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Glial Cells in Alzheimer's Disease Models

Doctoral dissertation

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ABSTRACT

Alzheimer's disease (AD) is a progressive neurodegenerative disorder leading to severe dementia. The major histopathological hallmarks of AD include extracellular plaques composed of aggregated beta-amyloid (Aβ), intracellular inclusions of abnormally phosphorylated tau protein and neuroinflammation as manifested by the recruitment and activation of microglia and astrocytes around Aβ plaques. However, the exact role of glial cells in the development of AD neuropathology remains elusive. Microglial cells are known to constantly renew by bone marrow (BM) derived monocytes, but the specific contribution of these cells to AD pathology has never been addressed before. The present study was carried out to investigate the role of glial cells in AD related neuroinflammation.

Novel BM transplantation techniques were applied to assess the role of BM-derived cells in transgenic AD mouse models. AD-like neuropathology significantly increased the infiltration of BM-derived cells into the brain. These cells displayed microglial markers and associated with Aβ deposits. Local inflammation induced by lipopolysaccharide injection further enhanced the infiltration with concomitant clearance of brain Aβ burden. Interestingly, a known anti-inflammatory drug minocycline, inhibited the activation of microglia and prevented the infiltration of BM-derived cells in the brain without inhibiting the clearance of Aβ burden.

The effect of direct Aβ exposure *in vivo* was applied to determine the role of inflammation in Aβ induced behavioral deficits. An intracerebroventricular infusion of Aβ resulted in behavioural deficits in mice and rats in the absence of clear neuroinflammation and Aβ plaque deposition. The level of Cu,Zn superoxide dismutase-1 was decreased in the Aβ infused mice suggesting a compromised antioxidant defence system. In addition, in Aβ infused rats, some of Aβ immunoreactivity was associated with astrocytes and the levels of metabolites in cerebrospinal fluid were decreased, suggesting altered energy metabolism.

Long term treatment of AD transgenic mice with another anti-inflammatory agent, pyrrolidine dithiocarbamate (PDTC), significantly ameliorated the cognitive decline independent of the ability of PDTC to inhibit markers of microglial activation and inflammation. The brain Aβ burden remained also unchanged. The protective effect of PDTC was mediated via inhibition of glycogen synthase kinase-3β, resulting a decrease in the phosphorylation of tau in hippocampal neurons and an increase in astrocytic glutamate transporter-1.

The results of this thesis show that BM-derived cells contribute to AD-related microglial recruitment and activation therefore offering a potential therapeutic target for the treatment of AD. In addition, our results support the hypothesis that Aβ may have direct effects on neuronal functions, and the modulation of the deleterious effects of Aβ may result in improvement in cognition independent of neuroinflammation and Aβ deposition.

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ABBREVIATIONS

Aβ	beta-amyloid
ACT	α1-antichymotrypsin
AD	Alzheimer's disease
ADDL	Aβ-derived diffusible ligand
APLP	APP like protein
apo E	apolipoprotein E
APP	amyloid precursor protein
BACE	β-site APP cleaving enzyme
BBB	blood brain barrier
BM	bone marrow
BrdU	bromo deoxyuridine
ChAT	choline acetyltransferase
CNS	central nervous system
CSF	cerebrospinal fluid
CTF	C-terminal fragment
ER	endoplasmic reticulum
eGFP	enhanced green fluorescent protein
FAD	familial Alzheimer's disease
FBS	fetal bovine serum
FTDP-17	frontotemporal dementia and parkinsonism linked to chromosome 17
GFAP	glial fibrillary acidic protein
GLT-1	glutamate transporter-1
GSH	glutathione
GSK-3	glycogen synthase kinase-3
HBSS	Hank's balanced salt solution
HDL	high density lipoprotein
HRP	horse radish peroxidase
IL	interleukin
LPS	lipopolysaccharide
LTP	long term potentiation
MAP	mitogen-activated protein
MCP-1	monocyte chemoattractant peptide-1
M-CSF	macrophage-colony stimulating factor
MHC II	major histocompatibility complex II
MIP-1α	macrophage inflammatory protein 1α
MWM	Morris Water Maze
NDA	naphtalenedicarboxaldehyde
NFκB	nuclear factor κB
NiDAB	nickel enhanced diamino benzidine
NO	nitric oxide
NOS	nitric oxide synthase
NSAID	non-steroidal anti-inflammatory drug
NSE	neuron specific enolase
PBS	phosphate buffered saline
PerCP	peridinin chlorophyll-a protein
PDTC	pyrrolidine dithiocarbamate
PHD	pyruvate dehydrogenase
PHF	paired helical filaments

PKA	protein kinase A
PKC	protein kinase C
PrP	Prion protein
PS	presenilin
R-PE	R-Phycoerythrin
SDS-PAGE	sodium dodecyl sulphate polyacrylamide gel electrophoresis
TGFβ1	transforming growth factor β1
TM	transmembrane
TNFα	transforming growth factor α
3 x Tg-AD	triple transgenic mouse model of Alzheimer's disease

LIST OF ORIGINAL PUBLICATIONS

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

I   **Malm TM**, Koistinaho M, Pärepalo M, Vatanen T, Ooka A, Karlsson S, Koistinaho J. Bone-marrow-derived cells contribute to the recruitment of microglial cells in response to beta-amyloid deposition in APP/PS1 double transgenic Alzheimer mice. Neurobiol Dis. 2005 Feb;18(1):134-42.

II   **Malm TM**, Koistinaho M, Vatanen T, and Koistinaho J. Minocycline reduces the engraftment and activation of bone marrow derived cells but has no effect on the clearance of β-amyloid in response to lipopolysaccharide treatment in a transgenic mouse model of Alzheimer's disease. Submitted.

III   **Malm T,** Ort M, Tähtivaara L, Jukarainen N, Goldsteins G, Puoliväli J, Nurmi A, Pussinen R, Ahtoniemi T, Miettinen TK, Kanninen K, Leskinen S, Vartiainen N, Yrjänheikki J, Laatikainen R, Harris-White ME, Koistinaho M, Frautschy SA, Bures J, Koistinaho J. β-Amyloid infusion results in delayed and age-dependent learning deficits without role of inflammation or beta-amyloid deposits. Proc Natl Acad Sci. USA. 2006 Jun 6;103(23):8852-7.

IV   **Malm TM**, Iivonen H, Goldsteins G, Keksa-Goldsteine V, Ahtoniemi T, Kanninen K, Salminen A, Van Groen T, Tanila H, Koistinaho J. PDTC activates Akt and improves spatial learning in APP/PS1 mice without affecting β-amyloid burden. Submitted

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1. INTRODUCTION

Alzheimer's disease (AD) is a progressive neurodegenerative disorder leading to severe dementia and eventually death. AD is the major cause of dementia in the elderly and the prevalence and incidence of AD increases with age. The disease affects approximately 4.9 million people in Western Europe, which comprises 5.5 % of the elderly population (Ferri et al., 2005). Considering that every 3rd 85-year-old has AD, this disorder has a huge impact on public economy. As the population ages in developing countries, it has been estimated that the prevalence of AD will double during the next 20 years, causing a heavy burden on public healthcare (Ferri et al., 2005; Jonsson and Berr, 2005). Despite extensive research in to the pathogenesis of AD, there are yet no curative therapies available for this devastating disorder.

AD pathology is complex and both genetic and environmental factors play important roles in the development of the disease. AD is characterized by the presence of two pathological hallmarks: extracellular beta-amyloid (Aβ) plaques and intracellular neurofibrillary tangles (Dickson, 1997a; McKhann et al., 1984; Price and Sisodia, 1998). Aβ, the cleavage product of amyloid precursor protein (APP), is thought to be directly neurotoxic and in AD it accumulates with various other proteins to form senile plaques in the brain (Rogers et al., 1988; Snow et al., 1988). On the other hand, neurofibrillary tangles are composed of abnormally phosphorylated tau protein, which after aberrant phosphorylation cannot function normally to stabilize microtubules. Instead, it forms highly insoluble filaments inside neurons and thereby affects their viability (Price and Sisodia, 1998).

Most of the AD cases occur as sporadic forms, in which specific genetic mutations do not underlie the disease pathogenesis. In these cases the cause of the disease remains largely unknown and the patients develop the first signs of disease in their seventh decade of life. In addition, approximately 10% of the AD cases occur due to inherited mutations in APP or presenilin (PS) genes, causing elevated levels of Aβ. In addition, triplication of chromosome 21 carrying the APP gene leads to elevated levels of Aβ. Therefore Down's syndrome patients develop AD. In addition, there are risk factors that may increase the incidence of late-onset, sporadic form of AD, such as the e4 allele of apolipoprotein E (apoE) (Price and Sisodia, 1998).

The use of animal models in the research of AD has yielded important knowlegde regarding the mechanisms underlying the disease pathogenesis. Several groups have attempted to model sporadic AD and study the toxicity of Aβ by direct intracranial infusion

of different forms of Aβ in mice and rats. These studies have reported some histopathological features of AD. Some studies have reported cognitive deficits in these models as analyzed by different behavioural tests. However, the reported pathological and behavioural deficits have often been inconsistent, varying from one study to another (Craft et al., 2004a; Craft et al., 2004b; Frautschy et al., 1998a; Frautschy et al., 1996; Hashimoto et al., 2002; Nakamura et al., 2001; Nitta et al., 1994). On the other hand, AD can also be modelled using transgenic mice overexpressing either wildtype or mutated APP and / or PS. These models typically develop more widespread pathology and behavioural deficits, and are widely used in the research of the disease pathogenesis.

Neuroinflammation, manifested by the accumulation of activated microglia and astroglia near Aβ plaques, is an invariant feature of AD (Akiyama et al., 2000). Despite extensive research, the exact role of inflammatory mediator cells, especially microglia and astrocytes, in the development and progression of AD has remained controversial. In the normal brain both microglia and astrocytes function in maintaining the brain in a normal condition. Astrocytes are capable of secreting neurotrophic factors, regulating synaptogenesis and neurogenesis, modulating synaptic networks, and removing excess neurotransmitters from the synaptic cleft (Ransom et al., 2003). Microglia, on the other hand, fight against foreign intruders and surveil the brain for possible pathogens (Davalos et al., 2005; Hanisch, 2002; Nimmerjahn et al., 2005). In AD, the chronic activation of glial cells has been associated with overt production of pro-inflammatory molecules and exacerbation of neuronal dysfunction (Akiyama et al., 2000; Hanisch, 2002). However, both microglia and astrocytes have been reported to be able to clear Aβ from the brain parenchyma (Bard et al., 2000; Wyss-Coray et al., 2003). In addition, despite the common knowledge that the brain microglial population is constantly renewing from bone marrow during adult life (Eglitis and Mezey, 1997; Simard and Rivest, 2004), the contribution of these bone marrow (BM) derived cells to AD pathogenesis and AD related inflammatory reactions has not beed addressed.

The aim of the present thesis was to further characterize the role and contribution of inflammatory mediator cells, especially microglia and astrocytes, in animal models of AD. By using novel bone marrow transplantation techniques we analyzed the role of BM-derived monocytic cells in a transgenic mouse model of AD overexpressing mutated APP and PS1 (APP+PS1 transgenic mouse). In addition, we studied the effects of continuous intracerebroventricular infusion of Aβ on cognitive abilities as well as on histopathology, mainly micro and astrogliosis, of mice and rats. Finally, the ability of a known anti-

inflammatory agent, pyrrolidine dithiocarbamate (PDTC) to ameliorate the behavioral deficits and AD-like pathology of APP+PS1 transgenic mouse model was analyzed.

2. REVIEW OF LITERATURE

2.1 Alzheimer's disease (AD)

2.1.1 General clinical features of AD

AD was first described by Alois Alzheimer in 1907 when he reported a case of a middle aged woman suffering from progressive memory impairment, loss of cognitive abilities, altered behavior including paranoia, delusions, loss of social appropriateness and a progressive decline in language function (Alzheimer, 1907 a, b). Many of the features originally described by Alois Alzheimer are nowadays still observed in AD patients, who show abnormalities in memory, problem solving, language, calculation, visuospatial perceptions, judgement, and behavior (McKhann et al., 1984; Price and Sisodia, 1998). Usually the patients become bedridden in the late stage of the disease and die of intercurrent medical illnesses. The disease diagnosis is established by clinical history, physical examinations and neuropsychological testing. However, excluding the pathological features that can be discovered by brain biopsy, there is still no test for definitive diagnosis of Alzheimer's disease in living patients (Price et al., 1998).

AD etiology can be divided into two groups. Approximately 10% of the AD cases are familial (FAD) and caused by the inheritance of mutations in specific genes. In these patients the clinical features of AD develop in midlife. However, most of the AD patients do not carry any AD-linked mutations and the cause of the disease remains largely unknown. These patients show clinical signs of the disease rather late, during the seventh decade of life (Mattson, 2004; Selkoe, 2001a).

2.1.2 Neuropathology of AD

AD affects various specific brain regions (Braak et al., 1996). These include the cortex, hippocampus, amygdala, basal forebrain, anterior thalamus and several brain stem monoaminergic nuclei (Whitehouse et al., 1982). Neuronal dysfunction and ultimately cell death lead eventually to the shrinkage of the affected brain areas and enlargement of ventricles. The progression of AD symptoms closely corresponds to the neuronal death. Neuronal loss begins in the layer II of enterorhinal cortex, in which the neuron loss is very prominent even in the early stages of AD (Gomez-Isla et al., 1996a). Indeed, the degeneration begins with the regions involved in the processing of learning and memory, and gradually spread into the areas responsible of higher cognitive functions, eventually affecting

also the control and coordination of movement. Degeneration occurs especially as a loss of cholinergic neurons and their markers (Davies and Maloney, 1976; Whitehouse et al., 1981). Neuropathological hallmarks of AD include intracellular neurofibrillary tangles, extracellular neuritic plaques as well as cerebrovascular angiopathy.

2.1.2.1 Neurofibrillary tangles

A distinct pathological feature of AD is the accumulation of intracellular filaments in neurons in the affected brain regions (Dickson, 1997a). These accumulations are composed primarily of hyperphosphorylated tau protein. The normal function of tau is to bind and stabilize microtubules, however, during AD pathogenesis tau becomes abnormally phosphorylated and subsequently loses its ability to bind to microtubules. Hyperphosphorylated tau aggregates to form paired helical filaments (PHFs) and straight filaments, and eventually neurofibrillary tangles (NFT). Since hyperphosphorylated tau no longer is able to bind and stabilize microtubules, intraneuronal transport, cellular geometry and neuronal viability are compromised.

Many neurons in the affected regions in AD brain exhibit abnormal accumulations of PHFs and straight filaments. NFTs are found in cell bodies and proximal dendrites. Neuropil threads are PHF accumulations found in dendrites and dystrophic neurites are PHF containing neuronal processes (Arnold et al., 1991; Price et al., 1998).

2.1.2.2 Senile plaques

Another distinct pathological feature of AD is the accumulation of senile plaques, especially in the amygdala, hippocampus and neocortex (McKhann et al., 1984; Price and Sisodia, 1998). These extracellular plaques consist of aggregated beta-amyloid (Aβ) peptides (Glenner and Wong, 1984b) and associated axonal and dendritic injury. Aβ peptides are cleavage products of amyloid precursor protein (APP). The accumulation of Aβ in AD may be due to enhanced production of these peptides caused by mutations in APP or PS genes. Down's syndrome patients, who suffer from the triplication of the chromosome that contains the APP gene, have elevated levels of Aβ and develop AD.

Even though the 40 amino acids long Aβ peptide (Aβ1-40) is the most abundant form of Aβ found in the brain, plaques are mainly composed of Aβ that is 42 amino acids in length (Aβ1-42). Aβ1-42 is much more prone to aggregation compared to Aβ1-40, however, Aβ1-40 often co-exists with Aβ1-42 in senile plaques (Gowing et al., 1994; Iwatsubo et al., 1994; Roher et al., 1986). In senile plaques the deposits of Aβ peptides are surrounded by

dystrophic neurites. These neurites are distinquished by ultrastructural abnormalities such as enlarged lysosomes, numerous mitochondria and PHFs of hyperphosphorylated tau (Dickson, 1997b). Senile plaques are also associated with activated microglia found within and adjacent to the lesions. Astrocytes are detected outside the plaques, often forming a ring around the plaque with processes extending toward the amyloid core (Wegiel and Wisniewski, 1990; Wisniewski and Wegiel, 1991). Aggregated $A\beta$ fibrils can also attract the accumulation of various other proteins, including α_1-antichymotrypsin (ACT), complement cascade components and apolipoproteins E and J (Abraham et al., 1988; Ghiso et al., 1993; LaDu et al., 1994; Ma et al., 1994; Price and Sisodia, 1998; Rogers et al., 1988; Snow et al., 1988; Strittmatter et al., 1993).

According to the current view the so called diffuse plaques or preamyloid deposits preceed the formation of neuritic plaques. These diffuse plaques are mainly composed of $A\beta1$-42 and represent immature lesions of senile plaques lacking neuritic dystrophy, gliosis and neurofibrillary tangles present in senile plaques (Joachim et al., 1989; Tagliavini et al., 1988; Yamaguchi et al., 1988). It has been shown that diffuse plaques exist before the development of dense core thioflavin S-positive and Congo red-positive neuritic plaques (Lemere et al., 1996). The time course of the development of senile plaques is not known, but most likely they gradually mature over a period of several years (Selkoe, 2001a).

2.1.2.3 Cerebral amyloid angiopathy

Cerebral amyloid angiopathy is the accumulation of $A\beta$ in the arterioles, venules and capillaries in patients with AD. Accumulated $A\beta$ peptides can be found in the abluminal basement membrane of vessel walls of the affected brain regions. Indeed, $A\beta$ was originally isolated from amyloid laden arteolies and venules of patients with AD or Down's syndrome (Glenner and Wong, 1984a; Glenner and Wong, 1984b). The extent of accumulation of cerebral amyloid varies among AD patients (Selkoe, 2001a). These vascular abnormalities may occasionally rupture, possibly leading to cerebral hemorrages (Luyendijk et al., 1988; van Duinen et al., 1987).

2.1.3 Proteins involved in the development of AD

2.1.3.1 APP

Expression of APP

Aβ peptides are cleavage products of APP. APP was first purified and partially sequenced by Glenner & Wong in 1984 from vascular deposits of Alzheimer's disease and Down's syndrome patients (Glenner and Wong, 1984b). Since then the APP gene has been identified to contain approximately 400 kb of DNA, and 19 exons that encode several alternatively spliced APP mRNAs. These splice variants give rise to three major isoforms: 695, 751 and 770 amino acid residues in length (Kang et al., 1987; Kitaguchi et al., 1988; Konig et al., 1992; Ponte et al., 1988; Tanzi et al., 1988).

APP is a typical type-I integral transmembrane (TM) glycoprotein that contains several different domains: an N-terminal signal peptide; a large ectodomain with N-glycosylation sites; an anternatively spliced Kunitz-type serine protease inhibitor domain; an Aβ region; a single membrane-spanning helix; and a short cytoplasmic domain that signals the trafficing of APP in the endocytic and secretory pathways (Price et al., 1998; Selkoe, 2001a). The longer 751 and 770 isoforms contain an exon that encodes a 56 amino acid domain homologous to the Kunitz-type of serine protease inhibitors (Kitaguchi et al., 1988; Ponte et al., 1988; Tanzi et al., 1988).

APP gene is located in chromosome 21 (Goldgaber et al., 1987; Kang et al., 1987). APP isoforms are expressed throughout the body in many different cell types. The longer isoforms 751 and 770 are mainly expressed in glial cells (Rohan de Silva et al., 1997) whereas 695 is mainly expressed in neurons (Koo et al., 1990; Sisodia et al., 1993). Down's syndrome individuals with dublication in chromosome 21 overexpress normal wild type APP, and thus it is not suprising that all patients with Down's syndrome develop AD in their mid-life (Petronis, 1999).

Proteolytic processing of APP

After translation into protein in endoplasmic reticulum (ER), APP undergoes several post translational modifications during its transit to the plasma membrane. N- and O-linked carbohydrates as well as sulphate moieties are added during the secretory pathway (Weidemann et al., 1989). When APP reaches the plasma membrane, it is rapidly internalized and trafficked through endosomes and recycled back to the cell surface. Additionally, APP can be directed to lysosomes where it can undergo degradation (Vetrivel and Thinakaran, 2006).

APP can undergo several different endoproteolytic cleavage events. Under normal conditions APP is preferentially cleaved by α-secretase releasing an extracellular amino-

terminal ectodomain of APP (sAPPα) (Esch et al., 1990). This cleavage retains an 83-amino acid residue C-terminal fragment (CTF) in the membrane (Selkoe, 2001a). Several zink metalloproteinases can cleave APP at the α-secretase cleavage site, including ADAM9, ADAM10, TACE/ADAM17, MDC-9 and an aspartyl protease BACE2, thus preventing the formation of amyloidogenic Aβ (Vetrivel and Thinakaran, 2006). This non-amyloidogenic processing of APP occurs primarily at the plasma membrane, where α-secretases are present (Vetrivel and Thinakaran, 2006).

Alternatively, a smaller amount of APP can be cleaved by a pathway resulting in amyloidogenic Aβ peptides. This pathway involves the sequential cleavage by β- and γ-secretases and occurs during the transit of APP through the endocytic organelles (Vetrivel and Thinakaran, 2006). Cleavage by β-secretase occurs in the extracellular domain of APP to generate a slightly smaller ectodomain derivative (βAPPs) and a retaining membrane bound 99-residue CTF (C99) (Seubert et al., 1993). β-secretase was simultaneuousy cloned by several laboratories (Hussain et al., 1999; Sinha et al., 1999; Vassar et al., 1999; Yan et al., 1999) and the main β-secretase is ß-site APP cleaving enzyme (BACE1), also called Asp-2 and memapsin-2. APP is thought not to be the only physiological substrate for BACE1, since the affinity of BACE1 towards APP is relatively low (Vetrivel and Thinakaran, 2006). C99 can be subsequently cleaved by γ-secretase at two different sites. This cleavage site is of major importance: if C99 is cleaved at residue 712 Aβ1-40 results; if it is cut on residue 714, the long Aβ1-42 is produced (Hardy, 1997). Thus, the γ-secretase plays an important role in the generation of amyloidogenic Aβ.

Physiological functions of APP

The exact role of APP is not yet known. APP has been thought to be important in several different functions such as cell adhesion, cell survival, promotion of neurite outgrowth, synaptogenesis, proliferation, and learning and memory (reviewed in (Mattson, 1997). sAPPα has been shown to protect neurons against excitotoxic and hypoglycemic damage (Mattson et al., 1993a) and promote neurite outgrowth of hippocampal neurons (Clarris et al., 1994; Qiu et al., 1995). sAPPα has been proposed to function in synapse formation, as intracerebroventricular injection of sAPPα peptide leads to increased synaptic density in frontoparietal cortex (Roch et al., 1994) and transgenic mice over-expressing human APP in neurons showed increased synaptic density (Mucke et al., 1994). Several lines

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of evidence also show that sAPPα regulates neuronal excitability and synaptic plasticity (Mattson, 1997) .

Despite the important functions of APP, knocking out the APP gene leads to only very subtle phenotypic changes (Zheng et al., 1996), suggesting that APP is not required in embryonic and early neuronal development. This may be due to fact that mammals express closely homologous proteins to APP, such as APP-like proteins (APLPs; (Slunt et al., 1994).

AD causing mutations of APP

The first direct evidence of genetic linkage in AD came from the observation of a missense mutation in APP in a British family (Goate et al., 1991). Since then more that 20 mutations in the gene encoding APP have been found and most of them affect the proteolytic cleavage of APP (Spires and Hyman, 2005).

One of the most widely studied mutations in the N-terminus of the APP gene was found in two Swedish families with early onset AD (Mullan et al., 1992). This double mutation was thus named the 'Swedish mutation' and it occurs immediately prior to β-secretase cleavage site, increasing the cleavage of β-secretase and subsequent production of Aβ peptides. Mutations occuring carboxy-terminal to the γ-secretase cleavage site, the APP_{717} or so called London or Indiana mutations, appear to enhance the production of Aβ1-42 species (Suzuki et al., 1994). APP_{692}, 'Flemish' mutation leads to presenile dementia and cerebral amyloid angiopathy (Hendriks et al., 1992) by inhibiting the cleavage by α-secretase and enhancing subsequent Aβ1-42 production (Hardy, 1997). Quite similarly, the 'Dutch' mutation causes accumulation of Aβ on the vessel walls, thus leading to cerebral hemorrhage and premature death (Levy et al., 1990). On the other hand, the 'Florida' (APP_{716}) mutation alters the site of γ-secretase cleavage (Hardy, 1997).

Aβ production and amyloid cascade hypothesis

The amyloid cascade hypothesis arises from the observations that Aβ is neurotoxic and that FAD mutations lead to increased Aβ accumulation (Hardy and Allsop, 1991). Aβ peptides are the major constituent of amyloid plaques seen in AD brain. Over a decade ago these peptides were suggested to be the major mediator of neuronal cell toxicity in AD pathogenesis. This hypothesis was addressed as 'amyloid cascade hypothesis' (Hardy and Allsop, 1991) based on the findings in familial AD cases and observations in Down's syndrome patients. The amyloid cascade hypothesis proposes that an increase in toxic Aβ

peptides leads to the formation of amyloid plaques and subsequently to the formation of neurofibrillary tangles, synapse loss and neuronal cell death. The increase in Aβ can arise due to an increase in the production of Aβ or a decrease in the clearance of the Aβ.

The amyloid cascade hypothesis has been revised over the years as research has progressed, and the most recent hypothesis is shown in figure 1. The major advancement in the hypothesis has arisen from studies showing that the most toxic form of Aβ is not actually the fibrillar form seen in plaques, but rather an oligomeric form of Aβ composed of 2 - 12 Aβ1-42 peptides. Oligomeric Aβ is prone to aggregation and the formation of extracellular deposits. Several lines of evidence support the toxicity of oligomeric Aβ in vitro (Dahlgren et al., 2002; Lambert et al., 1998; Oda et al., 1995). Oligomers have also been shown to inhibit long term potentiation (LTP) (Walsh et al., 2002) and in many cases, transgenic AD mice over-expressing wildtype or mutated APP develop behavioral deficits before the appearance or even in the absence of actual plaques (Dodart et al., 1999; Koistinaho et al., 2002). Also, Aβ plaque levels in the brains of AD patients and transgenic mice show poor correlation with behavioral abnormalities (Chishti et al., 2001; Nagy et al., 1995; Neve and Robakis, 1998). However, in transgenic mice Aβ oligomers, especially the dodecamers, correlate well with the behavioural deficits (Lesne et al., 2006).

The amyloid cascade hypothesis has strongly divided opinions in the scientific community. Several researchers believe that the Aβ itself is not promoting the disease, but rather that hyperphosphorylation of tau-protein is essential in the development of AD pathology (Brandt et al., 2005). The major critisism against the amyloid cascade hypothesis arises from the observations that non-demented elderly may have substantial levels of Aβ deposition (Hof et al., 1996). In addition, whilst deposition of Aβ poorly correlates with behavioral deficits, neurofibrillary tangles have been shown to better correlate with memory deficits (Crystal et al., 1988; Nagy et al., 1996; Neve and Robakis, 1998). However, the observation that in triple transgenic mice expressing mutated APP, presenilin and tau, Aβ pathology preceeds the formation of neurofibrillary tangles may favor the amyloid cascade hypothesis (Oddo et al., 2003a). In addition, Aβ immunotherapy by direct intracranial injection of Aβ antibody in the same triple transgenic mice was shown to reduce not only Aβ deposits, but also early tau pathology (Oddo et al., 2004). Both of these pathological lesions re-emerged eventually, however, the Aβ deposits re-emerged well before the phosphorylated tau, suggesting a causal relationship between the formation of these pathological lesions (Oddo et al., 2004).

APP

Non-amyloidogenic cleavage

α-secretase

β-secretase

Amyloidogenic cleavage

sAPPα

βAPPs

γ-secretase complex

Aβ40   Aβ42

Oligomers exhibit direct synaptotoxicity

Aβ42   Aβ42   Aβ42   Aβ42

Aβ42 oligomerizes

Aβ40
Aβ42   Aβ42
Aβ40   Aβ42   Aβ42
Aβ42

Aβ42 accumulates and forms diffuse deposits

Aβ aggregates finally forming dense core plaques. Other proteins associate with the plaques (ie complement)

Recruitment of activated microglia and astroglia around the plaques

Altered neuronal homeostasis and oxidative stress

Widespread neuronal dysfunction leading to dementia

Abnormal kinase activity, hyperphosphorylation of tau and tangle formation

Mutations affecting APP cleavage

β-secretase

Swedish mutation: Aβ40 and 42 production increased

Flemish mutation: Aβ42 increased

γ-secretase

Aβ42

Dutch mutation: Aβ aggregation increased

717 mutation: Aβ42 increased

Figure 1. Mutations affecting the processing of APP and the current view of amyloid cascade hypothesis. According to the hypothesis the proteolytic cleavage of Aβ from APP eventually leads to the accumulation of Aβ aggregates and subsequent glial activation. Mutations causing an increase in the Aβ production are shown in the inset. Modified from (Verdile et al., 2004).

In addition to the amyloid cascade hypothesis and tau-dysfunction, other mechanisms, such as oxidative stress (Joseph et al., 2001) or perpetuations in vesicular trafficking, cytoskeletal network and membrane cholesterol distribution have been suggested to underlie the AD pathogenesis (Drouet et al., 2000; Joseph et al., 2001). Neuroinflammation has been proposed to be a major contributor to the development of AD pathogenesis (Akiyama et al., 2000). On the other hand the 'bioflocculant' -hypothesis claims that Aβ that is normally produced binds to extracellular toxins, such as metal ions. The aggregation of Aβ and metal toxins into Aβ plaques thus serves as means for presenting the toxins to phagocytes in order to remove them from the brain (Robinson and Bishop, 2002).

Mechanisms of Aβ toxicity

Approximately 90% of the secreted Aβ is Aβ1-40, whereas 10% is highly fibrillogenic Aβ1-42. Aβ peptides are prone to aggregation and form a variety of structures: these include monomers (single peptide units), oligomers, which are 2 - 12 amino acid long Aβ1-42 peptide chains (Bitan et al., 2003), (Kirkitadze et al., 2001; Walsh et al., 2002), also called small diffusible Aβ oligomers or Aβ-derived diffusible ligands (ADDLs) (Lambert et al., 1998), protofibrils (Harper et al., 1997; Walsh et al., 1997), fibrils (Teplow, 1998) and spheroids (Hoshi et al., 2003; Westlind-Danielsson and Arnerup, 2001). Protofibrils are metastable intermediates in the assembly of Aβ into actual fibrils (Harper et al., 1997) and spheroids on the other hand are stable, toxic supramolecular assemblies of Aβ1-40, also referred as 'betaamy balls' (Westlind-Danielsson and Arnerup, 2001). The toxicity and structural relationship of these intermediates is under active research. Interestingly, Aβ peptides seem to gain their neurotoxicity upon oligomerization, since Aβ monomers have actually been shown to be neuroprotective (Zou et al., 2002). Initially the aggregated form of Aβ was suggested to cause AD, since it was shown to be toxic *in vitro* at high concentrations. However, according to the current view, the oligomeric form of Aβ is thought to cause the most neurotoxicity.

Aggregated Aβ peptides have been shown to be toxic *in vitro* by causing oxidative stress through the production of reactive oxygen species (Behl et al., 1994; Goodman and Mattson, 1994; Goodman et al., 1994; Huang et al., 1999a; Oda et al., 1995), inducing inflammatory response (London et al., 1996; Meda et al., 1995), and directly causing apoptosis (Anderson et al., 1996; Estus et al., 1997). Aβ peptides also cause membrane disruption (Hertel et al., 1997), disruption in Ca^{2+} homeostasis (Mattson et al., 1992; Mattson

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et al., 1993b; Sberna et al., 1997; Ueda et al., 1997) and activation of complement cascade (Afagh et al., 1996; Chen et al., 1996; Jiang et al., 1994; Rogers et al., 1992; Webster et al., 1997a; Webster et al., 1997b). In addition, Aβ peptides have been shown to induce abnormal tau phosphorylation and formation of dystrophic neurites (Ferreira et al., 1997; Rank et al., 2002). Aβ neurotoxicity can further cause more secondary neuronal death via activation of glial cells (Akiyama et al., 2000).

Recently, reseach has been focused on the role of oligomeric form of Aβ in AD. ADDLs have been shown to prevent the formation of long term potentiation (LTP) in hippocampal slice cultures (Lambert et al., 1998) as well as *in vivo* after intracranial injections (Walsh et al., 2002). The ability of Aβ oligomers to prevent LTP is even further supported by the observation in triple transgenic animals, which develop deficits in LTP before the appearance of Aβ deposits (Oddo et al., 2003b). Interestingly, immunization with antibodies recognizing oligomerized Aβ was shown to improve cognitive deficits in transgenic AD mice (Lee et al., 2006). Even though the exact mechanims by which oligomeric Aβ causes dementia in AD are unknown, it is likely that Aβ causes synaptic dysfunction rather than direct cell death (Terry, 2000). Oligomers isolated from human AD brain extracts have been shown to bind directly to synaptic sites in hippocampal neurons (Lacor et al., 2004). In support of this, a recent report from Lesne et al. showed that Aβ oligomers purified from the brains of AD transgenic mice caused transient memory disruptions without inducing neuron loss or plaques (Lesne et al., 2006).

2.1.3.2 Presenilins

Presenilin 1 (PS1) located in chromosome 14 (Sherrington et al., 1995) and presenilin 2 (PS2) located in chromosome 1 (Levy-Lahad et al., 1995; Rogaev et al., 1995), are TM proteins that have six to nine TM domains. Like APP, presenilins are ubiqutously expressed in various tissue and cell types, interestingly in higher levels especially in brain regions that are affected in AD (Lee et al., 1996). Presenilins are located mainly in the ER and to a lesser extent in the Golgi compartments (Kovacs et al., 1996; Walter et al., 1996). However, the putative functions of presenilins are poorly understood. PS1 has been suggested to be involved in Notch signaling during embryogenesis (Ray et al., 1999). Notch is a receptor that mediates several important cell fate events during embryogenesis. The role of PS1 in embryogenesis is supported by findings in PS1 knockout mice, since mice lacking PS1 show abnormal embryonic development and are not viable (Shen et al., 1997).

Deficiency in PS2 is not as crucial, since PS2 knockout mice are viable but with aging suffer from mild pulmonary fibrosis and haemorrhage (Herreman et al., 1999).

Mutations in APP are not underlying all cases of FAD. Also presenilins were linked to Alzheimer's disease and especially processing of APP when certain AD families were found to carry mutations in PS1 and PS2 (Levy-Lahad et al., 1995; Sherrington et al., 1995). Since then, more than 40 mutations in PS1 gene and 2 mutations in PS2 gene have been identified (reviewed in (Hardy, 1997). Most of these mutations are located in the TM domains of the presenilins and some especially cluster near the functionally and conformationally important sites. Therefore, it is not suprising that these mutations affect the function of presenilins.

The reason why presenilin mutations cause AD comes from the fact that presenilin homodimers have been suggested to be in the core of active γ-secretase complex (De Strooper et al., 1998; Schroeter et al., 2003; Xia et al., 1998; Xia et al., 1997), serving as catalytic components of γ-secretase. PS co-precipitates with APP (Xia et al., 1997) and the deletion of PS1 decreases the γ-secretase activity and subsequent cleavage of APP, and increases the accumulation of CTFs in the ER and Golgi (De Strooper et al., 1998; Xia et al., 1998). Later it was also shown that two TM aspartase residues are required for the γ-secretase activity of PS1 (Wolfe et al., 1999). Thus, presenilin mutations affect the metabolism of APP by increasing the production of amyloidogenic Aβ1-42. AD patients with a mutation in PS1 or PS2 have been shown to have increased amount of total brain and plasma Aβ levels (Gomez-Isla et al., 1997; Scheuner et al., 1996). Also, transgenic mice expressing mutated PS have elevated levels of Aβ1-42 (Borchelt et al., 1996; Duff et al., 1996).

2.1.3.3 Apolipoprotein E (ApoE)

Apolipoprotein E (ApoE) is a 34 kDa glycoprotein that exists in three different isoforms, ApoE2, ApoE3 and ApoE4 derived from the alleles (ε2, ε3 and ε4) at the same gene locus. These isoforms differ from each other only in two amino acid residues at positions 112 and 158; ApoE3 has cysteine at 112 and arginine at 158, ApoE4 has arginine and ApoE2 has cysteine in both residues (Mahley, 1988). ApoE3 is the most common isoform accounting for up to 80% of the population gene pool, whereas ApoE4 accounts for 10-15 % and ApoE2 5-10 %. ApoE is expressed in rodent brain mainly in astrocytes and microglia (Boyles et al., 1985; Nakai et al., 1996), but in humans ApoE protein has also been found in neurons (Han et al., 1994a; Han et al., 1994b).

ApoE4 has been shown to be a major susceptibility factor for the development of familial and sporadic forms of AD. Individuals carrying the ApoE4 allele are at a higher risk of developing AD, and the ApoE4 allele has been shown to decrease the mean age of the disease onset compared to individuals carrying ApoE2 or ApoE3 allele. Two copies of ApoE4 even furher increase the risk of AD (Corder et al., 1993; Saunders et al., 1993). On the other hand, ApoE2 allele has been shown to be protective (Corder et al., 1994). One mechanism by which ApoE4 can enhance the AD pathology is by increasing the brain Aβ burden (Gomez-Isla et al., 1996b; Schmechel et al., 1993). ApoE can bind Aβ and the three isoforms have different binding capacities, further explaining the difference in the susceptibility to AD (Strittmatter et al., 1993). Murine ApoE seems to enhance the deposition of Aβ, since AD transgenic mice lacking murine ApoE have been shown to have significantly less plaques compared to mice with both ApoE alleles (Bales et al., 1997). Even a 50 % reduction in ApoE gene dose reduces the deposition of Aβ in brain, suggesting that ApoE promotes the accumulation of Aβ (Bales et al., 1999). Several *in vitro* and *in vivo* studies show that ApoE4 inhibits Aβ clearance or stimulates the deposition of Aβ, thus leading to increased accumulation of Aβ (Holtzman et al., 2000; Ma et al., 1994; Sanan et al., 1994; Schmechel et al., 1993; Wisniewski et al., 1994). ApoE has also been shown to affect the brain Aβ deposition in an isoform specific manner, so that AD transgenic mice harbouring human ApoE3 developed significantly less plaques compared to mice carrying human ApoE4 alleles (Holtzman et al., 2000; Holtzman et al., 1999). Other mechanisms explaining ApoE4 as a susceptibility factor for AD include the decreased antioxidant activity of ApoE4 (Miyata and Smith, 1996) and alterations in tau phosphorylation and neurofibrillary tangle formation induced by ApoE4 (Huang et al., 2001; Strittmatter et al., 1994; Tesseur et al., 2000). ApoE4 has also been shown to exarcabate Aβ induced lysosomal leakage and apoptosis (Ji et al., 2002; Ji et al., 2006) and ApoE4 fragments have been hypothetized to cause mitochondrial dysfunction and cytoskeletal abnormalities (Mahley et al., 2006).

2.1.3.4 Tau

Physiological functions of tau

The neuronal cytoskeleton is responsible for the morphology and structural integrity of neurons. Microtubules are components of neuronal cytoskeleton, and are maintained by different proteins that bind to microtubules. Tau is one of the microtubule

associated proteins and in healthy adult brain is located mainly in axons. Tau has two physiological functions: it stabilizes assembled microtubules permitting neurite extension (Caceres and Kosik, 1990; Drubin and Kirschner, 1986); however, tau also competes with the motor protein kinesin for microtubule binding, thus inhibiting kinesin-dependent axonal transport (Ebneth et al., 1998; Stamer et al., 2002; Trinczek et al., 1999). To ensure proper axonal transport in neurons, these two mechanisms most likely exist in equilibrium (Avila, 2006).

Tau exist in six different isoforms in brain (Goedert et al., 1989). The tau gene contains 15 exons, from which the exons 2, 3 and 10 can be alternatively spliced (LaFerla and Oddo, 2005). The alternative splicing of tau gene leads to the generation of 6 splice variants that contain either 3 or 4 repeat domains (arising from alternative splicing of exon 10) and zero, one or two inserts at the N-terminus (arising from alternative splicing of exons 2 and 3). The 3 or 4 random repeats in the C-terminus are important for the microtubule binding and these microtubule-binding domains flank two proline rich regions. The phosphorylation of these proline rich regions has been suggested to affect the ability of tau to bind to microtubules (Heutink, 2000). All tau isoforms are capable of forming neurofibrillary tangles present in AD (Avila, 2006).

Phosphorylation and aggregation of tau

Tau protein can be phosphorylated at serine/threonine residues by two different classes of kinases: proline directed kinases, such as glycogen synthase kinase-3 (GSK3), cyclin dependent kinase5 and p38 or JNK belonging to the mitogen-activated protein (MAP) kinase family, as well as non proline kinases, such as protein kinase A (PKA), protein kinase C (PKC), Ca^{2+}/calmodulin dependent protein kinase II or casein kinase II (Avila, 2006; Baudier et al., 1987; Correas et al., 1992; Drewes et al., 1992; Goedert et al., 1997; Hanger et al., 1992; Morishima-Kawashima et al., 1995; Scott et al., 1993). Oxidative stress activates several kinases, which in turn can phosphorylate tau. An abnormal phosphorylation of tau at the C-terminal region leads to a loss of microtubule binding capacity of tau and subsequent formation of PHFs. After aggregation, PHF-tau is prone to multiple posttranslational modifications, including oxidation (Schweers et al., 1995), ubiquitination (Mori et al., 1987) and glycation (Ledesma et al., 1994; Yan et al., 1994). Since PHF-tau is not able to bind microtubules, the hyperphosphorylation of tau leads to disassembly of microtubules and compromised axonal or dendritic transport, finally affecting the viability of the neuron.
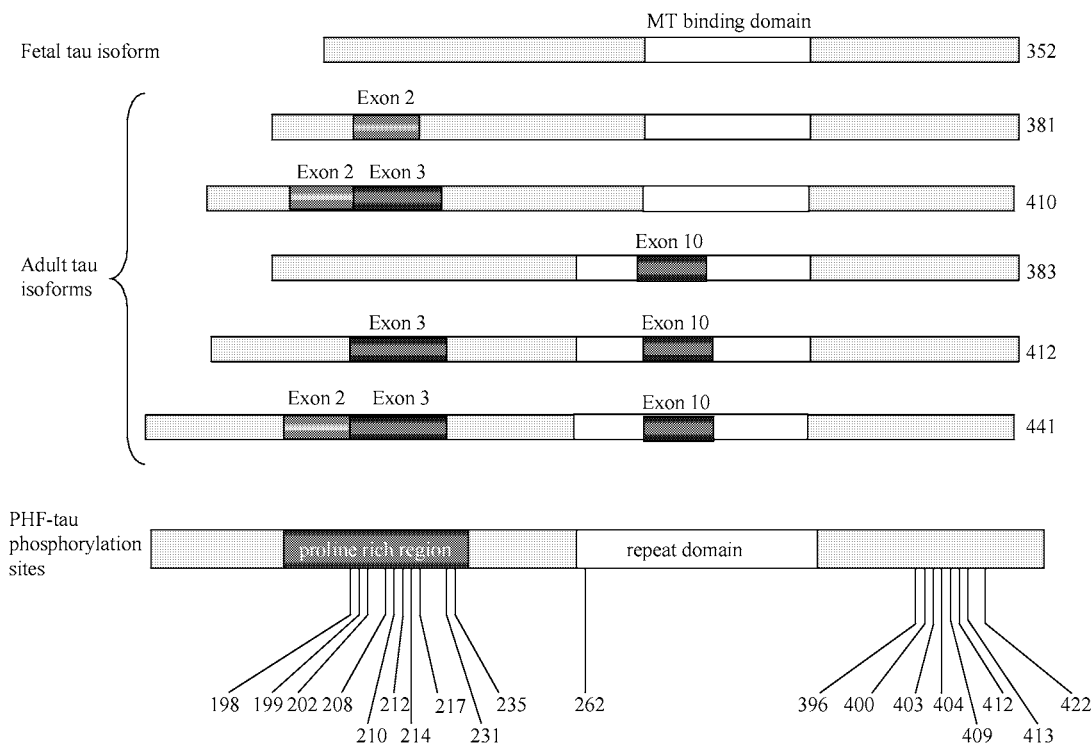
Figure 2. Different tau isoforms and the phosphorylation sites of PHF tau. Modified from (Brandt et al., 2005). Abbreviations: microtubule, MT; paired helical filament, PHF.

GSK-3β

One of the enzymes responsible for the phosphorylation of tau is GSK-3β. GSK-3β is a 45 kDa enzyme involved in glycogen metabolism. In addition, GSK-3β participates in signal transduction, gene expression, apoptosis and cell fate specification (Kim and Kimmel, 2000). GSK-3β is able to phosphorylate several AD associated epitopes in tau, such as Ser-199, Thr-212, Thr-214, and Ser-404.

GSK-3β is a unique enzyme in an aspect that it is activated by dephosphorylation in serine-9 and subsequently inactivated by phosphorylation of the same epitope. Several kinases are able to inactivate GSK-3β by phosphorylating serine-9 epitope. These kinases include Akt, p70 S6 kinase, PKA, PKC and p90Rsk (Grimes and Jope, 2001b).

GSK-3β has been linked to AD in many studies and the pathways by which GSK-3β may contribute to AD pathogenesis are summarized in figure 3. For example, active GSK-3β has been detected in neurofibrillary tangle bearing neurons in human AD brain (Pei et al., 1999) and in brains of patients with early Alzheimer' disease (Hye et al., 2005). Several *in vitro* studies show that Aβ can activate GSK-3β, eventually leading to increased tau phosphorylation and loss of microtubule binding (Busciglio et al., 1995; Takashima et al., 1996). GSK-3β has also been shown to interact with PS, thus increasing the production of APP and Aβ (Phiel et al., 2003; Takashima et al., 1995) and this has been confirmed also with *in vivo* findings showing that inhibition of GSK-3β reduces the plaque burden of transgenic AD mice (Phiel et al., 2003). GSK-3β may also directly phosphorylate APP *in vitro* (Aplin et al., 1996). Interestingly, AD-related mutated PS1 has been shown to bind to and activate GSK-3β (Takashima et al., 1998), but also indirectly regulate the activity of GSK-3β by regulating Akt in hippocampal neurons (Weihl et al., 1999). Transgenic mice with increased GSK-3β activity show increased tau phosphorylation and behavioral deficits, thus providing further evidence of the detrimental role GSK-3β activity in neuronal survival (Hernandez et al., 2002; Lucas et al., 2001; Spittaels et al., 2000). In addition, a recent report showed that passive immunization with antibodies targeted against oligomeric Aβ inhibited the activation of GSK-3β, suggesting that the protective effect may have been mediated through inhibition of GSK-3β (Ma et al., 2005).

Additionally, GSK-3β activated by Aβ1-42 has been shown to suppress acetylcholine synthesis in cholinergic neurons *in vitro* (Hoshi et al., 1997). This effect was shown to be mediated by phosphorylation of pyruvate dehydrogenase (PDH) by GSK-3β leading to a depletion of acetyl coenzyme A. Acetyl coenzyme A is a precursor for acetylcholine and therefore important in acetylcholine synthesis (Hoshi et al., 1997). This finding links the activity of GSK-3β into a well documented loss of cholinergic neurons in AD. GSK-3β activation may also affect cell survival by other mechanisms: by phosphorylating PDH, an important enzyme in the mitochondrial Krebbs cycle, GSK-3β can impair the neuronal energy supply in hippocampal neurons (Hoshi et al., 1996). GKS-3β has been hypothetized to prevent the expression of neurotrophic transcription factors, thus hampering the ability of cells to cope with subsequent cell stress. GSK-3β has been shown to negatively regulate transcription factors *in vitro*, such as heat shock factor-1 (Bijur and Jope, 2000; He et al., 1998) and cyclic AMP response element binding protein (Bullock and

Habener, 1998; Grimes and Jope, 2001a) which are important in cell survival mechanisms after potentially toxic insults (Davis et al., 1996; Grimes and Jope, 2001b; Jean et al., 1998; Struthers et al., 1991; Wilson et al., 1996). Thus, the impairment of these transcription factors can affect the ability of cells to respond to cell stress.
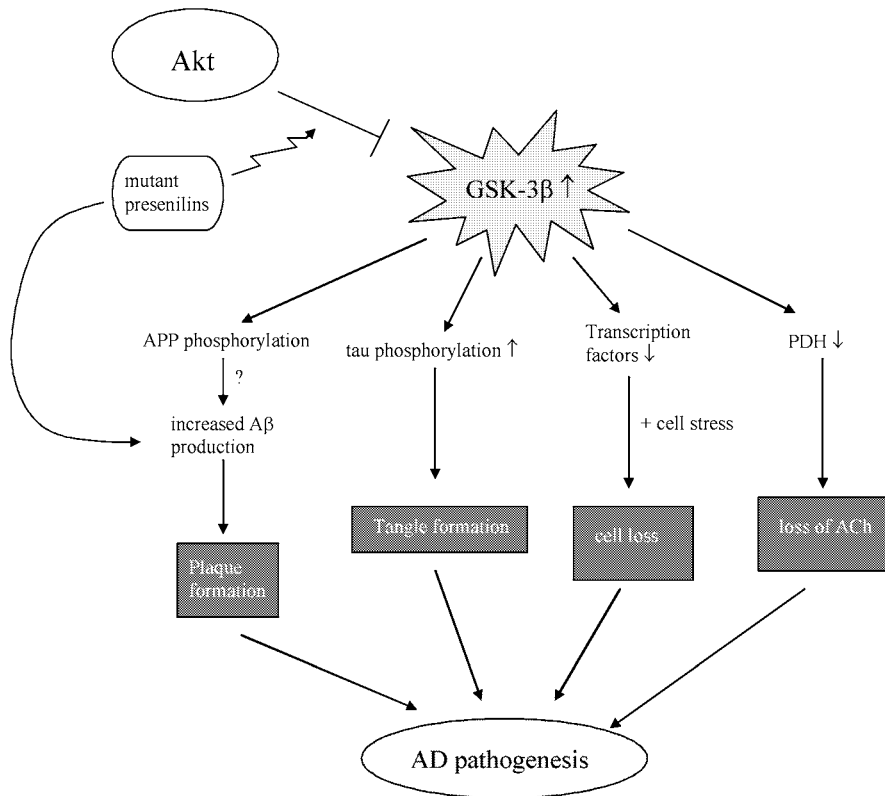
Figure 3. The role of GSK-3β in AD pathogenesis. GSK-3β may contribute to AD pathogenesis by enhancing the production of toxic Aβ peptides, thus promoting plaque formation. GSK-3β may also enhance tau phosphorylation, cause cholinergic neuron loss by phosphorylating pyruvate dehydrogenase (PHD) and diminish the ability of cells to respond subsequent cell stress. Modified from (Grimes and Jope, 2001b). Abbreviations: amyloid precurson protein, APP; pyruvate dehydrogenase, PHD; acetylcholine, ACh; glycogen synthase-3β, GSK-3β.

Mutations influencing the phosphorylation of tau

There are more than 30 known mutations in tau and many of them increase the phosphorylation and subsequent formation of PHFs (reviewed in (Brandt et al., 2005). While none of these mutations have been directly linked as the causative factor for AD, many of

them have been associated with frontotemporal dementia and parkinsonism linked to chromosome 17 (FTDP-17). FTDP-17 is an autosomal dominantly inherited neurodegenerative disease that is characterized by cognitive deficiency, and pathologically characterized by frontotemporal athrophy associated with neuronal loss, gliosis and formation of intraneuronal tau filaments in the absence of $A\beta$ plaques. The presence of tau mutations in FTDP-17 has suggested that hyperphosphorylation of tau and subsequent formation of fibrils may cause direct neuron damage. This would also implicate the neurotoxic mechanisms of hyperphosphorylated tau in AD pathology.

2.1.4 Animal models of Alzheimer's disease

AD animal models can be roughly divided into two categories: non-transgenic and transgenic animal models. Non-transgenic animal models are based on the generation of rather restricted pathological abnormalities seen in AD, and are generated by administration of agents causing neuron loss and memory impairments. The creation of transgenic animal models boosted after the discovery of FAD linked mutations and development of microinjection techniques. Each model has both advantages and disadvantages, but the use of preclinical animal models has proven to be extremely valuable for AD research.

2.1.4.1 Non-transgenic animal models of Alzheimer's disease

Neurotoxin induced animal models

The first animal models used for mimicking AD were based on the production of very restricted pathological findings in Alzheimer's disease, such as cholinergic neuron loss and defects in cholinergic function (Chapman et al., 2001). One of the earliest models utilized the injection of kainic acid to the ventral pallidum of rats (Friedman et al., 1983; Ksir and Benson, 1983), producing reasonably strict loss of cholinergic neurons and deficits in passive avoidance task. Also, ibotenic acid administration was shown to cause a loss of cholinergic neurons and subsequent memory impairment in passive avoidance behavior when administered into the cholinergic nucleus basalis magnocellularis (Fine et al., 1985; Schwarcz et al., 1979). A more selective loss in cholinergic neurons and a greated reduction in cortical ChAT activity were obtained with quisqualic acid (Robbins et al., 1989) or α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA) (Muir et al., 1994) injections into the nucleus basalis (Dunnett et al., 1987). In addition, a muscarinic receptor antagonist, scopolamine, has been used to mimic AD-like loss of cholinergic neurons (Smith, 1988). Intraventicular injections of monoclonal antibody 192 IgG saporin have also been described

to selectively destroy neurons expressing nerve growth factor receptors and induce deficits in passive avoidance learning (Wiley et al., 1995).

Alzheimer's disease infusion models

Direct Aβ infusion paradigms have been applied in an attempt to mimic the more common form of AD, the sporadic AD. It is fairly difficult to address the causal relationship between Aβ and the development of behavioural abnormalities in transgenic animal models, since the overexpression of wild type or mutated APP may induce overproduction of other cleavage products of APP in neurons. CTFs of APP may also contribute to brain dysfunction and development of cognitive deficits (Koistinaho and Koistinaho, 2005). Moreover, none of the transgenic AD models represent the full range of pathological or behavioral abnormalities of AD. In addition, all the models lack frank neuronal loss. The development of excess plaque pathology per se is most likely not causing the toxicity, since the plaque number tends to correlate poorly with behavioral deficits and behavioral deficits can occur in the absence of Aβ deposits (Chishti et al., 2001; Holcomb et al., 1999; Hsia et al., 1999; Koistinaho et al., 2001; Neve and Robakis, 1998). Recent reports have also shown that the oligomeric form of Aβ is actually more toxic than the fibrillar form of Aβ and correlates better with the severity of AD (Klein et al., 2001; McLean et al., 1999) .

Due to the limitations in transgenic animal models, several groups have attempted to develop AD animal models reflecting the sporadic forms of Alzheimer's disease. These animal models are produced by intracerebral or intracerebroventricular injections or infusions of different forms of Aβ. The attempts in trying to show the toxic effect of injection or infusion of Aβ have yielded some contradictory results and the reported deficits vary. Several groups have been able to demonstrate the *in vivo* toxicity (Craft et al., 2004b; Frautschy et al., 1996; Itoh et al., 1996; Nitta et al., 1994) of Aβ, whereas some have reported failed attempts to gain toxicity (Clemens and Stephenson, 1992; Games et al., 1992; Podlisny et al., 1993; Winkler et al., 1994). Most of the differences may be explained by different infusion paradigms and rodent species and the age of animals used in the studies. Most of the studies have been done on young rats. The nature of Aβ peptides makes them difficult to handle, since they are highly prone to aggregation. Whereas in some studies Aβ peptides have been dissolved in water (Nakamura et al., 2001), methods have been developed to keep the peptide in a constant and more toxic, oligomeric form (Dahlgren et al., 2002). Since the toxicity of

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Aβ depends on the conformation of the peptide, this may offer an explanation for some of the contradictory results.

In addition to a relevant approach for studying the sporadic form of AD, the advantages of the infusion models include the ability to study the toxicity of specific forms of Aβ peptide, their combinations, and co-infusion of various other agents afffecting the fibrillarization and degradation of Aβ (Frautschy et al., 1998a). The infusion paradigm also allows the infusion of Aβ into various transgenic mouse lines, thus avoiding time-consuming breeding to produce a double transgenic mouse line (Craft et al., 2005). However, most of the infusion models reported so far have been done in rats and the only mouse infusion model developed so far was reported by Craft et al (Craft et al., 2004a; Craft et al., 2004b).

Unfortunately, the mere infusion of Aβ has been able to capture only some pathological abnormalities seen in AD. Therefore, rather than modelling the neuropathology of AD, these infusion models offer a valuable tool to study the direct Aβ neurotoxicity without the possible effects of APP overexpression on neuronal viability.

The effects caused by infusion of Aβ1-40 in rats

The Aβ1-40 peptide has been widely used in the infusion studies, mainly because it is less prone to aggregation and therefore easier to maintain in oligomeric form. Continuous intracerebroventricular infusion of Aβ1-40 has been shown to induce learning and memory impairment, as well as deficits in the cholinergic system (Nitta et al., 1994). A similar infusion paradigm also showed that basal synaptic transmission and different forms of synaptic plasticity (long term potentiation, post-tetanic potentiation and paired-pulse facilitation) were deficient in the hippocampal slices of the Aβ1-40 infused rats (Chen et al., 2006). Others have reported that infusion of Aβ1-40 leads to decreased glutathione levels and glutathione reductase activity, and an increase in some markers of oxidative stress (Hashimoto et al., 2002). All of these defects were improved by the administration of docosahexaenoic acid, an antioxidant, further proving the involvement of oxidative damage in this particular infusion model. In a very recent study, continuos infusion of Aβ1-40 into lateral ventricles of rats caused impairments in behavior in Y-maze and water maze and reduced the choline acetyltransferase (ChAT) activity in specific brain regions and decreased the glutathion-S-transferase (GST) immunoreactivity in cortex (Yamaguchi et al., 2006). These deficits were ameliorated by treatment with azaindoliainone derivative ZSET1446.

It is of interest to note that few studies have actually shown clear deposition of infused Aβ, suggesting that the infused peptide was rapidly cleared from the parenchyma, or that the amount of deposited Aβ in the brain is under the detection limit of immunohistochemical methods. Frautscy et al. showed that bilateral coinjection of transforming growth factor β-1 (TGFβ1) into the anterodorsal thalamus markedly increases the deposited Aβ at 1 month after the start of the infusion (Frautschy et al., 1996). Also, coinfusion of protease inhibitor leupeptin significantly increased the deposition of Aβ1-40 after a 1 month infusion period, suggesting that age-related dysfunction of lysosomes/endosomes could promote Aβ deposition similar to seen in AD (Frautschy et al., 1998a)

The effects caused by infusion of Aβ1-42 in rats and mice

Infusion of Aβ1-42 has been shown to induce a dose and time dependent impairment in Y-maze, water maze and passive avoidance retention in rats (Nakamura et al., 2001). The effects became more prominent with time; 85 days following the infusion a reduction in ChAT activity was observed in specific brain areas, and brain athrophy, ventricular enlargement and neuronal damage in the CA1 region of the hippocampus were seen. However, Aβ deposition was not reported.

Recently Craft et al. reported a mouse infusion model, in which continuos infusion of Aβ1-42 resulted in AD-like neuroinflammation, including micro- and astrogliosis, neuron loss in the CA1 region, loss of synaptic markers, deposition of Aβ and hippocampal dependent memory deficits when analyzed two months after the start of the infusion (Craft et al., 2004a; Craft et al., 2004b). To ensure a wide penetration of Aβ1-42 into the brain parenchyma, high density lipoprotein (HDL) was co-infused as a carrier molecule. The neuroinflammation seen in this infusion paradigm appears to be upregulated 4-6 weeks after the start of the infusion (Craft et al., 2006).

The effects caused by coinfusion of Aβ1-42 and Aβ1-40 in rats

Since the brain normally produces both Aβ1-40 and 1-42, Frautschy et al. used coinfusion of these two peptides to obtain AD-like pathology. Coinfusion of Aβ 1-40 and 1-42 in a similar relationship than than found in brain (1 : 9), together with HDL as a carrier resulted in oxidative damage, synaptophysin loss, microglial response and widespread Aβ deposits in middle aged and aged rats at 3 months post-infusion (Frautschy et al., 2001).

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The shorter form of Aβ peptide, Aβ25-35 has been widely used in order to reveal the toxic mechanims of beta-amyloid in Alzheimer's disease. Even though Aβ25-35 is not normally detected in AD brain, it has been shown to be neurotoxic and is not prone to aggregation, making it easy to handle both in *in vitro* and *in vivo* research. Aβ25-35 has been shown to mediate oxidative events in fibroblasts from Alzheimer's disease patients (Choi et al., 2003) and impair hippocampal long term potentiation (Trubetskaya et al., 2003).

Direct single intrahippocampal injection of Aβ25-35 increased reactive oxygen species and lipid peroxidation and impaired learning responses in eight-arm radial maze and these changes were ameliorated by an anti-oxidant molecule, S-allylcysteine (Perez-Severiano et al., 2004). The results from a single intracerebroventricular infusion of aggregated Aβ25-35 suggested that the injection paradigm used caused memory impairments in short rather than long term memory (Stepanichev et al., 2003). Repetitive administration of Aβ25-35 into cerebral ventricles impaired spatial working memory in Y-maze and decreased the number of choline acetyltransferase (ChAT) positive neurons in paraventricular regions (Yamada et al., 2005). Continuous intracerebroventricular infusion of Aβ25-35 lead to Aβ deposition in various brain areas, cholinergic deficits and impairments in passive avoidance and alternation behavior (Maurice et al., 1996). In addition, cell death, decreased p-Akt levels and deficits in somatostatinergic system have been reported (Aguado-Llera et al., 2005; Hervas-Aguilar et al., 2005)

2.1.4.2 Transgenic animal models of Alzheimer's disease

The finding of mutations directly causing the familial form of AD greatly enhanced the development of transgenic animal models for AD research. Most of these models are based on the over-expression of human wild type or mutated APP (APP) under different promotes, hAPP together with mutations in presenilin genes, or triple transgenic mice expressing hAPP and mutated forms of presenilin and tau. These transgenic animal models are important in the understanding of molecular mechanisms leading to this devastating disease, such as processing of APP and formation of neurofibrillary tangles. Even though these animal models represent a variety of different pathological features of AD, none of them succeeded in developing the full range of neuropathology and behavioral deficits observed in AD patients. None of the transgenic models generated so far exhibit extensive neuronal loss. Interestingly, all the models differ from each other with respect to the

development and range of neuropathology, which depends on the transgene construct and promoter used, making them relevant for use in different applications in AD research.

Mice expressing APP

Mice expressing wild type APP

The first attempts to develop a transgenic AD mouse model were based on the amyloid cascade hypothesis: the overexpression of human APP would lead to increased levels of neurotoxic Aβ, tau fibrils and evidently neuron loss. Several groups developed mice expressing either wild type or FAD-linked mutant APP. One of the first models was developed by Quon et al. in 1991 (Quon et al., 1991), an APP751 mouse model in which the human wild type 751 amino acid form of APP was overexpressed under the control of neuron specific enolase (NSE) promoter. These mice developed some local gliosis and Aβ deposits, but dense core neuritic plaques were not apparent (Higgins et al., 1994). Interestingly, these mice developed behavioral deficits as early as 3 months of age and the deficits increased with aging (Koistinaho et al., 2001). A similar attempt was made by another group which overexpressed APP751 and APP695 under the same promoter, but in a different mouse background (Mucke et al., 1994). These transgenic mice showed no evidence of Aβ accumulation, but instead had increased numbers of synaptophysin immunoreactive presynaptic terminals and expression of growth cone marker GAP-43. Several groups also attempted to create transgenic mice by overexpressing CT100 fragments, which consist of the Aβ region and cytoplasmic domains of APP, under different promoters (Kammesheidt et al., 1992; Nalbantoglu et al., 1997; Sandhu et al., 1991). Overexpression of CT100 under viral JC early region promoter led to the accumulation of intraneuronal and extracellular Aβ (Sandhu et al., 1991). Nalbantoglu and coworkers reported a model created by overexpression of CT104 under human NF-L promoter which showed Aβ deposits, gliosis and deficits in LTP and Morris Water Maze. In addition, these mice exhibited neuron loss in the hippocampal CA1 region. However, neuron loss was not seen in other areas affected in AD, such as in cortical areas.

Mice expressing mutated APP

It soon became obvious that mere overexpression of wild type APP or APP fragments was not efficient in producing extensive AD like pathology. Thus, many groups

attempted to create transgenic mice over-expressing FAD-linked mutated forms of APP. One of the best characterized model was developed by Games et al. in 1995 (Games et al., 1995), called the PDAPP mouse (line 109). This model was created by the overexpression human APP minigene with FAD linked mutation V717F under the control of platelet derived growth factor promoter. The expression of the minigene allowed the formation of different splicing variants of APP: 770, 751 and 695. The promoter used was efficient enough to drive the expression of the transgene 10 x higher than the endogenous APP (Rockenstein et al., 1995), thus promoting the development of more extensive AD pathology compared to previous models. These mice exhibited diffuse $A\beta$ deposits starting at the age of 4 - 6 months and the accumulation was greatly enhanced between 7 and 9 months of age. The accumulation was regionally specific like observed in AD patients (Chen et al., 1998; Irizarry et al., 1997b), and was associated with micro- and astrogliosis (Chen et al., 1998; Irizarry et al., 1997b). Interestingly, the mice developed deficits in behavioral tests before the appearance of $A\beta$ deposits, but the deficits were also shown to correlate with the brain $A\beta$ burden in a different set of testing paradigms (Chen et al., 2000a; Dodart et al., 1999). Subseqent studies suggested that the behavioral deficits could be explained partly by $A\beta$ deposition and partly by hippocampal athrophy and changes in synaptic density which occur well before the $A\beta$ deposition (Dodart et al., 2000). In contrast to human AD brain, no global neuronal loss or neurofibrillary tangles were seen (Chen et al., 1998; Irizarry et al., 1997b; Masliah et al., 2001). The same transgene construct over-expressed in a different mouse backround, C57BL/6 x DBA/2 hybrid backround, and subsequently named PDAPP line H6, also caused extensive $A\beta$ pathology (Wyss-Coray et al., 1997). This line expressed slightly more $A\beta$ than the previously described line, and these mice were shown to have loss in presynaptic density and neurons before the development of $A\beta$ plaques (Hsia et al., 1999). These studies further supported the toxicity of $A\beta$ independent of plaque formation.

A year later from the original publication of the PDAPP mouse model, Hsiao and coworkers published another, a Tg2576 mouse line, carrying a double Swedish mutation (K670N and M671L) in human APP cDNA under the control of hamster prion protein (PrP) promoter (Hsiao et al., 1996). These mice developed an increase in $A\beta1-42$ / $A\beta1-40$ ratio which was accomppanied by an impairment in memory at 9 - 10 months of age. These mice showed similar neuropathology to PDAPP mice; the $A\beta$ plaques appeared in the hippocampus, cortex and subiculum (Hsiao et al., 1996) and were associated with gliosis. In addition, markers for oxidative stress were upregulated (Frautschy et al., 1998b; Smith et al.,

1998). Similar to other transgenic mice, these mice showed no neuronal loss (Irizarry et al., 1997a), however, age-dependent disruptions in hippocampal LTP may underlie the observed memory deficits (Chapman et al., 1999).

An industrial interest in transgenic AD mice in terms of drug discovery boosted the development of APP22 and APP23 mice, which was reported by a pharmaceutical company Novartis in 1997 (Sturchler-Pierrat et al., 1997). These mice carried the APP751 harboring either a Swedish mutation (APP23) or Swedish and V717I mutation (APP22). APP22 mice expressed the transgene at only 2-fold levels and developed a late and subtle neuropathology, whereas APP23 mice expressed the transgene 7-fold compared to mouse endogenous APP and developed plaques as early as at the age of 6 months. The lesions were accompanied with neuritic changes and loss of cholinergic fibers. Gliosis and tau hyperphosphorylation were apparent and unlike other transgenic models, these mice showed prominent neuron loss in hippocampal CA1 region (Calhoun et al., 1998).

In addition, others have generated transgenic mice harboring Swedish mutation (Borchelt et al., 1996; Moechars et al., 1999). The mouse model created by Borchelt et al. did not develop plaques until very late (at 18 months of age) (Borchelt et al., 1997; Borchelt et al., 1996), similar to the model created by Moechars (Moechars et al., 1999). Moechars et al. reported that the Aβ pathology developed at 18 months of age in mice carrying the Swedish mutation, and at 13 months of age in mice carrying the London mutation. The memory impairment, decreases in LTP and differences in glutamatergic responses developed well before the actual plaque pathology.

The advantage of mutations in APP was taken a step further, when Chishti et al. reported a transgenic mouse model over-expressing a double mutant APP695: Swedish and V717F mutations under the control of PrP promoter (Chishti et al., 2001). Due to an enhancement of the generation of Aβ peptides, these mice developed more severe Aβ pathology than any of the single mutant APP transgenic mouse lines. Fibrillar Aβ deposits were apparent as early as at 3 months of age with inflammatory response at 5 months of age. Deficits in behavior appreared at 3 months of age and did not correlate with the plaque pathology (Chishti et al., 2001).

In conclusion, the neuropathology of transgenic APP mice seems to be linked to the expression levels of the transgene. The expression levels, on the other hand, may depend on the promoter used. Furthermore, mutations in APP cause elevated Aβ1-42 / 1-40 ratio, thus enhancing the development of AD pathology. The plaque pathology of the transgenic mice

rarely correlates with the observed memory deficits, supporting the role of memory disrupting Aβ oligomers in the development of cognitive deficits.

Transgenic mice expressing APP and PS1

Even though overexpression of APP at high levels is sufficient to cause AD-like neuropathological changes, simultaneous overexpression of presenilins with FAD-linked mutations was discovered to greatly enhance the production of Aβ. Unlike in human FAD where mutations in presenilins have been directly linked to AD, the mere overexpression of mutated presenilins in mice is not sufficient to induce AD neuropathology despite the increase in Aβ1-42 / 1-40 ratio (Borchelt et al., 1997; Duff et al., 1996). However, crossing the mutant PS1 overexpressing mice with mice harboring APP Swedish mutation caused a marked acceleration in the development of AD-like pathology. In contrast to mice expressing APP Swedish mutation alone, the double transgenic mice developed plaques as early as at 9 months instead of 18 months of age (Borchelt et al., 1997; Borchelt et al., 1996). Similarly, crossing the Tg2576 mice with PS1 mice harboring M146L (named PSAPP mice) caused marked acceleration in the plaque pathology development (Holcomb et al., 1998). These mice develop Aβ deposits starting at the age of 3 months, and by the age of 6 months these mice show prominent Aβ deposition and related gliosis in cortical and hippocampal areas.

Even though the expression of both mutated APP and mutated presenilin is clinically not very relevant, double transgenic mice offer a valuable tool for experimental studies in which AD-like plaque pathology is required. Since the development of pathology in double transgenic mice is faster and more prominent compared to single transgenic mice, studies can be carried out more feasibly and the behavioral deficits occur at a reasonably young age. The APP+PS1 mice developed by Borchelt and coworkers (Borchelt et al., 1997; Borchelt et al., 1996) show Aβ deposits and plaques in hippocampal and cortical areas and associated gliosis. The age-dependent impairments in cognition are apparent at the age of 11 - 12 months and correlate with the total hippocampal Aβ (Puolivali et al., 2002). This mouse model was also used in the current study.

Triple transgenic mice

Triple transgenic mice (3 x Tg-AD) are produced by overexpression of mutated human APP, presenilin and tau. Although genetically not very relevant, this model offers valuable information on the interactions of these proteins and the relationship between the

different neuropathological lesions seen in AD brain: Aβ plaques and neurofibrillary tangles. Oddo and collagues reported the development and neuropathological characterization of 3 x Tg-AD harboring mutated APP (Swedish mutation), PS1 (M146V mutation), and tau (P301L mutation) genes (Oddo et al., 2003a; Oddo et al., 2003b). These mice develop both Aβ plaques and neurofibrillary tangles. The Aβ pathology starts with intracellular accumulation of Aβ and the extracellular Aβ plaques become apparent at 6 months of age, whereas the tau fibrils are not seen until 12 months of age. These mice were shown to develop synaptic dysfuntion and deficits in LTP before the appearance of plaques and tangles and interestingly, the deficits in the synaptic plasticity correlated with the accumulation of intraneuronal Aβ. The cognitive dysfunction in these mice appear as deficits in long term retention at 4 months of age, coinciding with the time of accumulation of intraneuronal Aβ (Billings et al., 2005).

2.2 Neuroinflammation

One of the major pathological hallmarks of Alzheimer's disease is chronic inflammation, characterized by the existence of inflammatory mediator cells surrounding the amyloid plaques. Inflammation in AD has been extensively studied, yet the exact role of inflammation in the disease pathogenesis remains elusive. Is neuroinflammation causative promoting the disease progression or merely an attempt of the diseased brain to fight the underlying cause of AD?

2.2.1 Inflammatory mediator cells

2.2.1.1 Physiological functions of microglia

Microglia are the immunological effector cells in the CNS that participate in the innate immune defence. Microglia originate from blood derived monocytes that develop in the bone marrow (BM) and migrate into the developing brain during early embryonic stages (Cuadros and Navascues, 1998). The engraftment of microglia is not restricted to embryonic stages, but occurs throughout the life. Indeed, it has been shown that the brain microglial population renews constantly from BM-derived monocytes (Eglitis and Mezey, 1997; Simard and Rivest, 2004).

In the CNS, microglia exist in two different forms. When the brain is maintained in healthy condition, microglia remain in the ramified morphology. However, in the case of brain injury, microglia turn into ameboid, activated form, and become proliferative (Kim and

de Vellis, 2005). The role of microglia in healthy brain is to protect the brain against possible pathogens and indeed, recent findings show that ramified microglia are constantly surveiling the brain parenchyma (Davalos et al., 2005; Nimmerjahn et al., 2005). The branches of resting microglia provide extensive and continuos surveillance of their cellular environment, ensuring that the entire brain parenchyma is throughly surveilled every two hours. The chemotactic response to tissue injury has been shown to be mediated by purine receptors and connexin hemichannels. Microglia also communicate with surrounding astrocytes in the normal and injured brain (Davalos et al., 2005).

2.2.1.2 Microglia in AD related inflammation

One of the main pathological features of Alzheimer's disease is the accumulation of activated microglia around and within the $A\beta$ deposits. Indeed, microglial activation seems to play an important part in the AD pathogenesis (Akiyama et al., 2000). Activated microglia around plaques are positive for CD11a, CD11b, CD11c (members of $\beta2$ integrin family), LCA (CD45), immunoglobulin $Fc\gamma$ receptors (CD64) and major histocompatibility class II glycoproteins (MHC II) and they also express complement proteins.

The exact role of microglia in AD is not clear. Even though they harbour phagocytic capacity, several studies have failed to show actual $A\beta$ laden vesicles inside microglia in AD models. Most likely the phagocytic capacity of microglia is very limited in AD brain and not sufficient to prevent the formation of $A\beta$ plaques. However, modulation of microglial activity may enable them to effectively phagocytose $A\beta$. This is evidenced by activation of microglia for example by $A\beta$ opsonization (Bard et al., 2000; Schenk et al., 1999) or induction of inflammation by lipopolysaccharide (LPS) injection (DiCarlo et al., 2001), as discussed below.

On the other hand, activated microglia can also be detrimental in AD as they are able to secrete a variety of neurotoxins and chemokines, and thus promote inflammatory reactions and the recruitment of other inflammatory mediator cells in brain (Hanisch, 2002). In the absence of effective clearance of $A\beta$, microglial activation and production of cytokines remains chronic, eventually contributing to neuronal death (Rogers et al., 2002). In support of this, Craft et al. have shown that suppression of inflammation and microglial activation by aminopyridazine reduced cognitive deficits obtained by intracerebroventicular injection of $A\beta$ (Craft et al., 2004a; Craft et al., 2004b).

Microglial activation by Aβ

Several lines of evidence suggest that microglia are activated in response to Aβ deposits in AD brain. Exposure of cultured human microglia isolated from post-mortem normal and AD-brain to fibrillar Aβ activates microglia as detected by dose-dependent secretion of pro-inflammatory cytokines interleukin-1β (IL-1β), interleukin-6 (IL-6) and tumor necrosis factor-α (TNF-α), macrophage inflammatory protein-1α (MIP-1α), monocyte chemoattactant peptide-1 (MCP-1) and the growth factor macrophage-colony stimulating factor (M-CSF) (Lue et al., 2001). Aβ also causes microglial chemotaxis and clustering of activated microglia around the Aβ deposits similar to AD brain (Rogers et al., 2002). Cultured human microglia isolated from post-mortem AD and normal brain show chemotaxis to aggregated Aβ deposits *in vitro* (Rogers et al., 2002) and similar findings have also been reported in murine macrophages and rat microglia (Davis et al., 1992; Maeda et al., 1997).

Fibrillar Aβ may activate microglia through binding to receptor for advanced glycation end-products (Yan et al., 1996), formyl peptide receptor (Lorton et al., 2000) and microglia scavenger receptor (El Khoury et al., 1996), all of which have Aβ as a ligand. Aβ can thus promote signals leading to microglial chemotaxis, proliferation, immobilization, increased macrophage scavenger receptor expression and secretion of reactive oxygen species (El Khoury et al., 1996; El Khoury et al., 1998; Lorton et al., 2000). Microglia express proteins of classical and alternative complement pathway components and both of these can be directly activated by Aβ (Akiyama et al., 2000; Haga et al., 1996; Tuppo and Arias, 2005; Walker, 1998; Walker et al., 1995; Veerhuis et al., 1999). Activation of the complement pathways results in the secretion of complement activation fragments, including anaphylatoxins and opsonins. Anaphylatoxins can cause further activation and chemotaxis, whereas opsonins mediate cell adherence reactions and phagocytosis for cells bearing the appropriate receptors. These include microglia, which express complement opsonin receptors CR3 and CR4 (Colten et al., 1979; Graeber et al., 1988; Rozemuller et al., 1989) and anaphylatoxin receptors C3a and C5a (Gasque et al., 1998; Lacy et al., 1995; Rogers et al., 2002; Yao et al., 1990).

Microglia as phagocytes

The phagocytic role of microglia is a well established feature and several *in vitro* studies have shown the ability of microglia to phagocytose aggregated forms of synthetic Aβ. Microglia express a number of cell surface receptors that bind to Aβ and mediate microglial activation. Aggregated Aβ binds to microglial scavenger class A receptors *in vitro* and the

class B scavenger receptors B1 and CD36, causing modest phagocytosis. Overexpression of M-CSF (Mitrasinovic et al., 2003) and M-CSF receptor (M-CSFR) in mouse and human microglia further increases the phagocytosis, in part through macrophage scavenger receptors (Mitrasinovic and Murphy, 2002). Estrogen increases the phagocytosis of aggregated synthetic Aβ by human cortical microglia (Li et al., 2000). Microglia have also been shown to clear Aβ from non-fixed human AD brain sections through Fc-receptor mediated phagocytosis after incubation with antibodies against Aβ (Bard et al., 2000). Nitric oxide synthase (NOS) inhibitors increase microglial phagocytosis (Kopec and Carroll, 2000) and interestingly, nitric oxide (NO) generating compounds (Kopec and Carroll, 2000) and C1q and MSR ligands (Webster et al., 2000) decrease the microglial phagocytosis, suggesting that microglial activation can be modulated from the cytokine production to phagocytosis in certain conditions.

Even though microglial phagocytosis is very limited in AD brain, several mechanisms have been suggested to induce it *in vivo*. Aβ immunization causes substantial reductions in plaque load in AD transgenic mouse models (Bard et al., 2000; Schenk et al., 1999) and a reduction in brain Aβ burden was also suggested in subset of patients involved in the clinical trial of Aβ immunization (Nicoll et al., 2003). The clearance of Aβ as a result of immunization (either passive or active) has also been linked to significant improvement of cognitive deficits in APP-overexpressing transgenic mice (Schenk et al., 1999). Even though the exact mechanism of action of immunization is still unclear, it has been suggested to be mediated by microglial Fc receptor mediated phagocytosis. Transforming growth factor-β1 (TGF-β1) has also been suggested, at least in part, to promote the phagocytosis by microglia, since transgenic AD mice co-expressing TGF-β1 in astrocytes show increased microglial activation, increased production of related inflammatory mediators, and reduced brain Aβ burden (Wyss-Coray et al., 2001). Local inflammation caused by intrahippocampal LPS injection has also been shown to cause significant activation of microglia and a subsequent reduction in Aβ burden (DiCarlo et al., 2001; Herber et al., 2006; Herber et al., 2004).

Some anti-inflammatory drugs have been shown to effectively modulate microglial activation. Treatment of Tg2576 transgenic mice with anti-inflammatory agent curcumin at a dose of 160 ppm reduces the overall microgliosis, but increases the microgliosis surrounding the Aβ deposits (Lim et al., 2001a). This is associated with a significant reduction in brain Aβ burden, suggesting that microglia may phagocytose Aβ upon curcumin treatment (Lim et al., 2001a). In that study, the effect of curcumin treatment on the behaviour of the Tg2576

mice was not reported, yet in another study, 500 ppm dietary curcumin treatment was shown to prevent the cognitive deficits obtained by intracerebroventricular infusion of Aβ (Frautschy et al., 2001). There is also some *in vitro* evidence that some non-steroidal anti-inflammatory drugs (NSAIDs) may reduce the Aβ induced secretion of potentially cytotoxic pro-inflammatory molecules without preventing the microglial chemotaxis or phagocytosis of Aβ (Strohmeyer et al., 2005).

CD40-ligation has also proven to be effective in modulating the microglial response to Aβ. Immune cells, including microglia, typically require co-stimulus from other cells to become activated. CD40L is an immunoregulatory molecule that is expressed by activated T-cells and CD40R is expressed by microglia. By preventing the CD40-CD40L interaction, for example via neutralizing antibodies to CD40L or knocking out CD40 or CD40L expression in AD transgenic mice (Laporte et al., 2006; Tan et al., 2002), the Aβ burden is reduced. Indeed, Aβ stimulation in the presence of CD40-CD40L interaction has been suggested to cause diminished microglial phagocytosis and a shift in balance towards an adaptive, antigen-presenting state (Town et al., 2005). In addition, co-expression of MCP-1 together with APP lead to an increase in brain Aβ load and in the number of microglia. The microglia however, did not express activated phenotype suggesting that defective Aβ clearance was responsible for the increase in deposited Aβ (Yamamoto et al., 2005).

Even though *in vivo* work often have failed to show actual Aβ laden intracellular vesicles in microglia to prove the phagocytosis without doubt, several lines of evidence arising from *in vitro* work show that once phagocytosed, microglia are able degrade Aβ intracellularly. Aβ phagocytosed by cultured rat microglia has been shown to be localized to both cell surface and to phagosome-like intracellular vesicles (Ard et al., 1996). Also, opsonized Aβ phagocytosed from non fixed human or rodent AD brain sections by cultured microglial cells has been shown to be localized in intracellular phagosomes (Bard et al., 2000).

Microglia in extracellular degradation of Aβ

Microglia can also play a role in the degradation of Aβ by releasing enzymes. Microglia have been shown to secrete insulin degrading enzyme, a protease with the capability to degrage Aβ (Chesneau et al., 2000; Qiu et al., 1998; Qiu et al., 1997). IDE is able to degrade specifically monomeric forms of Aβ, but is unable to degrade oligomeric or aggregated form of the peptide (reviewed in (Selkoe, 2001b).

Microglia as a source of soluble inflammatory mediators in AD brain

Microglia are able to secrete a variety of cytokines upon activation by Aβ (fig 4). These include IL-1, IL-6, IL-8, tumor necrosis factor-α (TNF-α), as well as chemokines such as MIP-1 and MCP-1 (Rogers and Lue, 2001). These secretory products have been postulated to contribute to neuronal death seen in AD brain. Several lines of evidence suggest that continuous cytokine production and inflammation-driven cascades cause further activation and recruitment of microglia and can exacerbate disease progression, or even sensitize to AD pathology (Akiyama et al., 2000; McGeer and McGeer, 2001). This continuous reactive microgliosis has been described as the cycle of neuronal death: as in AD brain the cause of microglial activation is not effectively cleared, microglia may enhance their secretion of inflammatory mediators, and thus promote toxicity to nearby neurons as shown in figure 4. However, the causal relationship between microglial activation, cytokine production, Aβ accumulation and neuronal death has not been resolved (Hanisch, 2002).

In addition to increased neuronal toxicity, cytokines produced by microglia have been suggested to enhance the production of Aβ by enhancing BACE1 activity (Heneka et al., 2005; Sastre et al., 2003). Indeed, several NSAIDs are capable of reducing brain Aβ burden and associated micro- and astrogliosis both in human AD patients and in preclinical animal models, possibly by activating peroxisome proliferator-activated receptor-γ thus decreasing BACE1 activity, targeting γ-secretase or by inhibiting IL-1β secretion and it's downstream target ACT (Eriksen et al., 2003; Lim et al., 2000; Lim et al., 2001b; McGeer et al., 1996; Morihara et al., 2005; Rogers et al., 1993; Sastre et al., 2006; Stewart et al., 1997).

Microglia as contributors in Aβ pathology

Some studies have suggested that microglia themselves may promote the formation of Aβ deposits. Even though the majority of Aβ is produced by neurons (Tuppo and Arias, 2005), microglia have also been shown to secrete Aβ under the influence of Aβ or pro-inflammatory stimulus (Bitting et al., 1996). Others have suggested that rather than phagocytosing Aβ, microglia and blood derived macrophages would promote the formation of plaques (Nagele et al., 2004; Wegiel et al., 2004). A study by Frackowiak and coworkers also showed that phagocytosed Aβ remained in microglial phagosomes for up to 20 days suggesting that microglial cells were fairly ineffective in degrading Aβ (Frackowiak et al., 1992). In addition, in AD brain sections, Aβ fibrils first appear in microglial endoplasmic reticulum and deep infoldings of cell membranes, supporting the hypothesis of microglial

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cells in the production of Aβ rather than their involvement in the clearance of Aβ (Frackowiak et al., 1992).
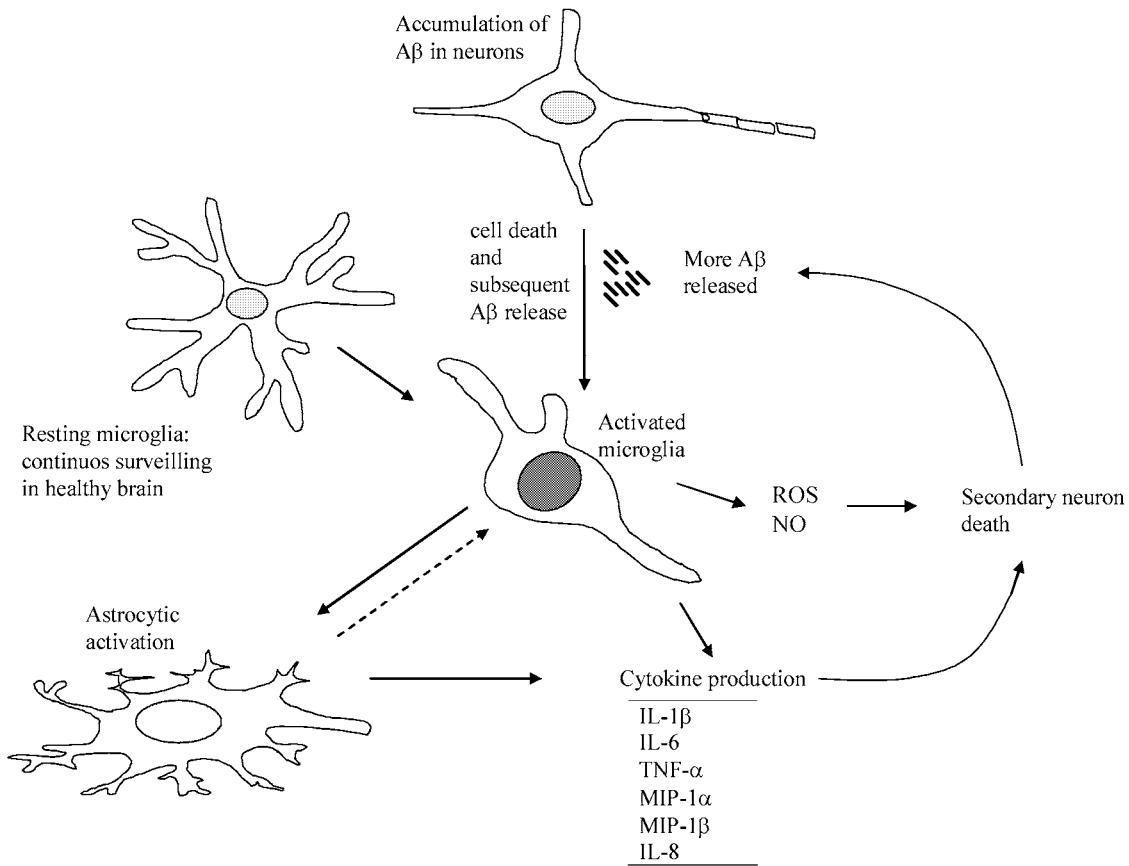
Figure 4. Proposed mechanims of microglial action upon stimulation by Aβ. Aβ can induce the cytokine, mainly IL-1β, IL-6, TNF-α, MIP-1α, MIP-1β and IL-8 production (Rogers and Lue, 2001). These cytokines may cause secondary damage to the neurons and also further activate microglia. Microglia may also secrete reactive oxygen and nitrogen species which cause neuronal damage. In addition, several factors released from dying neurons can further propagate mircroglial production of toxic cytokines. Abbreviations: interleukin-1β, IL-1β; interleukin-6, IL-6; interleukin-8, IL-8; tumor necrosis factor-α, TNF-α; macrophage inflammatory protein-1α and β, MIP-1α and MIP-1β; reactive oxyges species, ROS; nitric oxide, NO.

It has been long recognized that microglia originate from blood derived monocytes (Ling and Wong, 1993; Perry and Gordon, 1988) and recently, it has been shown that the infiltration of monocytes accross the BBB occurs also during the adult life (Eglitis and Mezey, 1997). The major obstacle in the research of microglia has been the inability to differentiate the infiltrated microglia from the endogenous ones which have been migrated and differentiated during early embryonic stages. In 1997 Eglitis and coworkers reported a chimeric mouse model showing microglial migration in healthy brain (Eglitis and Mezey, 1997). In this chimeric mouse model the mice underwent bone marrow transplantation receiving new bone marrow cells from enhanced green fluorescent protein (eGFP) overexpressing mice (Eglitis and Mezey, 1997). The transplanted eGFP positive bone marrow cells reconstituted the blood cell production of the recipient mice, thus allowing the visualization of monocytes transmigrated into the brain. Later it was shown that various pathological conditions, such as ischemia, transection of fimbria-fornix and even very mild injuries that leave the blood brain barrier (BBB) intact such as facial nerve axotomy, increase the migration of blood borne microglia / macrophages in brain (Priller et al., 2001). These studies raised the possibility of the use of peripheral monocytes as non-invasive gene delivery vehicles in neurological disorders. Indeed, bone marrow transplantation has been shown *in vivo* to ameliorate the pathology of lysosomal storage disease mouse models, such as globoid cell leukodystrophy and Sandhoff disease, in which the pathology is caused by a defect in a single gene in the CNS (Platt et al., 2001; Wada et al., 2000; Wu et al., 2000).

2.2.1.3 Physiological functions of astrocytes

Astrocytes greatly outnumber microglia in the brain (Savchenko et al., 2000) and they are classically linked to functions that support neurons. For long they have been known to organize the structural architecture of the brain and be involved in the removal of excess neurotransmitters from the synaptic cleft. However, recently astrocytes have been discovered to have even more important functions including regulation of synaptogenesis, neurogenesis and modulation of synaptic networks. Astrocytes seem to have dynamic and vital interactions with neurons (Ransom et al., 2003).

Astrocytes serve as uptaking glutamate from the synaptic cleft trough glutamate transporter-1 (GLT-1). One of the roles of astrocytes is to maintain the brain ion homeostasis, which is important for the normal neuronal function and excitability. Failure of astrocytes to keep the ion homeostasis within the normal range leads to abnormal neuronal membrane

depolarization, hyperexitability and excessive Ca^{2+} influx into the neurons contributing to excitotoxic damage. This can be further exacerbated by unsuccesful clearance of glutamate from the synaptic cleft by astrocytes. Indeed, glutamate excitotoxicity has been suggested to play an important role in AD pathogenesis. This is supported by findings that both AD patients (Masliah et al., 1996) and transgenic mice overexpressing APP have impaired GLT-1 activity (Masliah et al., 2000). Moreover, glutamate has been shown to promote Aβ induced impairment in LTP (Nakagami and Oda, 2002). Aβ has also been shown to inhibit Na^+-dependent glutamate uptake by astrocytes (Harris et al., 1996) Importantly, the extent of dementia correlates well with neuron loss caused by excess of glutamate (Sonkusare et al., 2005) and a non-competetive NMDA antagonist, Memantine, is used in the clinics for the treatment of AD (Molinuevo, 2003).

2.2.1.4 Astrocytes in AD related inflammation

In AD brain activated astrocytes cluster at the sites of Aβ deposits and form a ring-like structure outside the activated microglia, possibly forming a glial scar around the plaque (Akiyama et al., 2000). Astrocytes are mainly associated with dense core neuritic plaques and are rarely seen within diffuse, non-neuritic plaques (Mrak et al., 1996).

The function of astrocytes in AD is not clearly understood. When activated, astrocytic expression of glial fibrillary acidic protein increases. Activated astrocytes can secrete a variety of neurotrophic factors that are all related to neuronal survival, such as neuronal growth factor, neurotrophic signalling molecule S100β, brain derived neurotrophic factor, neurotrophin 3 and neurotrophin 4/5 (Blasko et al., 2004). Astrocytes may also serve beneficial functions by modulating the phagocytic capability of microglia (D'Andrea et al., 2004) and astrocytes have even been shown to produce Aβ degrading enzymes (Apelt et al., 2003), suggestive of their participation in the degradation of Aβ. In AD brain, astrocytes accumulate Aβ intracellularly (Thal et al., 1999), and unlike microglia (Bard et al., 2000), they have recently been shown to phagocytose Aβ even in the absence of any stimulus, such as opsonization (Wyss-Coray et al., 2003). Astrocytes not only phagocytose Aβ, but also clear it. The uptake and degradation of Aβ has been shown to be dependent on the expression of ApoE (Koistinaho et al., 2004).

On the other hand, astrocytic activity may also be harmful in AD brain, as overexpression of S100β may lead to excessive growth of dystropic neurites. In addition, the glial scar which astrocytes form may hinder the clearance of plaques (D'Andrea et al., 2004).

Astrocytes are also capable of releasing pro-inflammatory mediators, and thus contributing to chronic inflammation (Akiyama et al., 2000). APP holoprotein synthesis has even been shown to increase in cytokine stimulated astrocytes (Amara et al., 1999; Rogers et al., 1999). In addition, Aβ burdened astrocytes may undergo lysis similar to neurons (Nagele et al., 2003), and thus cause subsequent activation of microglia.

2.2.1.5 Neurons in AD related inflammation

Dying neurons not only promote microgliosis and subsequent inflammation, but - almost suprisingly - neurons themselves are also capable of producing inflammatory mediators, such as the complement, TNF-α, IL-1, IL-6, M-CSF and IL-6 (Akiyama et al., 2000). It is therefore possible that neurons themselves also promote the inflammatory reactions seen in AD brain, and thus contribute to their own damage and further activation of non-neuronal cells. Alternatively, release of pro-inflammatory mediators such as TNF-α may act as defence mechanisms against the glial mediated inflammation (Akiyama et al., 2000).

2.2.2 Minocycline and pyrroline dithiocarbamate as immunomodulatory agents

2.2.2.1 Minocycline

Minocycline is a tetracycline and well known anti-inflammatory agent that has been shown to be protective in various pathological conditions of the CNS, distinct from it's antibiotic abilities. These include stroke (Yrjanheikki et al., 1999), amyotrophic lateral sclerosis (Zhu et al., 2002), Huntington's disease (Chen et al., 2000b), traumatic brain injury (Sanchez Mejia et al., 2001), Parkinson's disease (Du et al., 2001) and intracerebral hemorrage (Power et al., 2003). The neuroprotective effects of minocycline have been hypothetized to be based on the suppression of microglial activity and subsequent production of cytotoxic molecules (Tikka et al., 2001; Tikka and Koistinaho, 2001), inhibition of cytochrome C release from mitochondria and inhibition of caspase activation (Chen et al., 2000b; Zhu et al., 2002).

Minocycline in AD

The effect of minocycline in Alzheimer's disease has been addressed in few studies. Wang et al. showed that Aβ mediated inhibition of LTP was associated with increased microglial activity and their production of reactive nitrogen and oxygen species (Wang et al., 2004). Minocycline was shown to prevent the activation of microglia and the inhibition of

LTP (Wang et al., 2004). It has also been reported that minocycline binds to human Aβ peptides and inhibits the formation of amyloid fibrils (Forloni et al., 2001; Tagliavini et al., 2000). In addition, minocycline has been shown to inhibit the microglial cytokine production caused by Aβ, as well as Aβ fibril formation in combination with serum amyloid P component and complement factor C1q (Familian et al., 2006). Hunter and coworkers reported that minocycline was able to attenuate the behavioral deficits and related neuroinflammation induced by lesioning of forebrain cholinergic neurons in mice (Hunter et al., 2004). The neuroprotection was suggested to be mediated by the ability of minocycline to suppress microglial activation and subsequent production of pro-inflammatory cytokines. Seabrook and coworkers also reported that minocycline was able to improve the cognitive abilities of transgenic AD mice when administered before the development of Aβ pathology (Seabrook et al., 2006). Suprisingly, the improvement in cognition was associated with an increase in hippocampal Aβ load and the treatment did not affect microglial activation. On the other hand, when the treatment was started after Aβ deposition had begun, minocycline suppressed microglial activation but had no effect on the Aβ burden or cognitive abilities of the mice (Seabrook et al., 2006). These results point out the complex role of microglial activation in AD-related inflammatory mechanims.

2.2.2.2 Pyrrolidine dithiocarbamate

Pyrrolidine dithiocarbamate (PDTC) belongs to a group of dithiocarbamates that share a common feature of having a functional (R1)(R2)N-C(S)-S-R_3 group. PDTC is a water soluble compound that is relatively stable in aqueous solutions with physiological pH (Topping and Jones, 1988). It also readily crosses the blood brain barrier after oral administration, a property which makes it feasible for a number of applications. Originally dithiocarbamates were discovered as agricultural pesticides, but their use has been widened over the years as they nowadays are also clinically approved for the treatment of alcohol addiction (Antabus) and heavy metal poisoning. However, recently dithiocarbamates have been reported to harbour a wide variety of properties, such as anti-inflammatory, anti-apoptotic and anti-carcinogenic properties, which makes them attractive candicates for use in treatment of other diseases.

As well as with other dithiocarbamates, PDTC has been reported to have an effect on various different experimental models. One of the best described features of PDTC is its antioxidant function, which seem - in many cases - to be mediated by nuclear factor κB

(NFκB) inhibition. The antioxidant functions and NFκB inhibiting properties of PDTC have been demonstrated by many studies (Liu et al., 1997; Schreck et al., 1992; Ziegler-Heitbrock et al., 1993). PDTC has been shown to provide protection against ischemic neuronal death in several different ischemia models (Nurmi et al., 2004a; Nurmi et al., 2004b). The neuroprotective effect of PDTC was shown to be mediated by the inhibition of NFκB and by inhibition of Akt/GSK-3β pathway (Nurmi et al., 2006). In the above mentioned studies PDTC effectively decreased the ischemia induced inflammatory mediators as well as caspase-3. Also, others have demonstrated the anti-apoptotic effects of PDTC (Nobel et al., 1997a). Interestingly, PDTC is also able to inhibit NFκB independently of it's antioxidant functions (Hayakawa et al., 2003).

Studies on the mechanims-of-action of PDTC have yielded contradictory results. Despite the evidence showing the protective actions of PDTC, numerous studies have also shown that PDTC may act as a pro-oxidant (Nobel et al., 1995). Also in contrast to several studies showing PDTCs anti-apoptotic function, PDTC also induces apoptosis is several different *in vitro* models (Chinery et al., 1998; Liu et al., 1998; Tsai et al., 1996). Therefore, PDTC administration has been suggested as an anti-cancer treatment. A 15-day treatment of rats with low concentrations of PDTC also was reported to cause functional and morphological dearrangements in peripheral nerves (Calviello et al., 2005), suggesting that PDTC may also be toxic to neurons. Whether the action of PDTC is anti- or pro-oxidant on PC12 cells has been reported to depend on the dose of PDTC and the presence of metal ions (Chung et al., 2000).

PDTC in Alzheimer's disease

PDTC has been shown to provide protection against Aβ25-35 mediated toxicity; PDTC prevents the Aβ25-35 or Aβ1-40 induced apoptosis in lymphocytes *in vitro* by inhibiting the caspase mediated cell death through the inhibition of NFκB (Cardoso and Oliveira, 2003) or by inhibiting p53 (Jimenez Del Rio and Velez-Pardo, 2006). Kaltschmidt and co-workers also showed that PDTC inhibits the Aβ1-40 or 25-35 induced activation of NFκB in primary neurons (Kaltschmidt et al., 1997). Very recently PDTC was shown inhibit the Aβ accumulation, micro- and astrogliosis, COX-2 immunoreactivity and behavioral deficits following intracerebroventricular infusion of Aβ25-35 (Cheng et al., 2006).

3. AIMS OF THE STUDY

An important pathological hallmark of Alzheimer's disease is the accumulation of activated microglia and astrocytes around Aβ deposits. However, very little is known about the origin and exact role of these activated cells in AD. Some studies suggest that AD pathology is associated with the infiltration of peripheral phagocytes, yet the results were only suggestive due to the lack of methods to distinquish brain endogenous microglia from peripheral macrophages. In addition, the exact role of inflammation in AD brain has remained controversial as to whether inflammation contributes to the progression or aggravation of cognitive deficits and neuropathology in AD, or whether brain inflammation is beneficial with regards to the clearance of Aβ from the brain. This study was carried out to assess the role of inflammatory mediator cells, mainly microglia and astrocytes, in Alzheimer's disease pathogenesis and inflammatory mechanisms in preclinical animal models of AD. The aims of the current study were the following:

1. To assess the role of BM-derived monocytic cells in AD transgenic mouse model using novel transplantation techniques to distinquish endogenous microglia from blood derived cells; to investigate whether Alzheimer's disease-like pathology increases the infiltration of peripheral macrophages / microglia-like cells into brain parenchyma and whether these cells are associated with Aβ deposits, show activated phenotype and take part in AD-like inflammatory reactions seen in AD brain.

2. To address the contribution of BM-derived cells in LPS induced inflammatory reaction and Aβ clearance; to further spesify the roles of endogenous microglia and BM-derived cells in Aβ phagocytosis by inhibiting neuroinflammation using anti-inflammatory agent minocycline.

3. To study whether intracerebroventricular infusion of Aβ results in cognitive deficits in wild type mice and rats, and if so, would it be dependent on Aβ deposition and micro- and astrogliosis; to address whether Aβ1-42 alone would be sufficient to induce an inflammatory response *in vivo* and related *in vitro* models.

4. To analyze whether PDTC treatment of AD transgenic mice, harbouring a significant amount of Aβ deposits and relative gliosis, would be protective against cognitive decline, and if so, would it alter the Aβ deposition and associated micro- and astrogliosis.

4. MATERIALS AND METHODS

4.1 Animals

All animal experiments were carried out according to the National Institute of Health (NIH) guidelines for the care and use of laboratory animals, and approved by the Ethical Committee of the National Laboratory Animal Center, University of Kuopio, Finland. Female mice were housed in groups of 3 - 5 mice in one cage, whereas male mice were housed in individual cages. Rats were housed in groups of 2 - 3 rats in one cage. All animals were kept in a light and temperature controlled environment with ad libitum food and water.

Wild type male C57BL/6j mice and male spontaneuously hypertensive rats (SHR) were used in study III. All mice received standard laboratory rodent chow and rats (III) were fed with diet containing 2 % cholesterol and 1 % sodium cholate (LabDiet TestDiet Cat # 57UJ) starting 1 month before the Aβ infusion and continuing until the start of behavioural testing to ensure the development of Aβ histopathology and behavioural deficits. SHR rats were chosen to maximize the toxic effects of Aβ. Animals were randomly divided into treatment groups within the same genotype. Altogether 34 9-month-old, 38 2.5-month-old male C57BL/6J mice (University of Kuopio, Kuopio, Finland) and 20 2.5-month-old male SHR rats (Taconic, Denmark) were used (III).

The APP+PS1 mice used in studies I and II were carrying chimeric mouse/human APP695 harboring the Swedish mutation and human PS1 with familial AD-linked A246E mutation controlled by prion promoter element (Borchelt et al., 1997). The parental APP695Swe and PS1 (A246E) mice had been backcrossed 13-14 generations to C57BL/6 strain, after which they had been intercrossed to create the double transgenic mouse line (APP+PS1 mice). All together 16 APP + PS1 mice were used (I, II).

eGFP overexpressing mice (Okabe et al., 1997) used in studies I and II were purchased from Jackson Laboratories (Maine, USA) and were maintained in C57BL/6J strain in the Animal Facilities of the National Public Health Institute in Kuopio, Finland.

The APP/PS1-dE9 mice used in studies I and IV were carrying chimeric mouse/human APP695 harboring the Swedish mutation and human PS1-dE9 vectors, both controlled by their own mouse prion protein promoter element (Jankowsky et al., 2004; Jankowsky et al., 2001). The double transgenic mice (APP/PS1-dE9) were backcrossed to the C57BL/6J strain for six generations. All together 20 APP/PS1-dE9 mice and 20 of their wild type controls were used in study IV and 5 APP/PS1-dE9 and 5 of their wild type contros were used in study I.

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Table 1. The mouse and rat strains used in the present thesis.

Mouse line	Study	no of used	Reference
C57BL/6j	III	34	
SHR rats	III	20	
APP+PS1	I, II	16tg	Borchelt et al., 1997
eGFP	I, II	15	Okabe et al., 1997
APP/PS1-dE9	I, IV	20tg + 20wt	Jankowsky et al., 2001, 2004

4.2 Drug treatments

APP/PS1-dE9 -mice and their wild type controls were treated with PDTC (Sigma, St Louis, MO, USA) in drinking water (20 mg / kg / day) for 7 months starting at the age of 9 months (IV). Control groups of APP/PS1-dE9 mice and their wild type controls received normal drinking water. APP+PS1 (II) mice were treated with intraperitoneal injections of bromodeoxy udiride (BrdU, Sigma) every 24 hours (dissolved in 10 mg / ml concentration in saline) at the dose of 50 mg / kg / day. The same mice were also given intraperitoneal injections of either saline or minocycline at the dose of 50 mg / kg starting at 2 hours before surgery and continuing every 12 hours for 3 days and thereafter once a day until the mice were sacrificed at the day 7.

4.3 Bone marrow transplantation

APP+PS1 and APP/PS1-dE9 double transgenic mice and their age-matched wild type controls were lethally irradiated with two doses of 550 cGy 3 hours apart with the dose rate 2.37 Gy / min (Varian 600 C Radiotherapy Accelerator, 4 MV high-energy x-rays). To ensure sufficient surface dose a 1-cm-thick custom-made polymethylmetacrylate lucite beam spoiler (scatterer) was used. On the next day the irradiated mice were transplanted with BM cells (5 x 10^6 cells) isolated from 6 to 8-week-old eGFP overexpressing donor mice by tail vein injection in a protocol similar to described earlier (Kennedy and Abkowitz, 1997). Shortly, the donor mice were sacrificed by carbon dioxide and femur and tibias were dissected out. The bones were placed in ice cold Hank's balanced salt solution (HBSS, Bio Whittaker Europe, Belgium) containing 2 % fetal bovine serum (FBS, Gibco, BRL /

LifeSciences). Bone marrow cells were flushed out by using a 26 G needle and a 10 ml syringe using ice cold HBSS / 2% FBS. The cells were filtered though a 70 μM nylon mesh filter, counted and suspended in appropriated concentration. Each recipient animal received 5 x 10^6 cells in 0.3 ml volume i.v.

4.4 Flow cytometric analysis of eGFP expression in peripheral blood cells

The ability of BM-derived cell to engraft the bone marrow and produce new blood cells was analyzed from blood samples taken 4 or 8 weeks after the transplantation using a dual laser flow cytometer (Becton Dickinson, Mountain View, CA, USA). Blood samples were stained with following antibodies: R-Phycoerythrin (R-PE) conjugated Ly-6G detecting granulocytes, R-PE-conjugated CD11b detecting monocytes, Peridinin chlorophyll-a protein (PerCP) conjugated CD3e detecting mature T-cells and PerCP-conjugated CD45R / B220 detecting B-cells (all from BD Biosciences, NJ, USA). Briefly, 50 μl blood samples were collected from femoral vein into heparinized eppendorf tubes. Blood samples were incubated with above mentioned antibodies in the presence of a blocking antibody mouse IgG1 (Sigma; 10 μg / ml) on ice for 30 min. The cells were centrifuged and lysed with 150 mM NHCl4, 10 mM KHCO3, 0.1 mM EDTA pH 7.4 and washed twice with phosphate buffered saline (PBS) containing 2 % FBS (Gibco). Finally, the cells were resuspended in PBS / 2 % FBS and fixed with 2 % formaldehyde for FACS analysis. Data were evaluated using the Cellquest™ software (BD immunocytometry systems, CA, USA).

4.5 Surgical procedures

4.5.1 Intrahippocampal LPS injection

The transplanted mice received intrahippocampal injections of 4 μg of LPS (4 μg / μl in saline; Lipopolysaccharide from Salmonella typhimurium, Sigma) into the right hippocampus, and an equal volume of 0.9 % NaCl (saline) into the left hippocampus according to the following coordinates: medial / lateral ± 2.5 mm, anterior / posterior -2.7 mm, dorsal / ventral -3 mm 16 weeks (I) or 12 months (II) after the transplantation. The mice were anesthetized with halothane (initial dose of 5 % which was reduced to 1.2 % for the time of surgery) and placed in a stereotaxic apparatus (David Kopf, model 940, Tujunga, CA, USA). Two holes were drilled in the cranium above the hippocampi and injections were made using a 5 μl syringe (Hamilton, Reno, Nevada) over a period of 10 minutes. After the

injection the needle was left in place for additional 4 minutes before pulling it up slowly. Finally, the incision was cleaned with saline and closed with silk sutures.

4.5.2 Aβ infusions

4.5.2.1 Preparation of oligomerized Aβ

The oligomerized Aβ was prepared as previously described (Dahlgren et al., 2002). Briefly, 1 mg of Aβ1-42 (American Peptide. Sunnyvale, CA, USA) was dissolved in 220 μl of 1,1,1,3,3,3-hexafluoro-2-propanol (HFIP, Sigma) and incubated for 30 minutes at room temperature. The HFIP was evaporated by speed vacuum for 1 hour. The dry pellet was further dissolved in 20 μl of DMSO (Sigma, Germany) and 480 μl of 4 mM Hepes pH 8.0. The concentration of the peptide was determined by DC protein assay kit (Bio Rad, Hercules, CA, USA). For infusion, the peptide was mixed with HDL (Calbiochem, Germany) so that each pump (in 100 μl volume) contained 40 μg of oligomerized peptide and 1mg of HDL. The control pumps were prepared similarly but in the absence of Aβ and contained (in 100 μl volume) 1 mg of HDL in 4 mM Hepes (pH 8.0).

For the analysis of Aβ oligomerization and the time course of Aβ release, extra pumps were prepared in each experiment. Sample solution was collected to eppendorf tubes at 37°C, crosslinked with glutaraldehyde, and analyzed on sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). Briefly, 4 μg samples were collected from the eppendorf tubes attached to infusion pumps and crosslinked with glutaraldehyde as described previously (Levine, 1995). The samples were incubated 10 minutes at room temperature and the reaction was stopped by adding 2 x sample buffer. 0.5 μg samples were run on the 18 % multiphasic buffer system SDS-PAGE gel without boiling in order to preserve the conformation of Aβ peptides. After the run the proteins were transferred to Hybond-P membrane (Amersham Biosciences, UK). The membranes were blocked in 5% skimmed milk solution in phosphate buffered saline containing 5 % Tween 20 (PBST) and incubated with human Aβ antibody (clone 6E10, 1:1000 dilution, Signet, MA, USA) overnight at 4°C. This was followed by incubation with horseradish peroxidase (HRP) -conjugated secondary anti-mouse antibody (Amersham Biosciences) and detection by ECL Plus -kit (Amersham-Pharmacia Biotech). Finally, the membranes were scanned on STORM (Molecular Dynamics) fluoroimager.

4.5.2.2 Infusion surgery

The anesthetized animals were placed in a stereotaxis (David Kopf model 940), the head of the animal was shaved and incision was made to expose the skull. A hole was drilled in the cranium above the pump implatation site. Stainless steel catheters were attached to miniosmotic pumps (model 1002, Alzet, Palo Alto, USA) and implanted into the following coordinates: mouse (unilaterally): +1.0 mm medial / lateral, -0.46 mm anterior / posterior, -1.75 mm dorsal / ventral; rat (bilaterally): ±1.9 mm medial / lateral, -0.9 mm anterior / posterior, -4.5 mm dorsal / ventral. The pump contents were released over a period of 2 weeks consisting of either the total 40 µg Aβ with 1.0 mg HDL (n = 18 for mice, n = 10 for rats) or 1.0 mg of HDL only (n = 16 for mice, n = 10 for rats) per each pump.

4.6 Behavioral testing

Mouse Morris Water Maze (MWM). The spatial navigation and reference learning of the mice was assessed by MWM (III, IV). The mice infused with Aβ (III) at the age of 9 months were randomly divided into two groups. The first group was tested at 6 weeks postinfusion. Since no behavioural deficits were detected at that time, the first group was retested at 12 weeks and the second group was tested only at 12 weeks after the start of the infusion. The effect of PDTC treatment (IV) on the spatial learning and memory was assessed by at the age of 16 months. For all experiments, a similar protocol of MWM was used.

A plastic pool with a diameter of 120 cm was used for the testing contained a hidden black square platform (14 x 14 cm) 1 cm below the water surface. The water temperature was kept at 20 ± 0.5°C throughout the experiment. The mice were trained to find the invisible platform for 2 days by using an alley (1 m x 14 cm x 25 cm) leading to the platform located 1 cm below the water and were allowed to have a 10 minute recovery period between the trials. The position of the platform was kept constant. The animal was placed in the pool with the nose pointing towards the wall at one of the starting points in a random manner. The training consisted of 8 (III) or 4 (IV) consecutive days of testing with five trials per day. The mice were given 60 seconds to find the platfom and if not found within that time, the mouse was placed on the platform for 10 seconds by the experimenter.

On the last day of the trial, the platform was removed and the mice were allowed to search for it for 60 seconds (III) or 30 seconds (IV) in order to determine their search bias. The swimming pattern was monitored by a computer connected to an image analyzer (HVS Image, Hampton, UK) and swimming speed and latency to find the platform were measured.

Search bias during the probe trial was measured by calculating the time the mice spent in the vicinity of where the platform was previously located. On day 7 the platform was placed into the opposite side of the pool and the mice were allowed 6 trials to find it for a reversal experiment. To exclude occasional floating mice, only mice with a swimming speed of 8 cm / s were included into the analysis (IV).

Spontaneous alteration Y-maze. The behavioral deficits of the infused rats were assesed by spontaneous alteration Y-maze. The rats were placed in one arm of the t-maze test apparatus (made of plastic with following dimensions: arm lenght 35 cm, height 25 cm, width 10 cm) and allowed to freely move in the maze for 8 minutes. The sequence of arm entries was recorded manually by the observer. Since the spontaneous alteration behavior is considered to reflect a primitive form of spatial short term working memory, the behavior was defined as the sequential entry into all three arms on consecutive choises in overlapping triplet sets. The percent spontaneous alternation score was calculated as the ratio of actual to possible alternations.

4.7 Immunohistochemistry

Mouse brains. Mice were anesthetized and perfused with heparinized saline (2500 IU / 1 liter saline). For biochemical analysis and western blotting one brain hemisphere was dissected out into hippocampal and cortical samples and snap frozen in liquid nitrogen. For immunohistochemistry, the whole brains or one of the hemispheres were immersion-fixed with 4 % paraformaldehyde for 20 hours, followed by cryoprotection in 30 % sucrose for 3 days. Frozen brains were cut into 10 μm (I) or 20 μm (II-IV) thick cryosections. The brains were analyzed by the following antibodies: microgliosis was analyzed by CD11b (1:500 dilution, Serotec, UK), CD45 (1:100 dilution), and I-A/I-E antibody recognizing MHC class II alloantigens (1:100 dilution, Serotec), mouse macrosialin lysosomal associated protein CD68 (1:2000 dilution, Serotec) and Iba1 (Wako, Germany). Astrogliosis was analyzed by GFAP (glial fibrillar acidic protein, 1:500, DAKO, Denmark or Chemicon, Temecula, CA, USA) antibody. T-cells were deteted by CD3e antibody (1:1000 dilution, Serotec) and brain Aβ burden was analyzed by pan-Aβ (1:250 dilution, Biosource, Belgium) or 6E10 antibody (1:1000 dilution, Signet, MA, USA). For Aβ antibodies the sections were boiled in sodium citrate buffer (pH 6.0) for 30 minutes prior to primary antibody incubation. All antibodies used are listed in table 3. For study III the brain sections were reacted against phospho-tau AT8 antibody (1:100 dilution, Innogenetics, Belgium).

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For fluorescence detection (I - IV), primary antibody incubations were followed by incubation with Alexa-fluor 488 or 568 -conjugated secondary antibody (1:200 dilution, Molecular Probes, Eugene, OR, USA) or alternatively by treatment with biotinylated secondary antibodies (Vector Laboratories, Burlingame, CA, USA) followed by the use of a TSA-amplification system (Perkin-Elmer, Boston, MA, USA) according to manufacturer's instructions. For colorimetric detection (I - IV) the primary antibody incubations were followed by incubation with biotinylated secondary antibody and avidin-biotin complex (Vectastain Elite Kit, Vector Laboratories) system. Immunoreactivities were visualized with H_2O_2 and nickel enhanced diaminobenzidine (NiDAB).

Congo Red or thioflavin staining were used to visualize $A\beta$ plaques in β-pleated sheet configuration. For congo red staining (I, II, III) the cryosections were dried at room temperature for 1 hour, after which they were rehydrated in PBS for 2 minutes following a 20-min treatment in 80 % EtOH containing 0.1 % sodium hydroxide and saturated NaCl. The sections were stained with alkaline 0.2 % Congo Red (Sigma) in saturated NaCl in 80 % EtOH for 20 min followed by dehydratation in an ascending series of EtOH and xylene. The staining was visualized under red fluorescence. For thioflavin staining (IV), the dried sections were rehydrated in PBS for 2 minutes following a 20 min treatment in 10 % thioflavin solution in distilled H_2O. The sections were washed in distilled H_2O and dehydrated in an ascending series of EtOH and xylene. The staining was visualized under green fluorescence.

The ratio of eGFP positive cells out of CD45 and MHC II positive cells in different brain regions and as well as their distribution relative to $A\beta$ deposits (I, II) were analyzed immunohistochemically using an antibody against human $A\beta$ (clone 6E10) and visualizing the green fluorescent cells under appropriate filter sets with a fluorescence (Olympus AX70) or a confocal microscope (BioRad Radiance Laser Scanning Systems 2100, Bio-Rad Microscience Ltd, UK) running LaserSharp 2000 software (Bio-Rad Microscience Ltd). Counting of eGFP expressing cells (I, II) was done by an observer blind to the genetic and/or treatment status of the mice based on the visibility of a cell soma from either 10 μm (I) or 20 μm thick (II) cryosections. Four to six coronal sections at 200 μm intervals at the hippocampal level were evaluated per animal and the cells were counted from the whole section. Immunoreactive cells were counted in 4 - 6 hippocampal sections per mouse from an 0.475 mm^2 area containing the hippocampal subfields stratum pyramidale, radiatum, laconosum-moleculare and molecular layers of the dentate gyrus.

Rat brains. All rat brains were processed for immunocytochemistry similar to the mouse brains. For immunohistochemical analysis, 20 μm thick coronal sections were cut in a 200 μm intervals from anterioposterior levels +2.0 − -2.0, and with 500 μm interval from levels +2.0 − +0.5 and -2.0 − -3.5. Analyses of the Aβ immunoreactivity and gliosis in the rat brain sections were similar to those in mouse sections, except that Aβ immunoreactivity was visualized using NiDAB, and for microgliosis an ED1 antibody (Serotec, 1:200) was used as a marker. To analyze the association of Aβ immunoreactivity and gliosis, Aβ-GFAP and Aβ-ED1 double-labelling was performed.

For the detection of dividing cells (II), double stainings with anti-BrdU and anti-eGFP or anti-GFAP were performed. Sections were first incubated with mouse monoclonal anti-BrdU antibody (6 μg / ml concentration, Roche Applied Sciences, Indianapolis, IN, USA). Unspecific antibody binding was blocked by incubating the sections with Mouse-on-mouse blocking reagent (Vector Laboratories) followed by biotinylated anti-mouse secondary antibody (1:200 dilution, Vector Laboratories), and avidin-biotin complex (Vectastain Elite Kit, Vector Laboratories), all done according to manufacturer's instructions. Immunoreactivity was visualized with H_2O_2 and NiDAB. Subsequently, the same sections were reacted with either rabbit polyclonal anti-GFP antibody (1:200 dilution, BD Biosciences, Palo Alto, CA, USA) or rabbit polyclonal anti-GFAP antibody (1:200 dilution, DAKO, Denmark), followed by biotinylated anti-rabbit secondary antibody (1:200 dilution, Vector Laboratories) and avidin-biotin complex (Vectastain Elite Kit). The staining was visualized with NovaRed (Vector Laboratories).

Quantification of immunoreactive areas. For quantification of immunoreactive areas, four to six sections at 200 μm intervals through the hippocampi were evaluated per animal. The areas of interest were imaged using an Olympus AX70 microscope (Olympus, NY, USA) equipped with a digital camera (Color View 12 or F-View, Soft Imaging System, Germany) running an Analysis Software (Soft Imaging System). The immunoreactive area was quantified using ImagePro Plus software (Media Cybernetics, Silver Spring, MD, USA). Data are expressed by the area of hippocampi occupied by immunoreactivity and represented as the mean ± SEM.

Table 2. Antibodies, their source and manufacturers used in the present thesis.
Abbreviations: immunohistochemistry, IHC; western blotting, WB

Antibody	Source	Use	Dilution	Manufacturer	Study
CD11b	rat	IHC	1:500	Serotec	I, III, IV
CD45	rat	IHC	1:100	Serotec	I - IV
MHC II	rat	IHC	1:100	Serotec	I, II
CD68	rat	IHC	1:2000	Serotec	II
GFAP	mouse, rabbit	IHC	1:500	Chemicon, DAKO	I - IV
CD3e	rat	IHC	1:1000	Serotec	II
pan-Aβ	rabbit	IHC	1:200	Biosource	I - IV
6E10	mouse	IHC	1:1000	Signet	I
eGFP	rabbit	IHC	1:500	BD Biosciences	I, II
anti-BrdU	mouse	IHC	1:250	Roche Applied Sciences	II
AT8	mouse	IHC	1:100	Innogenetics	IV
pGSK-3β	rabbit	WB	1:1000	Cell Signalling	IV
pAkt	rabbit	WB	1:1500	Cell Signalling	IV
totGSK-3β	rabbit	WB	1:1000	Cell Signalling	IV
totAkt	rabbit	WB	1:1000	Cell Signalling	IV
GLT-1	rabbit	WB	1:1000	Calbiochem	IV
Actin	mouse	WB	1 : 5000	Sigma	III, IV

4.8 Western blotting

For detection of phospho-GSK-3β, phospho-Akt, total GSK-3β, total Akt and glutamate transporter-1 (GLT-1) proteins, the freshly frozen hippocampal or cortical samples were homogenized in 50 mM Tris pH 7.4, 150 mM NaCl, 1 mM EGTA, 1 mM EDTA, 1 % NP-40, 1 mM Na_3NO_4 and 20 mM NaF containing complete protease inhibitor coctail (Roche Diagnostics). The samples were centrifuged for 20 min at 13000 rpm and the supernatant was mixed with standard Laemmli buffer and separated in 10 % SDS-PAGE on Mini-Protean III electrophoresis device (Bio-Rad, Hercules, CA, USA). Separated proteins

were transferred onto Hybond-P membrane (Amersham Biosciences) and the membranes were blocked in 5 % skimmed milk solution in PBST before incubation with primary antibodies (p-GSK-3β, p-Akt from Cell Signalling, Danvers, MA, USA and GLT-1 from Calbiochem, Germany, total Akt and GSK-3β from Cell Signalling) in blocking buffer. Secondary anti-rabbit IgG horseradish peroxide (HRP) conjugate (Amersham) was diluted 1:2000 in blocking buffer and the HRP label detected using ECL Plus kit (Amersham Biosciences). Membranes were directly scanned on Storm 860 (Amersham Biosciences) fluoroimager, and detected bands quantified using ImageQuant software (Molecular Dynamics). The blots were then stripped and reprobed with anti-actin antibody (Sigma, Saint Louis, USA) as loading control.

4.9 In vitro Aβ toxicity

The toxicity and inflammatory potential of Aβ1-42 preparation was tested in mixed cortical cultures prepared from E15 rat fetal brains and mouse microglia cultures, respectively. Briefly, the cortices of the embryos were dissected out and the tissues were minced and trypsinized using 0.25 % trypsin - EDTA in 0.1 M PBS (Gibco). After centrifugation for 5 minutes at 280 x g, the tissues were resuspended into modified Eagle's medium (MEM) containing 10 % FBS (Gibco). The cells were triturated using a pasteur pipette, counted with a Bürker hemocytometer and plated on poly-D-lysine coated plates. The mixed cortical cultures were exposed to Aβ1-42-HDL or HDL preparations for 12 or 48 h (5 µM and 10 µM) at 11 days in culture. Cell death was assessed by cell count after NeuN staining and by measuring kinetically the release of lactate dehydrogenase (LDH) using pyruvate and NADPH as substrates.

The microglia cultures were prepared from postnatal day P0 - P1 mice. The animals were decapitated and cortices dissected out into HBSS / Hepes (Gibco). The tissues were trypsinized with 2 % trypsin (Roche) containing DNase (3600 IU / ml, Sigma) in Hepes and triturated using a pasteur pipette. After 15 minute incubation at 37 °C, the cells were centrifuged at 780 x g for 5 minutes, resuspended in DMEM (Gibco) containing 10 % FBS (Gibco) and plated on poly-L-lysine (Sigma) coated flasks. After 12 days in cultivation, the loosely attached microglia were shaken off and replated on poly-L-lysine coated plates. The microglia cultures were exposed to Aβ preparations and the released TNFα was measured by ELISA kit (Endogen, Woburn, MA) and NO metabolites in a Multiskan ELISA reader (Labsystems) after adding Griess reagent.

4.10 Brain enzyme studies

For SOD1 activity measurements, tissues were homogenized in 10 x volume of 20 mM Tris buffer, pH 7.4. Samples were centrifuged (12000 x g at 4°C) and tissue extracts were collected as supernatants. SOD1 activity was assayed as previously described (Beauchamp and Fridovich, 1971). Briefly, the activity was measured as inhibition of the rate of nitroblue tetrazolium (NBT) reduction by superoxide anion radical, generated by xanthine / xanthine oxidase. The reaction mixture consisted from 50 µl of xanthine oxidase diluted 1:250 in extraction buffer, 40 µl of 2 mM xanthine, 60 µl of 0.32 mM NBT and 90 µl of tissue extract diluted 1:100 in extraction buffer. The optical density was measured in 96-well mictotitration plate on Victor2 multilabel reader (Perkin Elmer Life Sciences) at wavelength of 595 nm. SOD1 activity was expressed as percent of inhibition by tissue extract in comparison to blank sample.

Reduced glutathione (GSH) concentration in the tissues extracts was measured with 2,3-naphthalenedicarboxaldehyde derivatization as previously described (Orwar et al., 1995). Briefly, the brain tissues were homogenized in 10 x volume of extraction buffer (20 mM phosphate buffer pH 6.6) in terms of their wet weight. Tissue extracts were collected as supernatants after 15 min centrifugation at 12000 x g at 4°C. GSH concentration was measured with 2,3-naphtalenedicarboxaldehyde (NDA) derivatization, yielding a fluorescent product. The reaction mixture consisted from 50 µl of tissue sample homogenate diluted 1:10 in PBS, 150 µl PBS and 50 µl of 100 µM NDA. The fluorescence was measured in 96-well mictotitration plate on Victor2 multilabel reader (Perkin Elmer Life Sciences) at wavelenght of 535 nm upon excitation at the wavelenght of 485 nm. GSH concentration was calculated from the standard calibration curve.

4.11 Electrophoretic mobility shift assay

Electrophoretic mobility shift assay (EMSA) was performed as described earlier in detail (Helenius et al, 2001). Nuclear proteins were isolated from fresh frozen cortical samples according to a modified protocol of Dignam et al (1983). Double-stranded consensus and mutated oligonucleotides for NF-κB and AP-1 -binding sites were from Santa Cruz Biotechnology. Probes were labelled with T4 polynucleotide kinase (Promega) and unspecific binding blocked by 2 µg of poly(dI-dC):polyI(dI-dC) (Roche Applied Science) in an assay volume of 20 µl. The binding assays were performed as described earlier (Helenius et al, 1996). Bound and free probes were separated in a native 4 % polyacrylamide gel and

radioactive bands were visualized with Storm 860 PhosphorImager (Molecular Dynamics). Pixel volumes of specific bands were calculated with ImageQuaNT 4.2a software (Molecular Dynamics).

4.12 Aβ1-40 and 1-42 ELISAs

The levels of Aβ1-40 and 1-42 were analyzed by ELISA from freshly frozen hippocampal samples. For analysis of soluble fraction of Aβ species, the brain samples were homogenized in 7 x volume of 20 mM TRIS pH 8.5 containing complete protease inhibitory coctail (Roche Diagnostics, Germany). Samples were centrifuged for 1 hour at 23000 rpm at 4°C. The supernatant was taken for analysis of soluble fraction of beta amyloid species. To analyze insoluble fraction of Aβ, the remaining pellet was resolved in guanidine buffer (5.0 M guanidine – HCl / 50 mM Tris – HCl, pH 8.0). The levels of Aβ1-40 and Aβ1-42 were quantified using Signal Select Beta Amyloid ELISA kits (BioSource International, Inc.) according to the manufacturer's protocol. The level of total Aβ40 and Aβ42 was standardized to brain tissue weight and expressed as nanograms of Aβ per gram (brain tissue).

4.13 Measurement of copper concentration

Copper content of the freshly frozen cortical samples was detected from pyrolyzed samples by graphite furnace atomic absorbtion mass spectroscopy (Hitachi Z-8100 Polarized Zeeman) at the City of Kuopio Environmental Health Laboratory. Similar copper concentrations were obtained in rat spinal cord tissues using the same method (Ahtoniemi et al., 2006) and also others have reported similar copper concentrations in mouse brain tissue (Bayer et al., 2003; Ilback et al., 2006).

4.14 Metabolite NMR Analysis

Cerebrospinal fluid (CSF) sampling. An anesthetized rat was placed prone on the stereotaxic instrument (David Kopf). A sagittal incision of the skin was made inferior to the occiput. After blunt dissection of the subcutaneous tissue and neck muscles through the midline, the dura was penetrated between the third and fourth vertebra by a 26G needle. Approximately 100 µl of CSF was collected and CSF from 1-3 similarly treated rats was pooled for each sample.

NMR analysis. The freeze-dried CSF samples were stored at -20°C in sealed vials and prior to measurement reconstituted in 75 µl of D_2O (99.98%-D, Merck). Five µl of 21.5 mM TSP-d4 (3-(trimethylsilyl)-propionic-2,2,3,3-d4 acid) in D_2O was added and used as

an internal standard. The spectra were measured using a Bruker AVANCE DRX 500 instrument operating at 500.13 MHz (Bruker-Biospin GmbH, Karlsruhe, Germany) equipped with a broadband inverse probe (2.5 mm BBI BB, ^{1}H, Z-Grad) for all 1D ^{1}H quantitative measurements. The Bruker XWIN-NMR software version 3.5 pl 6 was used for acquisition of all spectra. PERCH NMR Software (Orwar et al., 1995) was used for all spectral processing prior to the model creation. The assignation of the spectral signals was done according to the coupling constant information in the literature and the available chemical shift. Some compounds were verified from 2D spectra. The *constrained total-line-shape* (CTLS) fitting method was used to quantitate the spectra by the PERCH NMR Software (Laatikainen et al., 1996). The constraints defining relative line-positions and intensities were written on basis of the theoretical spectral structures of the metabolites. The following metabolites (alltogether 14), corresponding to nearly 90% of the total area of the spectrum, were quantified: acetate, α-hydroxy isovalerate, β-hydroxy butyrate, α- and β-glucose, citrate, creatine, glutamate, glutamine, hippurate, myo-inositol, lactate, lysine and valine.

4.15 Statistical analysis

The statistical analyses were performed either with the STATISTICA software (StatSoft, Tulsa, OK) or SPSS software (SPSS Inc. Chicago, IL, USA). The analysis of the number of infiltrated eGFP positive cells in the brains of transgenic and their wild type control mice was performed by Student's t-test (I). To determine the effect of unilateral LPS injection on brain Aβ deposition, microgliosis and infiltration of eGFP positive cells into the brain parenchyma, the statistical analysis was performed by repeated measures ANOVA (I), and the p values in study II were based on linear mixed model multiple comparisons. The effect of PDTC on inflammatory markers in brain was evaluated by using Student's t-test or ANOVA when appropriate (IV). The MWM data (III, IV) were evaluated by ANOVA with repeated measures on one factor followed by Newman–Keuls posthoc tests and individual contrasts where appropriate. The rat Y-maze data (III) were analyzed by Student's t-test. In all cases statistical significance was assumed if $p < 0.05$.

5. RESULTS

5.1 AD like pathology enhanced the infiltration of blood derived cells in to the brain

Study I was carried out to examine whether AD pathology influences the engraftment of bone marrow derived cells into the brain. All transplantations were successful judging from the ability of the transplanted eGFP-positive cells to engraft the BM of the recipient mice and produce a variety of blood cells as analyzed by flow cytometry. In all transplantation experiments over 75% of the CD11b positive peripheral monocytes were eGFP-fluorescent. No differences between the wild type and transgenic mice were detected (not shown).

When transplantations were performed on young, 2.5-month-old APP/PS1-dE9 transgenic mice in order to study the engraftment of donor-derived cells into the brains of AD transgenic mice, the AD transgenic mice had significantly more eGFP-positive cells in brain compared to their age matched wild type controls. At the time of transplantation these young mice had no AD pathology. By the time of sacrifice at the age of 9 months, all mice showed Aβ deposition in the forebrain. Moreover, in these transgenic mice, approximately 6 % of the eGFP-positive cells were associated with Aβ deposits.

However, when transplantations were done on old APP+PS1 transgenic mice, which at the time of transplantation were 21-month-old and harboured excessive AD-like pathology, there were no differences in the number of infiltrated eGFP positive cells in brain parenchyma between the transgenic and control mice.

When the number of eGFP-positive cells detected near Aβ-deposits was calculated in proportion to brain Aβ burden, the number of eGFP positive cells associated with Aβ deposits was over 17-fold higher in AD transgenic mice transplanted before the development of AD-like pathology (51.5 ± 9.8 Aβ associated cells / Aβ pan immunoreactive area) compared to AD transgenic mice transplanted after the Aβ deposition was fully maturated (3.0 ± 1.0 Aβ associated cells / Aβ pan immunoreactive area, p < 0.01). Both in old and young AD transgenic mice the morphology of the eGFP positive cells associated with Aβ deposits was different from that found in the areas with no Aβ or in the brains of wild type controls. The eGFP positive cells in areas with no Aβ had a ramified phenotype, whereas the eGFP positive cells associated near Aβ deposits resembled ameboid, activated microglial cells by morphology. The majority of the eGFP positive cells found in the brains of these transgenic mice were also positive for a monocytic marker isolectin B4.

5.2 Local inflammation triggered by LPS enhanced the infiltration of blood derived cells in the brain and is associated with reduced Aβ burden.

To study whether local inflammation would increase the infiltration of eGFP positive cells into the brain and the role of BM-derived cells in Aβ clearance, old eGFP transplanted APP+PS1 transgenic mice were injected with LPS into the hippocampus. The contralateral side received an equal volume of saline. LPS injection significantly increased the infiltration of eGFP positive cells into the hippocampus compared to the saline injected side. The majority (< 90 %) of the eGFP positive cells were positive for a microglial marker CD11b and Isolectin B4.

In conjunction with the increased infiltration of eGFP positive cells into the hippocampus of Aβ plaque-bearing transgenic mice, LPS injection decreased Aβ burden in the hippocampus. This decrease was detected with two different antibodies against Aβ: a polyclonal Aβ pan recognizing amino acids 15 - 30 of human Aβ (24% reduction, p < 0.01) and monoclonal 6E10 recognizing amino acids 1 - 17 of human Aβ (42% reduction, p < 0.01). Especially the amount of diffuse Aβ was decreased by LPS injection. Indeed, congo red staining of aggregated β-pleated sheet fibrillar Aβ failed to show difference between LPS and saline injected hippocampi.

In addition, LPS injection increased microglial activation as detected by enhanced MHC-II immunoreactivity within the hippocampi. Cell counts revealed that 68% of the MHC II immunoreactive microglia were eGFP fluorescent. Moreover, induction of local inflammation by LPS significantly increased the association of eGFP-positive cells with Aβ deposits when compared to the saline injected contralateral side.

5.3 Minocycline inhibited the infiltration of blood derived cells in the brain without affecting the clearance of Aβ burden

To analyze the role of blood derived cells in LPS-induced inflammation and Aβ clearance in more detail, LPS-injected transgenic APP+PS1 mice were treated with minocycline. The proliferation of infiltrating eGFP positive cells was detected by BrdU treatment. Indeed, many of the infiltrated eGFP positive cells were BrdU positive, indicating mitotic activity, and eGFP positive cells accounted for approximately 30 % of the mitotically active cells following LPS injection. Minocycline treatment significantly decreased the infiltration and secondary also the proliferation of eGFP-positive cells in hippocampus as well as directly the microglial expression of Iba-1 and MHC II. Also in this study, LPS

exposure resulted in decreased hippocampal Aβ burden and interestingly, minocycline treatment did not prevent the LPS-induced reduction in the hippocampal pan-Aβ immunoreactivity. Minocycline treatment did not affect the microglial CD45 and CD68 immunoreactivity. To detect the possible involvement of T-cells in LPS-mediated inflammatory reaction, the sections were reacted against an antibody recognizing CD3e antigen present in T-cells. The infiltration of T-cells in the brain parenchyma was extremely rare, as there were hardly any T-cells in the hippocampus (less than 0.5 %). Most of the T-cells in the LPS injected hemisphere were found in ventricles or subventricular area.

5.4 Infusion of oligomeric Aβ caused behavioral deficit as detected by Morris Water Maze in 9-month-old mice but not in 2.5-month old mice

Study III was carried out to investigate whether long term infusion of oligomeric forms of Aβ into the lateral ventricles of wild type mice could induce the pathological phenotype resembling AD. Biochemical analysis revelaled that the Aβ1-42 released from the pumps contained monomers, dimers, various forms on oligomers, and larger aggregates that moved very little or not at all in the gel. HDL was used as a carrier to ensure the proper delivery of the Aβ peptides into the brain parenchyma. The Aβ peptides underwent aggregation in the pump, as at the end of infusion period only faint amounts of oligomeric forms of Aβ were released and the majority of Aβ released from the infusion pump moved very little on the gel reflecting high molecular weight aggregates. In vitro studies showed that the Aβ1-42-HDL preparation was neurotoxic, as exposure of cortical neuronal cultures to 10 μM Aβ1-42-HDL preparation resulted in the loss of 80-90 % of the neurons. Interestingly, exposure of primary mouse microglia to the same Aβ1-42 preparation at 5 or 10 μM Aβ did not significantly increase NO or TNFα production.

Morris Water Maze revealed that our 2-week infusion paradigm resulted in alterations in memory in aged mice. These deficits become detectable not earlier than 12 weeks after the infusion. The first cohort of 9-month-old mice were tested 6 weeks after the infusion, when the performance of both Aβ infused and HDL control groups was significantly improved over the 6 training days $[F_{(1,14)} = 0.03, p < 0.1]$ and no significant difference in the escape latencies nor reversal trials was observed. When these same mice were retested 6 weeks later, i.e. 12 weeks after the infusion, the control HDL mice but not the Aβ1-42-HDL mice improved their performance significantly $[(F_{1,14}) = 8.49, P < 0.05]$, however, the reversal trials did not reveal any differences between the groups.

When another cohort of Aβ infused mice were tested for their behavior in Morris Water Maze only 12 weeks after the infusion, our 2-week infusion paradigm resulted in significant deterioration of memory compared to HDL infused control mice regarding both the learning curves during the 6 training days [$F(1,15) = 5.98$, $P < 0.05$) and reversal trials [$t(14) = 15.38$, $P < 0.05$]. In fact, the Aβ1-42-HDL mice did not improve significantly at all in the search of the new target position. The deteoration of memory was age-dependent, as for the mouse groups infused with Aβ1-42-HDL and HDL at the age of 2.5 months, no statistically significant differences were detected when the tests were performed 12 weeks after the infusion (not shown).

5.5 Infusion of oligomeric Aβ in rats resulted in cognitive dysfunction as detected by Y-maze

Infusion of the same Aβ1-42 / HDL preparation in rats induced alterations in behavior in Y-maze Aβ1-42-HDL –infusion. Control HDL-infused rates were as active as Aβ1-42 infused littermates and no differences in the number of arm entries were detected between the groups (Aβ1-42 infusion: 16.4 ±1.3; HDL -infusion 13.4 ± 0.7, mean ± SEM; $P = 0.06$). However, the Aβ1-42 infused rats performed less sequential arm entries (70.9 ± 2.3) compared to HDL –infused rats (81.7 ± 4.4; mean ± SEM t-test, $P = 0.04$) suggesting that the working memory of Aβ-infused rats was compromised.

5.6 The impairment in memory was independent of the development of Aβ deposits and inflammation

The Aβ infusion paradigm resulted in no obvious neuronal loss either in mice or rats infused with Aβ1-42 when compared to HDL-infused control groups. The infusion of Aβ1-42 resulted in the accumulation of Aβ deposits mainly around the infusion site surrounding the ventricular area. The number of deposits varied from 1 to 41 deposits / 6 sections analyzed in mice and 16 to 2159 deposits / 15 sections analyzed in rats; that is, from 0.2 – 7 deposits per one section in mice and 1 – 144 deposits per one section in rats. No Aβ immunoreactive deposits were seen in control HDL infused animals.

The Aβ1-42 infusion did not trigger microgliosis as analyzed by CD11b immunoreactivity in mice and ED-1 immunoreactivity in rats. In addition, we did not find any correlation between the amount of Aβ deposition and microgliosis or between the microgliosis or Aβ and performance in behavioral tests. Interestingly, about 18 % of Aβ

immunoreactivity in Aβ1-42 infused rats was closely associated with astrocytes but not with microglia as evidenced by Aβ- GFAP or Aβ - ED1 double-labelling.

5.7 Deficits in cognition in Aβ infused mice were associated with the reduction in hippocampal SOD1 activity

The altered performance of Aβ-infused mice in the Morris Water Maze was associated with a small but significat reduction in hippocampal SOD1 activity, a phenomenon which in earlier studies has been associated with oxidative stress. However, we observed no corresponding reduction in another marker of oxidative stress, GSH activity. We also saw no difference in the phosphorylation of GSK-3β, which have been suggested to mediate oxidative stress and Aβ-induced neuronal damage in some in vitro studies and some animal models of AD (Farias et al., 2005; Suhara et al., 2003).

5.8 Aβ infusion resulted in altered metabolic processes as detected by ^{1}H-NMR

^{1}H-NMR analysis of CSF of the infused rats revealed that Aβ1-42 infusion caused alterations in several metabolites. The average concentration of metabolites was decreased by up to 60 % in Aβ1-42 infused rats when compared to HDL-infused rats for all metabolites except glutamate. The decrease was the most prominent for valine, myo-inositol, creatine, acetate, glutamine, lactate, glucose and hippurate as it was larger than 20%, and for α-hydroxy isovalerate and citrate the decrease was still larger than 10%. However, due to limited number of samples the difference of a single component between the two groups was statistically significant only for acetate (with p = 0.01).

5.9 Long term PDTC treatment ameliorated behavioral deficits in transgenic AD mice

To assess whether long term treatment of transgenic AD mice with a well known NFκB inhibitor and antioxidant / anti-inflammatory drug PDTC would be able to ameliorate behavioral deficits and AD-like pathology, APP/PS1-dE9 transgenic mice were treated with PDTC for 7 months starting at the age of 9 months. At the age of 16 months the transgenic mice performed significantly worse in MWM compared to their age matched wild type controls. Long term treatment with PDTC indeed proved to be significantly protective against deterioration of cognitive abilities. Since the effect of PDTC was dependent on the genotype (interaction nearly significant, p = 0.0055), wild type mice and transgenic mice were evaluated separately. Whereas in wild type mice PDTC treatment had no effect, PDTC

significantly improved the behavior of transgenic mice, bringing the latency of finding the platform to the level of non treated wild type mice (p = 0.015).

The probe trial was performed on day 4. The transgenic mice spent significantly less time in the correct quadrant on day 4 compared to their wild type controls (p = 0.047). However, PDTC had no significant effect on the ability of the mice to remember the position of the platform.

5.10 The effect of PDTC was not mediated through inhibition of NFκB, AP-1 or gliosis

Even though PDTC has been reported to efficiently inhibit NFκB, EMSA did not reveal any differences in the DNA binding activity of NFκB between PDTC treated and control animals. This was also true for DNA binding activity of AP-1. However, NFκB binding activity was not dependent on the genotype and thus did not show any phenotype difference between the transgenic water treated and wild type water treated mice. This indicates that NFκB upregulation is not apparent in whole tissue lysates or NFκB upregulation may not be a feature of these particular AD mice.

At the age of 16 months the APP/PS1-dE9 transgenic mice exhibited significant microgliosis and astrogiosis in the frontal cortex when compared to water treated wild type controls. PDTC treatment had no effect on the gliosis as analyzed by CD11b and CD45 staining for microglia as well as GFAP staining for astrocytes.

5.11 PDTC treatment had no effect on the brain Aβ burden

The brain Aβ burden in study IV was analyzed by both immunohistochemistry and Aβ ELISA. PDTC treatment did not alter the cortical or hippocampal pan-Aβ immunoreactive area or thioflavin positive Aβ plaque load. PDTC also had no effect on the hippocampal soluble or insoluble Aβ levels as analyzed by ELISA for both Aβ1-40 and Aβ1-42.

5.12 PDTC increases the cortical copper concentration

The cortical copper concentration was measured by atomic absorption spectrophotometer, which revealed a statistically significant increase in copper concentration (+ 20%) in cortex of the PDTC treated transgenic mice. AD pathology itself did not have any effect on the brain copper concentration, as there was no difference in the copper concentration between the water treated transgenic and wild type mice.

5.13 PDTC inhibited the GSK-3β activity most likely through Akt pathway

GSK-3β is an unique enzyme by beeing active when dephosphorylated at serine 9 and is subsequently inactivated by phosphorylation. Quantification of western blots revealed that the active form of GSK-3β, as detected by the decrease in the phosphorylation of GSK-3β at serine 9, was significantly increased in water treated transgenic mice compared to water treated wild type mice. Interestingly, PDTC treatment recovered this decrease in phosphorylation in transgenic mice, bringing it back to the levels of the water treated wild type, mice suggesting that PDTC treatment inactivates GSK-3β. The effect of GSK-3β phosphorylation was at least partially mediated through activation of Akt. No genotype effect of Akt activity between water treated transgenic and wild type mice was detected as analyzed by western blotting by phosphorylation of Akt at Ser473, however, PDTC treatment significantly increased the levels of phosphorylated Akt in transgenic mice indicating that PDTC treatment activates Akt. In contrast, PDTC had no effect on Akt activity in wild type mice. The changes observed in the phosphorylated forms of GSK-3β and Akt were not due to changes in the overall levels of these enzymes, since no differences were detected in the total amounts of GSK-3β and Akt.

5.14 PDTC increases the levels of GLT-1

Western blotting revealed significantly lower amounts of glutamate transporter-1 (GLT-1) in water treated transgenic mice compared to water treated wild type mice, indicating a possible effect of glutamate excitotoxicity in this particular AD transgenic animal model. Interestingly, PDTC treatment significantly increased the levels of GLT-1 in transgenic mice.

5.15. PDTC treatment reduces hippocampal AT8 immunoreactivity

Western blotting did not reveal any difference between transgenic and wild type mice in AT8 immunoreactivity. Immunohistochemical analysis showed some phosphotau AT8 immunoreactivity in cortical areas surrounding the putative Aβ deposits. AT8 immunoreactivity in hippocampus was region specific; CA1 neurons did not stain with AT8 at all, however neurons in CA3 region contained cytosolic AT8 immunoreactivity. PDTC treatment reduced the intraneuronal phosphotau immunoreactivity of transgenic mice in CA3 region neurons when compared to their water treated controls.

6. DISCUSSION

This study was carried out to investigate the role and contribution of microglia and astocytes, as well as BM-derived monocytic cells, in the pathogenic events related to AD using transgenic and infusion models of AD. Microglia and astroglia have been implicated in AD pathogenesis in many studies, yet their exact role remains controversial. While activated glial cells may promote the disease pathogenesis by secretion of proinflammatory cytokines and chemokines, they are also important in maintaining the brain homeostasis and in the battle against intrusions. The brain microglial population consists of endogenous microglia that have differentiated during the embryonic development. However, it has been recognized that the microglial population of the brain is constantly renewing (Eglitis and Mezey, 1997; Kennedy and Abkowitz, 1997; Priller et al., 2001; Simard and Rivest, 2004). Even though the newly migrated monocytic cells might provide an attractive therapeutic target in AD, their role in the development and progression of the neuropathology has not been previously addressed.

This study aimed at providing important knowledge regarding the role of BM-derived monocytic cells in AD and AD related inflammatory reactions, as well as the role of glial cells upon exposure to Aβ both *in vivo* and *in vitro*. The role of inflammation in AD was further evaluated by treating AD transgenic mice with a known anti-inflammatory agent, PDTC. This study demonstrated the following main findings which will be discussed below: (I) migration of BM-derived cells into the Aβ burdened brain was significantly increased and BM-derived cells associated with Aβ deposits. Intrahippocampal LPS injection, known to induce clearance of Aβ, even further enhanced the infiltration of BM-derived cells into the brain. Minocycline, a well known anti-inflammatory agent, was able to inhibit the migration of BM-derived cells without inhibiting the clearance of Aβ. (II) Direct intracerebroventricular infusion of Aβ caused behavioral deficits without promoting the activation of glial cells, and (III) PDTC treatment reduced the behavioral deficits of AD transgenic mice but this beneficial effect occurred without detectable anti-inflammatory effects on glia.

6.1 Bone marrow derived cells contribute to microgliosis in a transgenic model of AD (I, II)

A strong body of evidence from both post mortem human brain and AD transgenic mouse models shows that inflammation, as assessed by microglial activation, plays a major

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part in AD pathogenesis (Akiyama et al., 2000). Activated microglia are detected near Aβ deposits, yet previous studies have not addressed the role of brain endogenous vs. renewing microglial / monocytic cells in AD or AD related inflammatory reactions. This is mostly due to limitations in markers that would enable the distinquishment of newborn microglia from endogenous ones that have already differentiated during the early embryonic stages. Previous studies have shown that BM-derived cells populate the adult brain and that the infiltration of BM-derived cells is continuous (Eglitis and Mezey, 1997; Kennedy and Abkowitz, 1997). The migration of monocytes across the BBB is enhanced in the case of acute injury, such as ischemic stroke or axotomy (Chopp and Li, 2002; Priller et al., 2001). A novel bone marrow transplantation technique used in the present study enabled us to differentiate the endogenous microglia from the BM-derived cells. We show here that the infiltration of BM-derived cells in Aβ burdened brain is significantly increased. These cells have a phenotype that is typical for microglia, many of them associate with Aβ deposits, and have ameboid morphology. Noteworthy is that the infiltration of BM-derived cells in the brain was enhanced in response to even fairly mild pathology, such as increased Aβ burden which does not induce frank neuronal loss, axonal injury and massive distruption of BBB in contrast to ischemic brain injury.

Simard and collegues very recently reported findings supporting our initial observation of the increased infiltration of BM-derived microglia in response to AD pathology (Simard et al., 2006). Interestingly, in contrast to endogenous microglia, confocal microscopy indicated that some of the Aβ immunoreactive fragments colocalized with markers of microglia and eGFP-positive, BM-derived cells (Simard et al., 2006). However, this finding should be specified using electron microscopy. Another recent study found increased numbers of blood derived cells engrafting the AD transgenic mouse brain, yet in this study only the number of T-cells was increased (Stalder et al., 2005). This is in contrast with our findings, which show that less than 0.5 % of the infiltrated BM cells upon LPS induced inflammation were T-cells. The discrepancy between the results may be explained by different research settings, since in the study conducted by Stalder et al. the stem cells were isolated from fetal liver instead of bone marrow. In addition, since no evidence of phagocytosis was found (Stalder et al., 2005), more studies are clearly needed in order to determine the role of BM-derived microglia in AD.

To study the role of BM-derived cells in Aβ clearance, LPS was injected into the hippocampus of the transplanted mice. Intrahippocampal LPS injection has previously been

shown to result in activation of microglia, and subsequently a reduction in hippocampal Aβ burden (DiCarlo et al., 2001). This clearance of Aβ was thought to be microglia-mediated. Interestingly, in the present study local inflammation caused by LPS injection further boosted the infiltration of BM-derived cells into the hippocampus. In addition, we confirmed previous findings that LPS injection increased microglial activation and reduced hippocampal Aβ burden. Importantly, we showed that a significant amount of these activated microglia were actually blood derived, as 68% of MHC II positive monocytic cells in hippocampus were eGFP positive. Interestingly, LPS injection enhanced also the association of eGFP positive cells with Aβ deposits, suggesting a chemotactic response to Aβ. We also showed that following LPS injection, a significant portion of mitotically active cells were eGFP positive, yet minocycline had no direct effect on the proliferation of BM-derived cells in the brain.

Whether the blood derived microglia are detrimental to AD pathogenesis and related inflammation, or are perhaps capable of phagocyting Aβ deposits remains unclear. As in several other *in vivo* studies, we, by using confocal microscopy, also failed to show direct evidence of phagocytosis of Aβ either by BM-derived cells or endogenous microglia following LPS injection. Therefore, to further distinquish between the roles of BM-derived and endogenous microglia in LPS-induced clearance in brain Aβ burden, the transplanted and LPS injected APP+PS1 mice were treated with an anti-inflammatory agent, minocycline. We showed that minocycline was able to decrease the infiltration of BM-derived cells following intrahippocampal LPS injection without preventing the clearance of Aβ. Minocycline inhibited microglial Iba-1 and MHC II immunoreactivity, but did not have an effect on microgliosis as detected by CD45 or CD68 stainings, suggesting that a population of microglial cells remained active and may have retained their phagocytic capacity despite minocycline treatment.

In earlier studies, it has been shown that minocycline is able to prevent the Aβ induced production of pro-inflammatory molecules IL-6, IL-1β and TNFα (Familian et al., 2006; Seabrook et al., 2006). The effect of minocycline on microglial activation is not only suppressive: minocycline has been shown to inhibit the production of pro-inflammatory molecules after hypoxia induced activation *in vitro* without inhibiting the microglial release of pro-survival proteins brain derived neurotrophic factor and glial derived neurotrophic factor (Lai and Todd, 2006). The study by Seabrook and co-workers showed that minocycline has diverse effects on microgliosis in long term treatment of transgenic AD mice (Seabrook et al., 2006). The authors showed that when AD transgenic mice were treated

with minocycline before the development of AD-like pathology had started, the mice exhibited improved performance in MWM. This improvement was not associated with altered microgliosis, but increased brain Aβ burden. Interestingly, when the AD transgenic mice were treated with minocycline after the development of AD-like pathology, the mice did not perform better in MWM, but had decreased levels of microgliosis as analyzed by CD45 and CD11b stainings with unchanged levels of Aβ (Seabrook et al., 2006). In addition, a study conducted by Willcock et al. showed that intrahippocampal administration of Aβ antibodies reduced the hippocampal Aβ burden by a microglia-mediated mechanism. Interestingly, like in our study, minocycline treatment was unable to inhibit the clearance of Aβ (Wilcock et al., 2004). It may therefore be that in our study at least a proportion of infiltrated BM-derived cells contributed to LPS induced inflammation by secretion of proinflammatory cytokines rather than phagocytosis. However, since many of the infiltrated BM-derived cells also expressed CD68, they still may harbour phagocytic capacity.

Our results are in agreement with previous findings showing that many Aβ plaques in AD transgenic mouse models are associated with blood vessels, and that cells of the monocytic lineage are found surrounding the vessels and Aβ plaques (Wegiel et al., 2003), suggesting that blood derived cells contribute to the pool of inflammatory mediator cells in AD. The activation of T lymphocytes requires the expression of MHC II and the presentation of a specific antigen on the cell surface by an antigen presenting cell (APC). It has been shown that BM-derived monocytic cells express higher levels of MHC II than endogenous microglia (Priller et al., 2001; Simard and Rivest, 2004; Walker, 1999), further providing evidence that infiltrated cells are capable of activating the cells of the adaptive immune system. The fact that endogenous microglia are not as typical myeloid lineage cells as blood derived cells suggests that endogenous microglia are not as compentent APCs as BM-derived cells (Santambrogio et al., 2001; Simard and Rivest, 2006; Walker, 1999). Therefore, BM-derived monocytic cells may be able to produce higher amounts of proinflammatory cytokines in response to stimulus, and thus provoke a more severe inflammatory response in the CNS in comparison to endogenous microglia. However, the secretory products of BM-derived cells after LPS-injection were not detected in this study, and therefore this issue remains elusive.

Earlier studies have suggested the involvement of T-cells in AD pathology (Itagaki et al., 1988; Stalder et al., 2005; Togo et al., 2002), however, their role in the disease pathogenesis remains unknown. To detect the possible involvement of T-cells in the LPS-

induced inflammatory reaction, we used an antibody recognizing the CD3e antigen present on T-cells. Interestingly, we saw hardly any T-cells in the hippocampal brain parenchyma after LPS injection. The amount of T-cells present was too low (less than 0.5 %) to be reliably quantified and most of these solitary T-cells were located in ventricles and in the subventricular area. This finding suggests that local LPS-induced inflammation does not recruit T-cells in hippocampus, and that the majority of engrafted cells are peripheral monocytes / macrophages.

Taken together, our results suggest that BM-derived cells may act as contributors to the inflammatory reaction caused by LPS. In the present, study minocycline reduced the overall infiltration of BM-derived cells into the hippocampus in response to LPS, and possibly suppressed the secretion of proinflammatory mediators without preventing the clearance of Aβ. Minocycline treatment also completely blocked MHC II expression. These results require further investigation, since we did not analyze the possible changes in the amount secreted pro-inflammatory cytokines or expression the of pro-inflammatory molecules, however, our results together with previous studies suggest that minocycline may have potential to modulate microglial activity. The fact that many of the infiltrated BM-derived cells expressed CD68 despite minocycline treatment suggests that they may still harbour phagocytic capacity. The capacity of BM-derived cells to phagocytose Aβ could be addressed by additional *in vitro* and *ex vivo* studies in which cultured BM-derived cells would be plated on top of either fibrillar Aβ or non-fixed Aβ containing brain slices obtained from APP+PS1 transgenic mice. The ability of BM-derived cells to phagocytose Aβ could be detected as clearance of Aβ. Also of interest would be to study the role of BM-derived cells using similar transplantation techniques in other experimental settings in which Aβ clearance has been suggested to be microglia-mediated. These include for example the immunization studies using Aβ antibodies (Wilcock et al., 2003).

Due to the fact that BM-derived monocytes are possibly involved in AD pathogenesis, AD related inflammation and even Aβ clearance, makes the blood derived microglia an attractive therapeutic target for Alzheimer's disease. Possibilitites lie in the modulation of the inflammatory response of blood borne monocytes in AD pathogenesis. One would expect a positive outcome in AD pathogenesis by shifting the activation of BM-derived cells from the proinflammatory secretion of cytokines to the phagocytosis of Aβ deposits. In addition, since the brain microglial population renews constantly and AD

pathology further increases the infiltration of new monocytes into the brain, the infiltrating cells could be used as vehicles for gene delivery into the CNS.

6.2 Infusion of Aβ causes behavioral deficits without promoting inflammation (III)

Neuroinflammation, an invariant feature of AD neuropathology, has been detected in almost all transgenic and many of the infusion models of AD. The classical markers of neuroinflammation include the accumulation of astrocytes and microglia near Aβ deposits (Akiyama et al., 2000; Craft et al., 2004b; Frautschy et al., 2001). It has been hypothesized that the increased Aβ production triggers the formation of Aβ deposits with time, and after reaching a critical threshold, this disturbs neuronal functions and triggers inflammation (Akiyama et al., 2000; Koistinaho and Koistinaho, 2005). Small oligomeric forms of Aβ may underlie the disruption of synaptic functions, ultimately leading to neuronal damage (Dahlgren et al., 2002; Lambert et al., 1998; Oda et al., 1995; Walsh et al., 2002). It also is possible that aging renders the brain more vulnerable to Aβ induced damage: behavioural defects can occur without any detectable Aβ deposition in old transgenic AD mice in which the levels of total Aβ are only moderately increased or not even detectable (Holcomb et al., 1999; Koistinaho et al., 2001). Moreover, higher doses of intracerebroventricularly infused Aβ are required to obtain behavioral deficits in middle aged 9-month-old rats compared to 21-month-old rats (Frautschy et al., 2001). These observations challenge the hypothesis that the cognitive deficits are a result of increased concentration and long exposure to Aβ, and support the view that the brain may become more prone to the detrimental effects of Aβ when the nervous system ages. Supporting these observations, we show in this work that infusion of oligomeric Aβ caused behavioral deficits in aged but not in young mice (III). These learning deficits were not associated with microgliosis or astrogliosis. In both transgenic AD mice and infusion models the occurrence of behavioral deficits can occur in the absence of plaque pathology and related inflammation (Chishti et al., 2001; Jacobsen et al., 2006; Walsh et al., 2002). This implies that a low to moderate Aβ production is not sufficient in provoking inflammation and that the Aβ deposits per se are not needed in the development of behavioral deficits. Our infusion paradigm resulted in deficits in MWM, however, the infusion of Aβ did not induce high levels of Aβ deposition or detectable inflammation. Moreover, the Aβ deposits observed did not correlate with the performance in MWM. Alternatively, the relatively small amount of infused Aβ (20 μg per mouse and 40 μg per rat) may have been cleared out of the brain by the time the brains were analyzed.

Indeed, it has been shown that small amounts of Aβ are efficiently cleared out of the brain, possibly by glial mediated mechanisms (Frautschy et al., 1992).

In the present study two different methods for analyzing the behavioral deficits of the Aβ-infused animals were used: the Aβ-infused mice were tested by using Morris Water Maze and the rats by using spontaneous alteration Y-maze. The mice displayed significant deterioration in memory following our chronic Aβ infusion paradigm. MWM is a test that measures hippocampal dependent spatial navigation and reference memory. In addition, Aβ infused rats performed worse in Y-maze compared to HDL-infused control animals. Spontaneous alteration Y-maze detects short term working memory that is also hippocampal dependent. We chose to apply spontanous alteration Y-maze to our Aβ infused rats, because in our set up of behavioral tests the Y-maze proved to be more sensitive than Morris water maze to detect impaired cognitive functions in rats. In comparison to MWM, Y-maze does not include forcement of animals to swim, and therefore may be less stressfull. Y-maze also measures the activity of the animals, in which there were no differences between Aβ infused and control animals.

6.3 Intracerebroventricular infusion of Aβ may cause astrocytic dysfunction and oxidative stress (III)

In our infusion model of AD, the Aβ induced learning deficits were not associated with increased inflammation, however, we detected a mild reduction in the brain SOD1 activity and a moderate alteration in the metabolite pattern of the CSF. In relation to these observations, the learning deficits were observed in 9-month-old mice but not in young, 2.5-month-old mice, suggesting that aging renders the brain vulnerable to Aβ induced damage. The reduced SOD1 activity implies that the antioxidant defence system may be compromised in the Aβ infused mice even when neuroinflammation is not triggered. Indeed, many studies have shown age-related changes in the markers of oxidative stress in AD, and that oxidative stress plays a role in the early phases of AD pathogenesis and Aβ-induced learning deficits (Celsi et al., 2004; Chen et al., 2005; Cole et al., 2004; Jhoo et al., 2004; Li et al., 2004; Ouyang et al., 2005; Schuessel et al., 2006). Proteomic analyses have demonstrated oxidative damage of proteins in the CNS in advanced stages of AD (Castegna et al., 2002), yet in some proteins the stage of oxidation may also be decreased (Korolainen et al., 2002). In addition, the brain may become more prone to lipid peroxdidation with aging (de Haan et al., 1992). In this respect our results agree with previous data, since we found learning deficits in aged but

not young mice following intracerebroventricular infusion of Aβ. Changes in markers related to the antioxidant capacity of the brain may be diminished in Aβ-stressed conditions even when neuroinflammation is not triggered. Antioxidants have been suggested to mediate protective effects in AD transgenic mice, and in fact, the detrimental role of inflammation can be largely attributed to increased oxidative stress triggered in inflammatory cells (Celsi et al., 2004; Cole et al., 2004; Frautschy et al., 2001; Jhoo et al., 2004). In this respect, it is of interest that PDTC, a known anti-inflammatory and antioxidant compound (Nurmi et al., 2006; Nurmi et al., 2004b), was shown in the present work to protect AD transgenic mice against cognitive decline even though the mechanism of this beneficial effect may be more complex (IV).

Astrocytes serve to uptake excess glutamate from the synaptic cleft. In astrocytes, the glutamate is metabolised in several ways, of which the most important steps are glutamine formation and entry into the tricarboxylic acid (TCA) cycle (Anderson and Swanson, 2000). Interestingly, several of the intermediates produced by the uptake and metabolism of glutamate by astrocytes can serve as energy substrates for neurons (Anderson and Swanson, 2000). These intermediates include lactate, the levels of which were decreased by our Aβ infusion paradigm in rats (III). The finding of reduced lactate, acetate, alpha-hydroxyisovalerate, creatine and myo-inositol levels in the CSF of the Aβ infused rats implies impairment in astrocytic functions. Myo-inositol is known to reflect astrocytic metabolic activity (Brand et al., 1993) and lactate is mainly produced by astrocytes. In addition, Aβ deposits in rats were observed to be associated with astrocytes, but not with microglia (III). Since lactate is known to affect synaptic function in adult neurons (Meeks and Mennerick, 2003), it can be hypothetized that Aβ induced oxidative stress may have caused astrocytic dysfunction, and therefore impairment in the synaptic functions ultimately leading to the cognitive deficits detected in Aβ infused animals.

In the present study we tested the effect of direct intracerebroventricular infusion of Aβ on the development of behavioural deficits and the role of inflammation in Aβ-induced cognitive decline in mice and rats. We chose the direct infusion paradigm to exclude the possible effects of high over-expression of mutant proteins on the phenotype of the animals. Several fold APP over-expression results in increased levels of APP itself and $CT_{100-105}$ fragments. Earlier studies have shown that APP activates microglia and decreases antioxidant capacity (Barger and Harmon, 1997; Takahashi et al., 2000). In addition, $CT_{100-105}$ fragments have been shown to disrupt calcium homeostasis, render neurons vulneralbe to excitotoxicity

(Kim et al., 2000; Kim et al., 1998) and result in gliosis and cognitive impairment in vivo (Song et al., 1998). Since these fragments may cause unrelevant effects on AD-like pathology including neuroinflammation, we used the direct Aβ infusion paradigm in our study.

The involvement of oxidative stress in Aβ-infusion induced memory deficits could be further charaterized by treating the Aβ infused animals with antioxidants, which in previous studies have been shown to be protective in AD *in vitro* or *in vivo* models (Calon et al., 2005; Calon et al., 2004; Cheng et al., 2006; Cui et al., 2006; Rupniak et al., 2000; Yang et al., 2005), although mechanims of action between these compounds may vary. The involvement of SOD1 in Aβ-induced learning deficits could be specifically proved by infusing SOD1 over-expressing mice with our Aβ1-42 preparation. Indeed, earlier studies have shown that over-expression of SOD1 *in vitro* provides protection against Aβ-induced apoptosis (Celsi et al., 2004), and co-expression of APP together with wild-type SOD1 in transgenic mice rescues the mice from the Aβ-induced cerebrovascular toxicity (Iadecola et al., 1999). The *in vivo* Aβ infusion paradigm also enables the co-infusion of different factors and therefore the role of SOD1 could also be studied by co-infusion of SOD1 together with Aβ.

6.4 PDTC treatment inhibits GSK-3β activity through Akt and ameliorates behavioral deficits independent of inflammation (IV)

PDTC possess many protective functions in the CNS, including anti-inflammatory, antioxidant and anti-apoptotic functions (Kovacich et al., 1999; Nobel et al., 1997a; Nobel et al., 1997b; Nurmi et al., 2006; Nurmi et al., 2004b), and has been approved for clinical use in the treatment against alcohol addiction and metal intoxication. The anti-inflammatory and antioxidant functions of PDTC led us to investigate, whether the suppression of neuroinflammation would be sufficient to ameliorate the cognitive deficits in transgenic AD mice. We found that long term treatment with a moderately low concentration of PDTC led to a significant amelioration of cognitive deficits in AD transgenic mice compared to controls. Noteworthy in this study is that the treatment trial with PDTC was designed to reveal possible therapeutic and disease modifying properties of the drug. Therefore, the transgenic mice that already exhibited a substantial amount of Aβ plaques were selected. Indeed, the treatment was effective in preventing the cognitive decline, even though the actual AD pathology was already well established. This is important for possible clinical use,

as the AD diagnosis is made only after the behavioural abnormalities occur and the diseased brain contains significant amount of Aβ plaques.

Suprisingly, the effect of PDTC on the improved cognition of transgenic AD mice was independent of the ability of PDTC to act as an anti-inflammatory agent. Long term treatment of AD transgenic mice with a relatively low dose of PDTC failed to suppress micro- or astrogliosis. Moreover, despite the ability of PDTC to mediate the protective effects via inhibition of NFκB (Nurmi et al., 2004b), the amelioration of cognitive decline in our study was not mediated through the same pathway. The fact that we observed no genotype difference in the DNA binding activity of NFκB may be due to the fact that NFκB activation is not a part of the AD-like pathology in this particular mouse model, or is cell specific and thus not apparent in whole tissue samples. These observations together with the finding that PDTC did not affect the brain Aβ burden, suggests that the effect of PDTC was independent of Aβ deposition and neuroinflammation.

Our results showed that the AD transgenic mice had increased levels of the activated form of GSK-3β, and that PDTC treatment decreased GSK-3β activity. GSK-3β is an enzyme that is activated when it is dephosphorylated and inactivated through phosphorylation. GSK-3β activation has been linked to Alzheimer's disease pathogenesis also in many other studies (Avila, 2006). Some antibodies reducing the brain Aβ burden mediate their protective effects by reducing GSK-3β activity (Ma et al., 2005). GSK-3β activity has been shown not only to increase the abnormal phosphorylation of tau and APP (Aplin et al., 1996; Aplin et al., 1997), but to also affect cell viability by inhibiting the expression of transcription factors important to cell survival (Grimes and Jope, 2001b). Therefore, it has been hypothesized that GSK-3β activity may decrease the ability of cells to respond to subsequent stress. In addition, GSK-3β inhibits PDH, which is an important component of the mitochondrial Krebbs cycle, and may thus cause energy depletion in cells (Hoshi et al., 1996).

One well known kinase able to phosphorylate, and thus inactivate GSK-3β, is Akt (Grimes and Jope, 2001b). Since PDTC treatment in transgenic AD mice lead to an increase in the phosphorylated form Akt, the protective effect of PDTC was most likely mediated through activation of Akt pathway (Pei et al., 1999; Pei et al., 1997) as depicted in figure 5. This is in accordance with previous studies showing the importance of Akt - GSK-3β pathway in events related to memory: mutant presenilins are able to downregulate Akt and activate GSK-3β (Weihl et al., 1999), and learning deficits may be regulated by GSK-3β

(Engel et al., 2006; Hernandez et al., 2002). In addition, activation of Akt pathway is required for expression of LTP (Sanna et al., 2002), and Akt inhibitors impair long-term consolidation and recognition memory in rats (Horwood et al., 2006).

AD has been linked with copper ions in many studies. APP and Aβ can bind (Curtain et al., 2001) and reduce copper (Fuentealba et al., 2004). Moreover, senile plaques in the AD brain have been shown to be enriched with copper ions (Lovell et al., 1998) and serum copper levels are increased in AD patients (Squitti et al., 2002). Even though copper ions have been implicated in the Aβ mediated production of reactive oxygen species and subsequent oxidative stress (Huang et al., 1999b; Opazo et al., 2002), copper can be partially disassembled by copper chelators, and treatment with copper chelating agents has been shown to be protective in AD (Cherny et al., 2001; Lee et al., 2004) and in models of excitotoxicity (Rothstein et al., 2005). Copper ions may also promote cell survival signalling through the activation of Akt (Ostrakhovitch et al., 2002). Moreover, APP and Aβ overproduction have been reported to lead to intracellular copper insuffiency, and increase in tissue copper concentrations have been shown to be beneficial in AD transgenic mice (Bayer et al., 2003; Maynard et al., 2002; Phinney et al., 2003). Since PDTC is able to chelate copper and transport it inside cells (Verhaegh et al., 1997), it may have increased the total amount of copper and thus compensated for the possible reduction in intracellular copper. The actual souce of the increase, however, remained unknown. Copper ions have been shown to activate Akt and subsequently inhibit GKS-3β, thereby promoting cell survival signaling (Ostrakhovitch et al., 2002). The increased Akt activity may have therefore resulted from an increase in cortical copper concentration of PDTC treated mice when compared to water treated controls.

The finding the PDTC treatment had no effect on activation of Akt in wildtype mice remained unclear. Both mutant PS1 and Aβ may induce several alterations in neuronal and astrocytic functions that may result in differences in the cellular responses to PDTC. Indeed, PS1 and Aβ are able to inhibit the activation of Akt pathway (Baki et al., 2004; Magrane et al., 2005) and the inflammatory mediators in the AD transgenic mice may regulate Akt (Grzelkowska-Kowalczyk and Wieteska-Skrzeczynska, 2006). The low dose of PDTC used in this study may therefore affect only stressed cellular functions and cause changes only in diseased brain. In support of this, we found no overall adverse effects or changes in the body weights of the mice following the PDTC treatment.

GSK-3β is widely expressed in both developing and adult brain and has important roles in neuronal signaling (Leroy and Brion, 1999). GSK-3β is the major kinase that phosphorylates tau protein (Avila, 2006). Interestingly, the phosphorylation of tau by GSK-3β has also been shown to be dependent on copper (Lovell et al., 2004). Tau is a microtubule-associated protein that functions in regulation of neuronal microtubule assembly and stabilization. The hyperphosphorylation of tau has been linked as one mechanism leading to neuronal degeneration in AD, since hyperphosphorylated tau readily forms fibrillary tangles, and thus distracts the normal cellular transport and stability (Avila, 2006). We did not observe any difference in the total amount of phosphorylated tau as analyzed by western blotting, yet immunohistochemistry revealed that PDTC treated transgenic mice displayed significantly less cytosolic AT8 immunoreactivity in the neuronal layer of the hippocampal CA3 region, when compared to water treated transgenic mice. This indicates that inhibition of GSK-3β may have affected tau phosphorylation in the hippocampus (fig 5.). Since the overall amount of AT8 immunoreactivity in transgenic mice was low and not significantly increased compared to wild type controls, the effect of tau phosphorylation on the pathogenesis of AD-related events in this particular model should be interpreted with caution. Of importance is that in human AD, PHFs correlate with cognitive decline (Giannakopoulos et al., 2003).

6.5 PDTC increased the expression of GLT-1 (IV)

Glutamate excitotoxicity is one of the pathways hypothesized to contribute to AD pathogenesis. Glutamate is most the prevalent excitatory neurotransmitter in the brain, and is important in the formation of LTP and memory through its ability to bind and activate NMDA and AMPA / kainate receptors (Collingridge and Lester, 1989). Astrocytes prevent glutamate excitotoxicity by removing excess glutamate from the synaptic cleft via glutamate transporter-1 (GLT-1) (Hatten et al., 1991). Glutamate excitotoxicity occurs when excess glutamate is released from impaired or damaged neurons, or the astrocytic clearance of glutamate from the synaptic cleft is impaired. Importantly, the extent of dementia correlates well with neuron loss caused by excess of glutamate (Sonkusare et al., 2005). A non-competetive NMDA antagonist, Memantine, is also used in the clinics for the treatment of AD (Molinuevo, 2003).

In addition to the increased activity of GSK-3β, we detected decreased levels of GLT-1 in transgenic AD mice compared to their wild type controls. Moreover, PDTC treatment increased the levels of GLT-1 transporters in transgenic AD mice. Despite the fact

that there is little information available on the role of excitotoxicity in our particular transgenic AD model, impairment in GLT-1 has been reported in other models of AD (Harris et al., 1996; Liang et al., 2002; Masliah et al., 2000). In addition, earlier work in a similar double transgenic AD mouse model shows significant improvement against cognitive decline by treatment with Memantine (Minkeviciene et al., 2004). Moreover, in another neurodegenerative disease involving glutamate toxicity, β-Lactam antibiotics were shown to be protective against excitotoxicity through increased GLT-1 expression (Rothstein et al., 2005) and of interest is that β-Lactams are also metal ion chelators. GLT-1 expression is linked with Akt, since an earlier study has shown that overexpression of Akt in astrocytes is able to induce the expression of GLT-1 (Li et al., 2006).

Even though not directly shown in the present study, we hypothesize that the increase in GLT-1 levels may have been mediated through activation of Akt by PDTC as depicted in figure 5, yet it remains unknown whether this is sufficient or needed to promote protection against cognitive decline. Importantly, glutamate excitotoxicity is a well characterized feature of ischemia and a major contributor to ischemic cell death, and PDTC treatment was shown to activate Akt in ischemic brain, thereby possibly providing significant protection (Nurmi et al., 2006).

The involvement of GLT-1 upregulation after PDTC treatment could be adderssed by additional *in vitro* studies. Treatment of astrocytes with PDTC may cause upregulation in GLT-1 *in vitro*. Indeed, earlier studies have shown an upregulation in astrocytic GLT-1 expression following exposure to β-lactam antibiotics (Rogers et al., 2002). Also *in vitro* exposures of neuronal cultures to Aβ and PDTC could provide additional knowledge regarding the role of GSK-3β on direct Aβ mediated neuronal death. Figure 5 summarizes our hypothesis of how PDTC mediates the protective functions in the transgenic AD mouse model used in this study (IV).

Even though PDTC has anti-inflammatory abilities, at the given dose it failed to suppress neuroinflammation. Previous studies have reported beneficial effects of anti-inflammatory agents on AD pathology. Several NSAIDs are capable of reducing brain Aβ burden and associated micro- and astrogliosis both in human AD patients and in preclinical animal models (Eriksen et al., 2003; Lim et al., 2000; Lim et al., 2001b; McGeer et al., 1996; Rogers et al., 1993; Stewart et al., 1997). More selective glial inhibitors, such as aminopyridazine (Craft et al., 2004b), might have revealed the exact effect of supression of microglial activity on the behavioural deficits in our transgenic mouse model. On the other

hand the effect chronic PDTC treatment in AD transgenic mice has never been addressed before, and PDTC possesses superior protection in brain ischemia models involving neuroinflammation (Nurmi et al., 2006; Nurmi et al., 2004b).
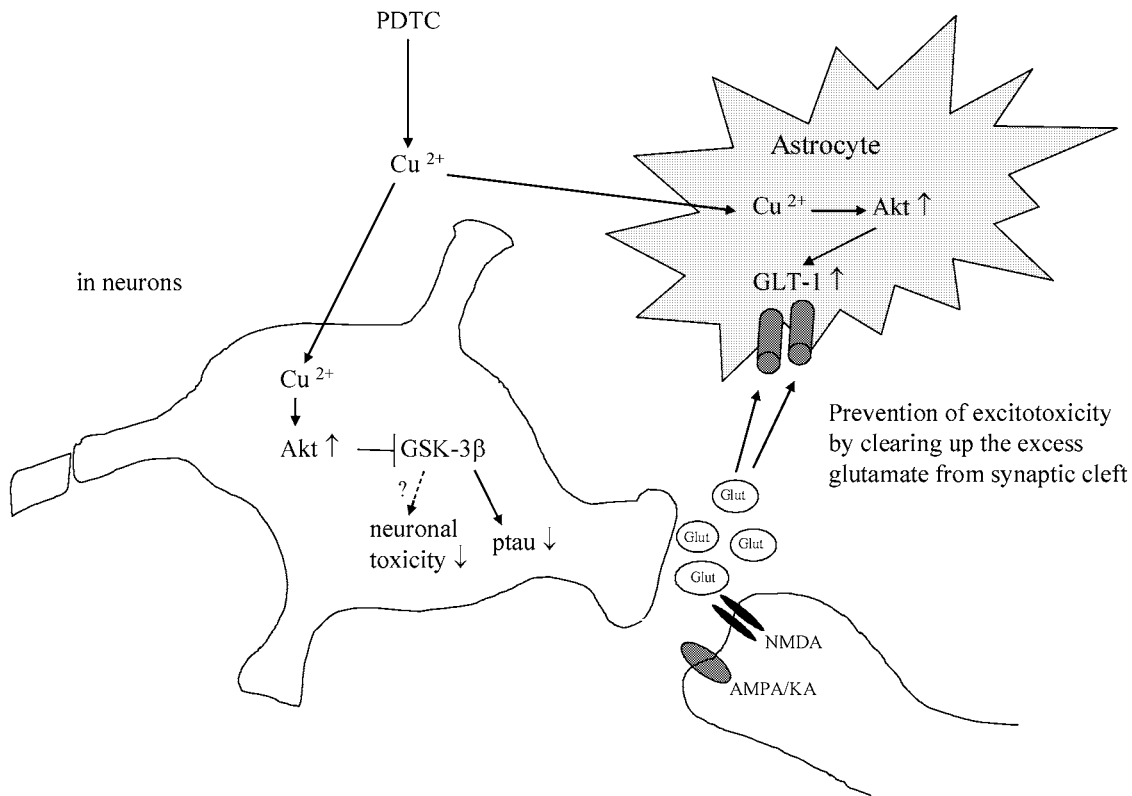
Figure 5. The proposed mechanisms underlying the protective effects of PDTC in APP/PS1 mice. PDTC transports copper inside the cells. In neurons copper activates Akt, which in turn phosphorylates and subsequently inactivates GSK-3β. This results in decreased phosphorylation of tau and possibly enables the cells to better respond to toxicity caused by Aβ. In astrocytes Akt activation causes upregulation in GLT-1 receptors, which prevent excitotoxicity by clearing up the excess glutamate from the synaptic cleft.

6.6 Hypothesized role of glial cells in preclinical models of AD

The reports in the present thesis work provide important information regarding the role of inflammatory cells in AD animal models. These reports show for the first time that the BM-derived cells take part in the recruitment and activation of inflammatory cells in AD-like pathology and related inflammation in AD transgenic mouse models. The recruitment of BM-derived cells in the brain was significantly enhanced by Aβ neuropathology. The AD-like neuropathology in the APP+PS1 mouse model used does not result in frank neuronal loss, yet it is associated with activation of brain endogenous microglia. To study the involvement of BM-derived cells in Aβ clearance, we induced local inflammation by intrahippocampal LPS injection, which previously has been reported to result in a microglia-mediated clearance in hippocampal Aβ burden (DiCarlo et al., 2001). LPS injection heavily increased the infiltration of BM-derived cells in hippocampus, and these cells showed activated phenotype as detected by their morphology and expression of MHC II. As addressed by the expression of CD68, a lysosomal marker for phagocytic activity, both the endogenous microglia and BM-derived cells may harbour the capacity to phagocytose Aβ upon LPS injection. The fact that we failed to detect any intracellular Aβ in either BM-derived cells or brain endogenous microglia by using confocal microscopy, may be explained by rapid degradation of phagocytosed Aβ. This issue however, remains unclear.

The finding that minocycline treatment following LPS injection inhibited the engraftment of BM-derived cells in the hippocampus, without preventing the reduction in brain Aβ burden, suggests that BM-derived cells in LPS induced inflammatory reaction may function to promote the inflammatory cascade. It is plausible that the brain endogenous microglial population is mostly responsible for the clearance of Aβ. However, since many infiltrated eGFP positive cells also expressed CD68 despite minoycline treatment, their role in the phagocytosis cannot be ruled out. Taken together, our results point out that BM-derived cells contribute to AD-related inflammatory reactions and total brain microglia population in AD-like neuropathology.

Our results from the Aβ infused mice and rats showed that Aβ infusion alone was not sufficient to induce micro- or astrogliosis *in vivo*. In support of this, our Aβ preparation was also ineffective in inducing microglial production of NO or TNFα *in vitro*. Instead, our findings suggest that compromized antioxidant capacity caused by oligomeric Aβ infusion may lead to dysfunction in astrocytic metabolism and subsequent behavioral deficits. On the other hand, since astrocytes have also been shown to be able to clear Aβ

(Koistinaho et al., 2004; Wyss-Coray et al., 2003), the decreased metabolic activity may also reflect the unsuccesful attempt of astrocytes to clear Aβ. Since no overall microgliosis was detected following intracerebroventricular infusion of Aβ, it may be that the behavioral deficits arise from the compromised antioxidant capacity and possible synaptotoxicity of infused Aβ, as well as direct toxicity to astrocytes independently of microgliosis.

We also show for the first time that inhibition of GSK-3β in an AD transgenic mouse model is associated with the amelioration of cognitive decline. The improvement may be explained partially by the decrease in tau phosphorylation and increase in the clearance of glutamate, as detected by increase in astrocytic GLT-1. Despite the anti-inflammatory abilities of PDTC, the oral administration of moderate dose of PDTC used in this study failed to suppress micro- or astrogliosis. This may be secondary to the fact that we did not observe any difference in the brain Aβ burden following PDTC treatment. Our results suggest that cognitive deficits may be directly modulated by PDTC through the Akt pathway without affecting the activation of glial cells.

Taken together, our results suggest that BM-derived cells contribute to AD-related inflammatory reactions, once the neuroinflammtion is triggered in AD transgenic mice. On the other hand, our findings from Aβ1-42 infused mice and rats as well as PDTC treated transgenic mice support the hypothesis that human Aβ may have direct effects on vital neuronal functions and that development of cognitive deficits, as well as protection against cognitive decline, may be independent of brain Aβ burden and neuroinflammation, as detected by unchanged astro- and microgliosis.

7. SUMMARY AND CONCLUSIONS

The present study was carried out to study the role of microglia and astrocytes, in animal models of AD. The role of BM-derived microglia in a transgenic AD mouse model was assessed by using novel transplantation techniques. The effect of direct intracerebroventricular infusion of Aβ and treatment of AD transgenic mice with a well-known anti-inflammatory and antioxidant agent, PDTC, was used with the emphasis on the role inflammatory mediator cells. The following results were obtained:

1. AD pathology increased the infiltration of BM-derived monocytic cells into the brain. These cells displayed the characteristics of microglia by morphology and surface receptors, associated with Aβ deposits, and showed an activated phenotype upon AD-related inflammatory reactions.

2. The infiltration and activation of BM-derived cells into the brain was diminished by treatment with minocycline. Minocycline did not prevent the clearance in Aβ burden, suggesting that endogenous microglia may be more prominent in Aβ phagocytosis compared to BM-derived cells upon LPS injection.

3. Direct intracerebroventricular infusion of Aβ caused behavioral abnormalities that were independent of Aβ deposition and were not accomppanied by micro- or astrogliosis. Our Aβ preparation was also ineffective in inducing inflammatory response *in vitro*. The infusion of Aβ caused a mild reduction in SOD1 activity, implying a compromised antioxidant defence system. The deposited Aβ was associated with astrocytes, which together with a reduction in levels of CSF metabolites, suggests a decreased astrocytic function or unsuccessful attempt of astrocytes to clear Aβ. The lack of microglial activation following Aβ infusion suggests that the antioxidant capacity of the brain may become impaired in Aβ-stressed conditions even when neuroinflammation is not triggered.

4. PDTC ameliorated cognitive dysfunction in transgenic AD mice even when the treatment was initiated after the development of plaque pathology. The effect of PDTC was independent of its' anti-inflammatory and antioxidant abilities. The protection was most likely mediated through an increase in Akt activity, and subsequent inhibition of GSK-3β activity. PDTC also increased the expression of GLT-1 transporters in transgenic AD mice, suggesting an enhanced astrocytic activity in glutamate uptake.

In summary, these reports provide new insights into the roles of non-neuronal cells in AD related events. We show for the first time that BM-derived monocytes are recruited into the brains of transgenic AD mice and are able to activate upon AD related inflammation. Targeting blood derived monocytes may therefore have potential therapeutic implications. Our results also suggest that astrocytic dysfunction and the compromized antioxidant capacity caused by oligomeric Aβ infusion may underlie the observed behavioural deficits. We also show for the first time that inhibition of GSK-3β by treatment with PDTC in an AD transgenic mouse model significantly ameliorates cognitive decline. Targeting GKS-3β may offer a potential therapeutic strategy for the treatment of AD in the future.

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