accounting for over 50% of the typical, late-onset cases of dementia. The estimated number of patients is approximately 20 million worldwide and is expected to keep growing as the current population ages. Epidemiological studies on the prevalence of AD estimate that not more than 10% of the population over 65 years but 25% of the people over 80 years of age are affected with AD (Pfeffer et al., 1987; Bachman et al., 1992; Lautenschlager et al., 1996; Hy and Keller, 2000). Problems with memory and cognition appear during the seventh decade in most individuals with AD, but may appear earlier, particularly in familial cases (FAD)(Albert and Drachman, 2000). The largest proportion of AD cases are sporadic, occurring with no clearly defined etiology. However, it has been known for several decades that AD can occur in a familial form that is autosomal dominant inherited. Estimates of the proportion of Alzheimer cases that are genetically based varied from as low as 5% to as high as 15% (Selkoe, 1994; Lendon et al., 1997). Familial AD (FAD) is clinically and pathologically indistinguishable from sporadic AD, except for the early age of onset (Lehtovirta et al., 1996; Lippa et al., 1996).

1.2.1 Neuropathological hallmarks

The brains of AD patients contain two hallmark pathological features: neurofibrillary tangles (NFT) and neuritic plaques. Classical senile or neuritic plaques are multicellular lesions containing extracellular deposits of amyloid-β protein (Aβ) that include abundant amyloid fibrils (7-10 nm) intermixed with non-fibrillar forms of the Aβ peptide. Neuritic plaques are surrounded by variable numbers of dystrophic neurites, both axonal terminals and dendrites. Many such plaques contain activated microglial cells situated near the amyloid core, as well as reactive astrocytes around the periphery of the plaque (Itagaki et al., 1989; Wisniewski et al., 1989). Although many plaques with these features can be found in the AD brain, an even larger number of deposits seem to lack surrounding dystrophic neurites or glia. These lesions are called diffuse or

preamyloid plaques, where the A occurs in a non-fibrillar, less dense and amorphous form in the neuropil (Tagliavini et al., 1988; Yamaguchi et al., 1988; Yamaguchi et al., 1989). In brain regions that are generally not implicated in the clinical syndrome of AD, such as cerebellum, striatum and thalamus, almost all A deposits are diffuse with little or no glial and neuritic reactions (Joachim et al., 1989; Ogomori et al., 1989; Yamaguchi et al., 1989). Besides diffuse and compact plaques, extracellular A deposits often occur in another morphological form in AD, in the walls of cerebral and leptomeningeal blood vessels (Mandybur, 1975; Vinters et al., 1988). According to the amyloid cascade hypothesis, accumulation and deposition of A in the brain is the primary influence and early process driving AD pathogenesis leading to neuronal dysfunction (Hardy and Higgins, 1992).

Increasing attention is turning towards small oligomeric aggregates that form initially before they assemble into fibrils or plaques (Fig. 1). These oligomeric A assemblies intermediates are potent neurotoxins and therefore are thought to be the key effectors of neurotoxicity in AD (Klein et al., 2001). Importantly, such oligomeric forms of A are neurotoxic *in vitro* (Oda et al., 1995; Walsh et al., 1997; Lambert et al., 1998; Hartley et al., 1999; Walsh et al., 1999; Nilsberth et al., 2001) and *in vivo* (Walsh et al., 2002). In transgenic mice expressing the human APP, neurological deficits develop even before amyloid deposits occur (Mucke et al., 2000). It has been shown recently that oligomers of A even inhibit long-term potentiation *in vivo* (Walsh et al., 2002). Another intermediate in the pathway of A fibril formation is the protofibril. Protofibrils are short assemblies of 5 nm in diameter and up to 200 nm in length (Harper et al., 1997; Walsh et al., 1997). They are not only important in terms of amyloid fiber formation but also in causing neuronal cell death (Hartley et al., 1999; Walsh et al., 1999). The role of these intermediates in amyloid fiber assembly will be discussed further in section 1.3.2.

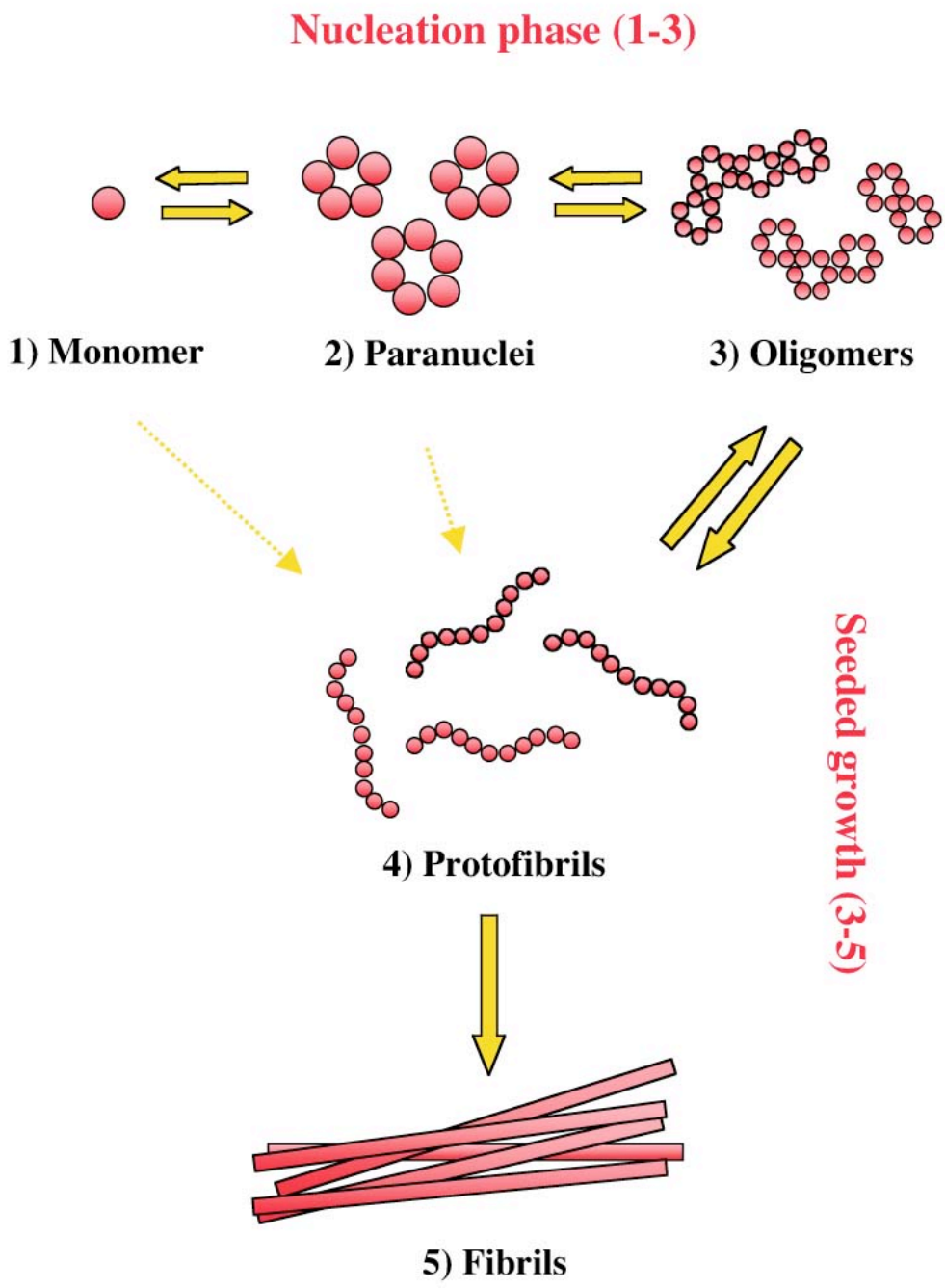

Figure 1. Model of A assembly via nucleation-dependent polymerization. Monomers form via several steps an unstable oligomeric nucleus. Once the critical protein concentration is reached, seeded growth starts and generates amyloid fibrils via protofibrils. This direct pathway may not be the only one since the addition of monomers or paranuclei to protofibrils could also lead to the maturation into fibrils (adapted from Harper and Lansbury, 1997; Bitan et al., 2003).

Neurofibrillary tangles are intraneuronal cytoplasmic lesions consisting of hyperphosphorylated isoforms of the microtubule-associated protein tau, which assemble into poorly soluble paired helical 10-nm filaments (Goedert, 1998). Six tau isoforms are produced in the adult human brain by alternative mRNA splicing from a single gene located on chromosome 17 (Goedert et al., 1989; Andreadis et al., 1992). The six resulting polypeptides have a molecular mass ranging from 45kDa to 65kDa (Buee et al., 2000). Neurofibrillary tangles generally occur in large numbers in the AD brain, particularly in limbic and paralimbic structures such as the entorhinal cortex, hippocampus and amygdala. Intensive studies have shown that the tau protein, which normally enhances the polymerization of tubulin into microtubules and acts to stabilize these organelles in neurons, becomes excessively phosphorylated which reduces binding to microtubules (Lovestone and Reynolds, 1997). The tau and tangle hypothesis argues that in AD the normal role of tau in stabilizing microtubules is impaired, and in diseased neurons microtubules are replaced by tangles (Gray et al., 1987). The resultant cytoskeletal dysfunction in these neurons eventually leads to the degeneration of dendrites and a loss of synapses at their axonal projection targets. The observation that neurofibrillary tangles form in some cell bodies whose axons terminate in regions containing amyloid-bearing neuritic plaques suggests that neurofibrillary tangle formation in some perikarya and neurites may be related to events associated with amyloid plaque formation in the AD brain (Hyman et al., 1986; Rasool et al., 1986).

In addition to plaques and tangles, the AD brain is characterized by extensive neuronal degeneration. The cortex and hippocampus of AD patients undergo a marked atrophy (Mouton et al., 1998), region specific neuron and synapse loss (Terry et al., 1991; West et al., 1994; Gomez-Isla et al., 1996) and a loss of cholinergic innervation (Coyle et al., 1983). Yet, despite intensive interest in a potential association between the tangles, amyloid plaques, vascular amyloid and neurodegeneration observed in the AD brain, the relationship remains poorly understood.

1.2.2 Genetics of Alzheimer's disease

While the complete etiological picture of AD remains unresolved, the inheritance of predisposing genetic factors appears to play a major role. After age, family history is the second greatest risk factor for AD. To date, three genes have been identified whose mutations cause early-onset familial AD (FAD). These genes are the amyloid precursor protein (APP), presenilin 1 (PS1) and presenilin 2 (PS2) (Schellenberg, 1995). Studies on the amyloid precursor protein (APP) as a genetic determinant of AD have begun with the observation that individuals with Down's syndrome develop the clinical and pathological features of AD (Mann et al., 1985; Mann, 1988). These data pointed to the involvement of chromosome 21 in AD leading to the first genetic linkage discovery between a locus on chromosome 21q and autosomal dominant early-onset familial AD (St George-Hyslop et al., 1987). Sequencing of the APP gene and screening for mutations led to the discovery of several missense mutations in families with early-onset AD (Goate et al., 1991; Hendriks et al., 1992; Mullan et al., 1992). Although the APP mutations account for less than 0.1% of all AD cases (Tanzi and Bertram, 2001), they carry virtually complete penetrance leading to AD between the fourth and seventh decades of life.

Since most early–onset AD families do not have mutations in the APP gene, it was expected that other AD loci might exist. A second locus was linked to chromosome 14, and the gene was later identified and named presenilin 1 (PS1) (Van Broeckhoven et al., 1992; Sherrington et al., 1995). A second gene was found based on its homology to PS1 and mapped on chromosome 1 (Levy-Lahad et al., 1995b; Levy-Lahad et al., 1995a; Rogaev et al., 1995). Mutations in these two genes (PS1 and PS2) are thought to cause up to 80% of familial early-onset AD cases. To date there are more than 120 different mutations identified in the PS1 gene (http://molgen-www.uia.ac.be/ADMutations), while only 8 missense mutations have been identified in PS2. Early-onset familial AD mutations in PS1, PS2 and APP lead to an increase of secreted Aβ42, the primary component of β-amyloid plaques in the brain (Price and Sisodia, 1998; Haass and De Strooper, 1999).

In addition to the mutations that have been associated with early-onset FAD, other genetic factors have been identified that increase the risk of developing sporadic AD. The ε4 allele of the apolipoprotein E (APOE) gene on chromosome 19 (Olaisen et al., 1982) was first discovered as a major risk factor for AD (Strittmatter et al., 1993a). In humans there are three common alleles of the APOE gene: ε2, ε3 and ε4. APOE normally plays a role in cholesterol transport and lipid metabolism (Mahley, 1988; Vancea et al., 2000). While the ε4 allele is strongly associated with increased neuritic plaques and cerebral amyloid angiopathy in AD (Olichney et al., 1996), the ε2 allele has been shown to have an impact on longevity and may confer protection against AD (Corder et al., 1994). Distinct binding properties to the Aβ peptide (Strittmatter et al., 1993b) have suggested ways by which APOE might mediate its action. Less than 50% of non-familial AD cases are carriers of the APOEε4 allele (Corder et al., 1993) suggesting that other susceptibility genes must be involved in the pathogenesis of the disease (Shastry and Giblin, 1999). Most of these genes are located on chromosome 9, 10 and 12 (Okuizumi et al., 1995; Ertekin-Taner et al., 2000; Lambert et al., 2000) and are proteolytic enzymes, plasma proteins, growth factors or membrane receptors such as α2-macroglobulin (α2-M) (Blacker et al., 1998), low density lipoprotein receptor-related protein (LRP) (Okuizumi et al., 1995), angiotensin converting enzyme (ACE) (Kehoe et al., 1999) and insulin degrading enzyme (IDE) (Bertram et al., 2000; Ertekin-Taner et al., 2000). α2-M is thought to mediate the clearance and IDE has been implicated in the degradation of Aβ peptides (Vekrellis et al., 2000).
Thus, all AD genetic factors discovered so far point to an alteration of Aβ production or clearance as a direct cause of the disease.

1.2.3 APP processing and Aβ

The isolation of Aβ from senile plaques and cerebrovascular deposits of AD brains (Glenner and Wong, 1984; Masters et al., 1985) and the cloning of the full-length cDNA of APP (Kang et al., 1987) were the beginning of the modern AD research era. APP is a type-I integral transmembrane glycoprotein that is ubiquitously expressed. Three major isoforms of APP are produced by alternative splicing: the two longer isoforms of 751 and 770 amino acids contain a protease inhibitor domain (Kitaguchi et al., 1988; Ponte et al., 1988; Tanzi et al., 1988), whereas the shorter form of 695 amino

acids lacks this Kunitz domain and is the main isoform expressed in neurons (Kang et al., 1987; Golde et al., 1990). The physiological function of APP in the brain remains unclear, although it has a putative function in transmembrane signal transduction (Nishimoto et al., 1993), calcium regulation (Mattson et al., 1993), cell proliferation (Saitoh et al., 1989), regulation of cell/cell or cell/matrix interactions (Breen et al., 1991), cell-adhesion (Jin et al., 1994), neurite outgrowth (Milward et al., 1992) and synaptogenesis (Mucke et al., 1994). Initial studies of APP knockout mice did not reveal an obvious disease phenotype (Zheng et al., 1995), however, other studies have suggested cognitive deficits (Dawson et al., 1999; Phinney et al., 1999) and impaired synaptic plasticity (Seabrook et al., 1999).

APP can be processed by several different proteases, namely the α-, β- and γ-secretases (Fig. 2). The α-secretase cleavage pathway corresponds to the default secretory pathway and is considered as non-amyloidogenic. α-secretase cleaves within the Aβ region to produce the soluble α-APPs (Esch et al., 1990; Sisodia, 1992) and the 83-residue COOH-terminal fragment C83. The identity of the α-secretase is unknown, although more than one enzyme may be involved as the preferential cleavage site varies in different cell types (Zhong et al., 1994). TACE, ADAM-10 and MDC-9, which all belong to the family of adamlysines have been postulated as α-secretase candidates (Buxbaum et al., 1998; Koike et al., 1999; Lammich et al., 1999; Lopez-Perez et al., 2001). The β-secretase cleavage corresponds to the alternative cellular pathway that process APP and constitutes the first step in the formation of Aβ peptides. In contrast to the α-secretase, the β-secretase generates the NH_2-terminus of Aβ, cleaving APP to produce a soluble version of APP and C99 which remains membrane-bound (Seubert et al., 1993). Two novel aspartyl protease homologous, BACE and BACE-2 were recently identified that cleave APP at the β-secretase sites (Sinha et al., 1999; Vassar et al., 1999). In addition, genetic experiments have demonstrated that BACE is required for Aβ peptide formation, confirming its identity as the β-secretase gene (Cai et al., 2001; Luo et al., 2001). Both the C83 and C99 fragments remain anchored in the membrane and may become degraded or further processed by γ-secretase to produce p3 from C83 or Aβ from C99. The γ-secretase cleavage is not sequence specific and can produce a variety of Aβ peptides. Aβ40 is the most common Aβ peptide, while Aβ42 is the

second most common form. The longer the peptide the more insoluble it gets and the more likely it is to form toxic fibrils and aggregates (Jarrett et al., 1993b). The identity of the γ-secretase remains elusive. However, it has been proposed that γ-secretase forms a large complex composed of presenilin, nicastrin, PEN-2, and APH-1, and that the activity of the γ-secretase depends on these proteins (Steiner and Haass, 2000; Yu et al., 2000; Francis et al., 2002; Lee et al., 2002; Steiner et al., 2002). Most recent studies have shown that APP is cleaved by PS-dependent γ-secretase not only in the middle of the transmembrane domain (γ-cleavage) but also inside the cytoplasmic membrane boundary (ε-cleavage) (Gu et al., 2001; Sastre et al., 2001; Yu et al., 2001a; Weidemann et al., 2002). However, the functional significance of ε-secretase cleavage remains to be clarified.

Many different pathogenic mutations such as Swedish (Mullan et al., 1992), Flemish (Hendriks et al., 1992), Dutch (Levy et al., 1990), Florida (Eckman et al., 1997) and London (Goate et al., 1991) have been identified in APP, all of which are missense mutations lying at the three secretase cleavage sites or within the Aβ region, as shown in Figure 2. AD-causing mutations in APP near the β-and γ-secretase cleavage sites all increase Aβ42. Those near the γ-secretase cleavage site augment ε-site proteolysis, leading to elevation of both Aβ40 and Aβ42 (Citron et al., 1992), whereas those near the ε-site increase the production of Aβ42 (Suzuki et al., 1994).

While Aβ40 is the major Aβ product of APP processing and the major component of the amyloid deposits of congophilic angiopathy (Joachim et al., 1988; Miller et al., 1993; Gravina et al., 1995), Aβ42/43 has a much greater tendency to aggregate spontaneously into amyloid fibrils (Hilbich et al., 1991; Burdick et al., 1992; Jarrett and Lansbury, 1993) and is the predominant form of Aβ in senile plaque cores (Roher et al., 1993; Fukumoto et al., 1996). In addition, Aβ42 displays enhanced neurotoxicity relative to Aβ40 (Younkin, 1995; Selkoe, 1999; Dahlgren et al., 2002). Interestingly, Aβ42 levels are reduced in the cerebrospinal fluid (CSF) of AD patients (Motter et al., 1995), which has been attributed to reduced clearance. Taken together, these findings implicate Aβ42 in the pathogenesis of AD. The development of antisera specific for Aβ40 and for Aβ42 enabled plaque evolution and composition to be studied

systematically (Iwatsubo et al., 1994; Gravina et al., 1995). The results are consistent with a nucleation or seeding hypothesis (Jarrett et al., 1993a; Jarrett and Lansbury, 1993), whereby A□42 forms the nucleus of a plaque initially, leading to the subsequent deposition of soluble A□40 (Iwatsubo et al., 1994). The seeding model and its role in the pathogenesis of AD will be described in section 1.3.2.

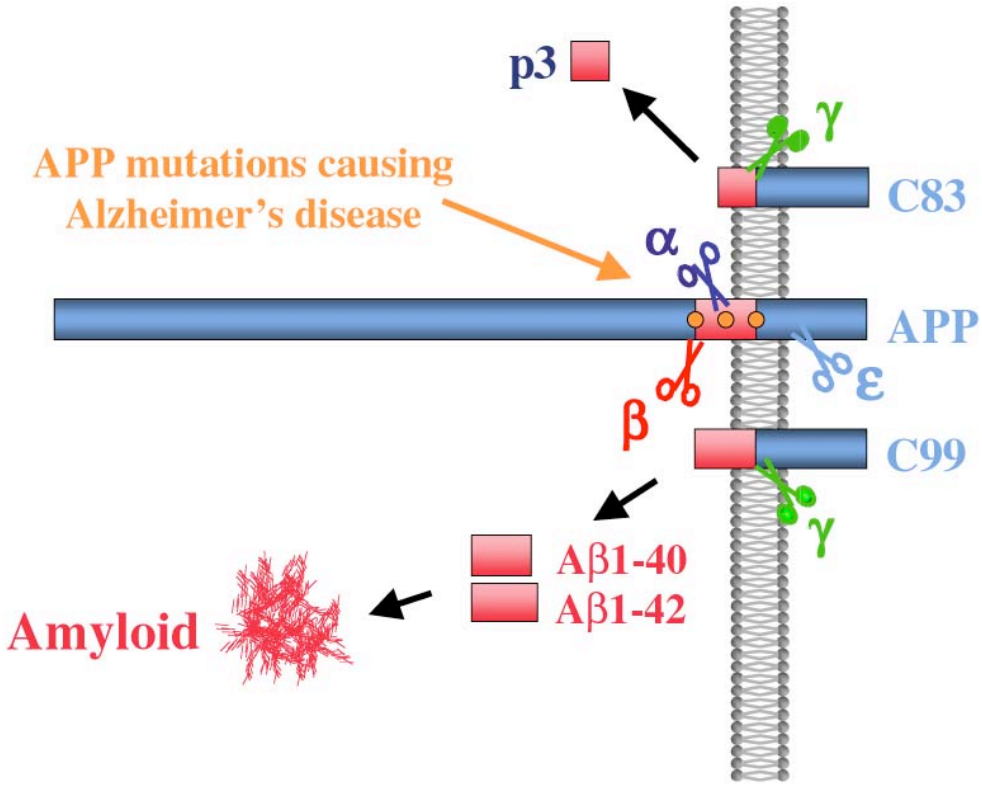

Figure 2. Proteolytic processing of APP. APP is a 770 amino acid type I transmembrane protein which is cleaved by different proteases. The initial cleavage by □-secretase is the most common cleavage, resulting in the release of the large extracellular domain. Since the □-secretase cleavage is in the middle of the A□ domain, no A□ can be generated. Instead, the remaining fragment (C83) is cleaved by □-secretase and releases the small peptide p3. Less frequently, APP is cleaved by □-secretase and then in the transmembrane domain either at position 40 or 42 by □-secretase. This □-secretase pathway is the first step in the formation of A□ peptides and amyloid. There are many APP mutations known that cause familial AD. Note that these mutations are localized within the A□ domain near the cleavage sites of the secretases.

1.2.4 Mouse models of Alzheimer's disease

The identification of mutations in specific genes has provided new opportunities to explore pathogenic mechanisms using genetically engineered mice. To generate animal models of A amyloidosis, many groups have produced transgenic mice that express wild-type APP, APP fragments, A and FAD linked mutant APP and PS1. Although early efforts were disappointing because transgenic mice did not exhibit abnormalities characteristic of AD, more recent work has shown that multiple lines of APP transgenic mice now exist that develop age-related deposition of cerebral amyloid similar to AD (Games et al., 1995; Hsiao et al., 1996; Sturchler-Pierrat et al., 1997). These mice have mild neuron loss, dystrophic neurites, glial responses, learning impairments and deficits in synaptic transmission and/or long-term potentiation (Hsiao et al., 1996; Irizarry et al., 1997; Calhoun et al., 1998; Frautschy et al., 1998; Chapman et al., 1999; Stalder et al., 1999; Bondolfi et al., 2002; Kelly et al., 2003). The disease severity is influenced by the level of transgene expression and the specific mutation. This is in line with the observation that mice expressing both mutant PS1 and mutant APP showed accelerated A amyloidosis well before APP transgenic mice (Borchelt et al., 1997; Holcomb et al., 1998). APP transgenic mice have also been crossed with mice deficient in APOE. In contrast to the APP/PS1 double transgenic mice, these mice show a remarkable decrease in congophilic plaques, suggesting that APOE promotes the deposition and fibrillization of A (Bales et al., 1997; Holtzman et al., 2000).

As APP transgenic models failed to develop tau filaments, several research groups have generated human wildtype tau transgenic mice (Gotz et al., 1995; Brion et al., 1999; Ishihara et al., 1999; Spittaels et al., 1999; Probst et al., 2000). However, none of these transgenic mice developed tau filaments. Only one group reported that their human wildtype tau transgenic mice developed tangle like structures at high age (Ishihara et al., 2001). The expression of P301L mutation was more successful in terms of pathology since these tau transgenic mice showed numerous neurofibrillary tangles (Lewis et al., 2000; Gotz et al., 2001b).

However, appropriate mouse models that display the entire neuropathological spectrum of AD have so far not been entirely successful (Wong et al., 2002). In an attempt to

obtain mice with both plaques and tangles, APP transgenic mice were breed with mice expressing the P301L mutant tau. Although the NFT pathology was substantially enhanced, plaque formation was unaffected by the presence of the tau lesions (Lewis et al., 2001). Similarly, tau pathology can be induced by introducing Aβ42 fibrils into P301L tau mutant mice (Gotz et al., 2001a). Together, both experiments demonstrated pathological interactions between Aβ and tau that lead to increased NFT formation. Recently, triple-transgenic mice (PS1, APP and P301L) have been created that develop extracellular Aβ deposits prior to tangle formation in AD relevant brain regions (Oddo et al., 2003). However, more appropriate models of AD might require co-expression of mutant APP and all six isoforms of wildtype human tau.

In an effort to understand the functions of the AD related genes, researchers have ablated most of them. As mentioned above, homozygous APP -/- mice show only a subtle phenotype (Zheng et al., 1995). PS1-/- mice fail to survive beyond the early postnatal period, but exhibit severe problems in the development of the axial skeleton and ribs (Shen et al., 1997). The lack of PS1 expression in the forebrain in conditional PS1-targeted mice resulted in decreased Aβ generation, further establishing that PS1 is critical for γ-secretase activity in the brain (Feng et al., 2001; Yu et al., 2001b). BACE1 -/- mice are healthy and have also no obvious phenotype, but secretion of Aβ peptides is abolished suggesting that BACE1 is the principal β-secretase in neurons (Cai et al., 2001; Luo et al., 2001; Roberds et al., 2001).

1.2.5 Therapeutic approaches

According to the amyloid cascade hypothesis, the accumulation of Aβ42 in certain brain regions is an early and critical event in the pathogenesis of AD (Hardy and Selkoe, 2002). Therefore, anti-amyloid strategies are currently being investigated to lower the production of Aβ (either by inhibiting β- and γ-secretases or by favouring the α-secretase pathway over the β-secretase pathway), or to clear the amyloid peptides by the use of defibrillating agents or by immunization (Fig. 3).

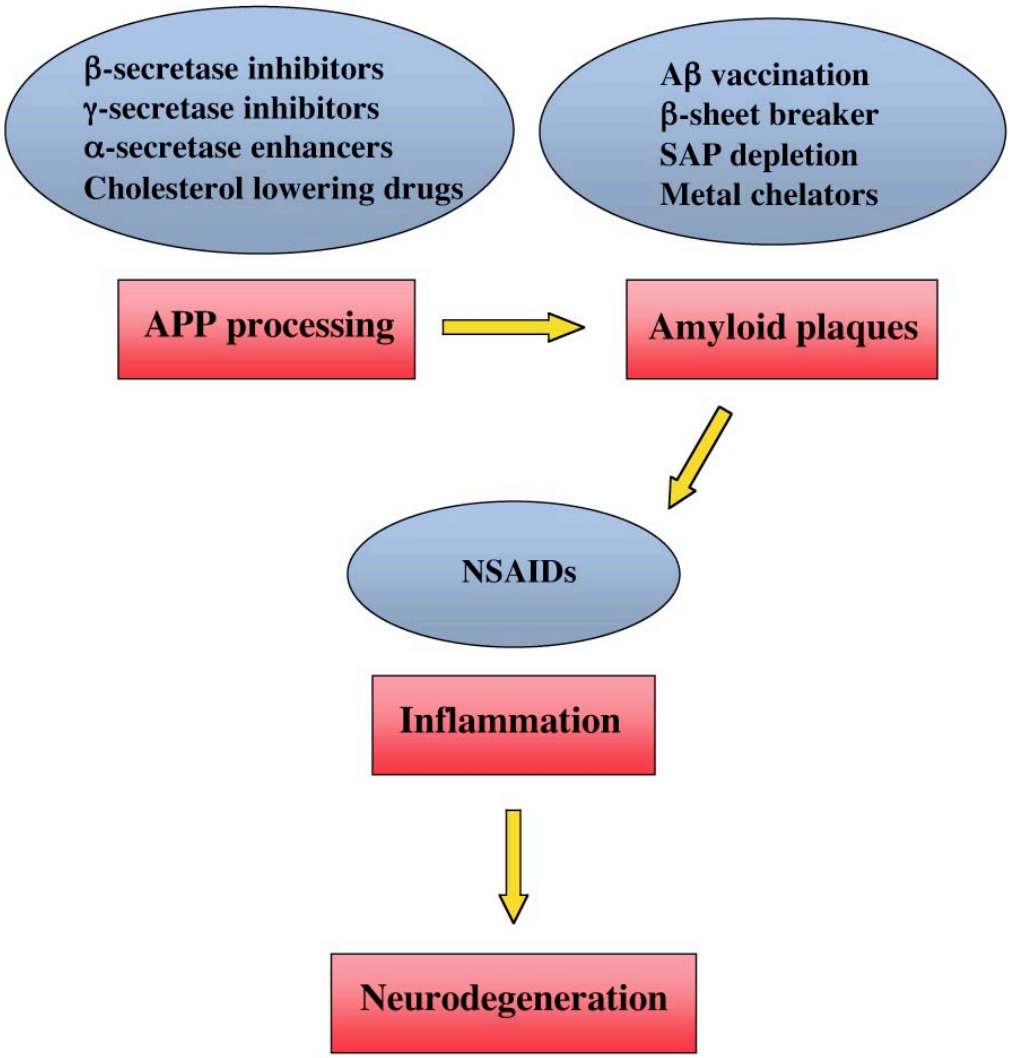

Figure 3. Summary of the main pathological processes and the related therapeutic approaches in AD. These approaches target the pathogenesis of the disease such as APP processing, amyloid plaques and inflammation. The treatment strategies include β- and γ-secretase inhibitors, α-secretase enhancers, cholesterol-lowering drugs and NSAIDs but also amyloid-β-peptide vaccination, β-sheet breakers and metal chelators (adapted from Scarpini et al., 2003).

The β-secretase BACE is thought to be an ideal therapeutic target as it catalyses the first step of Aβ production. Inhibitors described so far are polypeptides that are effective *in vitro* (Sinha et al., 1999; Ghosh et al., 2001), however, these peptidic compounds do not penetrate the blood-brain barrier to a sufficient extent (Citron, 2002). γ-secretase also represents a potential therapeutic target. Potent inhibitors have recently been developed that are effective in preventing the formation of both Aβ40 and Aβ42 (Wolfe et al.,

1999; Esler et al., 2000; Seiffert et al., 2000; Shearman et al., 2000; Dovey et al., 2001). However, a limitation to the use of γ-secretase inhibitors may be that they interfere with Notch signaling and possibly other cellular functions (De Strooper et al., 1999). A recent report that described compounds that inhibit Aβ formation without affecting Notch cleavage may offer new leads for the design of APP-specific γ-secretase inhibitors (Petit et al., 2000). It has also been shown that γ-secretase inhibitors reduce amyloid burden in animal models (Dovey et al., 2001). A subset of non-steroidal anti-inflammatory drugs (NSAIDs) are allosteric inhibitors of γ-secretase (Weggen et al., 2001). This effect was independent of the intended effect of the NSAIDs, namely cyclooxygenase inhibition. Metabolism by γ-secretase can be stimulated by the activation of several cell-surface receptors coupling to protein kinase C (Lee et al., 1995). Estrogens and testosterone were found to promote γ-secretase cleavage of APP in cell culture and to reduce Aβ formation. Thus, hormone replacement therapy may have positive effects in preventing AD. Estradiol treatment is currently being investigated in animal models (Petanceska et al., 2000). *In vitro* and *in vivo* experiments have proposed a molecular link between AD and cholesterol (Simons et al., 1998; Frears et al., 1999; Puglielli et al., 2001). High cholesterol uptake increased Aβ deposition in transgenic mice and in rabbits (Sparks et al., 1994; Refolo et al., 2000). It has also been shown in animals that a cholesterol synthesis inhibitor reduced plaque formation in transgenic mice (Refolo et al., 2000). Therefore, cholesterol lowering drugs such as simvastatin could be an attractive treatment strategy for AD (Fassbender et al., 2001).

Another approach to reduce amyloid accumulation and toxicity in AD is the use of chemical agents that prevent Aβ oligomerization and the formation of neurotoxic protofibrils. A variety of compounds including Congo red (Lorenzo and Yankner, 1994), rifampicin (Tomiyama et al., 1996) and benzofurans (Soto, 1999) have been proposed but are not applicable *in vivo* due to their toxicity. The design of short synthetic peptides homologous to the central hydrophobic region of Aβ has been suggested to disrupt β-sheet stabilisation (Soto, 2001). Administration of this β-sheet breaker reduced the plaque load and ameliorated astrogliosis, microglial activation as well as neurodegeneration in APP transgenic mice (Permanne et al., 2002). Moreover,

this peptide did not induce antibody production and could cross the blood-brain barrier. Serum amyloid P component (SAP) is a glycoprotein that is present in amyloid deposits and seems to protect amyloid fibrils from degradation and clearance (Tennent et al., 1995). Recently, a small compound has been developed that dimerizes SAP, reduces its concentration in the plasma and destabilizes A deposits *in vivo* (Pepys et al., 2002). Oligosaccharide (Fraser et al., 2001) and metal chelators (Cherny et al., 1999; Cherny et al., 2000) can also inhibit plaque formation and constitute potential plaque busters. The administration of clioquinol to transgenic mice reduced A deposition (Cherny et al., 2001).

Apart from the prevention of plaque formation, the removal of deposited plaques has become possible through immunotherapeutic approaches. Transgenic mice have been successfully immunized either by active or passive immunization against A (Schenk et al., 1999; Bard et al., 2000). This vaccination not only prevents the development of A plaques, neuritic dystrophy and astrogliosis, but also markedly reduces the extent and progression of AD-like pathology. Importantly, further studies revealed that such vaccination can improve learning and memory deficits (Janus et al., 2000; Morgan et al., 2000; Younkin, 2001; Dodart et al., 2002). However, a recent phase IIa clinical trial for an AD vaccine (AN-1792) was stopped when 17 of 360 patients developed severe inflammatory side effects. One of the trial participants developed leptomeningeal infiltrates around amyloid-laden blood vessels with T lymphocytes (Nicoll et al., 2003).
In summary, the development of anti-A therapeutics remains a rational approach to treat AD, based on our current understanding of the earliest features of the disease. Despite all difficulties, current data from various laboratories indicates that A-targeted therapy is the most likely to be effective in reducing amyloid-related pathology in AD.

1.3 Studying cerebral amyloidosis *in vivo*

1.3.1 Intracerebral grafting

Grafting tissue into the brain as a possible therapy for neurodegenerative disease has been of enormous interest for many scientists in the past and present research. Parkinson's patients especially have benefited from such therapeutic approaches (Bjorklund, 1991; Freed et al., 1992; Lindvall et al., 1992; Freed et al., 1993; Koutouzis et al., 1994). What is often ignored is the enormous benefit grafting techniques can provide basic research with. For many scientists, intracerebral grafting is a tool used to learn more about neuronal plasticity of the adult nervous system, and as a technique to establish principles of neuronal development. To these ends, intracerebral grafting is established as a powerful method to study basic problems in neuroscience (Fig. 4).

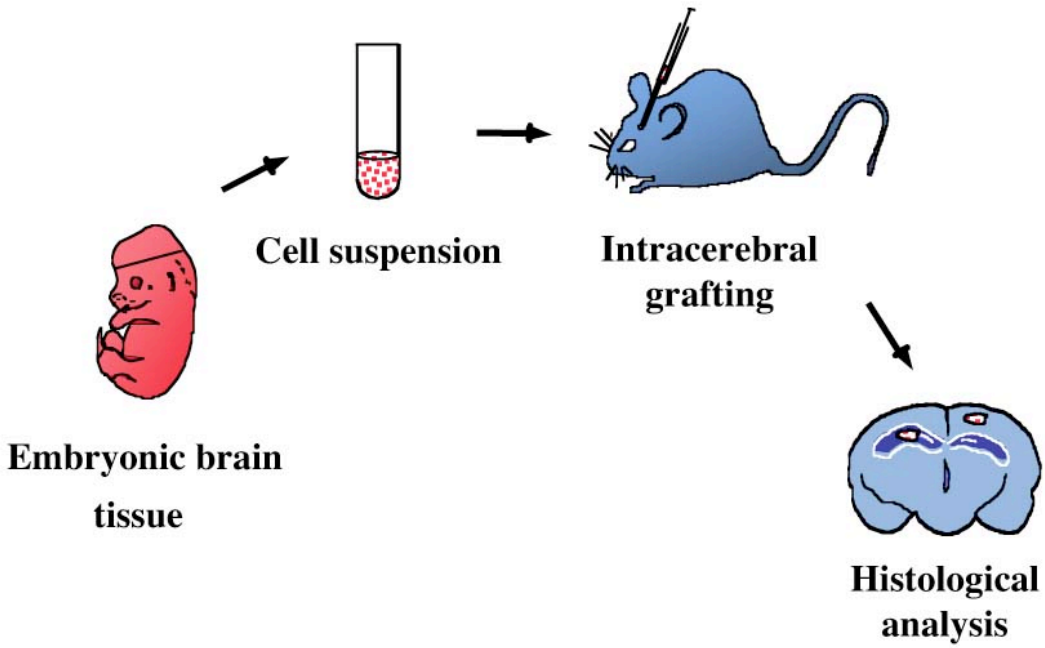

Figure 4. Schematic diagram of the transplantation procedure. Cortical and hippocampal tissue is dissected from E16-17 embryos and then mechanically dissociated into a cell suspension. Several 2.5 µl aliquots of this cell suspension are stereotactically injected via a microsyringe into the neocortex and hippocampus of the host animal. Histological analysis are performed after different time points.

Many important issues concerning the biological requirements for graft survival and optimal function of the graft have been identified. It has been postulated that for most donor tissues, the younger the cells are, the better their chance of survival (Gage et al., 1983; Brundin et al., 1985; Barker et al., 1995). This is particularly true for neuronal tissue, which explains the focus of attention on fetal cells in intracerebral grafting. An equally important factor is the age of the host brain. While grafts will survive in the adult and aged brain (albeit less well in the aged brain), significantly better survival is observed in the young neonatal brain. Both the limitations of donor age and host may be attributed in part to the availability of trophic factors which can address specifically the survival and growth of grafted tissue independently of many other issues (Gage et al., 1984; Mayer et al., 1993). In order for a graft to survive and integrate in the brain, it must be appropriately vascularized and thereby receive essential nutrients from the host environment (Broadwell et al., 1987; Dusart et al., 1989).

While the above factors can be used to influence the survival of the graft, a major objective is to establish graft function in the host brain. One such function is the interaction with the host brain, which should provide at least partial replacement for missing connections. Alternatively, the graft may serve as an endogenous minipump which secretes a missing transmitter (Horellou et al., 1991; Campbell et al., 1993; Cenci et al., 1994) or a trophic factor, which directly affect the survival and growth of damaged cells (Gage et al., 1984). Finally, the graft can function as a bridge that contributes to neuronal and axonal connections (Zimmer et al., 1986; Zimmer et al., 1988).

Before grafting can be considered as a likely approach to cure Alzheimer's disease, the cause of this neurodegenerative disease needs to be determined. With the availability of experimental animal models which mimic the pathological characteristics of the disease, intracerebral grafting could be used to address the pathophysiology of AD. Therefore, intracerebral grafting became a good tool to study the pathogenesis of plaque formation and the underlying and associated pathology.

1.3.2 Seeding mechanism

Kinetic studies have shown that the aggregation of A follows a seeding/nucleation mechanism (Jarrett and Lansbury, 1993), which resembles a crystallization process. The critical event is the formation of protein oligomers that act as a nucleus and which direct further growth of aggregates. Nucleation-dependent polymerization involves two stable states, the monomeric protein and the fibril (Harper and Lansbury, 1997). There is first a slow nucleation phase in which the protein forms an ordered oligomeric nucleus and then a growth phase in which the nucleus rapidly grows to form larger polymers. Polymers are not observed until the monomer concentration exceeds a certain level known as the critical concentration. Below this critical concentration, the monomer is the predominant species. At protein concentrations that exceed the critical concentration by a small amount, there is a lag time before polymerization occurs. During this lag time, addition of pre-formed nuclei or seeds will immediately induce fibril formation. This nucleation-dependent polymerization postulates that aggregation is dependent on protein concentration and time.

At least two intermediates have been identified in this process from the native monomeric protein to the fibrillar, fully aggregated structure *in vitro* (Teplow, 1998). The first are soluble oligomers, which have been detected in test tube experiments, in the medium of A secreting cells and in human cerebro-spinal fluid CSF and brain homogenate (Levine, 1995; Kuo et al., 1996; Lambert et al., 1998; Walsh et al., 2002). The second intermediates are short structures termed protofibrils, which have been studied by electron microscopy and atomic force microscopy (Walsh et al., 1997; Walsh et al., 1999). Protofibrils are 5 nm wide and up to 200 nm long. Protofibrils are the direct precursor of amyloid-like fibrils. Secondary structure studies show that protofibrils have a high -sheet content like fibrils (Walsh et al., 1999). Evidence indicates that these intermediates, the monomers, and the fibrils are all present in an dynamic equilibrium (Teplow, 1998).

1.3.3 Magnetic resonance imaging

Magnetic resonance imaging (MRI) is a special, non-invasive technique used to image internal stuctures of the body, particularly the soft tissues. Unlike conventional radiography and computed tomography (CT), which make use of potentially harmful X-rays to generate images, MRI is based on the magnetic spin properties of atoms. In essence, MRI produces a map of hydrogen distribution in the body. Hydrogen is the simplest element known and the most abundant in biological tissues. It will align itself within a strong magnetic field, like the needle of a compass. The earth's magnetic field is not strong enough to keep a person's hydrogen atoms pointing in the same direction, but the superconducting magnet of an MRI machine can. This comprises the "magnetic" part of MRI. Once the hydrogen atoms have been aligned in the magnet, they absorb energy emitted in the form of specific radio wave frequencies, which knocks them back out of the alignment. Thus, the atoms alternately absorb and emit radio wave energy, vibrating back and forth between their resting (magnetized) and their agitated (radio pulse) state. This comprises the "resonance" part of MRI. The equipment records the duration, strength, and source location of the signals emitted by the atoms as they relax and translates the data into an image on a television monitor. The state of hydrogen in diseased tissue differs from healthy tissue of the same type, making MRI particularly good for the diagnosis of many disease processes due to high soft-tissue contrast and discrimination.

Recent advances in MRI technology with high magnetic fields and the introduction of transgenic mouse models of neurodegenerative diseases in MRI studies produced a great interest for identifying alterations in cellular pathology. MRI can not only provide high-resolution anatomical details on the distribution of the pathology but also pathological information through additional parameters such as relaxion times, cerebral blood flow (CBF) and apparent diffusion coefficient (ADC). As the change of ADC in brain tissue was measured in one of the studies presented in this thesis, it will be briefly discussed further. ADC describes the self-diffusion of water molecules, which is characterized by Brownian motion in the restricted environment. In biological systems, most of the diffusive motion of water molecules is restricted by the structures in the microenvironment, including cell membranes. In many pathological conditions, ADC

has been shown to be a very sensitive parameter reflecting changes in the cellular environment including cell membrane disruption and cell swelling (Norris, 2001). A more recent variant of diffusion MRI is diffusion tensor imaging (DTI) (Basser et al., 1994), which is an important method to map and characterize white matter tracts in the brain (Mori et al., 2001). This new technique uses the anisotropy of ADC. When water molecules are unconstrained, as in the CSF, the diffusion is equal in all directions, i.e., isotropic. In contrast, when water molecules are in highly organized tissue structures, such as white matter fiber tracts that restrict the self-diffusion of water, the resulting diffusion is directional or so called anisotropic. DWI determines this anisotropy and plays therefore an important role in the characterization of neuronal disease in humans and in animal models (Kauppinen et al., 1993).

The recent technical advances of *in vivo* MRI, including the availability of higher magnetic fields permitting improved spectral and spatial resolution are of great use to examine morphological changes noninvasively. With the potential of MRI and the availability of a number of transgenic and rodent models of neurodegeneration, we can gain further understanding of the brain pathogenesis of neurodegenerative disorders and the therapeutic strategies, which will eventually benefit humans.

1.4 References

Albert MS, Drachman DA (2000) Alzheimer's disease: what is it, how many people have it, and why do we need to know? Neurology 55:166-168.

Alzheimer A (1907) Ueber eine eigenartige Erkrankung der Hirnrinde. (A characteristic disease of the cerebral cortex). Allgemeine Zeitschrift für Psychiatrie und Psychisch-Gerichtliche Medizin 64:146-148.

Andreadis A, Brown WM, Kosik KS (1992) Structure and novel exons of the human tau gene. Biochemistry 31:10626-10633.

Anfinsen CB (1973) Principles that govern the folding of protein chains. Science 181:223-230.

Bachman DL, Wolf PA, Linn R, Knoefel JE, Cobb J, Belanger A, D'Agostino RB, White LR (1992) Prevalence of dementia and probable senile dementia of the Alzheimer type in the Framingham Study. Neurology 42:115-119.

Bales KR, Verina T, Dodel RC, Du Y, Altstiel L, Bender M, Hyslop P, Johnstone EM, Little SP, Cummins DJ, Piccardo P, Ghetti B, Paul SM (1997) Lack of apolipoprotein E dramatically reduces amyloid beta-peptide deposition. Nat Genet 17:263-264.

Bard F, Cannon C, Barbour R, Burke RL, Games D, Grajeda H, Guido T, Hu K, Huang J, Johnson-Wood K, Khan K, Kholodenko D, Lee M, Lieberburg I, Motter R, Nguyen M, Soriano F, Vasquez N, Weiss K, Welch B, Seubert P, Schenk D, Yednock T (2000) Peripherally administered antibodies against amyloid beta-peptide enter the central nervous system and reduce pathology in a mouse model of Alzheimer disease. Nat Med 6:916-919.

Barker RA, Fricker RA, Abrous DN, Fawcett J, Dunnett SB (1995) A comparative study of preparation techniques for improving the viability of nigral grafts using vital stains, in vitro cultures, and in vivo grafts. Cell Transplant 4:173-200.

Basser PJ, Mattiello J, LeBihan D (1994) MR diffusion tensor spectroscopy and imaging. Biophys J 66:259-267.

Bertram L, Blacker D, Mullin K, Keeney D, Jones J, Basu S, Yhu S, McInnis MG, Go RC, Vekrellis K, Selkoe DJ, Saunders AJ, Tanzi RE (2000) Evidence for genetic linkage of Alzheimer's disease to chromosome 10q. Science 290:2302-2303.

Bitan G, Kirkitadze MD, Lomakin A, Vollers SS, Benedek GB, Teplow DB (2003) Amyloid beta -protein (Abeta) assembly: Abeta 40 and Abeta 42 oligomerize through distinct pathways. Proc Natl Acad Sci U S A 100:330-335.

Bjorklund A (1991) Neural transplantation--an experimental tool with clinical possibilities. Trends Neurosci 14:319-322.

Blacker D, Wilcox MA, Laird NM, Rodes L, Horvath SM, Go RC, Perry R, Watson B, Jr., Bassett SS, McInnis MG, Albert MS, Hyman BT, Tanzi RE (1998) Alpha-2 macroglobulin is genetically associated with Alzheimer disease. Nat Genet 19:357-360.

Bolton DC, McKinley MP, Prusiner SB (1982) Identification of a protein that purifies with the scrapie prion. Science 218:1309-1311.

Bondolfi L, Calhoun M, Ermini F, Kuhn HG, Wiederhold KH, Walker L, Staufenbiel M, Jucker M (2002) Amyloid-associated neuron loss and gliogenesis in the neocortex of amyloid precursor protein transgenic mice. J Neurosci 22:515-522.

Borchelt DR, Ratovitski T, van Lare J, Lee MK, Gonzales V, Jenkins NA, Copeland NG, Price DL, Sisodia SS (1997) Accelerated amyloid deposition in the brains of transgenic mice coexpressing mutant presenilin 1 and amyloid precursor proteins. Neuron 19:939-945.

Breen KC, Bruce M, Anderton BH (1991) Beta amyloid precursor protein mediates neuronal cell-cell and cell-surface adhesion. J Neurosci Res 28:90-100.

Brion JP, Tremp G, Octave JN (1999) Transgenic expression of the shortest human tau affects its compartmentalization and its phosphorylation as in the pretangle stage of Alzheimer's disease. Am J Pathol 154:255-270.

Broadwell RD, Charlton HM, Balin BJ, Salcman M (1987) Angioarchitecture of the CNS, pituitary gland, and intracerebral grafts revealed with peroxidase cytochemistry. J Comp Neurol 260:47-62.

Bruijn LI, Houseweart MK, Kato S, Anderson KL, Anderson SD, Ohama E, Reaume AG, Scott RW, Cleveland DW (1998) Aggregation and motor neuron toxicity of an ALS-linked SOD1 mutant independent from wild-type SOD1. Science 281:1851-1854.

Brundin P, Isacson O, Bjorklund A (1985) Monitoring of cell viability in suspensions of embryonic CNS tissue and its use as a criterion for intracerebral graft survival. Brain Res 331:251-259.

Buee L, Bussiere T, Buee-Scherrer V, Delacourte A, Hof PR (2000) Tau protein isoforms, phosphorylation and role in neurodegenerative disorders. Brain Res Brain Res Rev 33:95-130.

Burdick D, Soreghan B, Kwon M, Kosmoski J, Knauer M, Henschen A, Yates J, Cotman C, Glabe C (1992) Assembly and aggregation properties of synthetic Alzheimer's A4/beta amyloid peptide analogs. J Biol Chem 267:546-554.

Buxbaum JD, Liu KN, Luo Y, Slack JL, Stocking KL, Peschon JJ, Johnson RS, Castner BJ, Cerretti DP, Black RA (1998) Evidence that tumor necrosis factor alpha converting enzyme is involved in regulated alpha-secretase cleavage of the Alzheimer amyloid protein precursor. J Biol Chem 273:27765-27767.

Cai H, Wang Y, McCarthy D, Wen H, Borchelt DR, Price DL, Wong PC (2001) BACE1 is the major beta-secretase for generation of Abeta peptides by neurons. Nat Neurosci 4:233-234.

Calhoun ME, Wiederhold KH, Abramowski D, Phinney AL, Probst A, Sturchler-Pierrat C, Staufenbiel M, Sommer B, Jucker M (1998) Neuron loss in APP transgenic mice. Nature 395:755-756.

Campbell K, Kalen P, Wictorin K, Lundberg C, Mandel RJ, Bjorklund A (1993) Characterization of GABA release from intrastriatal striatal transplants: dependence on host-derived afferents. Neuroscience 53:403-415.

Carrell RW, Lomas DA (1997) Conformational disease. Lancet 350:134-138.

Cenci MA, Kalen P, Duan WM, Bjorklund A (1994) Transmitter release from transplants of fetal ventral mesencephalon or locus coeruleus in the rat frontal cortex and nucleus accumbens: effects of pharmacological and behaviorally activating stimuli. Brain Res 641:225-248.

Chapman PF, White GL, Jones MW, Cooper-Blacketer D, Marshall VJ, Irizarry M, Younkin L, Good MA, Bliss TV, Hyman BT, Younkin SG, Hsiao KK (1999) Impaired synaptic

plasticity and learning in aged amyloid precursor protein transgenic mice. Nat Neurosci 2:271-276.

Chen YC, Galpern WR, Brownell AL, Matthews RT, Bogdanov M, Isacson O, Keltner JR, Beal MF, Rosen BR, Jenkins BG (1997) Detection of dopaminergic neurotransmitter activity using pharmacologic MRI: correlation with PET, microdialysis, and behavioral data. Magn Reson Med 38:389-398.

Cherny RA, Legg JT, McLean CA, Fairlie DP, Huang X, Atwood CS, Beyreuther K, Tanzi RE, Masters CL, Bush AI (1999) Aqueous dissolution of Alzheimer's disease Abeta amyloid deposits by biometal depletion. J Biol Chem 274:23223-23228.

Cherny RA, Barnham KJ, Lynch T, Volitakis I, Li QX, McLean CA, Multhaup G, Beyreuther K, Tanzi RE, Masters CL, Bush AI (2000) Chelation and intercalation: complementary properties in a compound for the treatment of Alzheimer's disease. J Struct Biol 130:209-216.

Cherny RA, Atwood CS, Xilinas ME, Gray DN, Jones WD, McLean CA, Barnham KJ, Volitakis I, Fraser FW, Kim Y, Huang X, Goldstein LE, Moir RD, Lim JT, Beyreuther K, Zheng H, Tanzi RE, Masters CL, Bush AI (2001) Treatment with a copper-zinc chelator markedly and rapidly inhibits beta-amyloid accumulation in Alzheimer's disease transgenic mice. Neuron 30:665-676.

Citron M (2002) Alzheimer's disease: treatments in discovery and development. Nat Neurosci 5 Suppl:1055-1057.

Citron M, Oltersdorf T, Haass C, McConlogue L, Hung AY, Seubert P, Vigo-Pelfrey C, Lieberburg I, Selkoe DJ (1992) Mutation of the beta-amyloid precursor protein in familial Alzheimer's disease increases beta-protein production. Nature 360:672-674.

Corder EH, Saunders AM, Strittmatter WJ, Schmechel DE, Gaskell PC, Small GW, Roses AD, Haines JL, Pericak-Vance MA (1993) Gene dose of apolipoprotein E type 4 allele and the risk of Alzheimer's disease in late onset families. Science 261:921-923.

Corder EH, Saunders AM, Risch NJ, Strittmatter WJ, Schmechel DE, Gaskell PC, Jr., Rimmler JB, Locke PA, Conneally PM, Schmader KE, et al. (1994) Protective effect of apolipoprotein E type 2 allele for late onset Alzheimer disease. Nat Genet 7:180-184.

Coyle JT, Price DL, DeLong MR (1983) Alzheimer's disease: a disorder of cortical cholinergic innervation. Science 219:1184-1190.

Dahlgren KN, Manelli AM, Stine WB, Jr., Baker LK, Krafft GA, LaDu MJ (2002) Oligomeric and fibrillar species of amyloid-beta peptides differentially affect neuronal viability. J Biol Chem 277:32046-32053.

Davies SW, Turmaine M, Cozens BA, DiFiglia M, Sharp AH, Ross CA, Scherzinger E, Wanker EE, Mangiarini L, Bates GP (1997) Formation of neuronal intranuclear inclusions underlies the neurological dysfunction in mice transgenic for the HD mutation. Cell 90:537-548.

Dawson GR, Seabrook GR, Zheng H, Smith DW, Graham S, O'Dowd G, Bowery BJ, Boyce S, Trumbauer ME, Chen HY, Van der Ploeg LH, Sirinathsinghji DJ (1999) Age-related cognitive deficits, impaired long-term potentiation and reduction in synaptic marker density in mice lacking the beta-amyloid precursor protein. Neuroscience 90:1-13.

De Strooper B, Annaert W, Cupers P, Saftig P, Craessaerts K, Mumm JS, Schroeter EH, Schrijvers V, Wolfe MS, Ray WJ, Goate A, Kopan R (1999) A presenilin-1-dependent

gamma-secretase-like protease mediates release of Notch intracellular domain. Nature 398:518-522.

DiFiglia M, Sapp E, Chase KO, Davies SW, Bates GP, Vonsattel JP, Aronin N (1997) Aggregation of huntingtin in neuronal intranuclear inclusions and dystrophic neurites in brain. Science 277:1990-1993.

Dobson CM (1999) Protein misfolding, evolution and disease. Trends Biochem Sci 24:329-332.

Dodart JC, Bales KR, Gannon KS, Greene SJ, DeMattos RB, Mathis C, DeLong CA, Wu S, Wu X, Holtzman DM, Paul SM (2002) Immunization reverses memory deficits without reducing brain Abeta burden in Alzheimer's disease model. Nat Neurosci 5:452-457.

Dovey HF, John V, Anderson JP, Chen LZ, de Saint Andrieu P, Fang LY, Freedman SB, Folmer B, Goldbach E, Holsztynska EJ, Hu KL, Johnson-Wood KL, Kennedy SL, Kholodenko D, Knops JE, Latimer LH, Lee M, Liao Z, Lieberburg IM, Motter RN, Mutter LC, Nietz J, Quinn KP, Sacchi KL, Seubert PA, Shopp GM, Thorsett ED, Tung JS, Wu J, Yang S, Yin CT, Schenk DB, May PC, Altstiel LD, Bender MH, Boggs LN, Britton TC, Clemens JC, Czilli DL, Dieckman-McGinty DK, Droste JJ, Fuson KS, Gitter BD, Hyslop PA, Johnstone EM, Li WY, Little SP, Mabry TE, Miller FD, Audia JE (2001) Functional gamma-secretase inhibitors reduce beta-amyloid peptide levels in brain. J Neurochem 76:173-181.

Dusart I, Nothias F, Roudier F, Besson JM, Peschanski M (1989) Vascularization of fetal cell suspension grafts in the excitotoxically lesioned adult rat thalamus. Brain Res Dev Brain Res 48:215-228.

Eckman CB, Mehta ND, Crook R, Perez-tur J, Prihar G, Pfeiffer E, Graff-Radford N, Hinder P, Yager D, Zenk B, Refolo LM, Prada CM, Younkin SG, Hutton M, Hardy J (1997) A new pathogenic mutation in the APP gene (I716V) increases the relative proportion of A beta 42(43). Hum Mol Genet 6:2087-2089.

Ertekin-Taner N, Graff-Radford N, Younkin LH, Eckman C, Baker M, Adamson J, Ronald J, Blangero J, Hutton M, Younkin SG (2000) Linkage of plasma Abeta42 to a quantitative locus on chromosome 10 in late-onset Alzheimer's disease pedigrees. Science 290:2303-2304.

Esch FS, Keim PS, Beattie EC, Blacher RW, Culwell AR, Oltersdorf T, McClure D, Ward PJ (1990) Cleavage of amyloid beta peptide during constitutive processing of its precursor. Science 248:1122-1124.

Esler WP, Kimberly WT, Ostaszewski BL, Diehl TS, Moore CL, Tsai JY, Rahmati T, Xia W, Selkoe DJ, Wolfe MS (2000) Transition-state analogue inhibitors of gamma-secretase bind directly to presenilin-1. Nat Cell Biol 2:428-434.

Fassbender K, Simons M, Bergmann C, Stroick M, Lutjohann D, Keller P, Runz H, Kuhl S, Bertsch T, von Bergmann K, Hennerici M, Beyreuther K, Hartmann T (2001) Simvastatin strongly reduces levels of Alzheimer's disease beta -amyloid peptides Abeta 42 and Abeta 40 in vitro and in vivo. Proc Natl Acad Sci U S A 98:5856-5861.

Feng R, Rampon C, Tang YP, Shrom D, Jin J, Kyin M, Sopher B, Miller MW, Ware CB, Martin GM, Kim SH, Langdon RB, Sisodia SS, Tsien JZ (2001) Deficient neurogenesis in forebrain-specific presenilin-1 knockout mice is associated with reduced clearance of hippocampal memory traces. Neuron 32:911-926.

Fink AL (1998) Protein aggregation: folding aggregates, inclusion bodies and amyloid. Fold Des 3:R9-23.

Forno LS (1996) Neuropathology of Parkinson's disease. J Neuropathol Exp Neurol 55:259-272.

Forstl H, Kurz A (1999) Clinical features of Alzheimer's disease. Eur Arch Psychiatry Clin Neurosci 249:288-290.

Fox NC, Warrington EK, Seiffer AL, Agnew SK, Rossor MN (1998) Presymptomatic cognitive deficits in individuals at risk of familial Alzheimer's disease. A longitudinal prospective study. Brain 121 (Pt 9):1631-1639.

Francis R, McGrath G, Zhang J, Ruddy DA, Sym M, Apfeld J, Nicoll M, Maxwell M, Hai B, Ellis MC, Parks AL, Xu W, Li J, Gurney M, Myers RL, Himes CS, Hiebsch R, Ruble C, Nye JS, Curtis D (2002) aph-1 and pen-2 are required for Notch pathway signaling, gamma-secretase cleavage of betaAPP, and presenilin protein accumulation. Dev Cell 3:85-97.

Fraser PE, Darabie AA, McLaurin JA (2001) Amyloid-beta interactions with chondroitin sulfate-derived monosaccharides and disaccharides. implications for drug development. J Biol Chem 276:6412-6419.

Frautschy SA, Yang F, Irrizarry M, Hyman B, Saido TC, Hsiao K, Cole GM (1998) Microglial response to amyloid plaques in APPsw transgenic mice. Am J Pathol 152:307-317.

Frears ER, Stephens DJ, Walters CE, Davies H, Austen BM (1999) The role of cholesterol in the biosynthesis of beta-amyloid. Neuroreport 10:1699-1705.

Freed CR, Breeze RE, Rosenberg NL, Schneck SA (1993) Embryonic dopamine cell implants as a treatment for the second phase of Parkinson's disease. Replacing failed nerve terminals. Adv Neurol 60:721-728.

Freed CR, Breeze RE, Rosenberg NL, Schneck SA, Kriek E, Qi JX, Lone T, Zhang YB, Snyder JA, Wells TH, et al. (1992) Survival of implanted fetal dopamine cells and neurologic improvement 12 to 46 months after transplantation for Parkinson's disease. N Engl J Med 327:1549-1555.

Fukumoto H, Asami-Odaka A, Suzuki N, Shimada H, Ihara Y, Iwatsubo T (1996) Amyloid beta protein deposition in normal aging has the same characteristics as that in Alzheimer's disease. Predominance of A beta 42(43) and association of A beta 40 with cored plaques. Am J Pathol 148:259-265.

Gage FH, Bjorklund A, Stenevi U (1984) Denervation releases a neuronal survival factor in adult rat hippocampus. Nature 308:637-639.

Gage FH, Dunnett SB, Brundin P, Isacson O, Bjorklund A (1983) Intracerebral grafting of embryonic neural cells into the adult host brain: an overview of the cell suspension method and its application. Dev Neurosci 6:137-151.

Games D, Adams D, Alessandrini R, Barbour R, Berthelette P, Blackwell C, Carr T, Clemens J, Donaldson T, Gillespie F, et al. (1995) Alzheimer-type neuropathology in transgenic mice overexpressing V717F beta-amyloid precursor protein. Nature 373:523-527.

Ghosh AK, Bilcer G, Harwood C, Kawahama R, Shin D, Hussain KA, Hong L, Loy JA, Nguyen C, Koelsch G, Ermolieff J, Tang J (2001) Structure-based design: potent inhibitors of human brain memapsin 2 (beta-secretase). J Med Chem 44:2865-2868.

Glenner GG, Wong CW (1984) Alzheimer's disease: initial report of the purification and characterization of a novel cerebrovascular amyloid protein. Biochem Biophys Res Commun 120:885-890.

Glenner GG, Wong CW, Quaranta V, Eanes ED (1984) The amyloid deposits in Alzheimer's disease: their nature and pathogenesis. Appl Pathol 2:357-369.

Goate A, Chartier-Harlin MC, Mullan M, Brown J, Crawford F, Fidani L, Giuffra L, Haynes A, Irving N, James L, et al. (1991) Segregation of a missense mutation in the amyloid precursor protein gene with familial Alzheimer's disease. Nature 349:704-706.

Goedert M (1998) Neurofibrillary pathology of Alzheimer's disease and other tauopathies. Prog Brain Res 117:287-306.

Goedert M, Spillantini MG, Jakes R, Rutherford D, Crowther RA (1989) Multiple isoforms of human microtubule-associated protein tau: sequences and localization in neurofibrillary tangles of Alzheimer's disease. Neuron 3:519-526.

Golde TE, Estus S, Usiak M, Younkin LH, Younkin SG (1990) Expression of beta amyloid protein precursor mRNAs: recognition of a novel alternatively spliced form and quantitation in Alzheimer's disease using PCR. Neuron 4:253-267.

Gomez-Isla T, Price JL, McKeel DW, Jr., Morris JC, Growdon JH, Hyman BT (1996) Profound loss of layer II entorhinal cortex neurons occurs in very mild Alzheimer's disease. J Neurosci 16:4491-4500.

Gotz J, Chen F, van Dorpe J, Nitsch RM (2001a) Formation of neurofibrillary tangles in P301l tau transgenic mice induced by Abeta 42 fibrils. Science 293:1491-1495.

Gotz J, Chen F, Barmettler R, Nitsch RM (2001b) Tau filament formation in transgenic mice expressing P301L tau. J Biol Chem 276:529-534.

Gotz J, Probst A, Spillantini MG, Schafer T, Jakes R, Burki K, Goedert M (1995) Somatodendritic localization and hyperphosphorylation of tau protein in transgenic mice expressing the longest human brain tau isoform. Embo J 14:1304-1313.

Gravina SA, Ho L, Eckman CB, Long KE, Otvos L, Jr., Younkin LH, Suzuki N, Younkin SG (1995) Amyloid beta protein (A beta) in Alzheimer's disease brain. Biochemical and immunocytochemical analysis with antibodies specific for forms ending at A beta 40 or A beta 42(43). J Biol Chem 270:7013-7016.

Gray EG, Paula-Barbosa M, Roher A (1987) Alzheimer's disease: paired helical filaments and cytomembranes. Neuropathol Appl Neurobiol 13:91-110.

Group THsDCR (1993) A novel gene containing a trinucleotide repeat that is expanded and unstable on Huntington's disease chromosomes. The Huntington's Disease Collaborative Research Group. Cell 72:971-983.

Gu Y, Misonou H, Sato T, Dohmae N, Takio K, Ihara Y (2001) Distinct intramembrane cleavage of the beta-amyloid precursor protein family resembling gamma-secretase-like cleavage of Notch. J Biol Chem 276:35235-35238.

Gurney ME, Pu H, Chiu AY, Dal Canto MC, Polchow CY, Alexander DD, Caliendo J, Hentati A, Kwon YW, Deng HX, et al. (1994) Motor neuron degeneration in mice that express a human Cu,Zn superoxide dismutase mutation. Science 264:1772-1775.

Gutekunst CA, Li SH, Yi H, Mulroy JS, Kuemmerle S, Jones R, Rye D, Ferrante RJ, Hersch SM, Li XJ (1999) Nuclear and neuropil aggregates in Huntington's disease: relationship to neuropathology. J Neurosci 19:2522-2534.

Haass C, De Strooper B (1999) The presenilins in Alzheimer's disease--proteolysis holds the key. Science 286:916-919.

Hardy J, Selkoe DJ (2002) The amyloid hypothesis of Alzheimer's disease: progress and problems on the road to therapeutics. Science 297:353-356.

Hardy JA, Higgins GA (1992) Alzheimer's disease: the amyloid cascade hypothesis. Science 256:184-185.

Harper JD, Lansbury PT, Jr. (1997) Models of amyloid seeding in Alzheimer's disease and scrapie: mechanistic truths and physiological consequences of the time-dependent solubility of amyloid proteins. Annu Rev Biochem 66:385-407.

Harper JD, Wong SS, Lieber CM, Lansbury PT (1997) Observation of metastable Abeta amyloid protofibrils by atomic force microscopy. Chem Biol 4:119-125.

Hartley DM, Walsh DM, Ye CP, Diehl T, Vasquez S, Vassilev PM, Teplow DB, Selkoe DJ (1999) Protofibrillar intermediates of amyloid beta-protein induce acute electrophysiological changes and progressive neurotoxicity in cortical neurons. J Neurosci 19:8876-8884.

Hendriks L, van Duijn CM, Cras P, Cruts M, Van Hul W, van Harskamp F, Warren A, McInnis MG, Antonarakis SE, Martin JJ, et al. (1992) Presenile dementia and cerebral haemorrhage linked to a mutation at codon 692 of the beta-amyloid precursor protein gene. Nat Genet 1:218-221.

Hilbich C, Kisters-Woike B, Reed J, Masters CL, Beyreuther K (1991) Aggregation and secondary structure of synthetic amyloid beta A4 peptides of Alzheimer's disease. J Mol Biol 218:149-163.

Holcomb L, Gordon MN, McGowan E, Yu X, Benkovic S, Jantzen P, Wright K, Saad I, Mueller R, Morgan D, Sanders S, Zehr C, O'Campo K, Hardy J, Prada CM, Eckman C, Younkin S, Hsiao K, Duff K (1998) Accelerated Alzheimer-type phenotype in transgenic mice carrying both mutant amyloid precursor protein and presenilin 1 transgenes. Nat Med 4:97-100.

Holtzman DM, Fagan AM, Mackey B, Tenkova T, Sartorius L, Paul SM, Bales K, Ashe KH, Irizarry MC, Hyman BT (2000) Apolipoprotein E facilitates neuritic and cerebrovascular plaque formation in an Alzheimer's disease model. Ann Neurol 47:739-747.

Horellou P, Lundberg C, Le Bourdelles B, Wictorin K, Brundin P, Kalen P, Bjorklund A, Mallet J (1991) Behavioural effects of genetically engineered cells releasing dopa and dopamine after intracerebral grafting in a rat model of Parkinson's disease. J Physiol (Paris) 85:158-170.

Hsiao K, Chapman P, Nilsen S, Eckman C, Harigaya Y, Younkin S, Yang F, Cole G (1996) Correlative memory deficits, Abeta elevation, and amyloid plaques in transgenic mice. Science 274:99-102.

Hsiao KK, Scott M, Foster D, Groth DF, DeArmond SJ, Prusiner SB (1990) Spontaneous neurodegeneration in transgenic mice with mutant prion protein. Science 250:1587-1590.

Hy LX, Keller DM (2000) Prevalence of AD among whites: a summary by levels of severity. Neurology 55:198-204.

Hyman BT, Van Hoesen GW, Kromer LJ, Damasio AR (1986) Perforant pathway changes and the memory impairment of Alzheimer's disease. Ann Neurol 20:472-481.

Irizarry MC, Soriano F, McNamara M, Page KJ, Schenk D, Games D, Hyman BT (1997) Abeta deposition is associated with neuropil changes, but not with overt neuronal loss in the human amyloid precursor protein V717F (PDAPP) transgenic mouse. J Neurosci 17:7053-7059.

Ishihara T, Zhang B, Higuchi M, Yoshiyama Y, Trojanowski JQ, Lee VM (2001) Age-dependent induction of congophilic neurofibrillary tau inclusions in tau transgenic mice. Am J Pathol 158:555-562.

Ishihara T, Hong M, Zhang B, Nakagawa Y, Lee MK, Trojanowski JQ, Lee VM (1999) Age-dependent emergence and progression of a tauopathy in transgenic mice overexpressing the shortest human tau isoform. Neuron 24:751-762.

Itagaki S, McGeer PL, Akiyama H, Zhu S, Selkoe D (1989) Relationship of microglia and astrocytes to amyloid deposits of Alzheimer disease. J Neuroimmunol 24:173-182.

Iwatsubo T, Odaka A, Suzuki N, Mizusawa H, Nukina N, Ihara Y (1994) Visualization of A beta 42(43) and A beta 40 in senile plaques with end-specific A beta monoclonals: evidence that an initially deposited species is A beta 42(43). Neuron 13:45-53.

Jaenicke R (1995) Folding and association versus misfolding and aggregation of proteins. Philos Trans R Soc Lond B Biol Sci 348:97-105.

Janus C, Pearson J, McLaurin J, Mathews PM, Jiang Y, Schmidt SD, Chishti MA, Horne P, Heslin D, French J, Mount HT, Nixon RA, Mercken M, Bergeron C, Fraser PE, St George-Hyslop P, Westaway D (2000) A beta peptide immunization reduces behavioural impairment and plaques in a model of Alzheimer's disease. Nature 408:979-982.

Jarrett JT, Lansbury PT, Jr. (1993) Seeding "one-dimensional crystallization" of amyloid: a pathogenic mechanism in Alzheimer's disease and scrapie? Cell 73:1055-1058.

Jarrett JT, Berger EP, Lansbury PT, Jr. (1993a) The carboxy terminus of the beta amyloid protein is critical for the seeding of amyloid formation: implications for the pathogenesis of Alzheimer's disease. Biochemistry 32:4693-4697.

Jarrett JT, Berger EP, Lansbury PT, Jr. (1993b) The C-terminus of the beta protein is critical in amyloidogenesis. Ann N Y Acad Sci 695:144-148.

Jin LW, Ninomiya H, Roch JM, Schubert D, Masliah E, Otero DA, Saitoh T (1994) Peptides containing the RERMS sequence of amyloid beta/A4 protein precursor bind cell surface and promote neurite extension. J Neurosci 14:5461-5470.

Joachim CL, Morris JH, Selkoe DJ (1989) Diffuse senile plaques occur commonly in the cerebellum in Alzheimer's disease. Am J Pathol 135:309-319.

Joachim CL, Duffy LK, Morris JH, Selkoe DJ (1988) Protein chemical and immunocytochemical studies of meningovascular beta-amyloid protein in Alzheimer's disease and normal aging. Brain Res 474:100-111.

Kang J, Lemaire HG, Unterbeck A, Salbaum JM, Masters CL, Grzeschik KH, Multhaup G, Beyreuther K, Muller-Hill B (1987) The precursor of Alzheimer's disease amyloid A4 protein resembles a cell-surface receptor. Nature 325:733-736.

Katzman R, Terry R, DeTeresa R, Brown T, Davies P, Fuld P, Renbing X, Peck A (1988) Clinical, pathological, and neurochemical changes in dementia: a subgroup with preserved mental status and numerous neocortical plaques. Ann Neurol 23:138-144.

Kauppinen RA, Williams SR, Busza AL, van Bruggen N (1993) Applications of magnetic resonance spectroscopy and diffusion-weighted imaging to the study of brain biochemistry and pathology. Trends Neurosci 16:88-95.

Kehoe PG, Russ C, McIlory S, Williams H, Holmans P, Holmes C, Liolitsa D, Vahidassr D, Powell J, McGleenon B, Liddell M, Plomin R, Dynan K, Williams N, Neal J, Cairns NJ, Wilcock G, Passmore P, Lovestone S, Williams J, Owen MJ (1999) Variation in DCP1, encoding ACE, is associated with susceptibility to Alzheimer disease. Nat Genet 21:71-72.

Kelly PH, Bondolfi L, Hunziker D, Schlecht HP, Carver K, Maguire E, Abramowski D, Wiederhold KH, Sturchler-Pierrat C, Jucker M, Bergmann R, Staufenbiel M, Sommer B (2003) Progressive age-related impairment of cognitive behavior in APP23 transgenic mice. Neurobiol Aging 24:365-378.

Kitaguchi N, Takahashi Y, Tokushima Y, Shiojiri S, Ito H (1988) Novel precursor of Alzheimer's disease amyloid protein shows protease inhibitory activity. Nature 331:530-532.

Klein WL, Krafft GA, Finch CE (2001) Targeting small Abeta oligomers: the solution to an Alzheimer's disease conundrum? Trends Neurosci 24:219-224.

Koike H, Tomioka S, Sorimachi H, Saido TC, Maruyama K, Okuyama A, Fujisawa-Sehara A, Ohno S, Suzuki K, Ishiura S (1999) Membrane-anchored metalloprotease MDC9 has an alpha-secretase activity responsible for processing the amyloid precursor protein. Biochem J 343 Pt 2:371-375.

Koutouzis TK, Emerich DF, Borlongan CV, Freeman TB, Cahill DW, Sanberg PR (1994) Cell transplantation for central nervous system disorders. Crit Rev Neurobiol 8:125-162.

Kuo YM, Emmerling MR, Vigo-Pelfrey C, Kasunic TC, Kirkpatrick JB, Murdoch GH, Ball MJ, Roher AE (1996) Water-soluble Abeta (N-40, N-42) oligomers in normal and Alzheimer disease brains. J Biol Chem 271:4077-4081.

Lambert JC, Goumidi L, Vrieze FW, Frigard B, Harris JM, Cummings A, Coates J, Pasquier F, Cottel D, Gaillac M, St Clair D, Mann DM, Hardy J, Lendon CL, Amouyel P, Chartier-Harlin MC (2000) The transcriptional factor LBP-1c/CP2/LSF gene on chromosome 12 is a genetic determinant of Alzheimer's disease. Hum Mol Genet 9:2275-2280.

Lambert MP, Barlow AK, Chromy BA, Edwards C, Freed R, Liosatos M, Morgan TE, Rozovsky I, Trommer B, Viola KL, Wals P, Zhang C, Finch CE, Krafft GA, Klein WL (1998) Diffusible, nonfibrillar ligands derived from Abeta1-42 are potent central nervous system neurotoxins. Proc Natl Acad Sci U S A 95:6448-6453.

Lammich S, Kojro E, Postina R, Gilbert S, Pfeiffer R, Jasionowski M, Haass C, Fahrenholz F (1999) Constitutive and regulated alpha-secretase cleavage of Alzheimer's amyloid precursor protein by a disintegrin metalloprotease. Proc Natl Acad Sci U S A 96:3922-3927.

Lautenschlager NT, Cupples LA, Rao VS, Auerbach SA, Becker R, Burke J, Chui H, Duara R, Foley EJ, Glatt SL, Green RC, Jones R, Karlinsky H, Kukull WA, Kurz A, Larson EB, Martelli K, Sadovnick AD, Volicer L, Waring SC, Growdon JH, Farrer LA (1996) Risk of dementia among relatives of Alzheimer's disease patients in the MIRAGE study: What is in store for the oldest old? Neurology 46:641-650.

Lee RK, Wurtman RJ, Cox AJ, Nitsch RM (1995) Amyloid precursor protein processing is stimulated by metabotropic glutamate receptors. Proc Natl Acad Sci U S A 92:8083-8087.

Lee SF, Shah S, Li H, Yu C, Han W, Yu G (2002) Mammalian APH-1 interacts with presenilin and nicastrin and is required for intramembrane proteolysis of amyloid-beta precursor protein and Notch. J Biol Chem 277:45013-45019.

Lehtovirta M, Soininen H, Helisalmi S, Mannermaa A, Helkala EL, Hartikainen P, Hanninen T, Ryynanen M, Riekkinen PJ (1996) Clinical and neuropsychological characteristics in familial and sporadic Alzheimer's disease: relation to apolipoprotein E polymorphism. Neurology 46:413-419.

Lendon CL, Ashall F, Goate AM (1997) Exploring the etiology of Alzheimer disease using molecular genetics. Jama 277:825-831.

Levine H, 3rd (1995) Soluble multimeric Alzheimer beta(1-40) pre-amyloid complexes in dilute solution. Neurobiol Aging 16:755-764.

Levy E, Carman MD, Fernandez-Madrid IJ, Power MD, Lieberburg I, van Duinen SG, Bots GT, Luyendijk W, Frangione B (1990) Mutation of the Alzheimer's disease amyloid gene in hereditary cerebral hemorrhage, Dutch type. Science 248:1124-1126.

Levy-Lahad E, Wijsman EM, Nemens E, Anderson L, Goddard KA, Weber JL, Bird TD, Schellenberg GD (1995a) A familial Alzheimer's disease locus on chromosome 1. Science 269:970-973.

Levy-Lahad E, Wasco W, Poorkaj P, Romano DM, Oshima J, Pettingell WH, Yu CE, Jondro PD, Schmidt SD, Wang K, et al. (1995b) Candidate gene for the chromosome 1 familial Alzheimer's disease locus. Science 269:973-977.

Lewis J, Dickson DW, Lin WL, Chisholm L, Corral A, Jones G, Yen SH, Sahara N, Skipper L, Yager D, Eckman C, Hardy J, Hutton M, McGowan E (2001) Enhanced neurofibrillary degeneration in transgenic mice expressing mutant tau and APP. Science 293:1487-1491.

Lewis J, McGowan E, Rockwood J, Melrose H, Nacharaju P, Van Slegtenhorst M, Gwinn-Hardy K, Paul Murphy M, Baker M, Yu X, Duff K, Hardy J, Corral A, Lin WL, Yen SH, Dickson DW, Davies P, Hutton M (2000) Neurofibrillary tangles, amyotrophy and progressive motor disturbance in mice expressing mutant (P301L) tau protein. Nat Genet 25:402-405.

Lindvall O, Widner H, Rehncrona S, Brundin P, Odin P, Gustavii B, Frackowiak R, Leenders KL, Sawle G, Rothwell JC, et al. (1992) Transplantation of fetal dopamine neurons in Parkinson's disease: one-year clinical and neurophysiological observations in two patients with putaminal implants. Ann Neurol 31:155-165.

Linn RT, Wolf PA, Bachman DL, Knoefel JE, Cobb JL, Belanger AJ, Kaplan EF, D'Agostino RB (1995) The 'preclinical phase' of probable Alzheimer's disease. A 13-year prospective study of the Framingham cohort. Arch Neurol 52:485-490.

Lippa CF, Saunders AM, Smith TW, Swearer JM, Drachman DA, Ghetti B, Nee L, Pulaski-Salo D, Dickson D, Robitaille Y, Bergeron C, Crain B, Benson MD, Farlow M, Hyman BT, George-Hyslop SP, Roses AD, Pollen DA (1996) Familial and sporadic Alzheimer's disease: neuropathology cannot exclude a final common pathway. Neurology 46:406-412.

Lopez-Perez E, Zhang Y, Frank SJ, Creemers J, Seidah N, Checler F (2001) Constitutive alpha-secretase cleavage of the beta-amyloid precursor protein in the furin-deficient LoVo cell line: involvement of the pro-hormone convertase 7 and the disintegrin metalloprotease ADAM10. J Neurochem 76:1532-1539.

Lorenzo A, Yankner BA (1994) Beta-amyloid neurotoxicity requires fibril formation and is inhibited by congo red. Proc Natl Acad Sci U S A 91:12243-12247.

Lovestone S, Reynolds CH (1997) The phosphorylation of tau: a critical stage in neurodevelopment and neurodegenerative processes. Neuroscience 78:309-324.

Luo Y, Bolon B, Kahn S, Bennett BD, Babu-Khan S, Denis P, Fan W, Kha H, Zhang J, Gong Y, Martin L, Louis JC, Yan Q, Richards WG, Citron M, Vassar R (2001) Mice deficient in BACE1, the Alzheimer's beta-secretase, have normal phenotype and abolished beta-amyloid generation. Nat Neurosci 4:231-232.

Mahley RW (1988) Apolipoprotein E: cholesterol transport protein with expanding role in cell biology. Science 240:622-630.

Mandybur TI (1975) The incidence of cerebral amyloid angiopathy in Alzheimer's disease. Neurology 25:120-126.

Mangiarini L, Sathasivam K, Seller M, Cozens B, Harper A, Hetherington C, Lawton M, Trottier Y, Lehrach H, Davies SW, Bates GP (1996) Exon 1 of the HD gene with an expanded CAG repeat is sufficient to cause a progressive neurological phenotype in transgenic mice. Cell 87:493-506.

Mann DM (1988) The pathological association between Down syndrome and Alzheimer disease. Mech Ageing Dev 43:99-136.

Mann DM, Yates PO, Marcyniuk B (1985) Some morphometric observations on the cerebral cortex and hippocampus in presenile Alzheimer's disease, senile dementia of Alzheimer type and Down's syndrome in middle age. J Neurol Sci 69:139-159.

Martin JB (1999) Molecular basis of the neurodegenerative disorders. N Engl J Med 340:1970-1980.

Masliah E, Rockenstein E, Veinbergs I, Mallory M, Hashimoto M, Takeda A, Sagara Y, Sisk A, Mucke L (2000) Dopaminergic loss and inclusion body formation in alpha-synuclein mice: implications for neurodegenerative disorders. Science 287:1265-1269.

Masters CL, Simms G, Weinman NA, Multhaup G, McDonald BL, Beyreuther K (1985) Amyloid plaque core protein in Alzheimer disease and Down syndrome. Proc Natl Acad Sci U S A 82:4245-4249.

Mattson MP, Cheng B, Culwell AR, Esch FS, Lieberburg I, Rydel RE (1993) Evidence for excitoprotective and intraneuronal calcium-regulating roles for secreted forms of the beta-amyloid precursor protein. Neuron 10:243-254.

Mayer E, Fawcett JW, Dunnett SB (1993) Basic fibroblast growth factor promotes the survival of embryonic ventral mesencephalic dopaminergic neurons--II. Effects on nigral transplants in vivo. Neuroscience 56:389-398.

McLaurin J, Yang D, Yip CM, Fraser PE (2000) Review: modulating factors in amyloid-beta fibril formation. J Struct Biol 130:259-270.

Miller DL, Papayannopoulos IA, Styles J, Bobin SA, Lin YY, Biemann K, Iqbal K (1993) Peptide compositions of the cerebrovascular and senile plaque core amyloid deposits of Alzheimer's disease. Arch Biochem Biophys 301:41-52.

Milward EA, Papadopoulos R, Fuller SJ, Moir RD, Small D, Beyreuther K, Masters CL (1992) The amyloid protein precursor of Alzheimer's disease is a mediator of the effects of nerve growth factor on neurite outgrowth. Neuron 9:129-137.

Minton AP (2000) Implications of macromolecular crowding for protein assembly. Curr Opin Struct Biol 10:34-39.

Morgan D, Diamond DM, Gottschall PE, Ugen KE, Dickey C, Hardy J, Duff K, Jantzen P, DiCarlo G, Wilcock D, Connor K, Hatcher J, Hope C, Gordon M, Arendash GW (2000) A beta peptide vaccination prevents memory loss in an animal model of Alzheimer's disease. Nature 408:982-985.

Mori S, Itoh R, Zhang J, Kaufmann WE, van Zijl PC, Solaiyappan M, Yarowsky P (2001) Diffusion tensor imaging of the developing mouse brain. Magn Reson Med 46:18-23.

Morris JC (1996) Classification of dementia and Alzheimer's disease. Acta Neurol Scand Suppl 165:41-50.

Motter R, Vigo-Pelfrey C, Kholodenko D, Barbour R, Johnson-Wood K, Galasko D, Chang L, Miller B, Clark C, Green R, et al. (1995) Reduction of beta-amyloid peptide42 in the cerebrospinal fluid of patients with Alzheimer's disease. Ann Neurol 38:643-648.

Mouton PR, Martin LJ, Calhoun ME, Dal Forno G, Price DL (1998) Cognitive decline strongly correlates with cortical atrophy in Alzheimer's dementia. Neurobiol Aging 19:371-377.

Mucke L, Masliah E, Johnson WB, Ruppe MD, Alford M, Rockenstein EM, Forss-Petter S, Pietropaolo M, Mallory M, Abraham CR (1994) Synaptotrophic effects of human amyloid beta protein precursors in the cortex of transgenic mice. Brain Res 666:151-167.

Mucke L, Masliah E, Yu GQ, Mallory M, Rockenstein EM, Tatsuno G, Hu K, Kholodenko D, Johnson-Wood K, McConlogue L (2000) High-level neuronal expression of abeta 1-42 in wild-type human amyloid protein precursor transgenic mice: synaptotoxicity without plaque formation. J Neurosci 20:4050-4058.

Mullan M, Crawford F, Axelman K, Houlden H, Lilius L, Winblad B, Lannfelt L (1992) A pathogenic mutation for probable Alzheimer's disease in the APP gene at the N-terminus of beta-amyloid. Nat Genet 1:345-347.

Nicoll JA, Wilkinson D, Holmes C, Steart P, Markham H, Weller RO (2003) Neuropathology of human Alzheimer disease after immunization with amyloid-beta peptide: a case report. Nat Med 9:448-452.

Nilsberth C, Westlind-Danielsson A, Eckman CB, Condron MM, Axelman K, Forsell C, Stenh C, Luthman J, Teplow DB, Younkin SG, Naslund J, Lannfelt L (2001) The 'Arctic' APP mutation (E693G) causes Alzheimer's disease by enhanced Abeta protofibril formation. Nat Neurosci 4:887-893.

Nishimoto I, Okamoto T, Matsuura Y, Takahashi S, Murayama Y, Ogata E (1993) Alzheimer amyloid protein precursor complexes with brain GTP-binding protein G(o). Nature 362:75-79.

Norris DG (2001) The effects of microscopic tissue parameters on the diffusion weighted magnetic resonance imaging experiment. NMR Biomed 14:77-93.

Oda T, Wals P, Osterburg HH, Johnson SA, Pasinetti GM, Morgan TE, Rozovsky I, Stine WB, Snyder SW, Holzman TF, et al. (1995) Clusterin (apoJ) alters the aggregation of amyloid beta-peptide (A beta 1-42) and forms slowly sedimenting A beta complexes that cause oxidative stress. Exp Neurol 136:22-31.

Oddo S, Caccamo A, Shepherd JD, Murphy MP, Golde TE, Kayed R, Metherate R, Mattson MP, Akbari Y, LaFerla FM (2003) Triple-transgenic model of Alzheimer's disease with plaques and tangles: intracellular Abeta and synaptic dysfunction. Neuron 39:409-421.

Ogomori K, Kitamoto T, Tateishi J, Sato Y, Suetsugu M, Abe M (1989) Beta-protein amyloid is widely distributed in the central nervous system of patients with Alzheimer's disease. Am J Pathol 134:243-251.

Okuizumi K, Onodera O, Namba Y, Ikeda K, Yamamoto T, Seki K, Ueki A, Nanko S, Tanaka H, Takahashi H, et al. (1995) Genetic association of the very low density lipoprotein (VLDL) receptor gene with sporadic Alzheimer's disease. Nat Genet 11:207-209.

Olaisen B, Teisberg P, Gedde-Dahl T, Jr. (1982) The locus for apolipoprotein E (apoE) is linked to the complement component C3 (C3) locus on chromosome 19 in man. Hum Genet 62:233-236.

Olichney JM, Hansen LA, Galasko D, Saitoh T, Hofstetter CR, Katzman R, Thal LJ (1996) The apolipoprotein E epsilon 4 allele is associated with increased neuritic plaques and cerebral amyloid angiopathy in Alzheimer's disease and Lewy body variant. Neurology 47:190-196.

Pepys MB, Herbert J, Hutchinson WL, Tennent GA, Lachmann HJ, Gallimore JR, Lovat LB, Bartfai T, Alanine A, Hertel C, Hoffmann T, Jakob-Roetne R, Norcross RD, Kemp JA, Yamamura K, Suzuki M, Taylor GW, Murray S, Thompson D, Purvis A, Kolstoe S, Wood SP, Hawkins PN (2002) Targeted pharmacological depletion of serum amyloid P component for treatment of human amyloidosis. Nature 417:254-259.

Permanne B, Adessi C, Saborio GP, Fraga S, Frossard MJ, Van Dorpe J, Dewachter I, Banks WA, Van Leuven F, Soto C (2002) Reduction of amyloid load and cerebral damage in a transgenic mouse model of Alzheimer's disease by treatment with a beta-sheet breaker peptide. Faseb J 16:860-862.

Petanceska SS, Nagy V, Frail D, Gandy S (2000) Ovariectomy and 17beta-estradiol modulate the levels of Alzheimer's amyloid beta peptides in brain. Neurology 54:2212-2217.

Petit A, Barelli H, Morain P, Checler F (2000) Novel proline endopeptidase inhibitors do not modify Abeta40/42 formation and degradation by human cells expressing wild-type and swedish mutated beta-amyloid precursor protein. Br J Pharmacol 130:1613-1617.

Pfeffer RI, Afifi AA, Chance JM (1987) Prevalence of Alzheimer's disease in a retirement community. Am J Epidemiol 125:420-436.

Phinney AL, Calhoun ME, Wolfer DP, Lipp HP, Zheng H, Jucker M (1999) No hippocampal neuron or synaptic bouton loss in learning-impaired aged beta-amyloid precursor protein-null mice. Neuroscience 90:1207-1216.

Polymeropoulos MH, Lavedan C, Leroy E, Ide SE, Dehejia A, Dutra A, Pike B, Root H, Rubenstein J, Boyer R, Stenroos ES, Chandrasekharappa S, Athanassiadou A, Papapetropoulos T, Johnson WG, Lazzarini AM, Duvoisin RC, Di Iorio G, Golbe LI, Nussbaum RL (1997) Mutation in the alpha-synuclein gene identified in families with Parkinson's disease. Science 276:2045-2047.

Ponte P, Gonzalez-DeWhitt P, Schilling J, Miller J, Hsu D, Greenberg B, Davis K, Wallace W, Lieberburg I, Fuller F (1988) A new A4 amyloid mRNA contains a domain homologous to serine proteinase inhibitors. Nature 331:525-527.

Price BH, Gurvit H, Weintraub S, Geula C, Leimkuhler E, Mesulam M (1993) Neuropsychological patterns and language deficits in 20 consecutive cases of autopsy-confirmed Alzheimer's disease. Arch Neurol 50:931-937.

Price DL, Sisodia SS (1998) Mutant genes in familial Alzheimer's disease and transgenic models. Annu Rev Neurosci 21:479-505.

Probst A, Gotz J, Wiederhold KH, Tolnay M, Mistl C, Jaton AL, Hong M, Ishihara T, Lee VM, Trojanowski JQ, Jakes R, Crowther RA, Spillantini MG, Burki K, Goedert M (2000) Axonopathy and amyotrophy in mice transgenic for human four-repeat tau protein. Acta Neuropathol (Berl) 99:469-481.

Prusiner SB (1998) Prions. Proc Natl Acad Sci U S A 95:13363-13383.

Puglielli L, Konopka G, Pack-Chung E, Ingano LA, Berezovska O, Hyman BT, Chang TY, Tanzi RE, Kovacs DM (2001) Acyl-coenzyme A: cholesterol acyltransferase modulates the generation of the amyloid beta-peptide. Nat Cell Biol 3:905-912.

Rasool CG, Svendsen CN, Selkoe DJ (1986) Neurofibrillary degeneration of cholinergic and noncholinergic neurons of the basal forebrain in Alzheimer's disease. Ann Neurol 20:482-488.

Refolo LM, Malester B, LaFrancois J, Bryant-Thomas T, Wang R, Tint GS, Sambamurti K, Duff K, Pappolla MA (2000) Hypercholesterolemia accelerates the Alzheimer's amyloid pathology in a transgenic mouse model. Neurobiol Dis 7:321-331.

Roberds SL, Anderson J, Basi G, Bienkowski MJ, Branstetter DG, Chen KS, Freedman SB, Frigon NL, Games D, Hu K, Johnson-Wood K, Kappenman KE, Kawabe TT, Kola I, Kuehn R, Lee M, Liu W, Motter R, Nichols NF, Power M, Robertson DW, Schenk D, Schoor M, Shopp GM, Shuck ME, Sinha S, Svensson KA, Tatsuno G, Tintrup H, Wijsman J, Wright S, McConlogue L (2001) BACE knockout mice are healthy despite lacking the primary beta-secretase activity in brain: implications for Alzheimer's disease therapeutics. Hum Mol Genet 10:1317-1324.

Rogaev EI, Sherrington R, Rogaeva EA, Levesque G, Ikeda M, Liang Y, Chi H, Lin C, Holman K, Tsuda T, et al. (1995) Familial Alzheimer's disease in kindreds with missense mutations in a gene on chromosome 1 related to the Alzheimer's disease type 3 gene. Nature 376:775-778.

Roher AE, Lowenson JD, Clarke S, Wolkow C, Wang R, Cotter RJ, Reardon IM, Zurcher-Neely HA, Heinrikson RL, Ball MJ, et al. (1993) Structural alterations in the peptide backbone of beta-amyloid core protein may account for its deposition and stability in Alzheimer's disease. J Biol Chem 268:3072-3083.

Rosen DR, Siddique T, Patterson D, Figlewicz DA, Sapp P, Hentati A, Donaldson D, Goto J, O'Regan JP, Deng HX, et al. (1993) Mutations in Cu/Zn superoxide dismutase gene are associated with familial amyotrophic lateral sclerosis. Nature 362:59-62.

Saitoh T, Sundsmo M, Roch JM, Kimura N, Cole G, Schubert D, Oltersdorf T, Schenk DB (1989) Secreted form of amyloid beta protein precursor is involved in the growth regulation of fibroblasts. Cell 58:615-622.

Sastre M, Steiner H, Fuchs K, Capell A, Multhaup G, Condron MM, Teplow DB, Haass C (2001) Presenilin-dependent gamma-secretase processing of beta-amyloid precursor protein at a site corresponding to the S3 cleavage of Notch. EMBO Rep 2:835-841.

Scarpini E, Scheltens P, Feldman H (2003) Treatment of Alzheimer's disease: current status and new perspectives. Lancet Neurol 2:539-547.

Schellenberg GD (1995) Progress in Alzheimer's disease genetics. Curr Opin Neurol 8:262-267.

Schenk D, Barbour R, Dunn W, Gordon G, Grajeda H, Guido T, Hu K, Huang J, Johnson-Wood K, Khan K, Kholodenko D, Lee M, Liao Z, Lieberburg I, Motter R, Mutter L, Soriano F, Shopp G, Vasquez N, Vandevert C, Walker S, Wogulis M, Yednock T, Games D, Seubert P (1999) Immunization with amyloid-beta attenuates Alzheimer-disease-like pathology in the PDAPP mouse. Nature 400:173-177.

Seabrook GR, Smith DW, Bowery BJ, Easter A, Reynolds T, Fitzjohn SM, Morton RA, Zheng H, Dawson GR, Sirinathsinghji DJ, Davies CH, Collingridge GL, Hill RG (1999) Mechanisms contributing to the deficits in hippocampal synaptic plasticity in mice lacking amyloid precursor protein. Neuropharmacology 38:349-359.

Seiffert D, Bradley JD, Rominger CM, Rominger DH, Yang F, Meredith JE, Jr., Wang Q, Roach AH, Thompson LA, Spitz SM, Higaki JN, Prakash SR, Combs AP, Copeland RA, Arneric SP, Hartig PR, Robertson DW, Cordell B, Stern AM, Olson RE, Zaczek R (2000) Presenilin-1 and -2 are molecular targets for gamma-secretase inhibitors. J Biol Chem 275:34086-34091.

Selkoe DJ (1994) Amyloid beta-protein precursor: new clues to the genesis of Alzheimer's disease. Curr Opin Neurobiol 4:708-716.

Selkoe DJ (1999) Translating cell biology into therapeutic advances in Alzheimer's disease. Nature 399:A23-31.

Selkoe DJ (2001) Alzheimer's disease results from the cerebral accumulation and cytotoxicity of amyloid beta-protein. J Alzheimers Dis 3:75-80.

Seubert P, Oltersdorf T, Lee MG, Barbour R, Blomquist C, Davis DL, Bryant K, Fritz LC, Galasko D, Thal LJ, et al. (1993) Secretion of beta-amyloid precursor protein cleaved at the amino terminus of the beta-amyloid peptide. Nature 361:260-263.

Shastry BS, Giblin FJ (1999) Genes and susceptible loci of Alzheimer's disease. Brain Res Bull 48:121-127.

Shearman MS, Beher D, Clarke EE, Lewis HD, Harrison T, Hunt P, Nadin A, Smith AL, Stevenson G, Castro JL (2000) L-685,458, an aspartyl protease transition state mimic, is a potent inhibitor of amyloid beta-protein precursor gamma-secretase activity. Biochemistry 39:8698-8704.

Shen J, Bronson RT, Chen DF, Xia W, Selkoe DJ, Tonegawa S (1997) Skeletal and CNS defects in Presenilin-1-deficient mice. Cell 89:629-639.

Sherrington R, Rogaev EI, Liang Y, Rogaeva EA, Levesque G, Ikeda M, Chi H, Lin C, Li G, Holman K, et al. (1995) Cloning of a gene bearing missense mutations in early-onset familial Alzheimer's disease. Nature 375:754-760.

Simons M, Keller P, De Strooper B, Beyreuther K, Dotti CG, Simons K (1998) Cholesterol depletion inhibits the generation of beta-amyloid in hippocampal neurons. Proc Natl Acad Sci U S A 95:6460-6464.

Sinha S, Anderson JP, Barbour R, Basi GS, Caccavello R, Davis D, Doan M, Dovey HF, Frigon N, Hong J, Jacobson-Croak K, Jewett N, Keim P, Knops J, Lieberburg I, Power M, Tan H, Tatsuno G, Tung J, Schenk D, Seubert P, Suomensaari SM, Wang S, Walker D, John V, et al. (1999) Purification and cloning of amyloid precursor protein beta-secretase from human brain. Nature 402:537-540.

Sisodia SS (1992) Beta-amyloid precursor protein cleavage by a membrane-bound protease. Proc Natl Acad Sci U S A 89:6075-6079.

Small SA, Stern Y, Tang M, Mayeux R (1999) Selective decline in memory function among healthy elderly. Neurology 52:1392-1396.

Soto C (1999) Plaque busters: strategies to inhibit amyloid formation in Alzheimer's disease. Mol Med Today 5:343-350.

Soto C (2001) Protein misfolding and disease; protein refolding and therapy. FEBS Lett 498:204-207.

Soto C (2003) Unfolding the role of protein misfolding in neurodegenerative diseases. Nat Rev Neurosci 4:49-60.

Sparks DL, Scheff SW, Hunsaker JC, 3rd, Liu H, Landers T, Gross DR (1994) Induction of Alzheimer-like beta-amyloid immunoreactivity in the brains of rabbits with dietary cholesterol. Exp Neurol 126:88-94.

Spillantini MG, Schmidt ML, Lee VM, Trojanowski JQ, Jakes R, Goedert M (1997) Alpha-synuclein in Lewy bodies. Nature 388:839-840.

Spittaels K, Van den Haute C, Van Dorpe J, Bruynseels K, Vandezande K, Laenen I, Geerts H, Mercken M, Sciot R, Van Lommel A, Loos R, Van Leuven F (1999) Prominent axonopathy in the brain and spinal cord of transgenic mice overexpressing four-repeat human tau protein. Am J Pathol 155:2153-2165.

St George-Hyslop PH, Tanzi RE, Polinsky RJ, Haines JL, Nee L, Watkins PC, Myers RH, Feldman RG, Pollen D, Drachman D, et al. (1987) The genetic defect causing familial Alzheimer's disease maps on chromosome 21. Science 235:885-890.

Stalder M, Phinney A, Probst A, Sommer B, Staufenbiel M, Jucker M (1999) Association of microglia with amyloid plaques in brains of APP23 transgenic mice. Am J Pathol 154:1673-1684.

Steiner H, Haass C (2000) Intramembrane proteolysis by presenilins. Nat Rev Mol Cell Biol 1:217-224.

Steiner H, Winkler E, Edbauer D, Prokop S, Basset G, Yamasaki A, Kostka M, Haass C (2002) PEN-2 is an integral component of the gamma-secretase complex required for coordinated expression of presenilin and nicastrin. J Biol Chem 277:39062-39065.

Strittmatter WJ, Saunders AM, Schmechel D, Pericak-Vance M, Enghild J, Salvesen GS, Roses AD (1993a) Apolipoprotein E: high-avidity binding to beta-amyloid and increased frequency of type 4 allele in late-onset familial Alzheimer disease. Proc Natl Acad Sci U S A 90:1977-1981.

Strittmatter WJ, Weisgraber KH, Huang DY, Dong LM, Salvesen GS, Pericak-Vance M, Schmechel D, Saunders AM, Goldgaber D, Roses AD (1993b) Binding of human apolipoprotein E to synthetic amyloid beta peptide: isoform-specific effects and implications for late-onset Alzheimer disease. Proc Natl Acad Sci U S A 90:8098-8102.

Sturchler-Pierrat C, Abramowski D, Duke M, Wiederhold KH, Mistl C, Rothacher S, Ledermann B, Burki K, Frey P, Paganetti PA, Waridel C, Calhoun ME, Jucker M, Probst A, Staufenbiel M, Sommer B (1997) Two amyloid precursor protein transgenic mouse models with Alzheimer disease-like pathology. Proc Natl Acad Sci U S A 94:13287-13292.

Suzuki N, Cheung TT, Cai XD, Odaka A, Otvos L, Jr., Eckman C, Golde TE, Younkin SG (1994) An increased percentage of long amyloid beta protein secreted by familial amyloid beta protein precursor (beta APP717) mutants. Science 264:1336-1340.

Tagliavini F, Giaccone G, Frangione B, Bugiani O (1988) Preamyloid deposits in the cerebral cortex of patients with Alzheimer's disease and nondemented individuals. Neurosci Lett 93:191-196.

Tanzi RE, Bertram L (2001) New frontiers in Alzheimer's disease genetics. Neuron 32:181-184.

Tanzi RE, McClatchey AI, Lamperti ED, Villa-Komaroff L, Gusella JF, Neve RL (1988) Protease inhibitor domain encoded by an amyloid protein precursor mRNA associated with Alzheimer's disease. Nature 331:528-530.

Tennent GA, Lovat LB, Pepys MB (1995) Serum amyloid P component prevents proteolysis of the amyloid fibrils of Alzheimer disease and systemic amyloidosis. Proc Natl Acad Sci U S A 92:4299-4303.

Teplow DB (1998) Structural and kinetic features of amyloid beta-protein fibrillogenesis. Amyloid 5:121-142.

Terry RD, Masliah E, Salmon DP, Butters N, DeTeresa R, Hill R, Hansen LA, Katzman R (1991) Physical basis of cognitive alterations in Alzheimer's disease: synapse loss is the major correlate of cognitive impairment. Ann Neurol 30:572-580.

Tomiyama T, Shoji A, Kataoka K, Suwa Y, Asano S, Kaneko H, Endo N (1996) Inhibition of amyloid beta protein aggregation and neurotoxicity by rifampicin. Its possible function as a hydroxyl radical scavenger. J Biol Chem 271:6839-6844.

Van Broeckhoven C, Backhovens H, Cruts M, De Winter G, Bruyland M, Cras P, Martin JJ (1992) Mapping of a gene predisposing to early-onset Alzheimer's disease to chromosome 14q24.3. Nat Genet 2:335-339.

van den Berg B, Ellis RJ, Dobson CM (1999) Effects of macromolecular crowding on protein folding and aggregation. Embo J 18:6927-6933.

van Dorsten FA, Olah L, Schwindt W, Grune M, Uhlenkuken U, Pillekamp F, Hossmann KA, Hoehn M (2002) Dynamic changes of ADC, perfusion, and NMR relaxation parameters in transient focal ischemia of rat brain. Magn Reson Med 47:97-104.

Vancea JE, Campenotb RB, Vancec DE (2000) The synthesis and transport of lipids for axonal growth and nerve regeneration. Biochim Biophys Acta 1486:84-96.

Vassar R, Bennett BD, Babu-Khan S, Kahn S, Mendiaz EA, Denis P, Teplow DB, Ross S, Amarante P, Loeloff R, Luo Y, Fisher S, Fuller J, Edenson S, Lile J, Jarosinski MA, Biere AL, Curran E, Burgess T, Louis JC, Collins F, Treanor J, Rogers G, Citron M (1999) Beta-secretase cleavage of Alzheimer's amyloid precursor protein by the transmembrane aspartic protease BACE. Science 286:735-741.

Vekrellis K, Ye Z, Qiu WQ, Walsh D, Hartley D, Chesneau V, Rosner MR, Selkoe DJ (2000) Neurons regulate extracellular levels of amyloid beta-protein via proteolysis by insulin-degrading enzyme. J Neurosci 20:1657-1665.

Villringer A, Rosen BR, Belliveau JW, Ackerman JL, Lauffer RB, Buxton RB, Chao YS, Wedeen VJ, Brady TJ (1988) Dynamic imaging with lanthanide chelates in normal brain: contrast due to magnetic susceptibility effects. Magn Reson Med 6:164-174.

Vinters HV, Pardridge WM, Yang J (1988) Immunohistochemical study of cerebral amyloid angiopathy: use of an antiserum to a synthetic 28-amino-acid peptide fragment of the Alzheimer's disease amyloid precursor. Hum Pathol 19:214-222.

Walsh DM, Lomakin A, Benedek GB, Condron MM, Teplow DB (1997) Amyloid beta-protein fibrillogenesis. Detection of a protofibrillar intermediate. J Biol Chem 272:22364-22372.

Walsh DM, Klyubin I, Fadeeva JV, Cullen WK, Anwyl R, Wolfe MS, Rowan MJ, Selkoe DJ (2002) Naturally secreted oligomers of amyloid beta protein potently inhibit hippocampal long-term potentiation in vivo. Nature 416:535-539.

Walsh DM, Hartley DM, Kusumoto Y, Fezoui Y, Condron MM, Lomakin A, Benedek GB, Selkoe DJ, Teplow DB (1999) Amyloid beta-protein fibrillogenesis. Structure and biological activity of protofibrillar intermediates. J Biol Chem 274:25945-25952.

Weggen S, Eriksen JL, Das P, Sagi SA, Wang R, Pietrzik CU, Findlay KA, Smith TE, Murphy MP, Bulter T, Kang DE, Marquez-Sterling N, Golde TE, Koo EH (2001) A subset of NSAIDs lower amyloidogenic Abeta42 independently of cyclooxygenase activity. Nature 414:212-216.

Weidemann A, Eggert S, Reinhard FB, Vogel M, Paliga K, Baier G, Masters CL, Beyreuther K, Evin G (2002) A novel epsilon-cleavage within the transmembrane domain of the Alzheimer amyloid precursor protein demonstrates homology with Notch processing. Biochemistry 41:2825-2835.

West MJ, Coleman PD, Flood DG, Troncoso JC (1994) Differences in the pattern of hippocampal neuronal loss in normal ageing and Alzheimer's disease. Lancet 344:769-772.

Wisniewski HM, Wegiel J, Wang KC, Kujawa M, Lach B (1989) Ultrastructural studies of the cells forming amyloid fibers in classical plaques. Can J Neurol Sci 16:535-542.

Wolfe MS, De Los Angeles J, Miller DD, Xia W, Selkoe DJ (1999) Are presenilins intramembrane-cleaving proteases? Implications for the molecular mechanism of Alzheimer's disease. Biochemistry 38:11223-11230.

Wong PC, Cai H, Borchelt DR, Price DL (2002) Genetically engineered mouse models of neurodegenerative diseases. Nat Neurosci 5:633-639.

Yamaguchi H, Hirai S, Morimatsu M, Shoji M, Ihara Y (1988) A variety of cerebral amyloid deposits in the brains of the Alzheimer-type dementia demonstrated by beta protein immunostaining. Acta Neuropathol (Berl) 76:541-549.

Yamaguchi H, Nakazato Y, Hirai S, Shoji M, Harigaya Y (1989) Electron micrograph of diffuse plaques. Initial stage of senile plaque formation in the Alzheimer brain. Am J Pathol 135:593-597.

Younkin SG (1995) Evidence that A beta 42 is the real culprit in Alzheimer's disease. Ann Neurol 37:287-288.

Younkin SG (2001) Amyloid beta vaccination: reduced plaques and improved cognition. Nat Med 7:18-19.

Yu C, Kim SH, Ikeuchi T, Xu H, Gasparini L, Wang R, Sisodia SS (2001a) Characterization of a presenilin-mediated amyloid precursor protein carboxyl-terminal fragment gamma. Evidence for distinct mechanisms involved in gamma -secretase processing of the APP and Notch1 transmembrane domains. J Biol Chem 276:43756-43760.

Yu G, Nishimura M, Arawaka S, Levitan D, Zhang L, Tandon A, Song YQ, Rogaeva E, Chen F, Kawarai T, Supala A, Levesque L, Yu H, Yang DS, Holmes E, Milman P, Liang Y, Zhang DM, Xu DH, Sato C, Rogaev E, Smith M, Janus C, Zhang Y, Aebersold R, Farrer LS, Sorbi S, Bruni A, Fraser P, St George-Hyslop P (2000) Nicastrin modulates presenilin-mediated notch/glp-1 signal transduction and betaAPP processing. Nature 407:48-54.

Yu H, Saura CA, Choi SY, Sun LD, Yang X, Handler M, Kawarabayashi T, Younkin L, Fedeles B, Wilson MA, Younkin S, Kandel ER, Kirkwood A, Shen J (2001b) APP processing and synaptic plasticity in presenilin-1 conditional knockout mice. Neuron 31:713-726.

Zheng H, Jiang M, Trumbauer ME, Sirinathsinghji DJ, Hopkins R, Smith DW, Heavens RP, Dawson GR, Boyce S, Conner MW, et al. (1995) beta-Amyloid precursor protein-deficient mice show reactive gliosis and decreased locomotor activity. Cell 81:525-531.

Zhong Z, Higaki J, Murakami K, Wang Y, Catalano R, Quon D, Cordell B (1994) Secretion of beta-amyloid precursor protein involves multiple cleavage sites. J Biol Chem 269:627-632.

Zimmer J, Laurberg S, Sunde N (1986) Non-cholinergic afferents determine the distribution of the cholinergic septohippocampal projection: a study of the AChE staining pattern in the rat fascia dentata and hippocampus after lesions, X-irradiation, and intracerebral grafting. Exp Brain Res 64:158-168.

Zimmer J, Finsen B, Sorensen T, Poulsen PH (1988) Xenografts of mouse hippocampal tissue. Exchange of laminar and neuropeptide specific nerve connections with the host rat brain. Brain Res Bull 20:369-379.

Published in:
Nature Neuroscience (2003): 6, 370-377.

2. Extracellular amyloid formation and associated pathology in neural grafts

Melanie Meyer-Luehmann[1*], Martina Stalder[1*], Martin C. Herzig[1], Stephan A. Kaeser[1], Esther Kohler[1], Michelle Pfeifer[1], Sonia Boncristiano[1], Paul M. Mathews[2], Marc Mercken[3], Dorothee Abramowski[4], Matthias Staufenbiel[4], Mathias Jucker[1]

[1]Department of Neuropathology, Institute of Pathology, University of Basel, CH-4003 Basel, Switzerland; [2]Nathan Kline Institute for Psychiatric Research, New York University School of Medicine, Orangeburg, NY 10962, USA; [3]Johnson and Johnson Pharmaceutical Research and Development, B-2340 Beerse, Belgium; [4]Novartis Pharma AG, Nervous System Research, CH-4002 Basel, Switzerland.

[*] contributed equally to this work

2.1 Abstract

Amyloid precursor protein (APP) processing and the generation of β-amyloid peptide (Aβ) are important in the pathogenesis of Alzheimer's disease. While this has been studied extensively at the molecular and cellular levels, much less is known about the mechanisms of amyloid accumulation *in vivo*. We transplanted transgenic APP23 and wildtype B6 embryonic neural cells into the neocortex and hippocampus of both B6 and APP23 mice. APP23 transgenic grafts into wildtype hosts do not develop amyloid deposits up to twenty months post-grafting. In contrast, both transgenic and, surprisingly, wildtype grafts into young transgenic hosts develop amyloid plaques as early as three months post-grafting. Although largely diffuse in nature, some of the amyloid deposits in wildtype grafts were congophilic and were surrounded by neuritic changes and gliosis similar to the amyloid-associated pathology previously described in APP23 transgenic mice. Our results suggest that diffusion of soluble Aβ in the extracellular space plays an important role in the spread of Aβ pathology and that extracellular amyloid formation can lead to neurodegeneration.

2.2 Introduction

Alzheimer's disease (AD) is a late onset progressive neurodegenerative disorder that is characterized by aggregation of A into senile plaques and cerebrovascular amyloid. Genetic studies and transgenic (tg) mice support the view that the production and/or deposition of A is an early and critical process in AD pathogenesis that triggers a cascade of pathological events leading to neurofibrillary tangle formation, neuroinflammation, synapse and neuron loss, and dementia (Selkoe, 2001; Hardy and Selkoe, 2002). A matter of controversy, however, is whether this cascade is initiated by the accumulation of A in the extracellular space or by intraneuronal A generation (Martin et al., 1994; Hartmann et al., 1997; Geula et al., 1998; Gouras et al., 2000; Kane et al., 2000; Walsh et al., 2000; Bayer et al., 2001; D'Andrea et al., 2001; Selkoe, 2001; Wirths et al., 2001).

In an attempt to study mechanisms and early stages of abnormal protein deposition and related neurotoxicity, neural transplantation techniques have previously been applied to study prion encephalopathy (Brandner et al., 1996b). In AD research, similar approaches have been pursued, but no convincing amyloid pathology has been associated with the grafts (Richards et al., 1991; Holtzman et al., 1992; Mantione et al., 1995; Bayer et al., 1996). However, the recent generation of APP tg mice that develop robust cerebral amyloid with aging (Games et al., 1995; Hsiao et al., 1996; Sturchler-Pierrat et al., 1997) has opened new opportunities to study the mechanism of cerebral amyloidosis by using neurografting techniques. To test the hypothesis that transplanted neural tissue would retain its native properties regardless of host genotype, we transplanted wildtype (wt) and APP tg embryonic cortical and hippocampal brain tissue into the brains of both APP tg and wt mice. Our results suggest that the phenotype of the transplanted tissue is strongly influenced by the properties of the host, and suggest a key role of extracellular diffusion of A in cerebral amyloidogenesis. Moreover, we conclude that extracellular amyloid formation is closely associated with neurodegeneration.

2.3 Results

APP23 grafts in B6 hosts do not develop amyloid up to 20 months post-grafting

Cell suspensions of APP23 tg embryonic cortical and hippocampal tissue were injected into the neocortex and hippocampus, respectively, of young, 3 month-old B6 wt mice. Grafts were analyzed 3-24 months later. All grafts integrated well into the surrounding host tissue and appeared healthy and viable by morphological analysis. Only minimal gliosis was found at the graft-host interface. Nevertheless, grafts could easily be identified in cresyl-violet stained sections due to differences in cytoarchitecture between graft and host tissue (Fig. 1A,D,G). Immunohistochemistry revealed robust and exclusive expression of human APP (hAPP) in the graft, where hAPP was restricted to neurons and their processes, consistent with the neuron-specificity of the Thy-1 promoter used to generate APP23 tg mice (Fig. 1B,E,H). Cellular hAPP expression within the grafts looked very similar to that in adult APP23 mice.

Surprisingly, in light of the observation that amyloid deposition in neocortex and hippocampus of APP23 mice starts at the age of 6 months (Sturchler-Pierrat et al., 1997), no amyloid formation was observed in hippocampal or cortical grafts up to 20 months post-grafting (Fig. 1C,F and Table 1). Beyond 20 months, i.e. when hosts were more than 23 months of age, we identified two out of nine mice that developed massive amyloid in the parenchyma and vasculature within at least one graft (Fig. 1I). Interestingly, some amyloid was found outside the grafts, in particular in vessels. To study whether it is the age of the host or the post-grafting time that determines amyloid deposition in the grafts, we also placed tg grafts in adult and aged wt hosts (Table 1). Again, grafts appeared healthy, viable and, well integrated into the host. However, no amyloid formation was observed in such grafts, suggesting that it is not the age of the host but the time of the grafts residing in the host that determines amyloid formation.

APP expression and A□ levels in APP23 grafts

We have tested several hypotheses to explain the delay/lack of amyloid formation in neocortical and hippocampal APP23 tg grafts in wt hosts compared to the cerebral amyloidosis observed in APP23 mice at the age of 6 months. First, we have tested the hypothesis that hAPP expression may be downregulated in grafted neurons compared to

the expression in normal adult APP23 mice (Fig. 2A,B). However, no obvious differences in hAPP expression were found in micropunches taken from neocortical and hippocampal tg grafts compared to micropunches taken from neocortex and hippocampus of normal adult APP23 mice.

Second, we tested the hypothesis that alterations in the processing of hAPP in grafted neurons may lead to a reduction in Aβ production and/or a shift in the ratio of Aβ1-40 to Aβ1-42. To this end, Aβ was immunoprecipitated from micropunches taken from tg grafts and compared to micropunches taken from 4-6 month-old APP23 mice, at an age before these mice develop amyloid deposits. Results revealed similar Aβ levels in the grafts compared to young APP23 mice with a predominance of Aβ1-40 over Aβ1-42 (Fig. 2B). Differences of Aβ1-40 levels in the grafts compared to young APP23 mice ranged from –31% to +28% (mean –2% ± 17%; $P > 0.05$). Interestingly, in the oldest transplant analyzed (20 months post-grafting), there was a relative increase of Aβ1-42 compared to Aβ1-40 (Fig. 2C). This observation is consistent with the result obtained in aged amyloid-depositing APP23 mice. Overall, these results do not support the hypothesis that reduced Aβ production by the grafted tissue is responsible for the slow onset of amyloid deposition in APP23 grafts in wt mice vs. normal APP23 mice.

Third, we have followed the hypothesis that intracerebral grafting of tg hAPP/Aβ producing tissue might evoke a humoral immune response in a wt host that has never seen hAPP/Aβ before (Brandner et al., 1996a), which might prevent amyloid deposition in the transplant (Schenk et al., 1999). However, serum titers of anti-Aβ antibodies in engrafted mice were not different from serum titers of normal B6 mice (both < 1:100). As a positive control we have used sera of APP23 mice passively immunized with Aβ antibodies with titers of 1:4,000 to 1:20,000 (Pfeifer et al., 2002).

APP23 and B6 grafts in APP23 hosts develop amyloid 3 months post-grafting

To study the influence of the host upon amyloid deposition in the graft, we then intracerebrally grafted cortical and hippocampal APP23 tg and B6 wt control tissue into the cortex and hippocampus, respectively, of 6 month-old APP23 mice (Fig. 3 and Table 2). Again, grafts integrated well into the surrounding host tissue and appeared

healthy and viable by morphological analysis with no evidence of neurodegeneration. Strikingly, after only 3 months post-grafting, tg grafts developed significant amounts of amyloid (Fig. 3A-C). Even more surprising, amyloid deposition was also found in wt cortical and hippocampal grafts 3 months post-grafting (Fig. 3D-I and Table 2). Amyloid deposition occurred throughout the grafts, with the greatest accumulation along the border of the graft. At this age the host tissue has also developed amyloid plaques, but in most animals they were significantly fewer than in the grafts. Semiquantitative analysis of double-stained sections for Aβ and Congo red indicated that 50% and 12% of the amyloid was compact in tg and wt grafts, respectively (Fig. 3I insert). Immunohistochemical analysis with antibodies specifically to Aβx-40 and Aβx-42 revealed the presence of both Aβ species in the graft with a predominance of Aβx-40 over Aβx-42 (Fig. 4A,B). Immunolabeling with an antibody that recognizes mouse but not human Aβ showed very faint labeling of the amyloid deposits suggesting that minimal amounts of mouse Aβ may contribute to the amyloid in wt grafts (Fig. 4C), as was also seen for the host tissue amyloid. Aβ was also immunoprecipitated from micropunches taken from amyloid-bearing wt grafts. Results revealed again both human Aβ1-40 and Aβ1-42 in the graft similar to that seen in aged amyloid-depositing APP23 mice (Fig. 4D,E).

Mechanism of amyloid formation in B6 grafts

To study whether the site of the graft placement may influence Aβ formation in the grafts, we injected hippocampal and cortical wt tissue into either the thalamus or striatum of 6 month-old APP23 mice (n =4 for thalamus and n =8 for striatum). Grafts were analyzed 3 months later. Thalamus and striatum were selected because they markedly differ in amyloid deposition in APP23 mice. The thalamus develops significant amyloid deposition in aged APP23 mice, while amyloid deposition in the striatum is low even in aged APP23 mice (Sturchler-Pierrat et al., 1997; Calhoun et al., 1998). Consistent with the idea that the properties of the surrounding host tissue influence amyloid deposition in the graft, our results revealed that 2 out of 4 mice (50%) developed amyloid in the "thalamic" grafts, while 0 out of 8 mice (0%) developed amyloid in the "striatal" grafts (Fig. 5). To examine whether the influence of the surrounding host tissue may be explained by differences in soluble extracellular Aβ

levels among the engrafted brain regions, extracellular soluble A□x-40 and A□x-42 was measured by ELISA in thalamus, striatum, neocortex, and hippocampus of 6 month-old pre-depositing APP23 mice. Results revealed the highest A□ levels in hippocampus (A□x-40: 2.61 ng/g wet weight; A□x-42: 0.41 ng/g) and neocortex (A□x-40: 2.42 ng/g; A□x-42: 0.42 ng/g), followed by the thalamus (A□x-40: 1.94 ng/g; A□x-42: 0.32 ng/g). The levels in the striatum (A□x-40: 0.71 ng/g; A□x-42: 0.15 ng/g) were more than 50% lower compared to these other regions. Consistently, recent microdialysis analysis of A□ also revealed significant lower extracellular soluble A□ concentrations in striatum compared to hippocampus (Cirrito et al., 2002). Thus, soluble extracellular A□ levels in the host may determine amyloid deposition in the graft suggesting that A□ is transported extracellularly from the host into the graft. We also tested the hypothesis that the grafts in thalamus and striatum differ in levels of A□-degrading enzymes. However, no evidence of a difference was found using immunohistochemical analysis of insulin-degrading enzyme (Qiu et al., 1998) and neprilysin (Iwata et al., 2000) expression in graft vs. host (results not shown), although expression levels do not necessarily reflect enzyme activity.

We have also addressed several other hypothetical mechanisms to how A□ may be transported from the host into the graft. We considered that hAPP/A□ is anterogradely transported from host tg neurons into the wt graft. It has been described previously that host axons can penetrate a homotypic transplant, although in very low numbers (Clinton and Ebner, 1988). Moreover, APP is anterogradely transported to synaptic sites where it is released (Lazarov et al., 2002). Thus, we have used Holmes silver staining to visualize processes between host and graft. Overall, fiber density within the graft was much lower compared to the host. Although a few fibers were found to cross the host-graft border, most of the fibers were confined to their host or graft compartments (Fig. 6A). Moreover, immunohistochemistry did not reveal any hAPP-positive fibers that penetrated significantly into wt grafts (Fig. 6B, but see also Fig. 3E,H).

We also considered the possibility that microglia transport APP/A□ from the host into the graft. Thus, not the graft *per se* but the lesion-associated gliosis or inflammation may induce amyloid accumulation in wt grafts. In favor of this explanation is the

observation that amyloid was predominantly observed at the border of the graft, an area of a slightly higher density of microglia compared to the rest of the graft. To test this possibility, we performed stab wounds in the neocortex and hippocampus of 6 month-old APP23 mice and analyzed the mice 3 months later. However, in none of lesioned mice (n =4) was amyloid found despite appreciable microgliosis around the lesion site, which was more extensive compared to that seen at the host-graft border (Fig. 6C,D). Moreover, we have injected three APP23 mice with wt grafts in which cells were killed by freezing before transplantation. Again, no amyloid accumulation was found although microgliosis was clearly present (not shown). Thus, surgical trauma alone is insufficient to induce β-amyloidosis in APP23 mice.

These results favor the idea that soluble and diffusible Aβ is transported extracellularly via interstitial fluid from the host into the graft where Aβ undergoes fibril formation and deposition. To exclude the hypothesis that host-derived soluble extracellular Aβ is internalized by wt neurons in the graft and that such internalized Aβ is the nidus and a prerequisite for extracellular amyloid plaque formation, we have used immunohistochemical staining with a variety of antibodies to Aβ, including antibodies previously suggested to recognize intracellular human Aβ (Wirths et al., 2002). However, no evidence for intracellular human Aβ in the wt graft was found (Fig. 6E,F).

Amyloid-associated pathology in B6 grafts

The observation of amyloid in wt grafts allowed us to study the impact of host-derived extracellular amyloid formation on neurodegeneration. Although most of the amyloid in wt grafts was of the diffuse type and did not induce any notable neuropil changes, some compact and congophilic amyloid plaques were observed (Fig. 3I insert, 7A). Only a few neuronal cells were observed in the area proximal to compact plaques and some of them showed signs of degeneration (Fig. 7B). Moreover, plaques were surrounded by dystrophic synaptophysin-positive boutons (Fig. 7C) and abnormal thickened acetylcholinesterase-positive processes (Fig. 7D). A subpopulation of these distorted neuritic structures were positive for hyperphosphorylated tau (Fig. 7E). Signs of gliosis and neuroinflammation were also evident by clusters of darkly stained complement receptor 3 (CD11b)-positive and ionized calcium binding adaptor molecule 1 (Iba1)-positive microglia cells with hypertrophic processes that cover the amyloid core (Fig. 7F,G). Hypertrophic reactive glial fibrillary acidic protein (GFAP)-positive astrocytes

were also oberved around the amyloid deposits (Fig. 7H). Overall, these changes appeared similar to the amyloid-associated degeneration, microgliosis and reactive astrocytosis previously described in amyloid-depositing APP23 mice (Sturchler-Pierrat et al., 1997; Calhoun et al., 1998; Phinney et al., 1999; Stalder et al., 1999; Boncristiano et al., 2002; Bondolfi et al., 2002).

2.4 Discussion

Whether cerebral amyloidosis is initiated by a gradual increase in extracellular A or by the formation of intracellular A aggregates with subsequent accumulation in the extracellular space (ECS) is controversial (Martin et al., 1994; Hartmann et al., 1997; Geula et al., 1998; Gouras et al., 2000; Kane et al., 2000; Walsh et al., 2000; Bayer et al., 2001; D'Andrea et al., 2001; Wirths et al., 2001). Our results suggest that local intracellular A is not a prerequisite for extracellular amyloid deposition and that amyloid deposition is not necessarily confined to the region of A production. Rather, A can be transported and/or diffuses considerable distances in ECS of the brain before it aggregates, deposits extracellularely and causes neurodegeneration.

First, we have grafted APP23 tg tissue into wt hosts. Because cerebral amyloidosis in the neocortex and hippocampus of APP23 tg mice appears at six months of age (Sturchler-Pierrat et al., 1997), our initial expectation was that tg neocortical and hippocampal grafts placed in wt hosts would also develop amyloid at about six months post-grafting. However, no A deposition was observed in the tg grafts up to twenty months post-grafting despite APP/A production in the tg graft similar to APP23 tg mice. Thus, a reasonable explanation for this lack of or delay in amyloid formation is that extracellular A in the graft does not reach high enough concentrations for amyloid formation due to movement of extracellular soluble A out of the graft into the large volume of surrounding host tissue. It has previously been shown that extracellular A is transported via interstitial fluid to the vasculature where A is cleared by drainage along the perivascular spaces into the lymph nodes and/or by transport through the blood-brain-barrier into the blood (Weller, 1998; Calhoun et al., 1999; Shibata et al., 2000; DeMattos et al., 2002). That A is transported from the tg graft into the wt host is consistent with the observation of amyloid outside the tg graft, in particular in vessel walls.

Second, the most compelling evidence that A is extracellularely transported between host and graft comes from our observation that wt grafts in tg hosts exhibit amyloidosis. The amyloid in wt grafts is largely human A and thus transgene- and host-derived. Moreover, amyloid in wt graft only develops when grafts are placed in brain areas such

as neocortex, hippocampus, and to some extent thalamus, that have relative high levels of soluble extracellular A, but not when placed into the striatum, which has significant lower concentrations of extracellular soluble A. Finally, axonal transport and transport by microglia of A from the host into the graft did not appear to be significant. Thus, our results argue strongly that soluble A from the tg host is transported extracellularly via the interstitial fluid from the host into the graft where A then undergoes deposition. Recent findings have suggested that synaptic dysfunction may precede frank neuronal degeneration in AD and that this dysfunction may be caused by extracellular, diffusible oligomeric assemblies of A (Selkoe, 2002). Our results would also suggest that such soluble A aggregates may diffuse over considerable distances in the ECS and therefore may have an unexpected ability to impact synapses distal to the site of A production.

It could be argued that host-derived human A may be internalized by neurons within the wt graft (Bi et al., 2002; Nagele et al., 2002) and that such internalized human A may undergo fibril formation intracellularly prior to being released and providing the nidus for extracellular amyloid formation (Gouras et al., 2000; Walsh et al., 2000; Bayer et al., 2001; D'Andrea et al., 2001; Wirths et al., 2001). However, we found no evidence of intracellular human A in wt grafts. Additionally, we did not find evidence that the amyloid in wt grafts consisted of significant amounts of mouse A, which might have originated in neurons within the wt graft. Moreover, we have previously reported no difference in amyloid deposition between APP23 mice on a wt background and APP23 mice on a *App*-null background (Calhoun et al., 1999). Thus, intraneuronal A does not appear to be a prerequisite for extracellular amyloid formation. Nevertheless, a contributing role of intraneuronal A to extracellular amyloid deposition cannot be entirely ruled out. The relatively lower amount of compact amyloid in the wt grafts vs. tg grafts (both in tg hosts) may be explained by amyloid formation initiated by an intracellular nidus leading to predominantly compact amyloid plaques while the formation of amyloid in the ECS is largely of the diffuse type. However, these differences may simply be explained by an A concentration difference given that the tg graft produces A in addition to that provided by the surrounding host tissue.

The unexpected observation that wt grafts often had amyloid deposition prior to the tg host tissue, suggests that the graft provides a particularly favorable environment for

amyloidogenesis. Increased levels of extracellular matrix proteins in the ECS of neural grafts (Gates et al., 1996; Sykova et al., 1999) may act as chaperones for A fibril formation or promote amyloidogenesis by inhibiting interstitial fluid transport of A to the vessels and subsequent drainage (Snow et al., 1994; Gates et al., 1996; Weller, 1998; Sykova et al., 1999). Using diffusion-weighted MRI, we have recently shown that amyloidosis in APP23 mice is closely related to a decrease in brain ECS diffusion properties (Mueggler *et al.,* unpublished data). Interestingly however, the induction of extracellular matrix constituents by a stab wound or by the injection of dead cells does not promote amyloid deposition in APP23 tg mice. Thus, in future studies it will be important to characterize the factors that promote amyloidogenesis in the wt grafts. Identifying such amyloid-promoting factors may provide additional targets for amyloid-lowering therapies.

Whether neurodegeneration is related to intracellular A generation or to its extracellular deposition is key to understanding the pathobiology of AD (Selkoe, 2001). Oligomerization and accumulation of intracellular A1-42 in AD has been consistently reported (Hartmann et al., 1997; Wild-Bode et al., 1997; Gouras et al., 2000; Walsh et al., 2000; Wirths et al., 2001). Based on these findings, it has been suggested that accumulation of A1-42 in intracellular compartments may lead to neuronal death (Walsh et al., 2000; D'Andrea et al., 2001; Wirths et al., 2001). However, our findings of amyloid-associated nerve cell degeneration, with dystrophic neuritic and synaptic structures including hyperphosphorylated processes in the vicinity of compact amyloid deposits in wt grafts, suggest that intracellular A is not a prerequisite for neurodegeneration. In fact, amyloid pathology in wt grafts is very similar to that previously described in APP tg mice, including neuron death in the vicinity of amyloid plaques (Calhoun et al., 1998; Bondolfi et al., 2002; Urbanc et al., 2002), synaptic abnormalities (Phinney et al., 1999), dystrophic cholinergic processes (Wong et al., 1999; Boncristiano et al., 2002), and tau hyperphosphorylated distorted neuritic structures (Sturchler-Pierrat et al., 1997). Moreover, the gliosis and the increase in neuroinflammatory markers was also similar between wt grafts with amyloid and amyloid-depositing APP tg mice (Sturchler-Pierrat et al., 1997; Frautschy et al., 1998; Stalder et al., 1999).

In conclusion, our grafting experiments demonstrate that cerebral β-amyloidosis does not require locally generated intracellular Aβ to initiate Aβ deposition. Indeed, transport or diffusion of Aβ in the ECS of the brain is sufficient and can lead to amyloid pathology at a considerable distance from the site of Aβ generation. Overall, our findings demonstrate that factors beyond those directly involved in cellular Aβ production and intracellular Aβ metabolism are important for amyloidogenesis *in vivo*. The amyloid-associated pathology in wt grafts further suggests that neurodegeneration in AD is dependent, at least partially, upon extracellular amyloid accumulation.

2.5 Materials and Methods

Donor embryos for grafting

Donor mice were C57BL/6 (B6) mice (RCC, Füllinsdorf, Switzerland) and APP23 tg mice (Sturchler-Pierrat et al., 1997). APP23 mice overexpress mutated human APP (hAPP) with the Swedish double mutation under a neuron-specific murine Thy-1 promoter element. All APP23 mice were from generation 9-11 of backcrossing to B6. To produce tg grafts, homozygous male APP23 tg mice were bred with hemizygous female APP23 mice. For wt grafts B6 mice were bred. Pregnant females were staged by vaginal plugs and embryos at E15-17 (crown to rump length: 13-15 mm) were taken for grafting.

Intracerebral grafting

Embryonic neocortices and hippocampi were dissected on ice under semi-sterile conditions in Dulbecco's modified Eagle's Medium (Life Technologies, Basel, Switzerland). All neocortical tissue pieces from the entire litter were pooled. The same procedure was performed for the hippocampal pieces. Cell suspensions were prepared mechanically through repeated pipetting with Pasteur pipettes on ice. Total cell counts and cell viability were determined with a hemocytometer and 0.4% trypan blue stain (0.4%; Fluka, Buchs, Switzerland). Cell number was adjusted to 100,000 cells/μl and cell viability was 80%.

Host mice (3-24 month-old, females) were anaesthetized with a mixture of ketamine (10mg/kg body weight) and xylazine (20mg/kg body weight) in saline. Bilateral stereotaxic injections of 2.5µl cortical and hippocampal cell suspensions were placed with a Hamilton syringe into the neocortex (AP +1.0mm from bregma, L +/-2.0mm, DV -1.5mm) and hippocampus (AP -2.5mm, L +/-2.0mm, DV -2.5mm), respectively. In selected mice cell suspensions were injected into the striatum (AP +1.0mm, L +/-2.0mm, DV -3mm), and thalamus (AP -2.5mm, L +/-1.5.mm, DV -3mm). Injection speed was 1.25µl/minute and the needle was kept in place for an additional 2 minutes before it was slowly withdrawn. The surgical area was cleaned with sterile saline and the incision was sutured. As a control, some mice received stab wound lesions, i.e. the needle was lowered in the brain without injection of any cell suspension. All experiments were in compliance with protocols approved by the Animal Care and Use Committee (Veterinäramt, Basel).

Histology and immunohistochemistry

At various time points after grafting host mice were deeply anaesthetized and either perfused transcardially with 0.1 M PBS (pH 7.4), followed by 4% paraformaldehyde in PBS (pH 7.4) or brains were removed and immersion fixed for 2d in 4 % paraformaldehyde in PBS. Brains were then dehydrated overnight in 30% sucrose. After freezing, 20-30 µm serial coronal sections were cut through the transplants on a freezing-sliding microtome and collected in 0.1 M Tris-buffered saline (pH 7.4).

Sections were stained histologically with cresyl-violet and immunohistochemically according to previously published protocols (Stalder et al., 1999) with the following antibodies: polyclonal antibody NT12 (NT11) to Aβ (courtesy of P. Paganetti) (Sturchler-Pierrat et al., 1997); mouse monoclonal antibody W0-2 specific to human Aβ (Wirths et al., 2002) (courtesy of K. Beyreuther); mouse monoclonal antibody 6E10 specific to hAPP/Aβ (Signet Pathology Systems, Inc., Dedham, MA, USA); monolconal antibodies specific to Aβx-40 (JRF/cAβ40/10) and Aβx-42 (JRF/cAβ42/26) (Mathews et al., 2002); monoclonal antibody specific to rodent/mouse Aβ that does not cross-react with human Aβ (JRF7rAβ1-15/2) (Mathews et al., 2002); polyclonal antibody A4CT to the C-terminal 100 amino acid of hAPP (courtesy of K. Beyreuther); mouse monoclonal

antibody 56C6 to neprilysin (CD10; Novocastra Laboratories, Newcastle upon Tyne, U.K); polyclonal affinity-purified antibody IDE-1 to IDE protein (courtesy of D. Selkoe); polyclonal antibody to synaptophysin (Dako, Glostrup, Denmark), mouse monoclonal antibody AT8 which recognizes hyperphosphorylated tau (Innogenetics, Heiden, Germany), rat monoclonal antibody to CD11b (Mac-1; Serotec, Oxford, UK), polyclonal antibody to Iba1 (Ohsawa et al., 2000) (courtesy Y. Imai) and polyclonal antibody to GFAP (Dako). Some sections were double stained with NT12 and Congo Red. Additional sections were stained histochemically for acetylcholinesterase (Boncristiano et al., 2002) and Holmes silver staining.

Assessment of APP and A in graft and host using Western blotting

Under deep inhalation anesthesia, engrafted mice were sacrificed by decapitation. Brains were removed and sectioned on a vibratome (400 m thick slices) in ice-cold PBS. Slices were transferred onto a glass slide on ice. With the aid of a dissecting microscope and a Stoelting micropunch device (Stoelting, Wood Dale, IL, USA), micropunches were taken from the graft. For comparison, micropunches were also taken from normal APP23 and B6 mice. Punches had a diameter of 0.74 mm and thus consisted of 172 g wet weight tissue (Palkovits, 1983).

To determine hAPP expression, punched samples were diluted in 30 l of homogenization buffer (50 mM Tris, pH 8; 150 mM NaCl; 5 mM EDTA; protease inhibitor cocktail (Roche Diagnostic, Mannheim, Germany) and 90 l sample buffer (0.48 M Bistris; 0.21 M Bicine; 1.33% w/v SDS; 20% w/v sucrose; 3.33% v/v 2-mercaptoethanol; 0.0053% w/v bromophenol blue). Subsequently, samples were Dounce-homogenized, sonicated, and subjected to 10% Bicine/Tris 8M Urea SDS-PAGE (Wiltfang et al., 1997). Proteins were transferred onto a PVDF membrane and hAPP was detected using monoclonal mouse 6E10 antibody. The secondary antibody was horseradish peroxidase-conjugated goat anti-mouse IgG (Chemicon, Temecula, CA, USA). Bands were visualized using SuperSignal (Pierce, Rockford, Il, USA) and developed onto Kodak X-OMAT AR film (Rochester, NY, USA).

To analyze Aβ levels, tissues of two micropunches were combined and human Aβ was immunoprecipitated with antibody 6E10 and protein G Sepharose (Sigma, St Louis, MO, USA). Subsequent SDS-PAGE and blotting was done as described above. Synthetic Aβ1-40 and 1-42 (Bachem, Bubendorf; Switzerland) was used as control. Different exposures of the films were digitized and band density measurements for Aβ40 were made using NIH Image Version 1.61 (NIH, Bethesda, MD, USA). Only bands within the linear range of the film were analyzed.

ELISA to measure soluble extracellular Aβ in engrafted brain regions

Neocortex, hippocampus, striatum, and thalamus of 6 month-old male pre-depositing APP23 mice (n =8) were dissected on ice. Tissue of four mice was pooled and homogenized in 50mM Tris, pH 8, 150mM NaCl, and 5 mM EDTA and protease inhibitor cocktail (Roche Diagnostic). The homogenate was centrifuged at 107,000g for 1 hr at 4^0C and human Aβx-40 and Aβx-42 levels were determined in the supernatant by previously described sandwich ELISAs (Mathews et al., 2002). In brief, Aβ was captured with carboxy-terminal monoclonal antibodies that recognize exclusively either Aβx-40 (JRF/cAβ40/10) or Aβx-42 (JRF/cAβ42/26) and detected with horseradish peroxidase-conjugated JRF/Aβtot/17, which was raised against the amino-terminal 16 residues of human Aβ. All measurements were done in two or more replica wells. ELISA results reported are the mean of the two pooled tissue samples (ng Aβ per g wet weight), based on standard curves using synthetic Aβ1-40 and Aβ1-42 peptide standards (American Peptide Co. Sunnyvale, CA).

Serum titers of Aβ antibodies

At various time points post-grafting (30-510 days), retro-orbital blood samples were collected from selected engrafted mice by using heparin-coated capillary tubes. Samples were centrifuged and the sera were immediately frozen on dry ice. Antibody titers were assessed by ELISA. Serial dilutions of sera were made onto microtiter plates coated with Aβ1-40 (50 ng/ml; Bachem). Detection was through alkaline phosphatase-conjugated rabbit anti-mouse IgG (Calbiochem, San Diego, USA) and 4-nitrophenol phosphate disodium salt hexahydrate (Fluka). Titers were defined as dilution yielding 50% of the maximal signal.

Acknowledgements

We would like to thank S. Schmidt (New York, USA) for the advice with ELISA, and D. Ingram (Baltimore, USA) and S. Brandner and A. Aguzzi (Zürich, Switzerland) for the help with the grafting procedure. The antibody donations of P. Paganetti (Basel, Switzerland), D. Selkoe (Boston, USA), Y. Imai (Tokyo, Japan), and K. Beyreuther (Heidelberg, Germany) were greatly appreciated. Furthermore we thank L. Walker (Ann Arbor, USA), M. Calhoun (New York, USA), S. Sisodia (Chicago, USA), E. Sykova (Prague, Czech Republic), T. Saido (Saitama, Japan), M. Leissring (Boston, USA), G. Multhaup and T. Hartmann (Heidelberg, Germany), A. Probst, M. Tolnay, A. Renken, and D. Monard (Basel, Switzerland) for advice, help, and comments to this manuscript. This work has been supported by grants from the American Health Assistance Foundation (Alzheimer‘s disease program), the Horten Foundation (Madonna del Piano, Switzerland), the AETAS Foundation (Geneva, Switzerland), and the Swiss National Science Foundation.

2.6 References

Bayer TA, Fossgreen A, Czech C, Beyreuther K, Wiestler OD (1996) Plaque formation in brain transplants exposed to human beta-amyloid precursor protein 695. Acta Neuropathol (Berl) 92:130-137.

Bayer TA, Wirths O, Majtenyi K, Hartmann T, Multhaup G, Beyreuther K, Czech C (2001) Key factors in Alzheimer's disease: beta-amyloid precursor protein processing, metabolism and intraneuronal transport. Brain Pathol 11:1-11.

Bi X, Gall CM, Zhou J, Lynch G (2002) Uptake and pathogenic effects of amyloid beta peptide 1-42 are enhanced by integrin antagonists and blocked by NMDA receptor antagonists. Neuroscience 112:827-840.

Boncristiano S, Calhoun ME, Kelly PH, Pfeifer M, Bondolfi L, Stalder M, Phinney AL, Abramowski D, Sturchler-Pierrat C, Enz A, Sommer B, Staufenbiel M, Jucker M (2002) Cholinergic changes in the APP23 transgenic mouse model of cerebral amyloidosis. J Neurosci 22:3234-3243.

Bondolfi L, Calhoun M, Ermini F, Kuhn HG, Wiederhold KH, Walker L, Staufenbiel M, Jucker M (2002) Amyloid-associated neuron loss and gliogenesis in the neocortex of amyloid precursor protein transgenic mice. J Neurosci 22:515-522.

Brandner S, Raeber A, Sailer A, Blattler T, Fischer M, Weissmann C, Aguzzi A (1996a) Normal host prion protein (PrPC) is required for scrapie spread within the central nervous system. Proc Natl Acad Sci U S A 93:13148-13151.

Brandner S, Isenmann S, Raeber A, Fischer M, Sailer A, Kobayashi Y, Marino S, Weissmann C, Aguzzi A (1996b) Normal host prion protein necessary for scrapie-induced neurotoxicity. Nature 379:339-343.

Calhoun ME, Wiederhold KH, Abramowski D, Phinney AL, Probst A, Sturchler-Pierrat C, Staufenbiel M, Sommer B, Jucker M (1998) Neuron loss in APP transgenic mice. Nature 395:755-756.

Calhoun ME, Burgermeister P, Phinney AL, Stalder M, Tolnay M, Wiederhold KH, Abramowski D, Sturchler-Pierrat C, Sommer B, Staufenbiel M, Jucker M (1999) Neuronal overexpression of mutant amyloid precursor protein results in prominent deposition of cerebrovascular amyloid. Proc Natl Acad Sci U S A 96:14088-14093.

Cirrito PM, Alexander GM, Bales KR, Paul SM, DeMattos RB, Holtzman DM (2002) Using awake in vivo microdialysis to dynamically assess the exchangeable pool of amyloid beta within the brain interstitial fluid of young PDAPP mice. Pogram No 191152002 Abstract Viewer/Itinery Planner Washington, DC: Society for Neuroscience, 2002 CD-ROM.

Clinton RJ, Jr., Ebner FF (1988) Time course of neocortical graft innervation by AChE-positive fibers. J Comp Neurol 277:557-577.

D'Andrea MR, Nagele RG, Wang HY, Peterson PA, Lee DH (2001) Evidence that neurones accumulating amyloid can undergo lysis to form amyloid plaques in Alzheimer's disease. Histopathology 38:120-134.

DeMattos RB, Bales KR, Cummins DJ, Paul SM, Holtzman DM (2002) Brain to plasma amyloid-beta efflux: a measure of brain amyloid burden in a mouse model of Alzheimer's disease. Science 295:2264-2267.

Frautschy SA, Yang F, Irrizarry M, Hyman B, Saido TC, Hsiao K, Cole GM (1998) Microglial response to amyloid plaques in APPsw transgenic mice. Am J Pathol 152:307-317.

Games D, Adams D, Alessandrini R, Barbour R, Berthelette P, Blackwell C, Carr T, Clemens J, Donaldson T, Gillespie F, et al. (1995) Alzheimer-type neuropathology in transgenic mice overexpressing V717F beta-amyloid precursor protein. Nature 373:523-527.

Gates MA, Laywell ED, Fillmore H, Steindler DA (1996) Astrocytes and extracellular matrix following intracerebral transplantation of embryonic ventral mesencephalon or lateral ganglionic eminence. Neuroscience 74:579-597.

Geula C, Wu CK, Saroff D, Lorenzo A, Yuan M, Yankner BA (1998) Aging renders the brain vulnerable to amyloid beta-protein neurotoxicity. Nat Med 4:827-831.

Gouras GK, Tsai J, Naslund J, Vincent B, Edgar M, Checler F, Greenfield JP, Haroutunian V, Buxbaum JD, Xu H, Greengard P, Relkin NR (2000) Intraneuronal Abeta42 accumulation in human brain. Am J Pathol 156:15-20.

Hardy J, Selkoe DJ (2002) The amyloid hypothesis of Alzheimer's disease: progress and problems on the road to therapeutics. Science 297:353-356.

Hartmann T, Bieger SC, Bruhl B, Tienari PJ, Ida N, Allsop D, Roberts GW, Masters CL, Dotti CG, Unsicker K, Beyreuther K (1997) Distinct sites of intracellular production for Alzheimer's disease A beta40/42 amyloid peptides. Nat Med 3:1016-1020.

Holtzman DM, Li YW, DeArmond SJ, McKinley MP, Gage FH, Epstein CJ, Mobley WC (1992) Mouse model of neurodegeneration: atrophy of basal forebrain cholinergic neurons in trisomy 16 transplants. Proc Natl Acad Sci U S A 89:1383-1387.

Hsiao K, Chapman P, Nilsen S, Eckman C, Harigaya Y, Younkin S, Yang F, Cole G (1996) Correlative memory deficits, Abeta elevation, and amyloid plaques in transgenic mice. Science 274:99-102.

Iwata N, Tsubuki S, Takaki Y, Watanabe K, Sekiguchi M, Hosoki E, Kawashima-Morishima M, Lee HJ, Hama E, Sekine-Aizawa Y, Saido TC (2000) Identification of the major Abeta1-42-degrading catabolic pathway in brain parenchyma: suppression leads to biochemical and pathological deposition. Nat Med 6:143-150.

Kane MD, Lipinski WJ, Callahan MJ, Bian F, Durham RA, Schwarz RD, Roher AE, Walker LC (2000) Evidence for seeding of beta -amyloid by intracerebral infusion of Alzheimer brain extracts in beta -amyloid precursor protein-transgenic mice. J Neurosci 20:3606-3611.

Lazarov O, Lee M, Peterson DA, Sisodia SS (2002) Evidence that synaptically released beta-amyloid accumulates as extracellular deposits in the hippocampus of transgenic mice. J Neurosci 22:9785-9793.

Mantione JR, Kleppner SR, Miyazono M, Wertkin AM, Lee VM, Trojanowski JQ (1995) Human neurons that constitutively secrete A beta do not induce Alzheimer's disease pathology following transplantation and long-term survival in the rodent brain. Brain Res 671:333-337.

Martin LJ, Pardo CA, Cork LC, Price DL (1994) Synaptic pathology and glial responses to neuronal injury precede the formation of senile plaques and amyloid deposits in the aging cerebral cortex. Am J Pathol 145:1358-1381.

Mathews PM, Jiang Y, Schmidt SD, Grbovic OM, Mercken M, Nixon RA (2002) Calpain Activity Regulates the Cell Surface Distribution of Amyloid Precursor Protein.

Inhibition of calpains enhances endosomal generation of beta -cleaved C-terminal APP fragments. J Biol Chem 277:36415-36424.

Nagele RG, D'Andrea MR, Anderson WJ, Wang HY (2002) Intracellular accumulation of beta-amyloid(1-42) in neurons is facilitated by the alpha 7 nicotinic acetylcholine receptor in Alzheimer's disease. Neuroscience 110:199-211.

Ohsawa K, Imai Y, Kanazawa H, Sasaki Y, Kohsaka S (2000) Involvement of Iba1 in membrane ruffling and phagocytosis of macrophages/microglia. J Cell Sci 113:3073-3084.

Palkovits M (1983) Punch sampling biopsy technique. Methods Enzymol 103:368-376.

Pfeifer M, Boncristiano S, Bondolfi L, Stalder A, Deller T, Staufenbiel M, Mathews PM, Jucker M (2002) Cerebral Hemorrhage After Passive Anti-Abeta Immunotherapy. Science 298:1379.

Phinney AL, Deller T, Stalder M, Calhoun ME, Frotscher M, Sommer B, Staufenbiel M, Jucker M (1999) Cerebral amyloid induces aberrant axonal sprouting and ectopic terminal formation in amyloid precursor protein transgenic mice. J Neurosci 19:8552-8559.

Qiu WQ, Walsh DM, Ye Z, Vekrellis K, Zhang J, Podlisny MB, Rosner MR, Safavi A, Hersh LB, Selkoe DJ (1998) Insulin-degrading enzyme regulates extracellular levels of amyloid beta-protein by degradation. J Biol Chem 273:32730-32738.

Richards SJ, Waters JJ, Beyreuther K, Masters CL, Wischik CM, Sparkman DR, White CL, III, Abraham CR, Dunnett SB (1991) Transplants of mouse trisomy 16 hippocampus provide a model of Alzheimer's disease neuropathology. Embo J 10:297-303.

Schenk D, Barbour R, Dunn W, Gordon G, Grajeda H, Guido T, Hu K, Huang J, Johnson-Wood K, Khan K, Kholodenko D, Lee M, Liao Z, Lieberburg I, Motter R, Mutter L, Soriano F, Shopp G, Vasquez N, Vandevert C, Walker S, Wogulis M, Yednock T, Games D, Seubert P (1999) Immunization with amyloid-beta attenuates Alzheimer-disease-like pathology in the PDAPP mouse. Nature 400:173-177.

Selkoe DJ (2001) Alzheimer's disease: genes, proteins, and therapy. Physiol Rev 81:741-766.

Selkoe DJ (2002) Alzheimer's disease is a synaptic failure. Science 298:789-791.

Shibata M, Yamada S, Kumar SR, Calero M, Bading J, Frangione B, Holtzman DM, Miller CA, Strickland DK, Ghiso J, Zlokovic BV (2000) Clearance of Alzheimer's amyloid-ss(1-40) peptide from brain by LDL receptor-related protein-1 at the blood-brain barrier. J Clin Invest 106:1489-1499.

Snow AD, Sekiguchi R, Nochlin D, Fraser P, Kimata K, Mizutani A, Arai M, Schreier WA, Morgan DG (1994) An important role of heparan sulfate proteoglycan (Perlecan) in a model system for the deposition and persistence of fibrillar A beta-amyloid in rat brain. Neuron 12:219-234.

Stalder M, Phinney A, Probst A, Sommer B, Staufenbiel M, Jucker M (1999) Association of microglia with amyloid plaques in brains of APP23 transgenic mice. Am J Pathol 154:1673-1684.

Sturchler-Pierrat C, Abramowski D, Duke M, Wiederhold KH, Mistl C, Rothacher S, Ledermann B, Burki K, Frey P, Paganetti PA, Waridel C, Calhoun ME, Jucker M, Probst A, Staufenbiel M, Sommer B (1997) Two amyloid precursor protein transgenic mouse models with Alzheimer disease-like pathology. Proc Natl Acad Sci U S A 94:13287-13292.

Sykova E, Roitbak T, Mazel T, Simonova Z, Harvey AR (1999) Astrocytes, oligodendroglia, extracellular space volume and geometry in rat fetal brain grafts. Neuroscience 91:783-798.

Urbanc B, Cruz L, Le R, Sanders J, Ashe KH, Duff K, Stanley HE, Irizarry MC, Hyman BT (2002) Neurotoxic effects of thioflavin S-positive amyloid deposits in transgenic mice and Alzheimer's disease. Proc Natl Acad Sci U S A 99:13990-13995.

Walsh DM, Tseng BP, Rydel RE, Podlisny MB, Selkoe DJ (2000) The oligomerization of amyloid beta-protein begins intracellularly in cells derived from human brain. Biochemistry 39:10831-10839.

Weller RO (1998) Pathology of cerebrospinal fluid and interstitial fluid of the CNS: significance for Alzheimer disease, prion disorders and multiple sclerosis. J Neuropathol Exp Neurol 57:885-894.

Wild-Bode C, Yamazaki T, Capell A, Leimer U, Steiner H, Ihara Y, Haass C (1997) Intracellular generation and accumulation of amyloid beta-peptide terminating at amino acid 42. J Biol Chem 272:16085-16088.

Wiltfang J, Smirnov A, Schnierstein B, Kelemen G, Matthies U, Klafki HW, Staufenbiel M, Huther G, Ruther E, Kornhuber J (1997) Improved electrophoretic separation and immunoblotting of beta-amyloid (A beta) peptides 1-40, 1-42, and 1-43. Electrophoresis 18:527-532.

Wirths O, Multhaup G, Czech C, Blanchard V, Moussaoui S, Tremp G, Pradier L, Beyreuther K, Bayer TA (2001) Intraneuronal Abeta accumulation precedes plaque formation in beta- amyloid precursor protein and presenilin-1 double-transgenic mice. Neurosci Lett 306:116-120.

Wirths O, Multhaup G, Czech C, Feldmann N, Blanchard V, Tremp G, Beyreuther K, Pradier L, Bayer TA (2002) Intraneuronal APP/A beta trafficking and plaque formation in beta-amyloid precursor protein and presenilin-1 transgenic mice. Brain Pathol 12:275-286.

Wong TP, Debeir T, Duff K, Cuello AC (1999) Reorganization of cholinergic terminals in the cerebral cortex and hippocampus in transgenic mice carrying mutated presenilin-1 and amyloid precursor protein transgenes. J Neurosci 19:2706-2716.

2.7 Figure Captions

Figure 1 Neural grafting of APP23 tissue into B6 hosts. Embryonic APP23 hippocampal tissue was injected into the hippocampus of 3 month-old wt B6 hosts. Mice were analysed at various times post-grafting (see Table 1). Shown are mice analysed 3 months (A-C), 18 months (D-F), and 21 months (G-I) post-grafting. Cresyl violet staining was used to identify the grafts (asterisks in A,D,G). Immunohistochemistry revealed strong hAPP expression restricted to neurons in the grafts (B,E,H). Nevertheless, A□-immunostaining did not reveal any amyloid deposits 3 and 18 months post-grafting (C,F). At 21 months postgrafting massive amyloid deposition in both plaques and vessels was found that was to a great extend congophilic (i, insert). The bulk of the amyloid was confined to the graft, however, some vessels with amyloid (arrow) and some diffuse amyloid (arrowhead) were found outside the graft in the host tissue. Similar results were observed when cortical tg tissue was injected into the cortex of wt mice (not shown). Bars are 250 μm (A-C) and 400 μm (D-I).

Figure 2 APP and Aß levels in APP23 grafts placed in B6 hosts. (A) Hippocampus of a 15 month-old wt B6 mouse that received an APP23 tg graft at the age of 3 months. A micropunch (p) was taken from the graft (asterisk) and subjected to Western blot analysis. The remaining tissue was immersion-fixed and immunostained for hAPP demonstrating that hAPP expression was exclusively confined to the graft. Bar is 150 μm. (B) Western blotting of the micropunch taken from the APP23 hippocampal graft shown in (A) with hA□/APP-specific antibody 6E10. In addition micropunches from hippocampus were taken from young 4-6 month-old APP23 mice and control B6 mice for comparison. *Lane 1*: Synthetic A□40/42. *Lane 2*: punch from the hippocampal APP23 graft. *Lane 3*: punch from B6 hippocampus (CA1 area). *Lane 4*: punch from APP23 hippocampus (CA1 area). Note that hAPP expression and A□ levels in the graft (lane 2) were comparable to that in APP23 hippocampus (lane 4). In both graft and young APP23 mouse

A□1-40 was several fold higher than A□1-42. (C) Similar analysis as in (B) but for an APP23 cortical graft 20 months post-grafting. For comparison micropunches from neocortex were taken from a 12 month-old amyloid-depositing APP23 mouse and a control B6 mouse. *Lane 1*: Synthetic A□40/42. *Lane 2*: punch from the cortical APP23 graft. *Lane 3*: punch from B6 neocortex. *Lane 4*: punch from APP23 neocortex.

Figure 3 Neural grafting of APP23 and B6 tissue into APP23 hosts. Embryonic APP23 tg hippocampal tissue was injected into the hippocampus of a 6 month-old APP23 mouse and analyzed three months later (A-C). Cresyl violet staining reveals the graft (asterisk) in the dentate gyrus (A). Immunohistochemistry with an antibody to hAPP reveals robust neuronal expression of hAPP in the graft and host (B). Immunostaining for A□ in adjacent sections revealed considerable amyloid deposits in the graft (C). Strikingly, amyloid deposition in the graft was also found when B6 wt hippocampal tissue was injected into the hippocampus of a 6 month-old APP23 mouse and analyzed 3 months later (D-F). In this case no hAPP expression was found in the graft (E). The same was found when embryonic B6 wt cortical tissue was grafted into the neocortex of a 6 month-old APP23 mouse and analyzed 3 months later (G-I). Although the majority of the amyloid was of the diffuse type, some amyloid was compact and Congo red-positive. The insert in (I) shows an adjacent section stained for Congo red and viewed under cross-polarized light. Note, that in most cases amyloid deposition in the graft was more intense compared to the only scattered amyloid plaques in the 9 month-old host tissue (arrows in F and I). Bars are 90 μm (A-F) and 50 μm (G-I).

Figure 4 Amyloid in B6 grafts. Immunostaining with antibody JRF/cA□40/10 specific to A□x-40 (A) and antibody JRF/cA□42/26 specific to A□1-42 (B) of a B6 wt graft placed in neocortex of an APP23 host. Antibodies specific to rodent (mouse) A□ (JRF7rA□1-15/2) revealed only very faint labeling of the amyloid (C). Shown are adjacent sections with arrowheads indicating

the same amyloid deposits. Note that the faint labeling of vessels and microglia is due to unspecific labeling of the secondary anti-mouse IgG antibody. (D) A micropunch (p) was taken from a cortical wt graft (asterisk) and subjected to Western blot analysis. The remaining tissue was immersion-fixed and immunostained for hAPP demonstrating that hAPP expression was exclusively confined to the host. (E) Western blotting of the micropunch taken from the wt cortical graft shown in (A) with human Aβ-specific antibody 6E10. In addition micropunches were taken from the neocortex of a 12 month-old amyloid-depositing APP23 mouse and a control B6 mouse. *Lane 1*: Synthetic Aβ40/42. *Lane 2*: punch from the cortical wt graft. *Lane 3*: punch from B6 neocortex. *Lane 4*: punch from APP23 neocortex. Note the presence of both Aβ1-40 and Aβ1-42 in the wt graft. Bars are 20 μm (C) and 120 μm (D).

Figure 5 Neural grafting of B6 tissue into thalamus and striatum of APP23 hosts. (A,B) Embryonic B6 hippocampal tissue was injected into the thalamus of a 6 month-old APP23 mouse and analyzed 3 months later. Cresyl violet staining revealed that the grafts were nicely integrated into the host thalamus and are viable without any signs of neurodegeneration (asterisk in A). Immunostaining for Aβ in adjacent sections revealed amyloid deposits in the graft (B). In contrast, when embryonic B6 tissue was placed into the striatum of a 6 month-old APP23 mouse, no amyloid formation was observed 3 months later (C,D). Bars are 250 μm.

Figure 6 Mechanism of amyloid formation in B6 grafts. (A) Holmes silver staining of the graft-host border of a B6 wt graft (asterisk) in a 9 mo-old APP23 tg host. There is a lower density of fibers in the graft compared to the host. Only few fibers cross the host-graft border (dotted line) and most fibers were confined to either the host or the graft. (B) Consistent with the Holmes fiber staining, immunostaining for hAPP reveals many positive neurons and processes in the host tissue, but none in the wt graft. (C) Cortical stab wound in a tg 6 month-old APP23 host shows an appreciable increase in

CD11b-positive microglia around the lesion site 3 months post-surgery (arrowheads). (D) However, immunohistochemistry for Aβ in an adjacent section does not indicate any amyloid deposition in the vicinity of the lesion. (E) Aβ immunostaining with antibody NT12 in the neocortex of a 9 month-old APP23 tg host reveals punctate intraneuronal staining. (F) In contrast, no evidence of intraneuronal Aβ was found in neocortical wt grafts into APP23 hosts. An amyloid plaque in the wt graft is shown by an arrowhead. The same results were observed with monoclonal antibody W0-2 specific to human Aβ. Bars are 10 μm (A,B), 100 μm (C,D), and 15 μm (E,F).

Figure 7 Amyloid pathology associated with congophilic amyloid plaques in B6 grafts. (A) Compact plaque in B6 wt graft in a 9 month-old APPP23 tg host reveals birefringence when viewed under cross-polarized light (double staining for Aβ and Congo red). (B) Cresyl violet staining demonstrates only a few neuronal cell bodies at the plaque periphery and some of them with a dying phenotype (arrow). (C) Plaques are surrounded and interdigitated by synaptophysin-positive dystrophic boutons (arrows). (D) Acetylcholinesterase-staining reveals abnormal large cholinergic processes in vicinity of the plaque (arrow). (E) Some of these abnormal and distorted processes in the plaque periphery were positive for hyperphosphorylated tau (arrow). (F) CD11b-immunostaining demonstrates hypertrophic microglia clustered around compact amyloid plaques. (G) Amyloid-associated microglia were also intensively positive for Iba1 similar to those observed in amyloid-depositing APP23 mice. (H) GFAP-staining reveals hypertrophic and reactive astrocytes decorating the plaque periphery. Bars are 25 μm (A,B), 10 μm (C), 25 μm (D), 6 μm (E), 25 μm (F), 30 μm (G) and 25 μm (H).

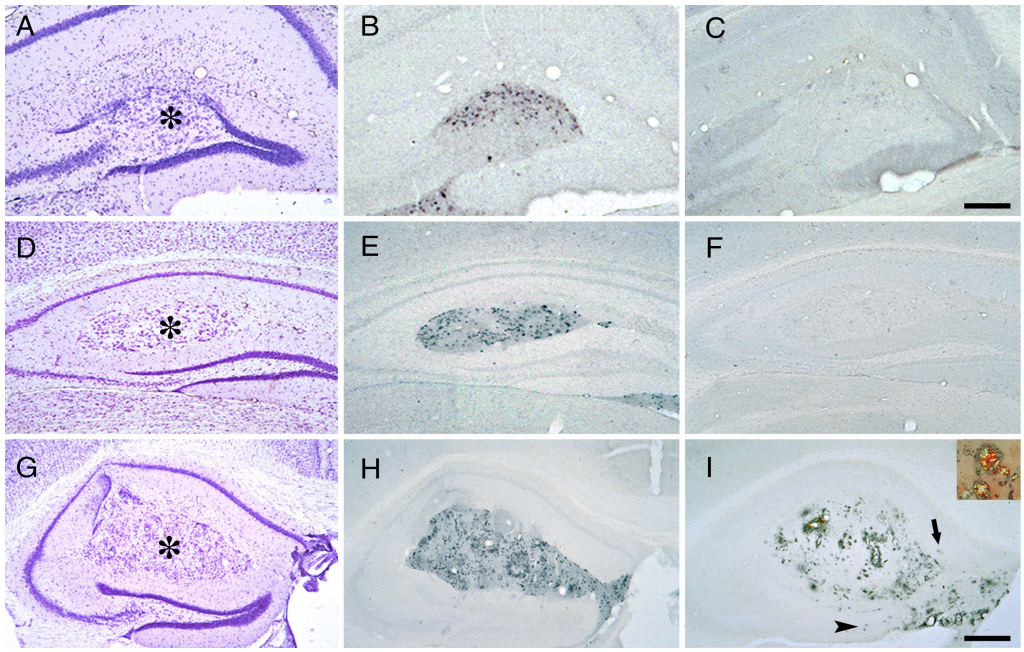

Figure 1

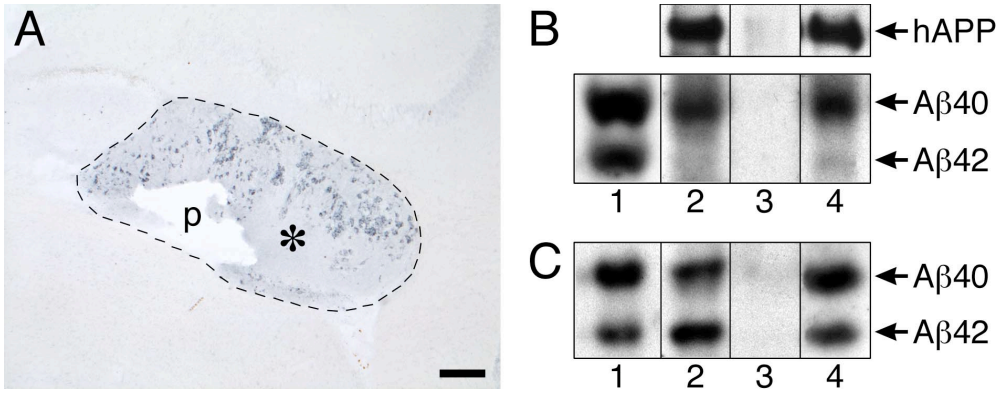

Figure 2

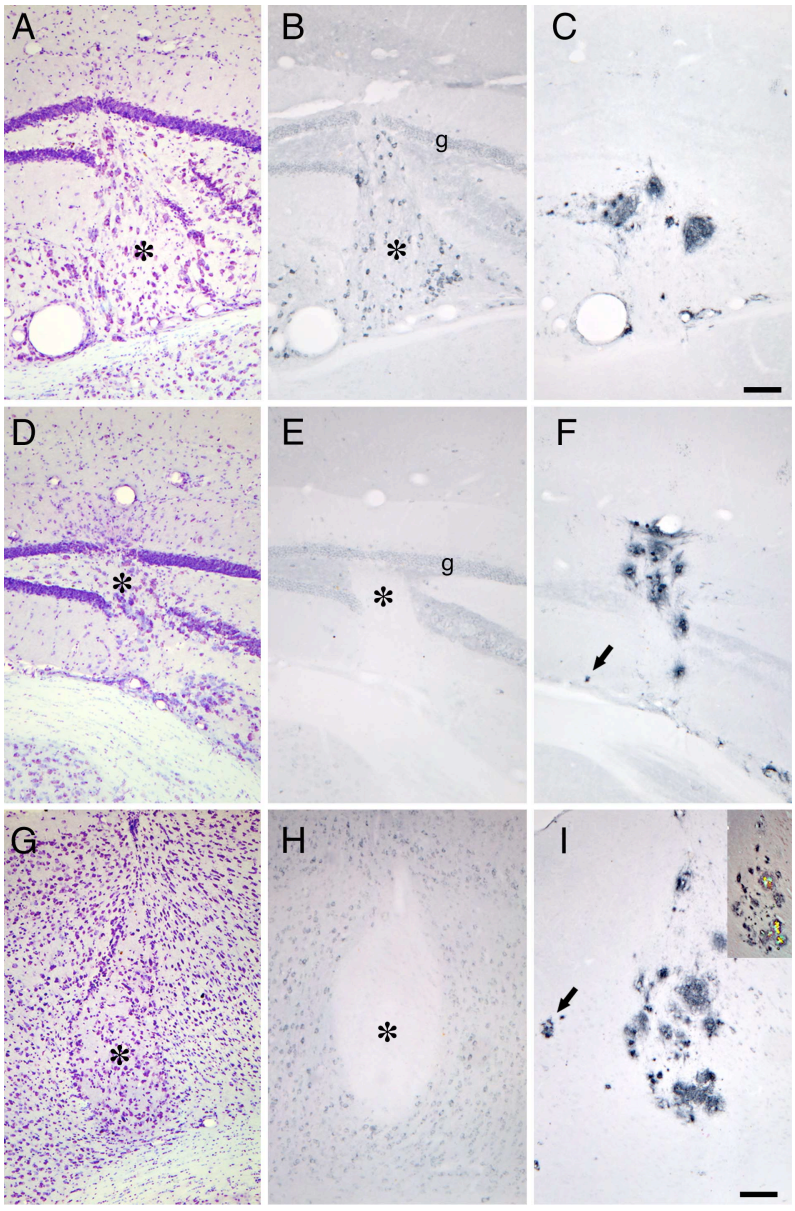

Figure 3

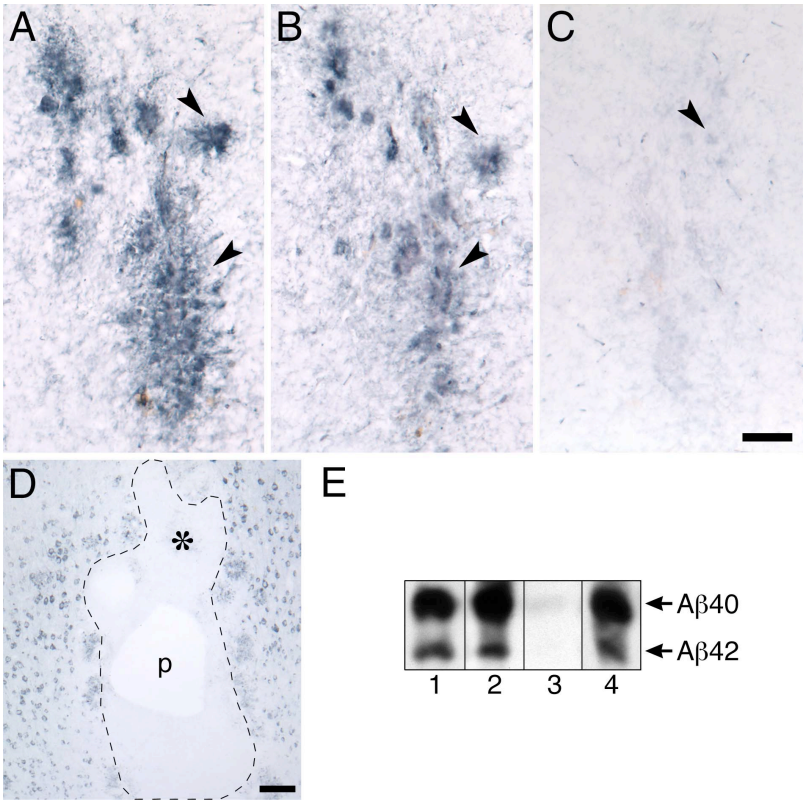

Figure 4

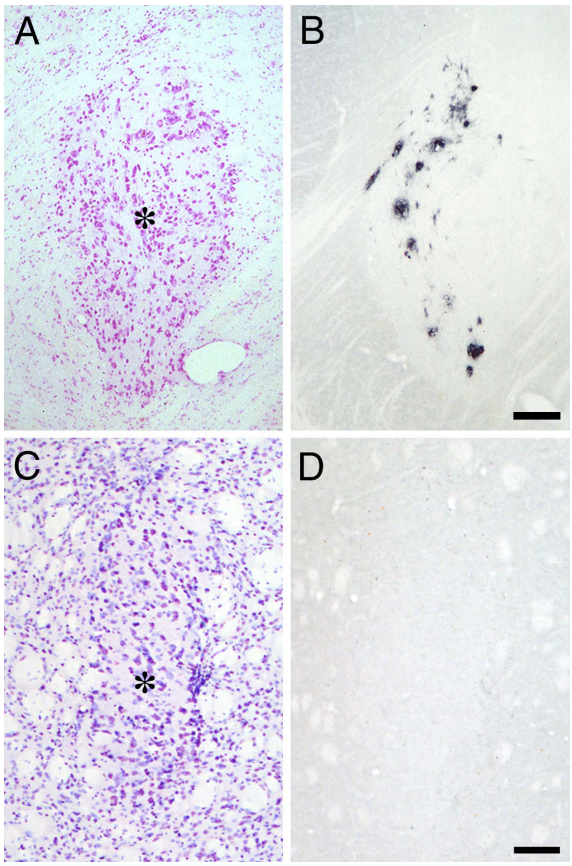

Figure 5

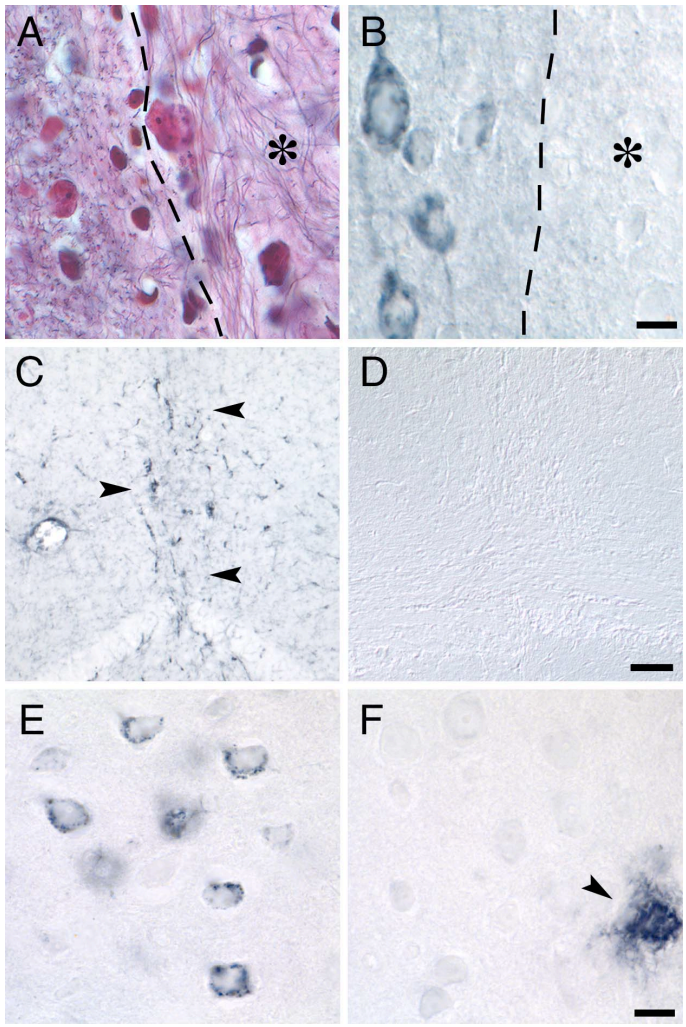

Figure 6

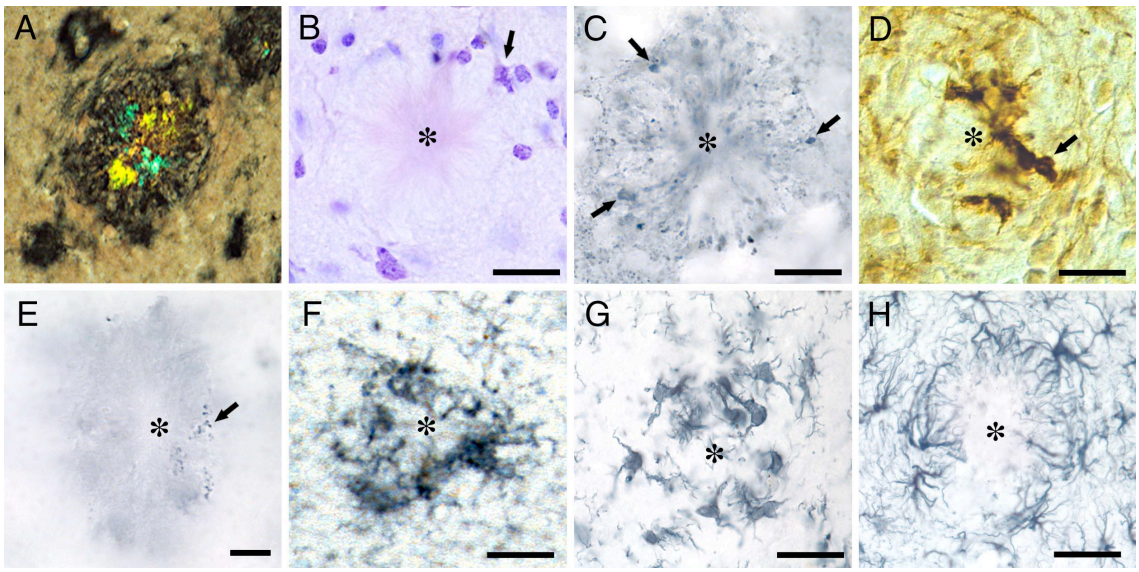

Figure 7

Table 1: *APP23 embryonic tissue transplanted in B6 brain*

Host	Age[a]	Post-grafting interval 3 mo	6 mo	12mo	18 mo	> 20 mo
B6	3 mo	0[b]/12[c]	0/6	0/6	0/20	2/9
B6	16 mo	0/1	0/2	0/6		
B6	24 mo	0/2	0/5			

[a] age of the host at time of transplantation
[b] number of mice with amyloid deposits in at least one graft
[c] total mice analyzed

Table 2: *APP23 and B6 embryonic tissue transplanted in APP23 brain*

		Grafts[b]	
Host	Age[a]	APP23	B6
APP23	6 mo	7[c]/7[d]	10/13

[a] age of the host at time of transplantation
[b] mice were sacrificed 3 months post-grafting
[c] number of mice with amyloid in at least one graft
[d] total mice analyzed

Manuscript in preparation

3. Seeding of cerebral amyloidosis in APP23 transgenic mice

Melanie Meyer-Luehmann[1,2], Lary C. Walker[3], Stephan A. Kaeser[1,2], Tristan Bolmont[1,2], Anton Neuenschwander[4], Peter Frey[4], Anneliese L. Jaton[4], Jeanmarie Vigouret[4], Matthias Staufenbiel[4], Mathias Jucker[1,2]

[1]Department of Neuropathology, Institute of Pathology, University of Basel, CH-4003 Basel, Switzerland; [2]Hertie-Institute for Clinical Brain Research, University of Tübingen, Tübingen, Germany; [3] Pfizer Ann Arbor Laboratories, Ann Arbor, MI, United States; [4]Novartis Pharma AG, Nervous System Research, CH-4002 Basel, Switzerland.

3.1 Abstract

The misconformation and self-assembly of specific proteins is well established as a disease process in Alzheimer's disease (AD), but little is known about how protein aggregation is initiated *in vivo*. Seeded polymerization of amyloid beta (Aβ) has been demonstrated *in vitro* and has recently also been achieved *in vivo* by intracerebral injection of AD brain extract into young amyloid precursor protein (APP) transgenic mice prior to plaque formation. We have confirmed and advanced these findings through studies involving injection of diluted brain extract from AD patients and from aged APP23 transgenic mice into young APP23 mice. Similar to the AD extract, transgenic mouse brain extract induced massive seeded Aβ deposits in the brain parenchyma and vessel walls four months post-infusion. This seeding was time- and concentration-dependent. These seeded amyloid deposits were mostly diffuse in nature, with some congophilic plaques and vessels. Surprisingly, intracerebral injection of synthetic Aβ resulted so far in only limited Aβ seeding deposition compared to that obtained with Aβ-rich brain extracts. Studies with fresh and aged synthetic Aβ40, Aβ42 and a mixture of Aβ40/42, with levels matching that in AD brain extracts, are ongoing. These studies will unequivocally determine whether Aβ alone is sufficient to induce seeding, and more specifically, which conformational state of Aβ has intrinsic properties to act as a seeding agent.

3.2 Introduction

Misfolding, aggregation and abnormal accumulation of proteins is a common feature of many neurodegenerative diseases such as Alzheimer's disease (AD), Parkinson's disease and Creutzfeldt-Jacob disease. Genetic studies support the view that A fibril formation is a central process in AD pathogenesis (Hardy et al., 1998) and accompanied by neurofibrillary tangle formation, neuron and synapse loss, neuroinflammation, and dementia (Yankner, 1996; Selkoe, 1999; Gotz et al., 2001; Lewis et al., 2001). However, little is known about how protein aggregation and deposition is initiated *in vivo*.

Protein aggregation *in vitro* is dependent on protein concentration and time (Jarrett and Lansbury, 1993; Harper and Lansbury, 1997; Lansbury, 1997). The relatively slow nucleation process of A can be accelerated by introducing an exogenous seed which then leads to rapid A aggregation (Koo et al., 1999). Similar to these *in vitro* findings, seeded aggregation may also determine the onset and progression of amyloid formation in amyloid precursor protein (APP) transgenic mice and in AD.

In the past, there have been many attempts to induce AD neuropathology in animal models by injection of A peptides. Microinjection of fibrillar A into the cerebral cortex of aged rhesus monkeys resulted in profound neuronal loss and tau phosphorylation (Geula et al., 1998). Moreover, amyloid cores, isolated from AD brains, caused neurodegeneration and neurotoxicity when injected into the cortex and hippocampus of rats (Frautschy et al., 1991; Frautschy et al., 1992). However, none of these studies has focused on the mechanism of A polymerization *in vivo*. Only recently has it been described that inoculation of dilute AD brain extracts leads to amyloid fibril formation in young Tg2576 mice (Kane et al., 2000; Walker et al., 2002). Nevertheless, it remains to be clarified which factors initiate and are involved in this seeding process. To this end, we have injected diluted brain extracts from AD patients and from aged APP23 transgenic mice into young APP23 mice prior to plaque formation. Our results indicate that A-containing human and transgenic mouse brain extracts can induce cerebral amyloidosis in APP23 transgenic mice *in vivo*.

3.3 Results

Intracerebral injection of brain extract of aged APP23 mice induces amyloid formation in brains of young APP23 mice similar to that of AD brain extract

To determine whether brain homogenate of aged APP23 mice is as potent in inducing seeding of A as AD brain homogenate, we injected both brain homogenates into the hippocampus of young, 5-month old APP23 mice. These mice were analyzed 4 months later and, similar to the AD extract, the APP23 brain extract induced robust seeding of -amyloid deposits, mainly in the hippocampus (Fig. 1A,B). As expected, intracerebral injection of control patient extract induced only few amyloid deposits (Fig. 1C). We observed no seeded amyloid deposits in APP23 transgenic mice injected with wt brain homogenate or PBS (Fig. 1D,E). Infusion of amyloid-containing APP23 brain homogenate into non-transgenic littermates did not produce A deposition, indicating that the observed immunoreactivity present at 4 months post-infusion was not due to the injected material itself (Fig. 1F).

Seeded amyloid is diffuse and occurs in vessels and plaques

Seeded amyloid exhibits a characteristic distribution pattern along the hippocampal fissure and the granular cell layer of the dentate gyrus, the dorsal lateral geniculate nucleus, in the vasculature of the thalamus and in pial vessels (Fig. 2A-D). In some cases, there was a spread of A deposits along the corpus callosum (Fig. 2A, right hemisphere). The seeded material in the parenchyma was mainly diffuse in nature (Fig. 2 A,C) with some congophilic compact plaques in the hippocampal area (Fig. 2E) and congo red positive vessels in the thalamus (Fig. 2F). Results revealed that not only tissue near the injection site was affected, but also brain regions away from the infusion site.

Seeding of A is time- and concentration-dependent

APP23 mouse brain homogenate was injected into 5-month-old APP23 transgenic mice and analyzed after different time points from 1 to 4 months post-infusion. Immunhistological analysis with an antibody against A revealed no amyloid deposits after 1 month (Fig. 3A). First signs of diffuse seeded amyloid were detectable 2 months

post-infusion (Fig. 3B). Clearly, 3 months after the inoculation of the brain homogenate, the typical seeding pattern was visible (Fig. 3C). The highest number of seeded amyloid plaques was obtained 4 months post-infusion (Fig. 3D). Stereological analysis also confirmed that the amount of Aβ deposition significantly increases with time (ANOVA, $p<0.0001$) (Fig. 3E).

To determine if Aβ concentration in the brain homogenate influences the seeding pattern, we injected different dilutions of the tg brain homogenate (0.5% vs. 10%) and compared the resulting hippocampal amyloid load. Immunohistochemical stainings and stereological analysis both revealed that the 10% extract exceeded seeding obtained with the 0.5% extract by a factor of 2 (Fig. 4A,B,C). Thus, these results clearly demonstrate that seeding of Aβ in APP23 mice is a time- and concentration-dependent process.

Seeded amyloid contains Aβ40 and Aβ42

The next set of experiments were designed to determine the nature of the seeded protein deposition. Aβ was immunoprecipitated from micropunches taken from hippocampus, lateral geniculate nucleus and thalamus out of formalin-fixed prestained tissue (Ikeda et al., 1998) (Fig. 5A-H). These micropunches did not only originate from different brain regions, but they also differed in the type of amyloid they contained. It was clearly visible that the hippocampus showed both, diffuse and compact plaques, in contrast to the only diffuse band in the lateral geniculate nucleus and the mainly congo red positive compact vessels in the thalamus (Fig. 5B,E,H). Western blot analysis revealed the presence of Aβ in the micropunches (Fig. 5C,F,I). There was a negligible amount of Aβ 1-42 detectable in the thalamus with a strong predominance of Aβ1-40 over Aβ1-42 compared to the hippocampus.

Seeding is an extracellular process

Next, we investigated the ultrastructure of Aβ depositions induced by the infusion of amyloid-containing brain extract. Seeded Aβ deposits were mostly of the diffuse type but some compact plaques were located along the granular cell layer in the dentate gyrus (Fig. 6A). Ultrastructural analysis revealed that Aβ deposits were limited to the

extracellular space (ECS) by observation under the electron microscope (Fig. 6B). We found no evidence for intracellular A, supporting the idea that seeded amyloid aggregates are located extracellularly.

Synthetic A injections induce only limited seeding properties

To identify the factors that initiated the seeding process in our model, we were wondering whether A itself may be sufficient to achieve the same degree of seeding seen with A-containing brain extracts. Therefore, we injected synthetic A40 and A42 peptides in APP23 transgenic mice and analyzed them 1 day and 4 months post-infusion. After 1 day up to 1 month, we found no immunhistological evidence of the dilute tg brain extract itself (Fig. 3A). In contrast, we observed big aggregates of the injected synthetic A40 and A42 material, respectively (Fig. 7A,B). After 4 months, a robust seeding pattern was only present in APP23 mice injected with A-containing brain homogenate (Fig. 1A,B). Synthetic A40 and A42 injections resulted in only a limited amount of amyloid deposits and failed to induce the same amount of seeding seen with the brain homogenates (Fig. 7C-F).

3.4 Discussion

Accumulation of highly insoluble protein aggregates is a hallmark of AD and other neurodegenerative diseases and considered to be a critical step in disease pathogenesis (Lansbury, 1997; Koo et al., 1999; Walker and LeVine, 2000). The present study was undertaken to characterize the mechanism that leads to amyloid formation in APP transgenic mice in more detail. First, we have confirmed the previous finding (Kane et al., 2000) that intracerebral injection of AD brain extract into APP transgenic mice induces A deposition. Thus, the AD brain extract provided an extremely potent seeding agent, but the factors that initiated amyloid formation in these mice still remains unknown.

Besides the typical extracellular amyloid deposits and the intracellular neurofibrillary tangles, the AD brain is characterized by a pronounced inflammation with an astroglial and microglial response and the presence of inflammatory cytokines (Itagaki et al., 1989; McGeer et al., 1994; Griffin et al., 1995). Research has shown that inflammation plays a role in plaque formation and it has also been suggested that infectious agents can initiate seeding (Yucesan and Sriram, 2001; Lin et al., 2002). To rule out the latter possibility, we also infused A-containing tg brain homogenate. Our results revealed robust seeding with both, human and tg animal brain homogenate 4 months post-infusion. The control experiments done in wt control littermates confirmed that it was not the tg injectate itself that was detected 4 month after injection.

The formation of A fibrils *in vitro* occurs via seeded polymerization (Jarrett and Lansbury, 1993). A aggregation is dependent upon A concentration and time and initiated by the formation of an oligomeric nucleus that is thought to be the rate-determining step of amyloid deposition (Lansbury, 1997). We have tested this theory of seeding -amyloid *in vivo* and found that seeding occurred after a lag phase of 2 months, which increases over time. In addition, plaque formation in our seeding model appears in a concentration-dependent manner. Thus, we show for the first time that the complex processes associated with misfolding and accumulation of -amyloid protein that emerge from *in vitro* studies are relevant for *in vivo* processes.

Next, we examined the biological properties of the seeded amyloid plaques. APP23 mice develop amyloid plaques and cerebrovascular amyloid throughout the neocortex, hippocampus and to a lesser extent in the thalamus with the majority of cerebrovascular amyloid (Sturchler-Pierrat et al., 1997; Calhoun et al., 1999; Winkler et al., 2001). In the experimental paradigm used here, we always observed a typical seeding pattern with affected brain areas identical to those of aged, untreated APP23 transgenic mice. Thus, not only the hippocampus, but also brain regions further away from the injection site such as thalamus developed seeded plaque formation. This observation is in line with our previous finding that diffusion of A in the extracellular space is an important parameter for cerebral amyloidosis (Meyer-Luehmann et al., 2003). Alternatively, axonal transport has also been proposed as possible explanation for the spread of seeded A pathology in the brain (Walker et al., 2002). Electron microspcopy of the dentate gyrus revealed A deposition located between neuron bodies in the extracellular space, indicating that amyloid formation was induced extracellularly. It has been postulated that amyloid deposits in vessels contain A 40 whereas the longer A 42 form is the predominant component of parenchymal plaques (Joachim et al., 1988; Gravina et al., 1995; Herzig et al., 2003). Western blot analysis confirmed that the seeded material consisted of both A peptides, the A 40 and A 42 depending on the form of aggregates. Thus, with the injection of A -containing brain homogenate we induced seeding of both A species, suggesting that the same initial seeding agent could seed amyloid deposits in the vessels and in brain parenchyma.

Finding out whether A is sufficient to induce seeding or whether this process requires additional factors, is key to the development of therapeutic strategies against cerebral amyloidosis. Previous attempts to generate consistent amyloid deposition causing neurodegeneration via injection studies have produced inconsistent results (Frautschy et al., 1991; Clemens and Stephenson, 1992; Emre et al., 1992; Frautschy et al., 1992; Games et al., 1992; Kowall et al., 1992; Rush et al., 1992; Stein-Behrens et al., 1992; Waite et al., 1992; Sigurdsson et al., 1997). Most of these studies used single injections of A peptides into rodent brains, indicating that the method of A preparation itself like peptide concentration or aggregation state represents a common source of variation. Our data so far favour the possibility of important cofactors because synthetic A 40 and A 42 injections resulted in fewer seeded amyloid deposits than those after tg or AD

brain homogenate infusion. We hypothesize that additional plaque enhancing factors are abundant in the brain homogenates since it has been reported that after immunodepletion of A□, similar seeding still occurs (Younkin, 1995). TGF-□, heparan sulfate proteoglycan, apolipoprotein E and A□43 have been successfully applied in combination with A□40 to promote plaque formation (Snow et al., 1994; Wisniewski et al., 1994; Frautschy et al., 1996; Stephan et al., 2001). Future studies will help to identify cofactors in the brain homogenate that are required for the initiation of cerebral amyloidosis *in vivo*. In particular, the identification of endogenous chaperone molecules for A□ seeding and/or conditions to transform synthetic A□ into a seeding agent for cerebral amyloidosis would give new important insights.

Studies with synthetic A□ at concentrations equivalent to those found in A□ brain extracts are ongoing. In the long run, these studies will hopefully answer the most fundamental question whether A□ itself is the seeding factor. Additionally, ongoing analyzes with fresh and aged samples will reveal what conformational state of A□ is most effective in inducing seeding. Such results will greatly enhance the understanding of A□ aggregation and early amyloidogenesis *in vivo* and will provide new therapeutic targets in the search for compounds that inhibit A□ aggregation.

3.5 Materials and Methods

Tissue extracts and synthetic A□

Human tissue for preparation of extracts was derived at autopsy from cortices of 2 patients who had died of confirmed AD and from a neurological normal control. Animal tissue was derived from neocortices of two APP23 transgenic mice and from a wildtype nontransgenic littermate (Table 1). The tissue samples were fresh frozen and stored at -80°C. Pieces of neocortex were homogenized at 10% (w/v) in sterile PBS, vortexed, sonicated for 5 seconds and centrifuged at 3000x g for 5 minutes. The supernatant was immediately frozen (-80°C) and sometimes further diluted 1:20 to a final concentration of 0.5%.

Synthetic A□40 and A□42 was prepared by resuspending lyophilized A□40 and A□42 (Bachem, Bubendorf, Switzerland) in phosphate-buffered saline (PBS), pH 7,4,

followed by an incubation period of 5 days at 37°C to allow them to aggregate. A was vortexed, sonicated and injected in the same manner as the brain homogenates. Congo red staining confirmed the formation of fibrils.

Western blot analysis

To determine A levels in the brain homogenates used in this study, samples were further diluted 1 to 4 in sample buffer (0,48 M Bis-Tris; 0,21 M Bicine; 1,32 % (w/v) SDS; 20% (w/v) sucrose; 3,33 % (v/v) 2-mercaptoethanol; 0,0053% (w/v) bromophenol blue). Samples were then subjected to 10% Bicine-Tris 8M Urea SDS-PAGE (Wiltfang et al., 1997). Synthetic A 1-40 and 1-42 (Bachem, Bubendorf, Schweiz) were used as controls. Proteins were transferred onto a PVDF membrane and probed with monoclonal antibody 6E10 (dilution 1:500). The secondary antibody was horseradish peroxidase–conjugated goat anti-mouse IgG (Chemicon,Temecula, California). Bands were visualized using SuperSignal (Pierce, Rockford, Illinois) and developed onto Kodak X-OMAT AR film (Rochester, New York).

Stereotaxic surgery

Host mice (5 month-old, males) were anaesthetized with a mixture of ketamine (10 mg/kg body weight) and xylazine (20 mg/kg body weight) in saline. Bilateral stereotaxic injections of 2.5 l brain homogenate were placed with a Hamilton syringe into the neocortex (AP -2.5mm from bregma, L +/-2.0mm, DV -1mm) and hippocampus (AP -2.5mm, L +/-2.0mm, DV -2.5mm), respectively. In an additional experiment 10 mice were injected unilaterally into the cortex and hippocampus. Injection speed was 1.25 l/minute and the needle was kept in place for an additional 2 minutes before it was slowly withdrawn. The surgical area was cleaned with sterile saline and the incision was sutured. As a control, some mice received stab wound lesions (n=5), i.e. the needle was lowered into the brain without injection of any cell suspension.

Histology and immunohistochemistry

Brains were removed and immersion-fixed for 24 h in 4% paraformaldehyde in PBS, then dehydrated overnight in 30% sucrose. After freezing, 25 µm serial coronal sections were cut through the brains on a freezing-sliding microtome and collected in 0.1 M Tris-buffered saline (pH 7.4). Sections were stained with cresyl-violet and immunohistochemically according to previously published protocols (Stalder et al., 1999) with the following antibodies: polyclonal antibody NT12 to Aβ (courtesy of P. Paganetti, Basel, Switzerland) (Schrader-Fischer and Paganetti, 1996); mouse monoclonal antibody 6E10 specific to hAPP/Aβ (Signet Pathology Systems, Inc., Dedham, MA, USA); polyclonal antibodies specific to Aβx-40 (FCA3340) and 42 (FCA3542) (Barelli et al., 1997), rat monoclonal antibody to CD11b (Mac-1; Serotec, Oxford, UK), and mouse monoclonal antibody AT8 which recognizes human and murine tau phosphorylated at S202 and T205 (Goedert et al., 1995). Additional sections were stained with congo red and viewed under cross-polarized light.

Ultrastructural analysis

Some mice (n=3) were deeply anaesthetized and perfused transcardially with fresh 0.1 M phosphate buffered 4% paraformaldehyde (PFA) and 0.5% glutaraldehyde. Brains were postfixed in 4% PFA in 0.1 M phosphate buffer (PB) overnight. The next day they were sectioned on a vibratome and the 40 μm slices were collected in 0.01 M PBS. Immunohistochemistry was performed as described in a previous publication (Stalder et al., 1999) except that they were treated with Triton and DMSO (5, 10 and 20%) instead of H_2O_2 to perforate the cells. The polyclonal antibody, NT12, served as primary antibody against human Aβ (Schrader-Fischer and Paganetti, 1996) and the visible reaction was achieved by means of diaminobenzoidine (DAB). For the background staining, slices were incubated in 0.5% OsO_4 and 6.8% sucrose in PBS. After washing them with PBS, slices were dehydrated in ethanol and acetone and subsequently incubated overnight in Durcupan® ACM (Fluka Chemie GmbH, Buchs SG, CH) according to the instructions of Fluka. Finally, the slices were embedded between a mould parting compound coated slide and coverslipped before incubation at 60°C for 48 hours. Coverslips were removed by means of a glass-cutter and pieces of brain tissue were excised with a scalpel and subsequently glued on Durcupan-blocs in order to

dissect them on the ultramicrotome. Semithin cuts (1 μm) were collected on Superfrost (gel coated) glass slides and some of them were stained with toluidine blue. Ultrathin cuts (at about 80 nm) were collected on standard 200 mesh copper grids (Provac AG, Balzers, FL). Investigation of the ultrastructure in the ultrathin brain samples was done on a Transmission Electron Microscope (Philips EM 900). The high-tension was set to 50 kV in order to achieve an optimal contrast.

Assessment of A in different brain regions using Western blotting

Under deep inhalation anesthesia, mice were sacrificed by decapitation. Hippocampi of unilateral injected mice (n=4) were dissected and homogenized at 10% (w/v) in sterile PBS, vortexed and sonicated for 5 seconds. In another group of animals (n=3), brains were removed and sectioned on a vibratome (400 m thick slices) in ice-cold PBS. Slices were transferred onto a glass slide on ice. With the aid of a dissecting microscope and a Stoelting micropunch device (Stoelting, Wood Dale, IL, USA), micropunches were taken from the hippocampus and thalamus of the injected and uninjected hemisphere, respectively. For comparison, micropunches were also taken from normal APP23 and B6 mice. Punches had a diameter of 0.74 mm and 0.96 mm and thus consisted of 172 g and 362 g wet weight tissue (Palkovits, 1983). To analyze A levels, tissues of five micropunches were combined and human A was immunoprecipitated with antibody 6E10 and protein G Sepharose (Sigma, St Louis, MO, USA). Subsequent SDS-PAGE and blotting was done as described above. Synthetic A 1-40 and A 1-42 (Bachem, Bubendorf; Switzerland) were used as controls.

3.6 References

Barelli H, Lebeau A, Vizzavona J, Delaere P, Chevallier N, Drouot C, Marambaud P, Ancolio K, Buxbaum JD, Khorkova O, Heroux J, Sahasrabudhe S, Martinez J, Warter JM, Mohr M, Checler F (1997) Characterization of new polyclonal antibodies specific for 40 and 42 amino acid-long amyloid beta peptides: their use to examine the cell biology of presenilins and the immunohistochemistry of sporadic Alzheimer's disease and cerebral amyloid angiopathy cases. Mol Med 3:695-707.

Calhoun ME, Burgermeister P, Phinney AL, Stalder M, Tolnay M, Wiederhold KH, Abramowski D, Sturchler-Pierrat C, Sommer B, Staufenbiel M, Jucker M (1999) Neuronal overexpression of mutant amyloid precursor protein results in prominent deposition of cerebrovascular amyloid. Proc Natl Acad Sci U S A 96:14088-14093.

Clemens JA, Stephenson DT (1992) Implants containing beta-amyloid protein are not neurotoxic to young and old rat brain. Neurobiol Aging 13:581-586.

Emre M, Geula C, Ransil BJ, Mesulam MM (1992) The acute neurotoxicity and effects upon cholinergic axons of intracerebrally injected beta-amyloid in the rat brain. Neurobiol Aging 13:553-559.

Frautschy SA, Baird A, Cole GM (1991) Effects of injected Alzheimer beta-amyloid cores in rat brain. Proc Natl Acad Sci U S A 88:8362-8366.

Frautschy SA, Cole GM, Baird A (1992) Phagocytosis and deposition of vascular beta-amyloid in rat brains injected with Alzheimer beta-amyloid. Am J Pathol 140:1389-1399.

Frautschy SA, Yang F, Calderon L, Cole GM (1996) Rodent models of Alzheimer's disease: rat A beta infusion approaches to amyloid deposits. Neurobiol Aging 17:311-321.

Games D, Khan KM, Soriano FG, Keim PS, Davis DL, Bryant K, Lieberburg I (1992) Lack of Alzheimer pathology after beta-amyloid protein injections in rat brain. Neurobiol Aging 13:569-576.

Geula C, Wu CK, Saroff D, Lorenzo A, Yuan M, Yankner BA (1998) Aging renders the brain vulnerable to amyloid beta-protein neurotoxicity. Nat Med 4:827-831.

Goedert M, Jakes R, Vanmechelen E (1995) Monoclonal antibody AT8 recognises tau protein phosphorylated at both serine 202 and threonine 205. Neurosci Lett 189:167-169.

Gotz J, Chen F, van Dorpe J, Nitsch RM (2001) Formation of neurofibrillary tangles in P301l tau transgenic mice induced by Abeta 42 fibrils. Science 293:1491-1495.

Gravina SA, Ho L, Eckman CB, Long KE, Otvos L, Jr., Younkin LH, Suzuki N, Younkin SG (1995) Amyloid beta protein (A beta) in Alzheimer's disease brain. Biochemical and immunocytochemical analysis with antibodies specific for forms ending at A beta 40 or A beta 42(43). J Biol Chem 270:7013-7016.

Griffin WS, Sheng JG, Roberts GW, Mrak RE (1995) Interleukin-1 expression in different plaque types in Alzheimer's disease: significance in plaque evolution. J Neuropathol Exp Neurol 54:276-281.

Hardy J, Duff K, Hardy KG, Perez-Tur J, Hutton M (1998) Genetic dissection of Alzheimer's disease and related dementias: amyloid and its relationship to tau. Nat Neurosci 1:355-358.

Harper JD, Lansbury PT, Jr. (1997) Models of amyloid seeding in Alzheimer's disease and scrapie: mechanistic truths and physiological consequences of the time-dependent solubility of amyloid proteins. Annu Rev Biochem 66:385-407.

Herzig MC, Winkler DT, Burgermeister P, Pfeifer M, Kohler E, Schmidt SD, Danner S, Abramowski D, Bürki K, van Duinen SG, Maat-Schieman MLC, Staufenbiel M, Mathews PM, Jucker M (2004) The APP Dutch mutation targets neuronally produced Abeta to the vasculature. Pogram No 731102003 Abstract Viewer/Itinery Planner Washington, DC: Society for Neuroscience, 2003 CD-ROM.

Ikeda K, Monden T, Kanoh T, Tsujie M, Izawa H, Haba A, Ohnishi T, Sekimoto M, Tomita N, Shiozaki H, Monden M (1998) Extraction and analysis of diagnostically useful proteins from formalin-fixed, paraffin-embedded tissue sections. J Histochem Cytochem 46:397-403.

Itagaki S, McGeer PL, Akiyama H, Zhu S, Selkoe D (1989) Relationship of microglia and astrocytes to amyloid deposits of Alzheimer disease. J Neuroimmunol 24:173-182.

Jarrett JT, Lansbury PT, Jr. (1993) Seeding "one-dimensional crystallization" of amyloid: a pathogenic mechanism in Alzheimer's disease and scrapie? Cell 73:1055-1058.

Joachim CL, Duffy LK, Morris JH, Selkoe DJ (1988) Protein chemical and immunocytochemical studies of meningovascular beta-amyloid protein in Alzheimer's disease and normal aging. Brain Res 474:100-111.

Kane MD, Lipinski WJ, Callahan MJ, Bian F, Durham RA, Schwarz RD, Roher AE, Walker LC (2000) Evidence for seeding of beta -amyloid by intracerebral infusion of Alzheimer brain extracts in beta -amyloid precursor protein-transgenic mice. J Neurosci 20:3606-3611.

Koo EH, Lansbury PT, Jr., Kelly JW (1999) Amyloid diseases: abnormal protein aggregation in neurodegeneration. Proc Natl Acad Sci U S A 96:9989-9990.

Kowall NW, McKee AC, Yankner BA, Beal MF (1992) In vivo neurotoxicity of beta-amyloid [beta(1-40)] and the beta(25-35) fragment. Neurobiol Aging 13:537-542.

Lansbury PT, Jr. (1997) Structural neurology: are seeds at the root of neuronal degeneration? Neuron 19:1151-1154.

Lewis J, Dickson DW, Lin WL, Chisholm L, Corral A, Jones G, Yen SH, Sahara N, Skipper L, Yager D, Eckman C, Hardy J, Hutton M, McGowan E (2001) Enhanced neurofibrillary degeneration in transgenic mice expressing mutant tau and APP. Science 293:1487-1491.

Lin WR, Wozniak MA, Cooper RJ, Wilcock GK, Itzhaki RF (2002) Herpesviruses in brain and Alzheimer's disease. J Pathol 197:395-402.

McGeer PL, Walker DG, Akiyama H, Yasuhara O, McGeer EG (1994) Involvement of microglia in Alzheimer's disease. Neuropathol Appl Neurobiol 20:191-192.

Meyer-Luehmann M, Stalder M, Herzig MC, Kaeser SA, Kohler E, Pfeifer M, Boncristiano S, Mathews PM, Mercken M, Abramowski D, Staufenbiel M, Jucker M (2003) Extracellular amyloid formation and associated pathology in neural grafts. Nat Neurosci 6:370-377.

Mueggler T, Meyer-Luehmann M, Rausch M, Staufenbiel M, Jucker M, Rudin M (2003) Reduced apparent diffusion coefficient (ADC) in the cortex of aged APP23 mice. submitted.

Palkovits M (1983) Punch sampling biopsy technique. Methods Enzymol 103:368-376.

Rush DK, Aschmies S, Merriman MC (1992) Intracerebral beta-amyloid(25-35) produces tissue damage: is it neurotoxic? Neurobiol Aging 13:591-594.

Schrader-Fischer G, Paganetti PA (1996) Effect of alkalizing agents on the processing of the beta-amyloid precursor protein. Brain Res 716:91-100.

Selkoe DJ (1999) Translating cell biology into therapeutic advances in Alzheimer's disease. Nature 399:A23-31.

Sigurdsson EM, Lee JM, Dong XW, Hejna MJ, Lorens SA (1997) Bilateral injections of amyloid-beta 25-35 into the amygdala of young Fischer rats: behavioral, neurochemical, and time dependent histopathological effects. Neurobiol Aging 18:591-608.

Snow AD, Sekiguchi R, Nochlin D, Fraser P, Kimata K, Mizutani A, Arai M, Schreier WA, Morgan DG (1994) An important role of heparan sulfate proteoglycan (Perlecan) in a model system for the deposition and persistence of fibrillar A beta-amyloid in rat brain. Neuron 12:219-234.

Stalder M, Phinney A, Probst A, Sommer B, Staufenbiel M, Jucker M (1999) Association of microglia with amyloid plaques in brains of APP23 transgenic mice. Am J Pathol 154:1673-1684.

Stein-Behrens B, Adams K, Yeh M, Sapolsky R (1992) Failure of beta-amyloid protein fragment 25-35 to cause hippocampal damage in the rat. Neurobiol Aging 13:577-579.

Stephan A, Laroche S, Davis S (2001) Generation of aggregated beta-amyloid in the rat hippocampus impairs synaptic transmission and plasticity and causes memory deficits. J Neurosci 21:5703-5714.

Sturchler-Pierrat C, Abramowski D, Duke M, Wiederhold KH, Mistl C, Rothacher S, Ledermann B, Burki K, Frey P, Paganetti PA, Waridel C, Calhoun ME, Jucker M, Probst A, Staufenbiel M, Sommer B (1997) Two amyloid precursor protein transgenic mouse models with Alzheimer disease-like pathology. Proc Natl Acad Sci U S A 94:13287-13292.

Waite J, Cole GM, Frautschy SA, Connor DJ, Thal LJ (1992) Solvent effects on beta protein toxicity in vivo. Neurobiol Aging 13:595-599.

Walker LC, LeVine H (2000) The cerebral proteopathies: neurodegenerative disorders of protein conformation and assembly. Mol Neurobiol 21:83-95.

Walker LC, Callahan MJ, Bian F, Durham RA, Roher AE, Lipinski WJ (2002) Exogenous induction of cerebral beta-amyloidosis in betaAPP-transgenic mice. Peptides 23:1241-1247.

Wiltfang J, Smirnov A, Schnierstein B, Kelemen G, Matthies U, Klafki HW, Staufenbiel M, Huther G, Ruther E, Kornhuber J (1997) Improved electrophoretic separation and immunoblotting of beta-amyloid (A beta) peptides 1-40, 1-42, and 1-43. Electrophoresis 18:527-532.

Winkler DT, Bondolfi L, Herzig MC, Jann L, Calhoun ME, Wiederhold KH, Tolnay M, Staufenbiel M, Jucker M (2001) Spontaneous hemorrhagic stroke in a mouse model of cerebral amyloid angiopathy. J Neurosci 21:1619-1627.

Wisniewski T, Castano EM, Golabek A, Vogel T, Frangione B (1994) Acceleration of Alzheimer's fibril formation by apolipoprotein E in vitro. Am J Pathol 145:1030-1035.

Yankner BA (1996) Mechanisms of neuronal degeneration in Alzheimer's disease. Neuron 16:921-932.

Younkin SG (1995) Evidence that A beta 42 is the real culprit in Alzheimer's disease. Ann Neurol 37:287-288.

Yucesan C, Sriram S (2001) Chlamydia pneumoniae infection of the central nervous system. Curr Opin Neurol 14:355-359.

3.7 Figure Captions

Figure 1 *Seeding of A by infusion of AD and Tg brain extract into APP23 mice.* Brain extract was intracerebrally injected into the hippocampus of 5 month-old transgenic APP23 hosts (A-E) and nontransgenic littermates of the same age (F). Mice were analyzed 4 month post-injection. A-immunoreactivity (A-F) in the hippocampus of APP23 transgenic mice after injection of AD (A) and tg (B) brain extract (10%) reveals similar amyloid deposits. In contrast, only few and no amyloid deposits are detected when brain extract of a control patient (C) and wildtype mouse (D) into APP23 transgenic mice was injected. Similarly, no amyloid is observed, when PBS was injected (E) into APP23 transgenic mice. No amyloid is observed, when APP23 brain extract is injected into a wildtype nontransgenic mouse (F). Scale bar: 350 μm.

Figure 2 *Regional distribution of seeded amyloid. (*A) A-immunostaining 4 months after bilateral injection of tg brain extract (10%) into APP23 mice with local distribution of seeded amyloid. Shown at a higher magnification (B-D) are the affected areas indicated by white arrowheads. A deposits are predominately located in the dentate gyrus, along the hippocampal fissure (B), as a band in the dorsal lateral geniculate nucleus (C) and in vessels in the thalamus (D). Congo red stained amyloid plaques in the hippocampus (E) and congo red positive vessels in the thalamus (F) are shown. Scale bars: 90 μm (B), 60 μm (C), 25 μm (D), 40 μm (E) and 100 μm (F).

Figure 3 *Time course study of seeding in APP23 mice*. Transgenic mice were analyzed after 1 to 4 months post-infusion. (A) A-immunoreactivity is absent 1 month after injection of APP23 brain extract. (B) In contrast, after 2 months seeded amyloid is clearly detectable, distributed along the hippocampal fissure, dentate gyrus and around blood vessels. The amount of A deposition further increases after 3 and 4 months of incubation (C and D). Scale bar: 350 μm. (E) Stereological analysis confirms a significant

increase in amyloid load with increasing post-injection time (ANOVA, $p<0.0001$).

Figure 4 *0.5% and 10% APP23 brain extract injections into APP23 mice.* (A) Moderate Aβ-immunoreactivity in APP23 transgenic mice injected with 0.5% APP23 brain extract compared to Aβ-immunoreactivity after injections with 10% APP23 brain extract (B). (C) Stereological analysis reveals that 10% APP23 brain extract injections resulted in a significantly higher seeded amyloid load than injections with 0.5 % tg brain extract (ANOVA, $p< 0.01$). Scale bar: 350 μm.

Figure 5 *Aß levels in different seeded brain regions.* (A,D,G) Hippocampus, dorsal lateral geniculate nucleus and thalamus of a 9 month-old APP23 mouse after injection of tg brain extract. Seeding resulted in mostly diffuse amyloid in hippocampus and dorsal lateral geniculate nucleus and vascular amyloid in the thalamus. (B,E,H) Micropunches were taken (asterisks) and subjected to Western blot analysis. Bar is 150 μm. (C,F,I) Western blotting of micropunches. (C) *Lane 1*: Synthetic Aβ40/42. *Lane 2*: punch from injected hippocampus of an APP23 mouse. Note the strong Aβ1-40 and Aβ1-42 band. (F) *Lane1*: Synthetic Aβ40/42. *Lane 2*: punch from the dorsal lateral geniculate nucleus of an APP23 mouse. (I) *Lane 1*: Synthetic Aβ40/42. *Lane 2*: punch from the thalamus of an APP23 mouse. Note the strong Aβ1-40 band and the weak Aβ1-42 suggesting that Aβ 1-40 is the predominant peptide in seeded vessels.

Figure 6 *Ultrastructure of seeded amyloid fibrils in APP23 transgenic mice.* (A) Semithin section of the boundary of the granular cell layer (GCL) and the hilus (H) of the dentate gyrus of an APP23 mouse injected with tg brain extract. Aβ-immunoreactivity seems to be limited to the extracellular space. Amyloid plaques are indicated by asterisks. Counterstaining was achieved by toluidine blue. (B) Ultrastructural analysis reveals that Aβ (indicated by

arrowheads) is located between neurons (N). A closer look suggests that A deposits are located extracellularly. Scale bars: 10 μm (A) and 1 μm (B).

Figure 7 *Synthetic A peptide injections at 1 day and 4 month post-infusion*. One day after the injection of synthetic A42 (A) and A40 (B) peptide a clot of the injected material in the hippocampus is detectable. (C,D) This clot disappeared and only minimal amount of amyloid deposits in the parenchyma and vessels were observed 4 month post-infusion. (E,F) Immunoreactivity is absent in nontransgenic control mice injected with synthetic A40 and A42. Scale bar: 350 μm.

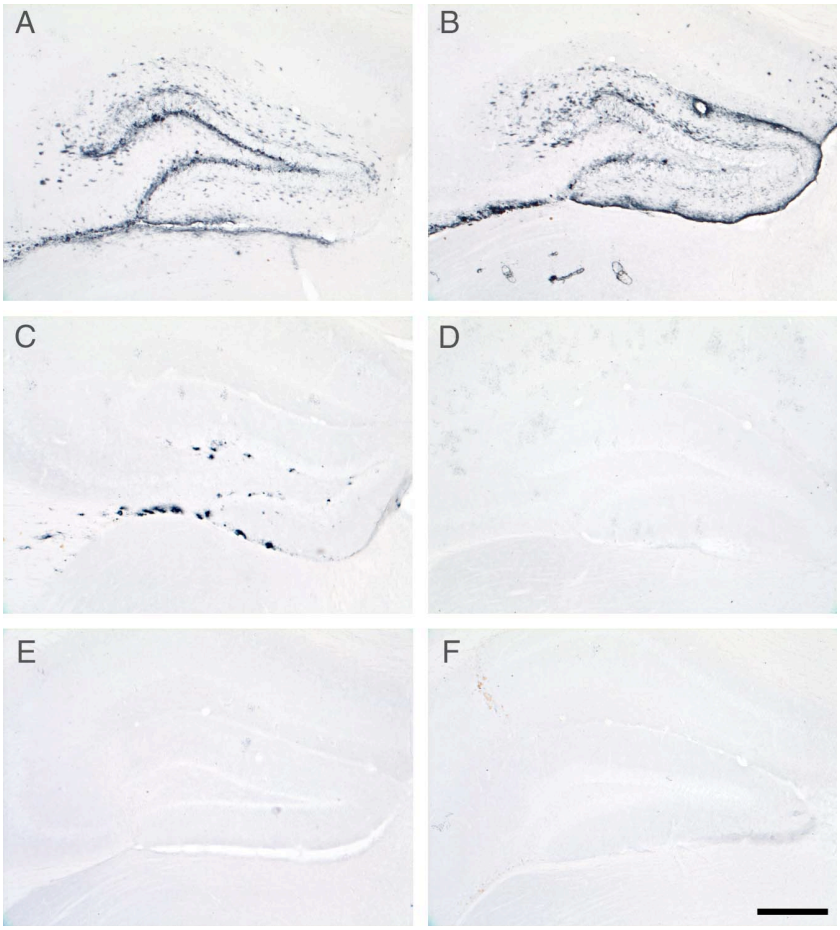

Figure 1

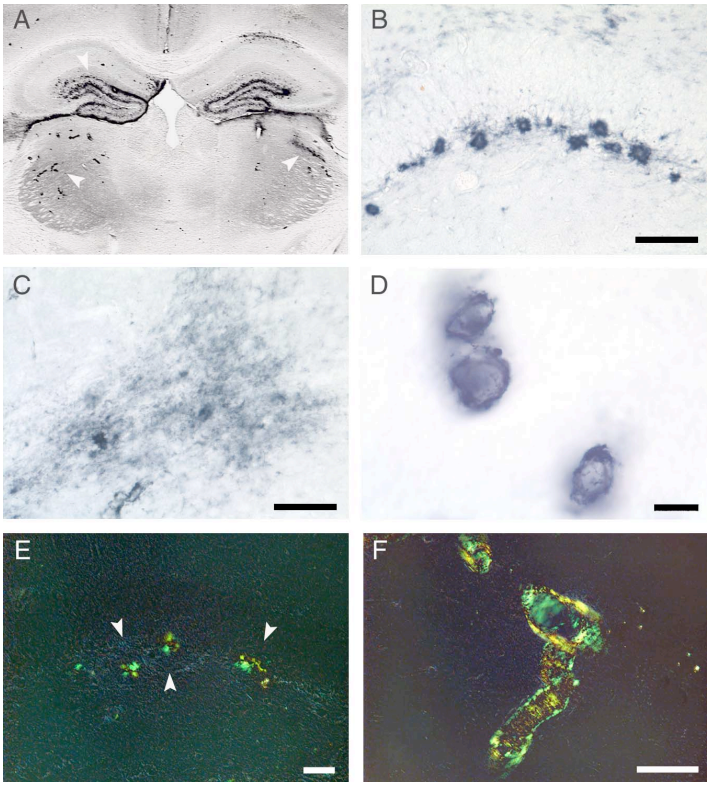

Figure 2

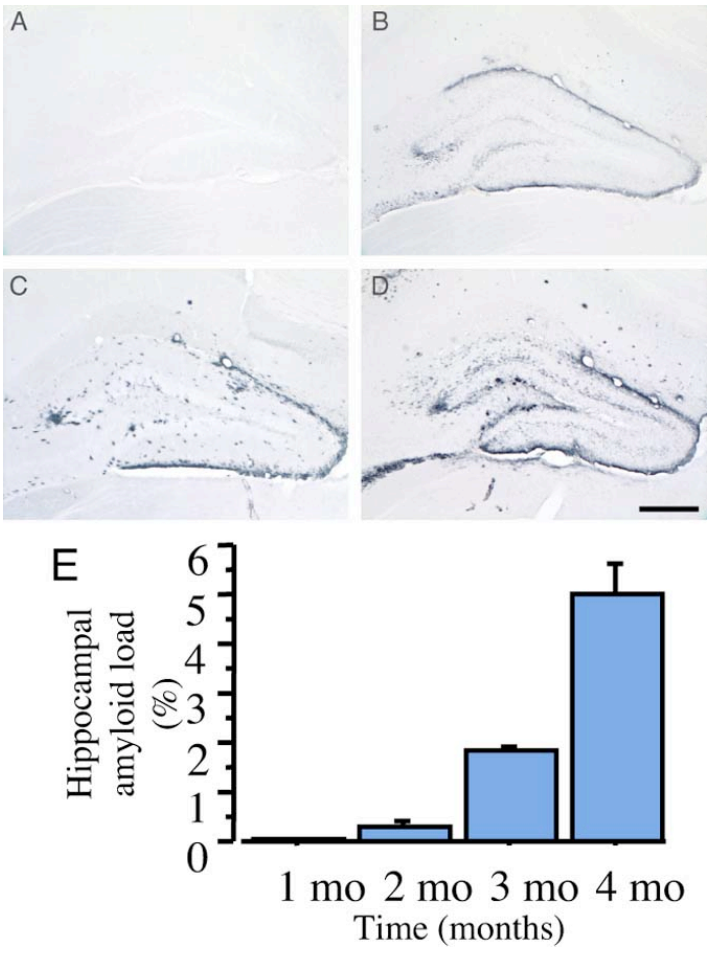
A
B
C
D
E
Hippocampal amyloid load (%)
0
1
2
3
4
5
6
1 mo
2 mo
3 mo
4 mo
Time (months)

Figure 3

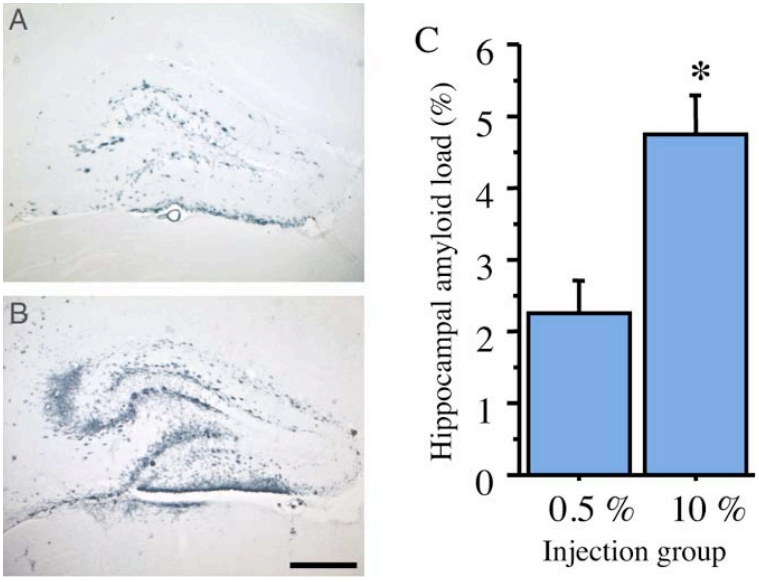
A
B
C
Hippocampal amyloid load (%)
0
1
2
3
4
5
6
*
0.5 %
10 %
Injection group

Figure 4

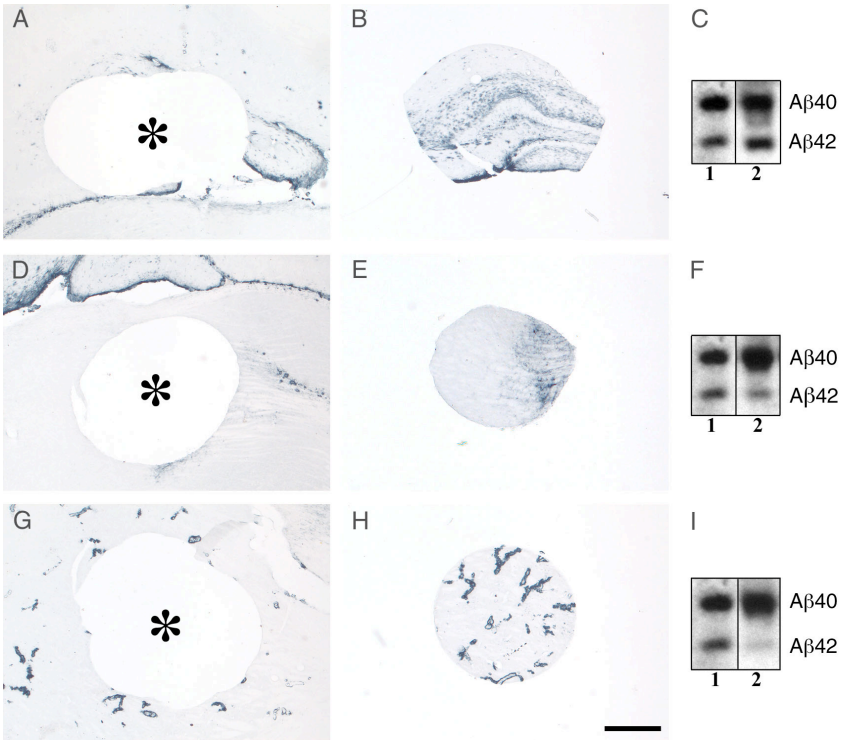

Figure 5

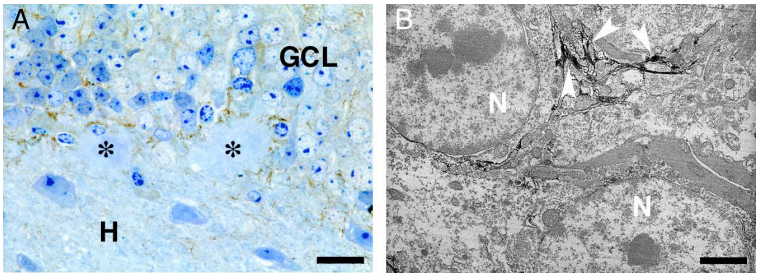

Figure 6

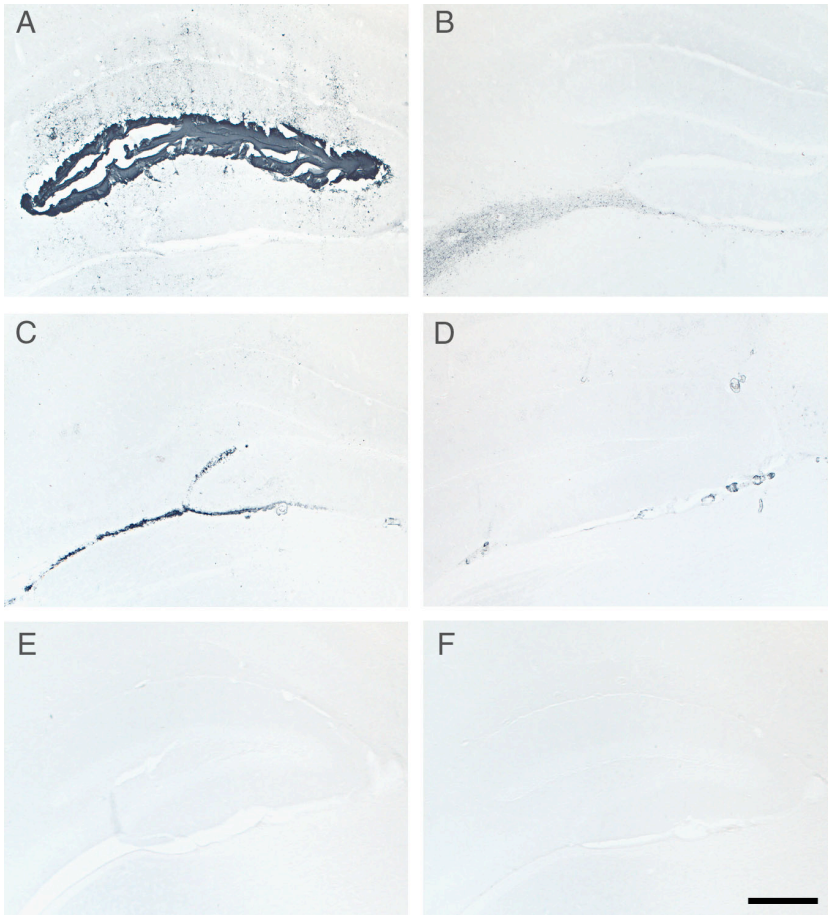

Figure 7

Table 1: *AD and animal brain extracts*

Case	Age	PMD[a]	Sex
AD case #1	85 y	6	f
AD case #2	74 y	7	m
Control #1	95 y	27	f
APP23 #1	26 mo	-	f
APP23 #2	26 mo	-	f
WT #1	24 mo	-	f

[a] Postmortem delay (hours)

Submitted

4. Restricted diffusion in the brain of transgenic mice with cerebral amyloidosis

Thomas Mueggler[1], Melanie Meyer-Luehmann[2,3], Martin Rausch[1], Matthias Staufenbiel[1], Mathias Jucker[2,3] and Markus Rudin[1]

[1]Discovery Technologies, Novartis Institute for Biomedical Research, CH-4002 Basel, Switzerland; [2]Department of Neuropathology, Institute of Pathology, University of Basel, CH-4003 Basel, Switzerland; [3]Department of Cellular Neurology, Hertie-Institute for Clinical Brain Research,University of Tübingen, D-72076 Tübingen, Germany; [4]Nervous System Research, Novartis Institute for Biomedical Research, CH-4002 Basel, Switzerland.

4.1 Abstract

A prominent hallmark of Alzheimer's disease (AD) pathology is cerebral amyloidosis. However, it is not clear how extracellular amyloid-β peptide (Aβ) deposition and amyloid formation compromise brain function and lead to dementia. It has been argued that extracellular amyloid deposition is neurotoxic and/or that soluble Aβ oligomers impair synaptic function. Amyloid deposits, on the other hand, may affect diffusion properties of the brain interstitium with implications for the transport of endogenous signalling molecules during synaptic and/or extrasynaptic transmission. We have used diffusion weighted magnetic resonance imaging (DWI) to study diffusion properties in brains of young (6-month-old) and aged (25-month-old) APP23 transgenic mice and control littermates. Our results demonstrate that fibrillar amyloid deposits and associated gliosis in brains of aged APP23 transgenic mice are accompanied by a reduction in the apparent diffusion coefficient (ADC). This decrease was most pronounced in neocortical areas with a high percentage of congophilic amyloid and was not significant in the caudate putamen, an area with only modest and diffuse amyloid deposition. These findings suggest that extracellular deposition of fibrillar amyloid and/or associated glial proliferation and hypertrophy cause restrictions to interstitial fluid diffusion. Reduced diffusivity within the interstitial space may impair volume transmission and therefore contribute to the cognitive impairment in AD.

4.2 Introduction

Alzheimer's disease (AD) is a late onset progressive neurodegenerative disorder that is characterized by aggregation of the amyloid-β peptide (Aβ) into senile plaques and cerebrovascular amyloid deposits (Selkoe, 1999). In the past few years, several transgenic mouse models of cerebral amyloidosis have been generated through overexpression of mutated amyloid precursor protein (APP). These mice develop amyloid plaques predominately in neocortex and hippocampus and share many features with the amyloid deposits in AD brain (Wong et al., 2002). In both AD patients and transgenic mice amyloid deposits are surrounded by activated microglia, and reactive astrocytes (Frautschy et al., 1998; Stalder et al., 1999; Akiyama et al., 2000). Diffusion and drainage of Aβ throughout the brain interstitium (Meyer-Luehmann et al., 2003) and along perivascular pathways (Calhoun et al., 1999; Weller et al., 2002; Preston et al., 2003) play an important role in the initiation and spread of cerebral amyloidosis. Once deposited, cerebral amyloid and accompanied pathophysiology may influence extracellular space volume and interstitial fluid (ISF) diffusion properties. A reduction in ISF diffusion impairs synaptic and/or extrasynaptic transmission and compromises neuronal function (Nicholson and Sykova, 1998).

The purpose of this study was to assess the apparent diffusion coefficient of water (ADC) in brains of APP23 transgenic mice using diffusion-weighted magnetic resonance imaging (DWI) followed by immunohistochemical analysis of cerebral amyloidosis. DWI is sensitive to microscopic incoherent motion of water molecules and can detect disturbances in ion and water homeostasis by measuring the ADC in the tissue (Moseley et al., 1990; Kauppinen et al., 1993).

4.3 Results

Reduced cortical Apparent Diffusion Coefficient in aged APP23 mice

Changes in ADC values in relation to cerebral amyloidosis were studied in young 6-month-old (pre-depositing) and aged 25-month-old male APP23 mice. We focused on the neocortex, a brain region that exhibits robust age-related congophilic amyloid deposition in aged APP23 mice, and on the caudate putamen, a brain region that develops only few and mostly diffuse amyloid in aged APP23 mice (Sturchler-Pierrat et al., 1997; Calhoun et al., 1998). Three coronal planes were selected and ROI within the caudate putamen and neocortex were defined (Fig. 1).

In caudate putamen ADC-values did not differ among the four groups of mice (Fig. 2). In contrast, ANOVA for the neocortex revealed a significant (age)x(genotype) interaction ($F(3,25) = 7.87$, $p<0.001$). Subsequent Tukey post-hoc analysis revealed that aged APP23 mice had significantly reduced cortical ADC value compared to both age-matched wt mice ($p=0.004$) and young APP23 mice ($p=0.017$) (Fig. 2).

Visual inspection of pseudo-coloured ADC maps (Fig. 3) confirmed the robust decrease in ADC values in the neocortex. However, a closer analysis indicated that this decrease was mainly confined to the medial and dorso-lateral part of the neocortex that contributed to this decrease. Subregional analysis of neocortical structures (medial, dorso-lateral and ventro-lateral part, Fig. 1) revealed substantial ADC reductions in the medial and dorso-lateral cortex of the aged APP23 mice compared to aged-matched control mice ($p=0.012$ and $p=0.001$, respectively). However, ADC-values were not reduced in the ventro-lateral cortex of aged APP23 mice (Table 1).

Amyloid burden and associated gliosis in aged APP23

Following ADC measurements mice were sacrificed and brains analysed for amyloid burden. No amyloid plaques were detected in the 6-month-old male APP23 transgenic mice and in wt control mice (Fig. 4). In the 25-month-old APP23 mice A□-immunostaining revealed prominent amyloidosis throughout the neocortex but only little amyloid in the caudate putamen (Fig. 4D). Quantitative stereological analysis

yielded an amyloid load of 12.6±1.2 % and 3.1±0.7 % in the neocortex and caudate putamen, respectively (Table 2). Combined Congo red and Aβ-immunostaining revealed that the amyloid in the neocortex consisted mainly of compact plaques with some diffuse amyloid, whereas all the amyloid in the caudate putamen was of the diffuse type (Table 2, Fig. 4; Fig.5 A, B). Interestingly, there was also considerable region-specific variability in diffuse amyloid in neocortex with the ventro-lateral cortex exhibiting more diffuse amyloid compared to the dorso-lateral and medial part of the neocortex (Fig. 4).

Amyloid-associated activation of astrocytes and microglia has been associated with congophilic amyloid deposition (Frautschy et al., 1998; Stalder et al., 1999). Consistently, in the neocortex of aged APP23 mice, abundant hypertrophic GFAP-immunoreactive astrocytes closely associated with Aβ deposits were found (Fig. 5C), whereas in the caudate putamen only modest activation of astrocytes occurred (Fig. 5D). No such astrocytosis was found in neocortex and caudate putamen of young APP23 mice and wt control mice (Fig. 5E, F). Similarly, numerous Iba1-immunreactive activated microglia were observed in the vicinity of compact plaques in neocortex of aged APP23 mice (Fig. 5G) while no such microgliosis was seen associated with the diffuse amyloid in the caudate putamen (Fig. 5H). No activated microglial cells were found in young APP23 mice and wt control mice (Fig. 5I, J).

4.4 Discussion

Diffusion-weighted MRI in APP23 mice revealed reduced ADC values in substantial portions of the neocortex of aged 25-month-old transgenic mice when compared to age-matched control mice and to young 6-month-old APP23 mice. In contrast, there was no reduction of ADC values in the caudate putamen of aged APP23 mice compared to age-matched wt control mice or young APP23 mice.

Consistent with previous findings (Sturchler-Pierrat et al., 1997; Calhoun et al., 1998) aged (25-month-old) APP23 mice revealed robust compact A plaques in the neocortex but only few and diffuse amyloid in the caudate putamen. No amyloid deposition was detected in 6-month-old APP23 mice and in wt control mice. This observation suggests that the ADC reduction in the neocortex of the aged APP23 mice is related to the amyloid deposition in the brain of these mice. However, ADC values were not reduced in all cortical areas containing amyloid. For example, the ventro-lateral part of the neocortex did not show a reduction in ADC although this region exhibited a significant amyloid load. Interestingly, this brain region develops more diffuse amyloid compared to the medial and dorso-lateral parts of the neocortex. Thus, one may argue that not only the amount of amyloid deposition but also its nature (compact versus diffuse) determines diffusivity within cerebral tissue.

ADC values *in vivo* are thought to be affected by the net movement of compartmental water, transmembrane molecular exchange and hence an alteration of viscosity in the intra- and extracellular compartments (Le Bihan, 2003). In addition it has been shown that an increase in the tortuosity of the diffusion paths in the extracellular space leads to a decrease of the extracellular ADC (Norris et al., 1994; van der Toorn et al., 1996; Le Bihan, 2003). Increased tortuosity is a characteristic feature of injured brain tissue (Nicholson and Sykova, 1998; Sykova, 2001). A higher tortuosity may be caused by macromolecules produced by activated glial cells that in turn and may form diffusion barriers and lead to an increased tissue viscosity. In addition, astrogliosis and microgliosis, characterized by glial hypertrophy and an increase in glial number, interposed between the cells, can form diffusion barriers (Roitbak and Sykova, 1999). Recently it has been suggested that increased tortuosity during ischemia, and other

conditions involving cellular swelling, is caused by the formation of dead-space microdomains in the extracellular space. Such dead-end pores occluding the gaps between the cells can be formed by astrocytic expansions occurring during gliosis (Hrabetova et al., 2003).

In APP23 mice a substantial inflammatory reaction is observed and exemplified by activated and hypertrophic astrocytes and microglia (Sturchler-Pierrat et al., 1997; Bornemann et al., 2001). This gliosis occurs in vicinity of congophilic compact but not diffuse amyloid (Stalder et al., 1999). Therefore, we investigated whether ADC changes found in the present study might be attributable to the gliosis and not the amyloid deposition *per se*. Indeed, prominent astrocytosis and microgliosis was observed in the neocortex with virtually no gliosis in the caudate-putamen in the aged APP23 mice. An expected difference in gliosis between the medial and dorso-lateral as compared to ventro-lateral neocortex was less obvious but might have been masked by a ceiling effect due to a prominent gliosis throughout all neocortical areas. Thus, the observed ADC reduction in neocortex of aged APP23 mice might be related to the gliosis associated with congophilic compact amyloid plaques.

ADC measurements in AD patients revealed a loss of diffusion anisotropy in the white matter and an increased diffusivity within the hippocampus (Sandson et al., 1999). This ADC increase has been related to a loss of neurons as it correlates with the brain atrophy observed in the hippocampus (Sandson et al., 1999). However, other studies reported similar ADC values in the hippocampus of AD patients copared to controls (Hanyu et al., 1998; Bozzao et al., 2001). These studies suggest that an increase in water diffusivity due to neuron loss is compensated for by the gliosis that accompanies neurodegeneration and the accumulation of senile plaques and tangles. Thus, the present observation of a significant reduction of diffusion in APP23 mice may be explained by the only modest neuron loss but prominent gliosis in the neocortex of these mice (Stalder et al., 1999; Bondolfi et al., 2002).

In summary we have shown that aged 25-month-old APP23 mice exhibit reduced cortical ADC values. This reduction goes in parallel with the formation of compact amyloid plaques and might be attributable to the pronounced gliosis associated with the

amyloid deposits. Alterations of ISF diffusion properties may change synaptic and/or extrasynaptic (volume) transmission and compromise neuronal function (Zoli et al., 1999). Thus, reduced ISF diffusion may contribute to the cognitive deficits previously reported in these mice (Lalonde et al., 2002; Kelly et al., 2003) and to the AD cognitive impairment that has primarily been attributed to neuron and synaptic loss.

4.5 Materials and Methods

Animals

APP23 mice express human mutated APP_{751} under the control of the murine Thy-1 promoter and exhibit the age-related deposition of cerebral amyloid (Sturchler-Pierrat et al., 1997). Mice have been generated on a B6D2 background and have subsequently been backcrossed for more than 8 generations to B6. For the present experiments 6-month-old (n=8) and 25-month-old (n=10) male APP23 mice and age-matched wild-type (wt) control mice were used (n=5 and n=6, respectively).

Animal preparation

All animal experiments were carried out in strict adherence to the Swiss Law for Animal Protection. For MRI experiments animals were anesthetized using an initial dose of 3 % isoflurane (Abbott, Cham, Switzerland) in air:0_2 (2:1), intubated with a tube made from polyethylene (PE; ID/OD: 0.4/0.8 mm) and artificially ventilated using a ventilator for small animals (KTR 3, Alfos Electronics, Biel-Benken, Switzerland). The animals were positioned in a cradle made from Plexiglas and kept anesthetized with 1.4 % isoflurane in air:O_2 (2:1). The animals were paralysed with 10 mg/kg (intravenously, i.v.) gallamine triethiodie (Aldrich, Milwaukee, USA) in saline (3 mg/ml). Blood carbon dioxide levels were monitored transcutaneously ($PtcCO_2$) during the experiment using a paediatric monitoring device (TCM3, Radiometer Copenhagen) as described in previous studies (Mueggler et al., 2001). Body temperature was maintained at 36.5 ±1ºC using warm air, regulated by a rectal temperature probe (MLT415, ADInstruments, Hastings, UK).

MRI protocol

DWI experiments were carried out on a Biospec system equipped with a PharmaScan 70/16 magnet (Bruker Medical System, Ettlingen, Germany) equipped with actively shielded gradient coils (maximum gradient strength: 200 mT/m; rise time <150 μs). The radiofrequency probe was a birdcage resonator of 28 mm inner diameter. A spin-echo sequence with echo time TE=50 ms and repetition time TR=1325 ms comprising a diffusion-sensitizing pulsed gradient pair according to Stejskal and Tanner (1965) was used for DWI (Stejskal and Tanner, 1965). Seven 1 mm thick coronal slices, separated by a 0.6 mm inter-slice gap, were imaged with a field-of-view of 2.56 x 2.56 cm^2 and a matrix size of 128 x 64. For the determination of the apparent diffusion coefficient (ADC) of water, diffusion-encoding was applied along slice gradient direction (for coronal images caudal to rostral) with five b-values covering a range from 0 to 2000 s/mm^2. ADC values are determined by the volume fraction of the various tissue compartments and by the water diffusion coefficient within each of these compartments, which by itself depends on fluid viscosity and tortuosity λ, which is a measure for the steric hindrance of diffusion within a compartment (λ^2 = free diffusion coefficient/ apparent diffusion coefficient).

Tissue preparation and immunohistochemistry

One day after the DW-MRI experiments mice were sacrificed by decapitation under deep inhalation anaesthesia. Brains were removed and post fixed for 4d in 4% paraformaldehyde then dehydrated overnight in 30% sucrose. After freezing, serial coronal sections of 25 μm thickness were cut on a freezing-sliding microtome throughout the brains. Sections were stained immunohistochemically according to previously published protocols (Stalder et al., 1999) by using the avidin-biotin-peroxidase complex method with SG blue as chromogen (Vector Laboratories, Burlingame, CA). The following antibodies were used: polyclonal antibody NT12 to Aβ (Sturchler-Pierrat et al., 1997); polyclonal antibody to glial fibrillary acidic protein (GFAP; Dako, Glostrup, Denmark); and polyclonal antibody to ionized calcium binding adaptor molecule 1 (Iba1; courtesy of Y. Imai, Tokyo, Japan) (Ohsawa et al., 2000). Some sections were double stained with NT12/GFAP and NT12/ Iba1 antibodies using SG blue and DAB as chromogens.

Stereological assessment of amyloid load

Stereological techniques were used to estimate the amyloid load in the neocortex and caudate putamen. To this end every 24th section throughout the brain was immunostained with NT12 yielding 10-12 sections for the neocortex and 4-5 section for the caudate putamen. The amyloid load was determined by calculating the area fraction occupied by amyloid in two-dimensional disectors on a single focal plane (20X objective; 0.45 numerical aperture (Calhoun et al., 1998). One set of sections was double stained for Congo red and NT12 and the percentage of congophilic (compact) amyloid was estimated. Stereological analysis was performed with the aid of Stereologer™ software and a motorized x-y-z stage coupled to a video-microscopy system (Systems Planning and Analysis, Inc., Alexandria, VA). Region definitions of neocortex and caudate putamen were based upon mouse brain atlas (Franklin and Paxinos, 1997).

Data analysis

Quantitative ADC maps were calculated on a pixel-by-pixel basis by linear regression analysis using the model function:

$$ln\left(S(b)/S_0\right) = -b \cdot ADC \qquad [1]$$

where $S(b)$ refers to the pixel intensity as a function of b (the diffusion-sensitizing gradient value), S_0 refers to the signal intensity of the pixel with zero diffusion gradient amplitude (b=0).

Analysis was carried out using in-house developed imaging analysis software (Biomap v3.1). To assess ADC values from different brain structures regions-of-interest (ROIs) were defined (Fig. 1).

Statistics

The ADC values of both hemispheres measured in the DWI experiments were averaged to yield one value per region. Data in text and figures are expressed as mean values ± standard deviation (SD), unless stated otherwise. Multiple comparisons were evaluated by the analysis of variance (ANOVA) and subsequent post-hoc group comparison

(Tukey test). Probability values of less than 0.05 were considered as statistically significant.

Acknowledgements

We would like to thank L. Bondolfi (Basel, Switzerland) and Y. Imai (Tokyo, Japan) for experimental help and antibody donation, respectively. This work was supported by grants to MJ from the Swiss National Science Foundation.

4.6 References

Akiyama H, Barger S, Barnum S, Bradt B, Bauer J, Cole GM, Cooper NR, Eikelenboom P, Emmerling M, Fiebich BL, Finch CE, Frautschy S, Griffin WS, Hampel H, Hull M, Landreth G, Lue L, Mrak R, Mackenzie IR, McGeer PL, O'Banion MK, Pachter J, Pasinetti G, Plata-Salaman C, Rogers J, Rydel R, Shen Y, Streit W, Strohmeyer R, Tooyoma I, Van Muiswinkel FL, Veerhuis R, Walker D, Webster S, Wegrzyniak B, Wenk G, Wyss-Coray T (2000) Inflammation and Alzheimer's disease. Neurobiol Aging 21:383-421.

Bondolfi L, Calhoun M, Ermini F, Kuhn HG, Wiederhold KH, Walker L, Staufenbiel M, Jucker M (2002) Amyloid-associated neuron loss and gliogenesis in the neocortex of amyloid precursor protein transgenic mice. J Neurosci 22:515-522.

Bornemann KD, Wiederhold KH, Pauli C, Ermini F, Stalder M, Schnell L, Sommer B, Jucker M, Staufenbiel M (2001) Abeta-induced inflammatory processes in microglia cells of APP23 transgenic mice. Am J Pathol 158:63-73.

Bozzao A, Floris R, Baviera ME, Apruzzese A, Simonetti G (2001) Diffusion and perfusion MR imaging in cases of Alzheimer's disease: correlations with cortical atrophy and lesion load. AJNR Am J Neuroradiol 22:1030-1036.

Calhoun ME, Wiederhold KH, Abramowski D, Phinney AL, Probst A, Sturchler-Pierrat C, Staufenbiel M, Sommer B, Jucker M (1998) Neuron loss in APP transgenic mice. Nature 395:755-756.

Calhoun ME, Burgermeister P, Phinney AL, Stalder M, Tolnay M, Wiederhold KH, Abramowski D, Sturchler-Pierrat C, Sommer B, Staufenbiel M, Jucker M (1999) Neuronal overexpression of mutant amyloid precursor protein results in prominent deposition of cerebrovascular amyloid. Proc Natl Acad Sci U S A 96:14088-14093.

Franklin KBJ, Paxinos G (1997) The Mouse Brain in Stereotaxic Coordinates. San Diego: Academic Press.

Frautschy SA, Yang F, Irrizarry M, Hyman B, Saido TC, Hsiao K, Cole GM (1998) Microglial response to amyloid plaques in APPsw transgenic mice. Am J Pathol 152:307-317.

Hanyu H, Sakurai H, Iwamoto T, Takasaki M, Shindo H, Abe K (1998) Diffusion-weighted MR imaging of the hippocampus and temporal white matter in Alzheimer's disease. J Neurol Sci 156:195-200.

Hrabetova S, Hrabe J, Nicholson C (2003) Dead-space microdomains hinder extracellular diffusion in rat neocortex during ischemia. J Neurosci 23:8351-8359.

Kauppinen RA, Williams SR, Busza AL, van Bruggen N (1993) Applications of magnetic resonance spectroscopy and diffusion-weighted imaging to the study of brain biochemistry and pathology. Trends Neurosci 16:88-95.

Kelly PH, Bondolfi L, Hunziker D, Schlecht HP, Carver K, Maguire E, Abramowski D, Wiederhold KH, Sturchler-Pierrat C, Jucker M, Bergmann R, Staufenbiel M, Sommer B (2003) Progressive age-related impairment of cognitive behavior in APP23 transgenic mice. Neurobiol Aging 24:365-378.

Lalonde R, Dumont M, Staufenbiel M, Sturchler-Pierrat C, Strazielle C (2002) Spatial learning, exploration, anxiety, and motor coordination in female APP23 transgenic mice with the Swedish mutation. Brain Res 956:36-44.

Le Bihan D (2003) Looking into the functional architecture of the brain with diffusion MRI. Nat Rev Neurosci 4:469-480.

Meyer-Luehmann M, Stalder M, Herzig MC, Kaeser SA, Kohler E, Pfeifer M, Boncristiano S, Mathews PM, Mercken M, Abramowski D, Staufenbiel M, Jucker M (2003) Extracellular amyloid formation and associated pathology in neural grafts. Nat Neurosci 6:370-377.

Moseley ME, Cohen Y, Mintorovitch J, Chileuitt L, Shimizu H, Kucharczyk J, Wendland MF, Weinstein PR (1990) Early detection of regional cerebral ischemia in cats: comparison of diffusion- and T2-weighted MRI and spectroscopy. Magn Reson Med 14:330-346.

Mueggler T, Baumann D, Rausch M, Rudin M (2001) Bicuculline-induced brain activation in mice detected by functional magnetic resonance imaging. Magn Reson Med 46:292-298.

Nicholson C, Sykova E (1998) Extracellular space structure revealed by diffusion analysis. Trends Neurosci 21:207-215.

Norris DG, Niendorf T, Leibfritz D (1994) Health and infarcted brain tissues studied at short diffusion times: the origins of apparent restriction and the reduction in apparent diffusion coefficient. NMR Biomed 7:304-310.

Ohsawa K, Imai Y, Kanazawa H, Sasaki Y, Kohsaka S (2000) Involvement of Iba1 in membrane ruffling and phagocytosis of macrophages/microglia. J Cell Sci 113 (Pt 17):3073-3084.

Preston SD, Steart PV, Wilkinson A, Nicoll JA, Weller RO (2003) Capillary and arterial cerebral amyloid angiopathy in Alzheimer's disease: defining the perivascular route for the elimination of amyloid beta from the human brain. Neuropathol Appl Neurobiol 29:106-117.

Roitbak T, Sykova E (1999) Diffusion barriers evoked in the rat cortex by reactive astrogliosis. Glia 28:40-48.

Sandson TA, Felician O, Edelman RR, Warach S (1999) Diffusion-weighted magnetic resonance imaging in Alzheimer's disease. Dement Geriatr Cogn Disord 10:166-171.

Selkoe DJ (1999) Translating cell biology into therapeutic advances in Alzheimer's disease. Nature 399:A23-31.

Stalder M, Phinney A, Probst A, Sommer B, Staufenbiel M, Jucker M (1999) Association of microglia with amyloid plaques in brains of APP23 transgenic mice. Am J Pathol 154:1673-1684.

Stejskal EO, Tanner JE (1965) Spin diffusion measurements: spin echoes in the presence of a time-dependent field gradient. J Chem Phys 42:288-292.

Sturchler-Pierrat C, Abramowski D, Duke M, Wiederhold KH, Mistl C, Rothacher S, Ledermann B, Burki K, Frey P, Paganetti PA, Waridel C, Calhoun ME, Jucker M, Probst A, Staufenbiel M, Sommer B (1997) Two amyloid precursor protein transgenic mouse models with Alzheimer disease-like pathology. Proc Natl Acad Sci U S A 94:13287-13292.

Sykova E (2001) Glial diffusion barriers during aging and pathological states. Prog Brain Res 132:339-363.

van der Toorn A, Sykova E, Dijkhuizen RM, Vorisek I, Vargova L, Skobisova E, van Lookeren Campagne M, Reese T, Nicolay K (1996) Dynamic changes in water ADC, energy metabolism, extracellular space volume, and tortuosity in neonatal rat brain during global ischemia. Magn Reson Med 36:52-60.

Weller RO, Yow HY, Preston SD, Mazanti I, Nicoll JA (2002) Cerebrovascular disease is a major factor in the failure of elimination of Abeta from the aging human brain: implications for therapy of Alzheimer's disease. Ann N Y Acad Sci 977:162-168.

Wong PC, Cai H, Borchelt DR, Price DL (2002) Genetically engineered mouse models of neurodegenerative diseases. Nat Neurosci 5:633-639.

Zoli M, Jansson A, Sykova E, Agnati LF, Fuxe K (1999) Volume transmission in the CNS and its relevance for neuropsychopharmacology. Trends Pharmacol Sci 20:142-150.

4.7 Figure Captions

Figure 1 Coronal sections through mouse brain (1.6, 0, -1.6 mm anterior to Bregma) with regions-of-interest (ROI) selected for quantitative data analysis indicated. ROI 1 was defined for the lateral and medial caudate putamen. ROI 2 was defined for neocortex. In a subsequent analysis ROI 2 was further subdivided into medial (2a) cortex; dorso-lateral cortex (2b), and ventro-lateral cortex (2c).

Figure 2 Apparent diffusion coefficient (ADC) of water in neocortex (ROI 1) and caudate putamen (ROI 2) of 6- and 25-month-old APP23 mice and age-matched littermates (wt). Data reveal a significant reduction of cortical ADC in aged APP23 animals. Values are given as mean ±SD. *=$p<0.05$; **=$p<0.01$.

Figure 3 Colour-coded representative ADC maps of a coronal brain section at the level of Bregma for a 6- (A) and 25-month-old APP23 mouse (D) and age-matched controls (6 months (B), 25 months (C)). ADC values given as 10^{-6} cm^2s^{-1}. A significant ADC decline, colour-coded in blue, is detectable in the medial and dorso-lateral cortex of aged APP23 mice. In the dorso-lateral cortex of the 6-month-old APP23 mouse few areas with reduced ADC are visible.

Figure 4 Histological analysis of A deposition in APP23 and wt mice. A immunostaining with the NT12 antibody shows no amyloid deposits in young wt mice (A), young APP23 transgenic mice (B) and aged wt mice (C), while aged APP23 transgenic mice displayed massive amyloid deposits predominantly in neocortex (D).Histological analysis of A deposition in APP23 and wt mice. A immunostaining with the NT12 antibody shows no amyloid deposits in young wt mice (A), young APP23 transgenic mice (B)

and aged wt mice (C), while aged APP23 transgenic mice displayed massive amyloid deposits predominantly in neocortex (D).

Figure 5 Activation of plaque-associated astro- and microglia in 25-month-old APP23 transgenic mice. In aged APP23 neocortex massive compact amyloid plaque formation was observed (A) whereas in the caudate putamen only diffuse plaques was found (B). A related astrocytosis was extensive around plaques in the neocortex (C) compared to the weakly GFAP-positive astroglia in the caudate putamen (D) and in wt control mice (E,F). A deposits in the neocortex of 25-month-old APP23 mice were also associated with activated and Iba1-immunoreactive microglia (G). In contrast no activation of microglia was observed in the caudate putamen (H) and neocortex and caudate putamen of wt control mice (I, J). Bars are 100μm (A,B) and 60μm (C-J).

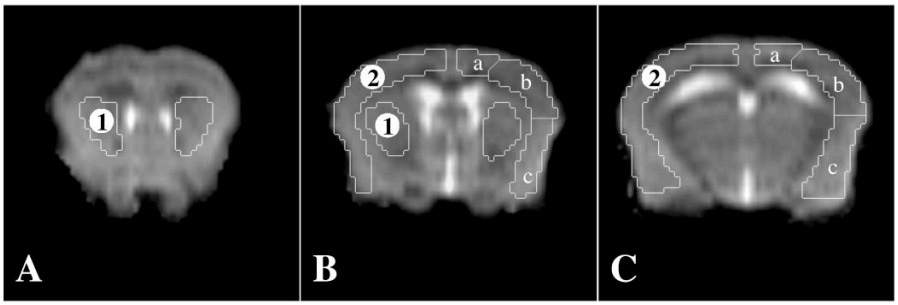

Figure 1

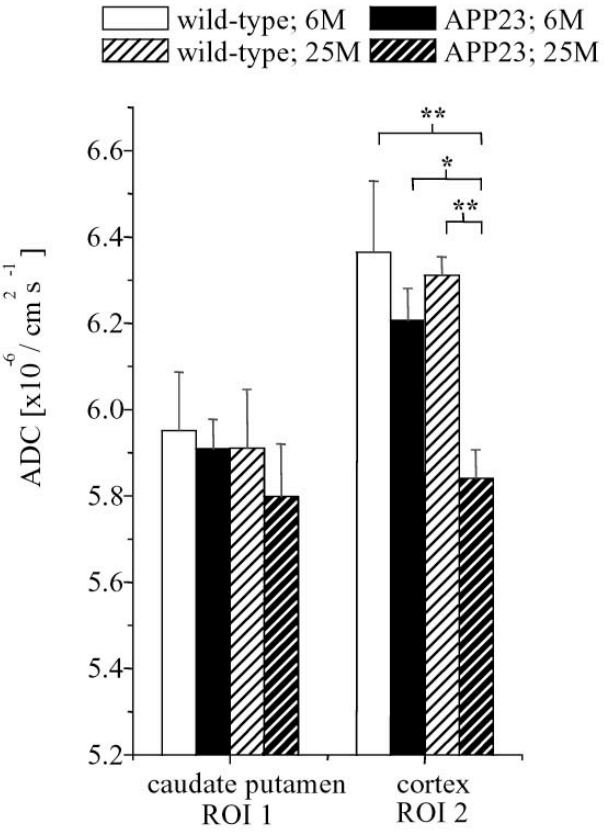
wild-type; 6M
APP23; 6M
wild-type; 25M
APP23; 25M
ADC [x10 -6 / cm 2 s -1]
6.6
6.4
6.2
6.0
5.8
5.6
5.4
5.2
**
*
**
caudate putamen
ROI 1
cortex
ROI 2

Figure 2

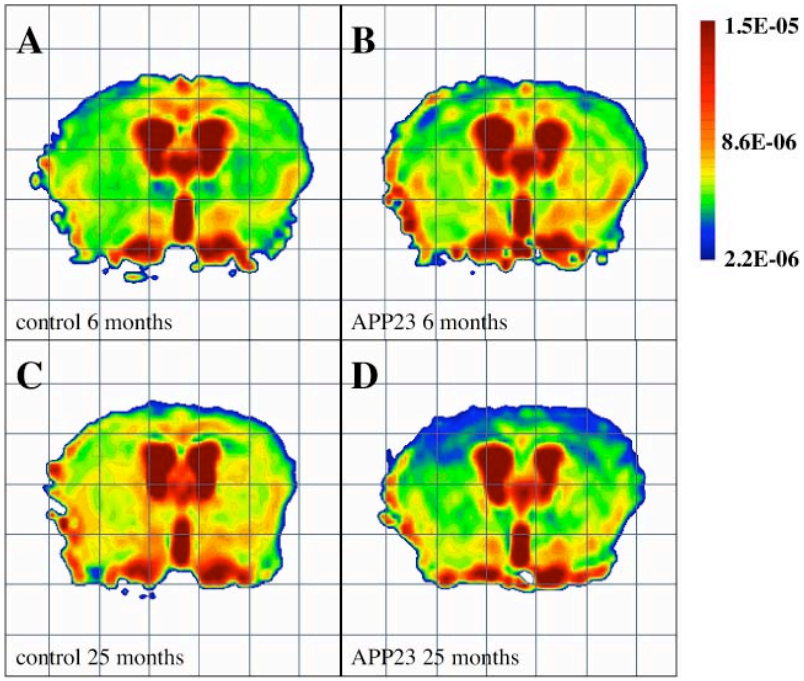
A
B
C
D
control 6 months
APP23 6 months
control 25 months
APP23 25 months
1.5E-05
8.6E-06
2.2E-06

Figure 3

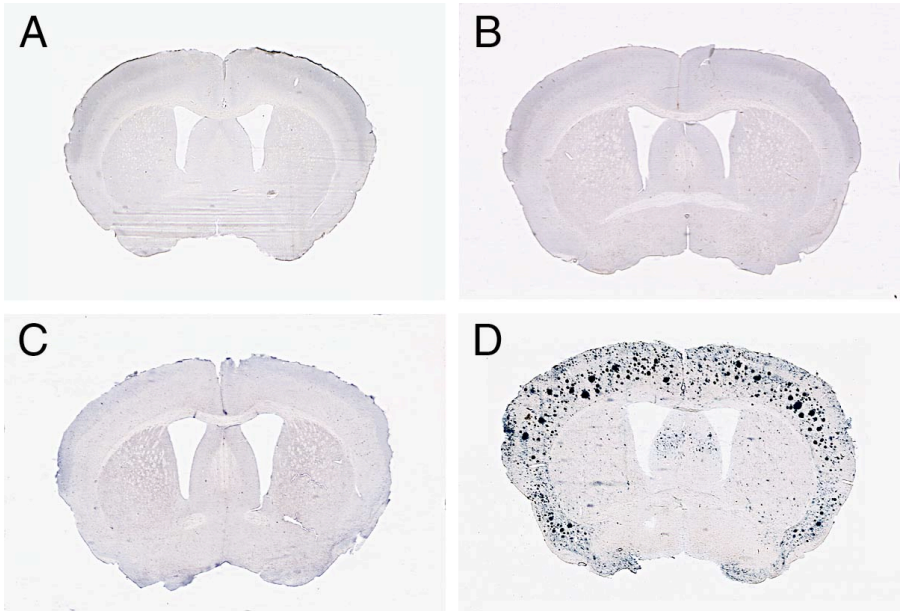

Figure 4

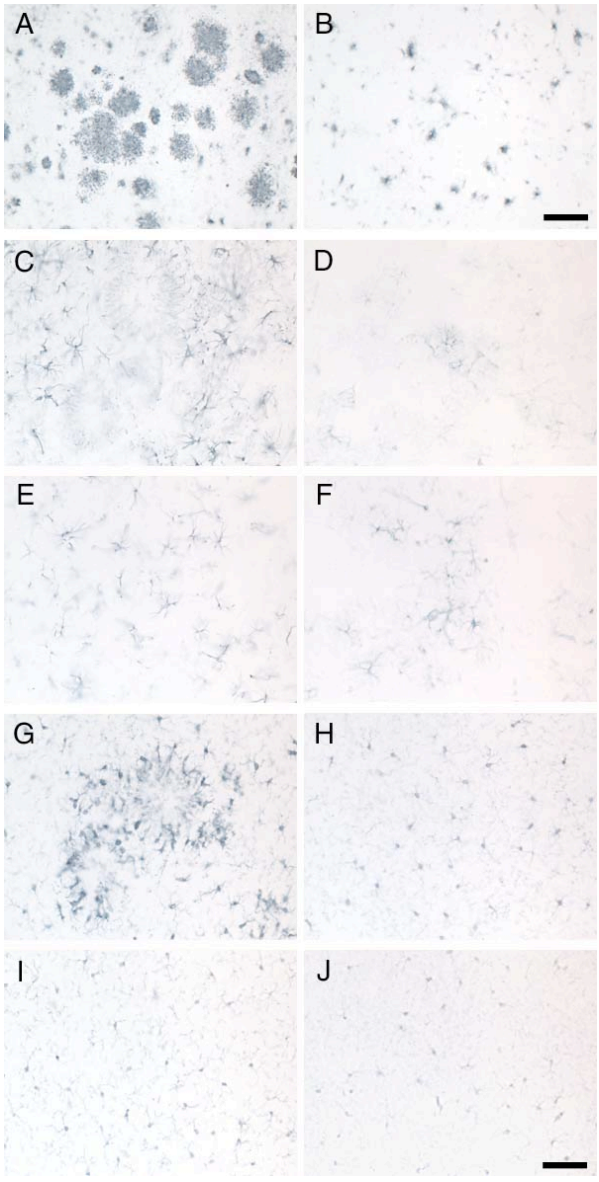

Figure 5

Table 1 ADC values for different brain regions of wild-type control and APP23 mice

	6 months				25 months			
	wildtype N=5		APP23 N=8		wildtype N=6		APP23 N=10	
region analyzed	mean	SD	mean	SD	mean	SD	mean	SD
striatum	**5.95**	0.3	**5.9**	0.19	**5.91**	0.57	**5.79**	0.43
ventro-lateral cortex	**6.96**	0.43	**6.91**	0.22	**6.88**	0.22	**6.86**	0.34
dorso-lateral cortex	**5.61**	0.45	**5.47**	0.25	**5.8**	0.21	**5.09**$^{\S, \&}$	0.33
medial cortex	**6.52**	0.54	**6.23**	0.38	**6.25**	0.29	**5.57**$^{\S, \&, *}$	0.37

Significant differences ($p<0.05$) are indicated (for p values see text). Aged APP23 compared to 6-month-old control§, 25-month-old control$^{\&}$ and 6-month-old APP23* (ANOVA Tukey test). Diffusion coefficient calculated as $x10^{-6}$ cm^2s^{-1}

Table 2 Amyloid load in 25-month-old APP23 mice

region analyzed		cortex		caudate putamen	
		mean [%]	SEM	mean [%]	SEM
total amyloid load		**12.6**	1.2	**3.2**	0.7
composed of	A. compact plaques	**92.8**	1.1	**0**	
	B. diffuse plaques	**7.2**	1.1	**100**	

5. Conclusion

This thesis aims at understanding some of the underlying mechanisms of plaque formation and associated neurodegeneration in cerebral amyloidosis. Although the generation of A and the processing of its precursor APP has been studied extensively at the molecular and cellular level, it still remains a mystery how neuronally derived A propagates and forms fibrillary amyloid deposition *in vivo* and what factors influence this pathological process. The availablilty of transgenic mice that reflect the neuropathological spectrum of AD enabled neuroscientists to explore the relationship between the pathological features, biochemical abnormalities, and memory dysfunctions occurring in the human AD brain.

In this thesis, several methods have been applied in order to identify the mechanisms of cerebral A accumulation *in vivo*. As a first attempt to investigate early stages of abnormal protein deposition and related neurotoxicity, we have used neural transplantation techniques. These experiments have shown that neuronally produced A diffuses over considerable distances in the extracellular space of the brain before it aggregates, deposits extracellularly and finally causes neurodegeneration. There has been a long debate in the field about the origin of plaques and a longstanding question in AD research is whether amyloid formation occurs intra- or extracellularly. Our experiments clearly indicate that cerebral amyloidosis does not require locally generated intracellular A to initiate A deposition. We conclude that the properties of the host influence plaque formation in the transplant and that extracellular amyloid can cause neurodegeneration in the absence of intracellular A production.

The second part of the present study has focused on the identification of factors which initiate or are involved in the seeding process and that induce A fibril formation in APP23 transgenic mice and in AD patients. Preliminary results indicate that cerebral amyloidosis can be induced by the infusion of A-containing brain extract into APP23 transgenic mice, and that this seeded polymerization of A is both time- and concentration-dependent. Interestingly, the infusion of synthetic A peptide has so far failed to induce amyloid seeding. Future experiments will have to examine whether A itself is the seeding factor, and in particular, what A conformation is important to

initiate plaque formation *in vivo*. The identification of the seeding agent and cofactors would open new therapeutic strategies to prevent cerebral A deposition in diseases such as AD.

The findings presented above highlight the importance of transport or diffusion of soluble A in neural tissue as a major factor implicated in amyloid formation and neurotoxicity. In order to characterize A diffusion properties in more detail, non-invasive diffusion–weighted MRI measurements have been applied to APP23 transgenic mice in the final part of this thesis. The results strongly suggest that amyloid deposition and the associated gliosis build a hindrance in the brain tissue leading to decreased interstitial fluid diffusion properties, which may finally change synaptic and extrasynaptic transmission. Diffusion measurements by MRI would therefore be a valuable tool to detect early alterations in the diffusion properties in AD patients.

What can we learn from these studies? The discovery of the underlying origin of cerebral amyloidosis is important in view of the increasing relevance of protein misfolding diseases for the aging society. Further progress on the key question about the mechanism of plaque formation and associated neurodegeneration in cerebral amyloidosis discussed in this thesis may also shed some light on other protein misfolding diseases. Uncovering some of the fundamental mechanisms of cerebral amyloidosis will give us the opportunity to develop new therapeutic strategies in the future to cure these devastating diseases.

6. Abbreviations

A□	□-amyloid
AD	Alzheimer's disease
ADC	apparent diffusion coefficient
ALS	amyotrophic lateral sklerosis
APP	amyloid precursor protein
APOE	apolipoprotein E
CBF	cerebrovascular blood flow
CSF	cerebrospinal fluid
CAA	cerebral amyloid angiopathy
DWI	diffusion weighted magnetic resonance imaging
ECS	extracellular space
FAD	familial Alzheimer's disease
HD	Huntington's disease
IDE	insulin degrading enzyme
ISF	interstitial fluid
MRI	magnetic resonance imaging
NFT	neurofibrillary tangle
NSAID	non-steroidal anti-inflammatory drugs
PD	Parkinson's disease
PrP	prion protein
PS	presenilin
SAP	serum amyloid P component
TSE	transmissible spongiform encephalopathy

7. Curriculum Vitae

Personal Data

Name:	Melanie Meyer-Lühmann
Address:	Kartäuserstr.48 79102 Freiburg Germany
Email:	M.Meyer-Luehmann@unibas.ch
Date of Birth:	May 11, 1974
Nationality:	German

Education

Since June 2000:	PhD student at the University of Basel, Neuroscience PhD program.
1999-2000:	Diploma thesis at the University of Michigan at Ann Arbor, USA.
1993-1999:	Graduate student in Biology at the University of Freiburg, Germany.
1984-1993:	High school in Lahr, Germany. Degree: "Allgemeine Hochschulreife".
1980-1984:	Elementary school in Heiligenzell, Germany.

8. Bibliography

Peer Reviewed Journal Articles

1. **Meyer-Luehmann, M.,** Thompson, J. F., Berridge, K. C., and Aldridge, J. W. (2002). Substantia nigra pars reticulata neurons code initiation of a serial pattern: implications for natural action sequences and sequential disorders.
Eur J Neurosci *16*, 1599-1608.

2. **Meyer-Luehmann, M.,** Stalder, M., Herzig, M. C., Kaeser, S. A., Kohler, E., Pfeifer, M., Boncristiano, S., Mathews, P. M., Mercken, M., Abramowski, D., Staufenbiel, M. and Jucker, M. (2003). Extracellular amyloid formation and associated pathology in neural grafts.
Nat Neurosci *6*, 370-377.

3. Mueggler, T., **Meyer-Luehmann, M.,** Rausch, M., Staufenbiel, M., Jucker, M., and Rudin, M. (2003). Reduced cortical apparent diffusion coefficient in amyloid precursor protein transgenic mice. Submitted

Presentations

Abstracts

1. **Meyer-Luehmann, M.,** Thompson, J.F., Berridge, K. C., and Aldridge, J.W. (2000). Neuronal activity in the substantia nigra pars reticulata codes the onset of grooming sequences in rats. Soc Neurosci Abstr. 26, 360.10.

2. Hadden, L.E., **Meyer-Luehmann, M.,** Berridge, K.C., Aldridge, J.W. (2000). Effective connectivity between neurons in the substantia nigra pars reticulata is modulated by grooming behavioral state. Soc Neurosci Abstr. 26, 360.9.

3. **Meyer-Luehmann, M.,** Stalder M., Pfeifer M., Boncristiano S., Abramowski D., Staufenbiel, M., Jucker, M. (2001). Wildtype grafts in young APP23 transgenic mice develop amyloidosis prior to amyloid plaque formation in the host. Soc Neurosci Abstr. 27, 355.1.

4. Mazel, T., Antonova, T., **Meyer-Luehmann, M.,** Staufenbiel, M., Jucker, M., Sykova, E. (2001). Extracellular space diffusion parameters are altered in the brain of the APP23 mouse model of Alzheimer's Disease. Soc Neurosci Abstr. 27, 860.3.

5. Jucker, M., Stalder, M., **Meyer-Luehmann, M.,** Pfeifer, M., Kaeser, S. A., Staufenbiel, M. (2001). Mechanism of cerebral amyloidosis in transgenic mice. J Neurochem 78 (Suppl. 1), 207.

6. **Meyer-Luehmann, M.,** Neuenschwander, A., Kaeser, S. A., Frey, P., Jaton, A. L., Vigouret, J. M., Walker, L. C., Staufenbiel, M., Jucker, M. (2002). Seeding of beta-amyloid in APP23 transgenic mice. 8th International Conference on Alzheimer`s Disease and Related Disorders, Stockholm. Neurobiol Aging 23, S192, 719.

7. **Meyer-Luehmann, M.,** Walker, L. C., Neuenschwander, A., Kaeser, S. A., Bolmont, T., Ghiso, J., Frey, P., Jaton, A. L., Vigouret, J. M., Staufenbiel, M., Jucker, M. (2003). Initiation of amyloid deposition in APP23 transgenic mice. Soc Neurosci Abstr. 29, 731.11.

Invited Presentations

1. **Meyer-Luehmann, M.** : Extracellular amyloid formation causes neurodegeneration. 1st Symposia of the Hertie-Institute of Clinical Brain Research, Tübingen, 2003.

2. **Meyer-Luehmann, M.** : Mechanism of cerebral amyloidosis. Departmental lecture series, Institute of Anatomy, Basel, 2003.

3. Meyer-Luehmann, M. : Initiation and spread of cerebral amyloidosis in transgenic mice. Alzheimer meeting Eltem, Neurex, Basel, 2003.

During my studies and PhD thesis, lectures and seminars of the following persons were taken: S. Arber, Y.-A. Barde, B. Bettler, H.-R. Brenner, M. Burger, P. Caroni, A. Gratwohl, N. Hynes, M. Jucker, J. Kapfhammer, A. Lüthi, A. Lüthi, A. Matus, T. Meier, A. Merlo, H. Moch, D. Monard, C. Moroni, D. Oertli, P. Piguet, U. Müller, H. Reichert, C. Rochlitz, M. Rudin, M. Rüegg, N. Schaeren-Wiemers, G. Spagnioli and E. Stöckli.