

From the Department of Neurotec,
Division of Experimental Geriatrics,
Karolinska Institutet, Huddinge, Sweden

Characterization of hippocampal slice cultures as model systems for neurodegenerative processes in Alzheimer's disease



Sara Johansson



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To my parents, Elisabeth and Leif, with love

*”Tänk om alla människor på jola vore västgötar,
vad lugnt och skönt det vore,
och tänk vad skôjigt vi sulle ha!”*

Trad. västgötsk vers

ABSTRACT

Alzheimer's disease (AD) is the most common cause of dementia and a growing health care problem worldwide. The disease affects different brain regions, including hippocampus, entorhinal cortex, amygdala, neocortex and certain basal forebrain nuclei. Macroscopically, shrinkage of gyri and widening of sulci characterize the AD brain. Histopathological changes consist of deposition of extracellular senile plaques, composed of the amyloid- β peptide ($A\beta$), and intracellular neurofibrillary tangles (NFTs), composed of hyperphosphorylated forms of the microtubule-binding protein tau, concomitant with a progressive loss of neurons. Inflammatory processes are believed to contribute to the pathophysiology of AD and may play an important role in the disease progression. One of the cytokines upregulated in AD is interleukin 1 (IL-1). IL-1 seems to be overexpressed early in the plaque formation process, possibly by activated microglia.

Organotypic slice cultures of the roller-drum and membrane techniques were applied to develop model systems of AD. The major advantages with these cultures are the well-preserved phenotypic cellular and structural organization as well as possibilities for long-term culturing. The following studies were performed using organotypic hippocampal cultures to characterize key pathophysiological components of AD:

In **study 1** roller-drum cultures were used for evaluating the effect of $A\beta_{25-35}$ on different nerve cell populations and glial cells. $A\beta_{25-35}$ gave rise to fibrillar aggregates, a time- and concentration-dependent reduction in the number of NMDA-R1 immunolabeled pyramidal cells and damaged GAD65 immunopositive interneurons. Also, neurons immunopositive for phosphorylated tau and GFAP-labeled astrocytes were affected, while GABA-immunopositive interneurons and glial cells seemed unaffected. In **study 2** roller-drum cultures were used to investigate mechanisms involved in neuronal cell death induced by IL-1 β . IL-1 β induced a concentration-dependent loss of NMDA-R1 immunoreactive pyramidal neurons, which could be inhibited by trolox and MK-801. These findings suggest the involvement of free radicals and NMDA receptor-mediated processes in IL-1 β -induced neurodegeneration. In **study 3** the neurodegenerative effect of lipopolysaccharide (LPS) in roller-drum cultures was investigated. The LPS-exposure caused an increased density of CD11b positive cells, indicating the occurrence of reactive microgliosis. A rapid loss of both NMDA-R1 and GABA-immunoreactive cells was induced by the LPS exposure. Concomitant with the pyramidal cell loss an increased number of p53/NMDA-R1 and NMDA-R1/TUNEL double-labeled cells were found, suggesting that apoptotic events were involved in the neurodegeneration. In **study 4** the relation between $A\beta$ and tau phosphorylation was characterized in a membrane organotypic culture system. Western blotting analysis showed that exposure to $A\beta_{25-35}$, increased the degree of tau phosphorylation at Tau [pS396] and [pS199] epitopes. Also protein levels of active GSK3 β [pY²¹⁶] were increased, while Cdk5 and p35 did not change significantly.

In this thesis different components of the amyloid cascade hypothesis were characterized in phenotypically advanced model systems. Links between $A\beta$ neurotoxicity, tau phosphorylation and alterations in activated kinases were established (study 1 and 4) as well as a role of inflammatory processes in neurodegeneration, using IL-1 β (study 2) and LPS (study 3) as inducers.

The developed models may serve as a useful platform for detailed characterization of key components AD pathophysiology and their interdependence in the disease process. Additional opportunities include the benefits of organotypic cultures as a crucial step between simple *in vitro* assays and *in vivo* models in the evaluation of disease modifying therapies for AD.

ORIGINAL PAPERS

This thesis is based on the four papers below, referred to by their Roman numerals (I-IV):

- I. Modelling of amyloid β -peptide induced lesions using roller-drum incubation of hippocampal slice cultures from neonatal rats.
Johansson S, Radesäter A-C, Cowburn RF, Thyberg J, and Luthman J.
Experimental Brain Research 2005, Sep 21: 1-14 [Epub ahead of print]
- II. The vitamin-E analog trolox and the NMDA antagonist MK-801 protect pyramidal neurons in hippocampal slice cultures from IL-1 β -induced neurodegeneration.
Radesäter A-C, **Johansson S**, Öberg C, and Luthman J.
Neurotoxicity Research, 2003, 5(6): 433-42.
- III. Salmonella lipopolysaccharide (LPS) mediated neurodegeneration in hippocampal slice cultures
Johansson S, Bohman S, Radesäter A-C, Öberg C, and Luthman J.
Neurotoxicity Research, 2005, 8(4): 001-014
- IV. Increased tau phosphorylation at the S³⁹⁶ and S¹⁹⁹ epitopes after A β ₍₂₅₋₃₅₎-exposure in organotypic hippocampal slice cultures.
Johansson S*, Jämsä A*, Vasänge M, Winblad B, Luthman J, and Cowburn RF
Manuscript.

* Shared first authorship

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LIST OF ABBREVIATIONS

ACh	acetylcholine
AChE	acetylcholine esterase
AChE-Is	acetylcholine esterase inhibitors
AD	Alzheimer's disease
ADDLs	A β -derived diffusible ligands
ApoE	apolipoprotein E
APP	amyloid precursor protein
ASPDs	Amylospheroids
A β	amyloid- β
BBB	blood brain barrier
CA	cornu ammonis
CERAD	Consortium to Establish a Registry of Alzheimer's disease
CSF	cerebrospinal fluid
DG	dentate gyrus
DIV	days <i>in vitro</i>
ECL	enhanced chemiluminescence
EM	electron microscopy
GBSS	Gey's balanced salt solution
GSK3 β	glycogen synthase kinase 3 β
HCS	high-information-content systems
HEK 293	human embryonic kidney cells
HF	hippocampal formation
HRP	horeseradish peroxidase
IFN- γ	interferon- γ
IL	Interleukin
IL-1	Interleukin-1
IL-1 β	Interleukin-1 β
IL-1RI	IL-1 receptors type I
IL-1RII	IL-1 receptors type II
LPS	lipopolysaccharide
MAP	mitogen activated protein kinase
MBDs	microtubule-binding repeat domains
MCI	mild cognitive impairment
MMSE	Mini-Mental State Examination
MRI	magnetic resonance imaging
NBM	nucleus basalis of Meynert
NFTs	neurofibrillary tangles
NINCDS-ADRDA	National Institute of Neurological and Communicative Disorders and Stroke and Alzheimer's disease and Related Disorders Association
NMDA	N-methyl-D-aspartate
NSAIDs	non-steroidal anti-inflammatory drugs
PDPK	proline-directed protein kinases
PET	positron emission tomography

PFA	paraformaldehyde
PHFs	paired helical filaments
PS	presenilin
ROS	reactive oxygen species
TMT	trimethyltin
TNF- α	tumor necrosis factor- α

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INTRODUCTION

Alzheimer's disease (AD) is a major cause of dementia with brain pathology that includes amyloid depositions, neurofibrillary degeneration and neuronal cell loss. AD has been proposed to involve a number of linked pathophysiological mechanisms, such as A β -toxicity, tau phosphorylation and inflammation. All three mechanisms were studied in this thesis using advanced *in vitro* systems based on organotypic roller-drum or membrane cultures of the rat hippocampus. The studies show that the developed models can be applied in the characterization of pathophysiological mechanisms in AD and present possibilities for evaluating how pathological processes interrelate. These models can also serve as a useful step between cell lines and animal models in the search for new, disease-modifying, therapeutic approaches for AD.

Background

AD is the most common cause of dementia, constituting approximately 60% of all dementia cases in Europe (Fratiglioni et al., 1999). The prevalence of AD is about 1% at the age of 65 and thereafter it doubles every five years to about 50% at the age of 95 (Jorm, 1987). The first AD case was described 1907 by the German neuropathologist and psychiatrist *Alois Alzheimer* (Alzheimer, 1907), who examined a 51-year old woman with memory loss, disorientation and hallucinations.

Well-established risk factors for the development of AD are increasing age, family history of dementia, and inheritance of the apolipoprotein E (ApoE) ϵ 4 allele (Fratiglioni, 1996). Other factors believed to increase the risk for AD include female gender, low education, hypertension and head trauma (Mayeux et al., 1993; Small, 1998; Munoz and Feldman, 2000). The duration of the disease varies between 5 and 15 years and the cause of death is usually associated with secondary diseases, such as pneumonia or other infections.

With an increasing age of the population, the number of demented people will increase, emphasizing the need for novel and effective treatments. Research in the AD-field has during the last decade been very expansive and has revealed genetic causes of the disease as well as identified cellular and molecular mechanisms linked to the disease. Until recently the pathological features of AD could only be observed in *post mortem* brain tissue. This inability to identify pathology in living patients has prompted research on biochemical markers of AD in serum and cerebrospinal fluid (CSF) that can complement clinical approaches for making an early and valid diagnosis (Khachaturian, 2002). Also, the possibility to visualize the pathological features of AD in the *in vivo* brain will be of importance for the validation of disease modifying therapies. Another challenge in the identification of novel therapies for AD has been the lack of preclinical models that closely mimic the disease and allow for efficient testing of therapeutic principles and compound screening.

Classification of Alzheimer's disease

AD cases can be classified into different subgroups, such as familial or sporadic and early or late onset forms. The familial and sporadic forms of AD are separated according to the presence or absence of other demented individuals in the family, or in rare cases the possible identification of specific genetic mutations. Early- versus late-onset AD is distinguished dependent on whether the patient presented symptoms before, or after, 65 years of age. The late-onset form of AD accounts for more than 90% of all cases. Both the familial early-onset and the late-onset (mostly sporadic) forms of the disease have similar neurohistopathological changes, suggesting that the different forms of the disease have a common pathophysiology and shared etiology.

Clinical symptoms and diagnosis

Symptoms

The most striking symptom of AD is impaired memory, followed by deterioration of language, motor and spatial abilities. AD usually begins with short-term memory impairment, and subsequently disorientation and aphasia. The disease progression leads to a global cognitive impairment and the patient becomes bedridden and totally dependent on caregivers.

Clinical diagnosis

The clinical diagnosis of AD is based on a number of different tests to determine the state of a possible dementia. The diagnostic procedures are extensive and involve an evaluation based on the patient's medical history combined with the exclusion of other diseases. The National Institute of Neurological and Communicative Disorders and Stroke and Alzheimer's disease and Related Disorders Association (NINCDS-ADRDA) have developed a set of criteria for diagnosing patients as possible, probable or definite AD (McKhann G et al., 1984). There are also neuropsychological procedures, such as the Mini-Mental State Examination (MMSE), which aid the clinical diagnosis of AD (Folstein et al., 1975). MMSE is one of the most widely used screening instruments for cognitive impairment and serves as a guide for the diagnosis of mild, moderate and severe AD. However, AD cannot be diagnosed before the disease has progressed to dementia. The current diagnostic tests are primarily important in identifying other possible causes of dementia before a definitive diagnosis of AD can be made with confidence (McKhann et al., 1984). Mild cognitive impairment (MCI), a clinical condition representing a transitional state between normal cognition and AD (Petersen et al., 1999), has attained increased attention in recent years. Patients with MCI have slight memory impairments. They are not demented, but possess an increased risk for developing AD, making these patients interesting for treatment trials of disease progression modifying therapies. Today, there is no clinical method to determine which MCI patients that will progress to AD. Therefore, it is of great importance to develop new diagnostic tools for diagnosing early AD and to identify incipient AD in MCI cases. The recent development of biochemical markers for MCI and AD in the CSF (Blennow, 2004) is a complement to the clinical diagnosis of AD. Levels of certain factors implicated in disease pathogenesis, such as phosphorylated tau protein or the amyloid β (A β)-peptide, may give an indication of an increased risk for AD, but a definitive

clinical diagnosis cannot be made and no exact differentiation from other dementias can be attained (for review see Blennow et al., 2001). The accuracy of a clinical diagnosis has improved over recent years by the use of techniques that enable visualization of the brain, such as computed tomography, magnetic resonance imaging (MRI) and positron emission tomography (PET). At specialized centers a correct diagnosis of AD can be made in 90% of all cases, when comparing clinical diagnosis with post mortem examination (Klatka et al., 1996).

Neuropathology

The macroscopic changes seen in brains from AD patients include shrinkage of gyri, widening of sulci and enlargements of ventricles. The brain regions most affected are hippocampus, enthorinal cortex, amygdala, neocortex and certain basal forebrain nuclei (Braak and Braak, 1994). Pyramidal glutamatergic hippocampal neurons seem to be especially vulnerable in AD and are affected early in the pathological process (Beach et al., 1982). As the disease progresses a pronounced neurodegeneration occurs in other regions, including the temporal and parietal lobes. In some cases the frontal and occipital lobes may also be affected (Braak and Braak, 1994). At the microscopical level histopathological observations include a widespread necrotic and apoptotic neuronal degeneration (see chapter “Neurodegeneration in AD”), loss of synapses, the presence of intracellular neurofibrillary tangles (NFTs) (see chapter “Neurofibrillary tangles and tau phosphorylation sites”) and amyloid deposits, occurring as extracellular plaques or in the cerebrovasculature (Glenner and Wong, 1984; Grundke-Iqbal et al., 1986) (Fig. 1) (see chapter “amyloid β -peptide”).

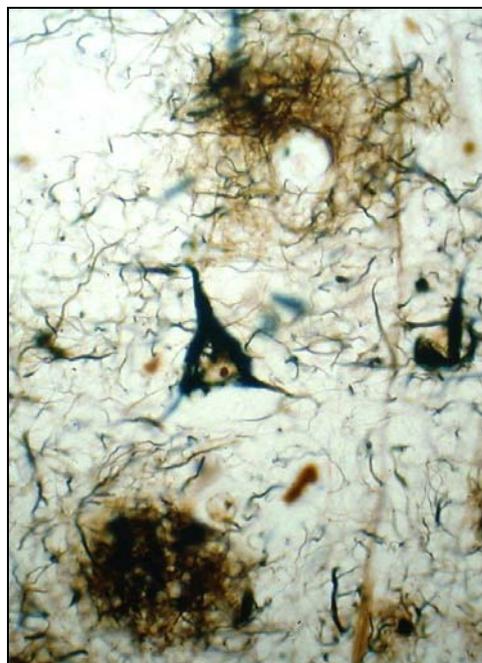


Figure 1. The main hallmarks of AD; extracellular senile plaques and intracellular neurofibrillary tangles. Bielschowsky silver staining (Courtesy of Dr. Nenad Bogdanovic).

Hallmarks and diagnosis

The main protein constituent of the amyloid plaques is the A β -peptide, a 39-43 amino acid proteolytic product of the amyloid precursor protein (APP). Other components of the plaque include proteoglycans, inflammatory molecules and apolipoproteins E and J (Atwood et al., 2002). The classical senile plaques consist of a central amorphous core that can be identified by the histological amyloid stains Congo red (Klunk et al., 1989) and Thioflavin S or T (Sage et al., 1983). Surrounding the plaques are dystrophic neurites, indicating neurodegenerative processes (Glennner, 1989), as well as reactive astrocytes (Pike et al., 1995) and microglia (Perlmutter et al., 1990), that are thought to cause an inflammatory reaction around the plaques by releasing complement factors and cytokines (McGeer and McGeer, 2001) (see chapter “Neuroinflammatory processes in AD”). Amyloid deposits may also appear as non-fibrillar diffuse plaques and in the walls of small cerebral blood vessels.

The NFTs are intracellular aggregates composed of abnormally hyperphosphorylated forms of the microtubule-binding protein tau. These aggregates are found within the neuronal perikaryon, the neuropil threads and within dystrophic neurites surrounding the plaques. In AD, tau is hyperphosphorylated and has a decreased ability to promote microtubule assembly and instead aggregates into paired helical filaments (PHFs) in the cytoplasm of degenerating neurons. Tangle formation is also a common feature in Parkinson’s disease, frontotemporal lobe dementia and other dementias (Lee et al., 2001).

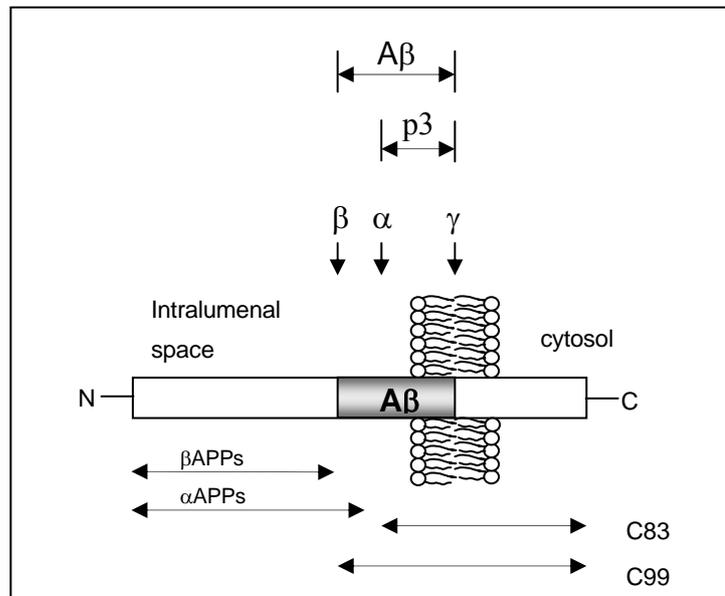
Certain neuropathological criteria must be fulfilled for a definitive diagnosis of AD. A number of different sets of criteria exist, including those developed by the Consortium to Establish a Registry of Alzheimer’s disease (CERAD), as well as Khachaturian’s criteria and the Tierney criteria (Mirra et al., 1991; Tierney et al., 1988; Khachaturian et al., 1985). These criteria, based on the presence of amyloid plaques and/or NFTs in the brain, are not by themselves sufficient for a definitive diagnosis. The neuropathological findings have to be supported by a clinical diagnosis, ruling out other types of dementias.

Amyloid β -peptide

Processing of amyloid precursor protein

APP, from which A β is derived, is an integral type 1 membrane-spanning glycoprotein with its N-terminal in the intraluminal space and the C-terminal in the cytosol. The A β -containing region of APP extends from the ectodomain into the transmembrane domain of the protein. APP is proteolytically processed by α -, β - and γ -secretases (Fig. 2). Close to the lipid bilayer α - or β -secretase cleave APP to generate the N-terminal fragments α APPs and β APPs, and the C-terminal fragments C83 and C99 respectively. Both C-terminal fragments are substrates for γ -secretase cleavage that generates the p3 or A β peptides.

Figure 2. APP processing pathways. APP is initially cleaved by α - or β - secretase generating the soluble fragments α APPs or β APPs and the C-terminal fragments C83 or C99. Additional cleavage by γ -secretase of the C-terminal fragments C83 and C99 releases p3 and A β , respectively.



The major APP processing route is via the α -secretase pathway that generates the C83 fragment that is the immediate precursor for the p3 peptide, containing the C-terminal region of A β . The role of p3 in amyloidogenesis remains unclear. It is generally considered that the α -secretase pathway is non-amyloidogenic, preventing the deposition of intact A β -peptide into plaques. The sAPP α released from the cell has neuroprotective and memory-enhancing effects (Meziane et al., 1998). Several protease candidates have been proposed to be involved in the α -cleavage, including ADAM-9, ADAM-10 and ADAM-17.

The cleavage of APP at the N-terminus of A β is executed by the β -site APP cleaving enzyme BACE (Vassar et al., 1999; Yan et al., 1999). Two homologs of BACE exist, BACE 1 and BACE 2, of which BACE 1 is mainly expressed in the brain and pancreas (Vassar et al., 1999), and is most likely responsible for the β -cleavage of APP.

The final step in the production of A β is cleavage of the C99-fragment of APP by γ -secretase. γ -Secretase is a multiprotein complex consisting of presenilin (PS), nicastrin, Aph-1 and Pen-2. All four proteins of the γ -secretase complex are necessary for full proteolytic activity (De Strooper B, 2003). PS1 and its homolog PS2 are multipass proteins that are mainly expressed in the brain. Several integral membrane proteins apart from APP can serve as substrates for γ -secretase, including Notch that controls cell fate decisions during embryogenesis and adulthood and can even influence apoptotic processes (Alvez da Costa et al., 2002). The A β -peptides generated by γ -secretase cleavage differ in length, with A β 1-40 being the most abundant, while A β 1-42 is produced to a lesser extent (Lichtenthaler et al., 1999; Selkoe D, 2001). However, A β 1-42 is more closely associated with the disease pathogenesis (Mills and Reiner, 1999) and senile plaques in diseased brains are composed primarily of A β 1-42. Indeed, in vitro A β 1-42 has been shown to form fibrils more rapidly than A β 1-40. The hydrophobicity of residues at positions 41 and 42 has been suggested to be a major contributor to the enhanced amyloidogenicity of A β 1-42, relative to A β 1-40 (Kim and Hecht, 2005). In familial AD, where there is a relative increase in the production of A β 1-42, increased

length of the hydrophobic C-terminus has been shown to promote early deposition of fibrillar A β (Citron et al., 1997).

Aggregation of the A β -peptide

More than 30 years ago, Terry et al. showed, using electron microscopy (EM), that amyloid plaques were composed of fibrils (Terry et al., 1964). 20 years later, in 1984, Glenner and Wong demonstrated that the main component of the plaques was A β (Glenner and Wong, 1984). The toxicity of A β is believed to require aggregation of native A β monomers (Pike et al., 1993, Lorenzo and Yankner, 1994, Hardy and Higgins, 1992). The aggregation of soluble A β -peptide into fibrillar cross- β pleated sheet conformation involves various polymerization steps from the innocuous monomers to the toxic fibrils. Detailed *in vitro* investigations of the polymerization of A β has revealed three intermediate oligomeric assembly states: 1) small oligomers ranging from dimer to hexamer size (LeVine, 1995; Garzon-Rodriguez et al., 1997); 2) A β -derived diffusible ligands (ADDLs), which are oligomers ranging from 17-42 kDa (Lambert et al 1998) and 3) protofibrils; short fibril intermediates that can be observed by EM, having a diameter of <8 nm and a length of <150 nm.

Slow rotation of A β 1-40 or A β 1-42 in solution produces spherical A β aggregates containing a stable and a highly toxic moiety. The size of the aggregates, called amylospheroids (ASPDs), appears to influence the toxicity. A β 1-42 forms ASPDs more rapidly and with a 100-fold higher toxicity. Since ASPDs are formed below the concentration required for fibril formation and in the presence of β -sheet breaking peptides, it has been suggested that A β aggregates into ASPDs in a pathway distinct from fibril formation (Hoshi et al., 2003).

The neurotoxic properties of the full length A β 1-42 sequence are retained by a synthetic A β peptide consisting of only residues 25-35 (Yankner et al. 1990; Pike et al. 1995), the A β 25-35 peptide. The A β 25-35 peptide has been shown to be highly toxic to neurons *in vitro* (Pike and Walencewicz-Wasserman et al. 1995; Forloni et al., 1993). This peptide represents the biologically active region of A β , in that it is the shortest fragment that exhibits large, β -sheet aggregated structures and retains the toxicity of the full-length peptide (Pike et al., 1995).

Neurofibrillary tangles and tau phosphorylation sites

Tau and NFTs

The microtubule-binding protein tau regulates neuronal microtubule assembly and stability and is expressed in both the fetal and adult human brain (Avila et al., 1994). Tau is highly phosphorylated in fetal brain and minimally phosphorylated in the normal adult brain (Seubert et al., 1995). The protein structure of tau is composed of four domains: the acidic N-terminal, the basic and proline-rich middle, the basic microtubule-binding repeats, and the C-terminal. In the human CNS, 6 isoforms of tau exist that are derived by alternative splicing of tau mRNA from a single gene, located on chromosome 17 (Goedert et al., 1989). The tau isoforms vary in having 3 or 4 microtubule-binding repeat domains (MBDs), regions that bind to microtubules and promote their assembly. The flanking domains protruding from MBDs (projecting domains) target tau to microtubules and assist to stabilize them (Preuss et al., 1997). Interactions of tau with

microtubules are regulated by the length and phosphorylation of MBDs (Morita-Fujimura et al., 1996).

Tau plays a significant role in the development of neuronal processes, in the establishment of cell polarity and in intracellular transport (Mandelkow and Mandelkow, 1998). Tau is also involved in signal transduction (Hwang et al., 1996; Jenkins and Johnson, 1998), anchoring enzymes such as protein kinases and protein phosphatases (Sontag et al., 1996; Liao et al., 1998) and interacts with the actin cytoskeleton (Cunningham et al., 1997) and plasma membranes (Brandt et al., 1995; Lee et al., 1998).

In AD, tau is hyperphosphorylated and has a decreased ability to promote microtubule assembly and instead aggregates into paired helical filaments (PHFs) in the cytoplasm of degenerating neurons. PHFs are the main constituent of NFTs found in neuronal cell bodies, neuropil threads within neuronal processes of the neuropil and dystrophic neurites associated with senile plaques (Buee et al., 2000). In AD, PHFs appear as twisted structures between 10-20 nm in width with a half-periodicity of 80 nm (Ksiazek-Reding and Wall, 1994). Bundles of PHFs form the NFTs, which are seen as extracellular ghost tangles after the neurons have died (Goedert and Klug et al., 1999).

Tau phosphorylation sites

PHF tau in the AD brain contains at least 30 serine and threonine phosphorylation sites that have been identified using mass spectrometry and phosphorylation-dependent tau antibodies (Hanger DP et al., 1998., see Fig. 3). Most of the phosphorylation sites are on Ser-Pro and Thr-Pro motives and are located outside the microtubule-binding domains with a few exceptions. Hyperphosphorylation of tau influences tau biology in a number of ways. There are specific phosphorylation sites that promote dissociation of tau from microtubules, including Ser199, Ser202, Thr205, Ser214, Ser231, Ser262, Ser356, Ser396 and Ser404 (Bramblett GT et al., 1992; Wagner U et al., 1996; Sontag E et al., 1996). In the AD brain there is also a considerable amount of normal tau (Pei et al., 2003). Dephosphorylation of PHF-tau disassembles PHFs, making it possible for PHF-tau to bind microtubules and promote microtubule assembly (Alonso et al., 1994; Iqbal et al., 1994; Alonso et al., 1996).

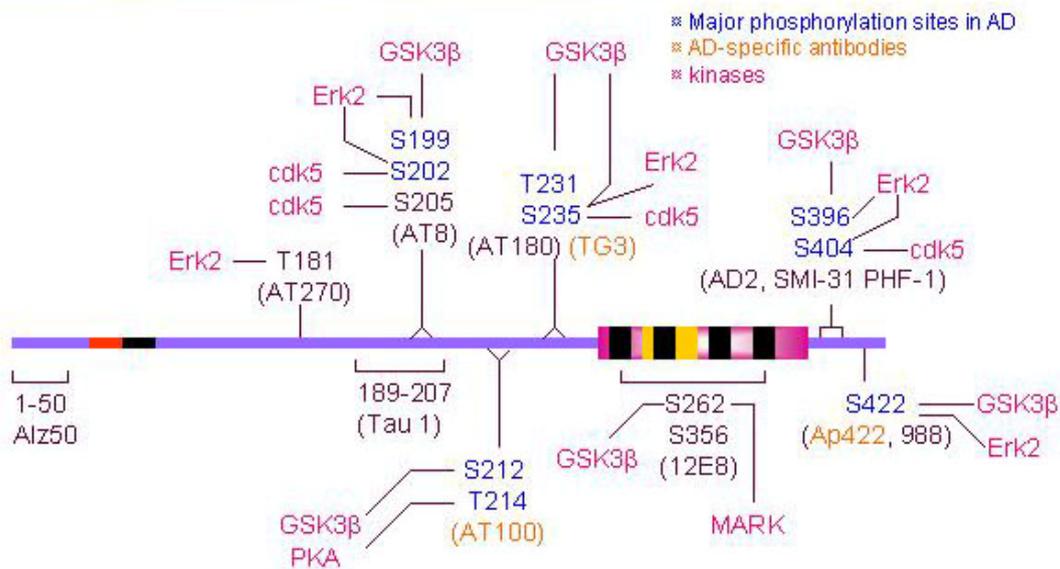


Figure 3. Schematic picture of the major tau phosphorylation sites in AD (Courtesy of Dr Ratan Bhat).

Kinases involved in tau phosphorylation

The different states of tau result from the activity of specific kinases and phosphatases acting at the different tau phosphorylation sites. Most of the kinases involved in tau phosphorylation are proline-directed protein kinases (PDPK), such as mitogen activated protein kinase (MAP), glycogen synthase kinase 3 β (GSK3 β), tau-tubulin kinase and the cyclin-dependent kinases Cdc2 and Cdk5. *In vitro* GSK3 β phosphorylates tau at most sites that are abnormally hyperphosphorylated in AD PHFs (Lovestone et al., 1994). When GSK3 β is overexpressed in mouse brain, tau becomes hyperphosphorylated at the PHF-1 site (pSer396/404) (Lucas et al., 2001), a site critical for PHF formation. GSK3 β has two phosphorylation sites that influence the catalytic activity of the protein. Phosphorylation of the residue Ser9 on GSK3 β inactivates GSK3 β and phosphorylation of Tyr216, located on the activation loop, increases its catalytic activity. The proline-directed kinase Cdk5 phosphorylates tau at residues Ser202, Thr205, Ser235 and Ser404. Studies in hippocampal cultures suggest that the initial tau phosphorylation by Cdk5 stimulates subsequent modifications by GSK3 β , thus preventing tau from becoming incorporated into microtubules (Alvarez, et al., 1999).

The amyloid cascade hypothesis

The prominent models of AD pathophysiology are those based on the amyloid cascade hypothesis. The amyloid cascade hypothesis was formulated more than a decade ago and states that the neurodegenerative process is a series of events triggered by the abnormal processing of APP and the subsequent production of A β (Hardy and Higgins, 1992). According to the amyloid cascade hypothesis deposition of A β in plaques is the main initiator of the disease (Fig. 4). Gliosis, inflammatory changes, oxidative injury, altered kinase/phosphatase activities leading to tangle formation, neuritic/synaptic injury and neuronal cell death are suggested to occur as secondary events to A β deposition and lead to onset of symptoms of dementia (Hardy J and Selkoe DJ, 2002).

The strongest evidence in support of the amyloid cascade hypothesis is the fact that AD-causing mutations in the APP and PS genes cause altered proteolytic processing of APP. This leads to increased A β production, in particular of A β 1-42 (Cai et al., 1993; Scheuner et al., 1996), or changes in A β stability or aggregation, resulting in a chronic imbalance between A β production and clearance.

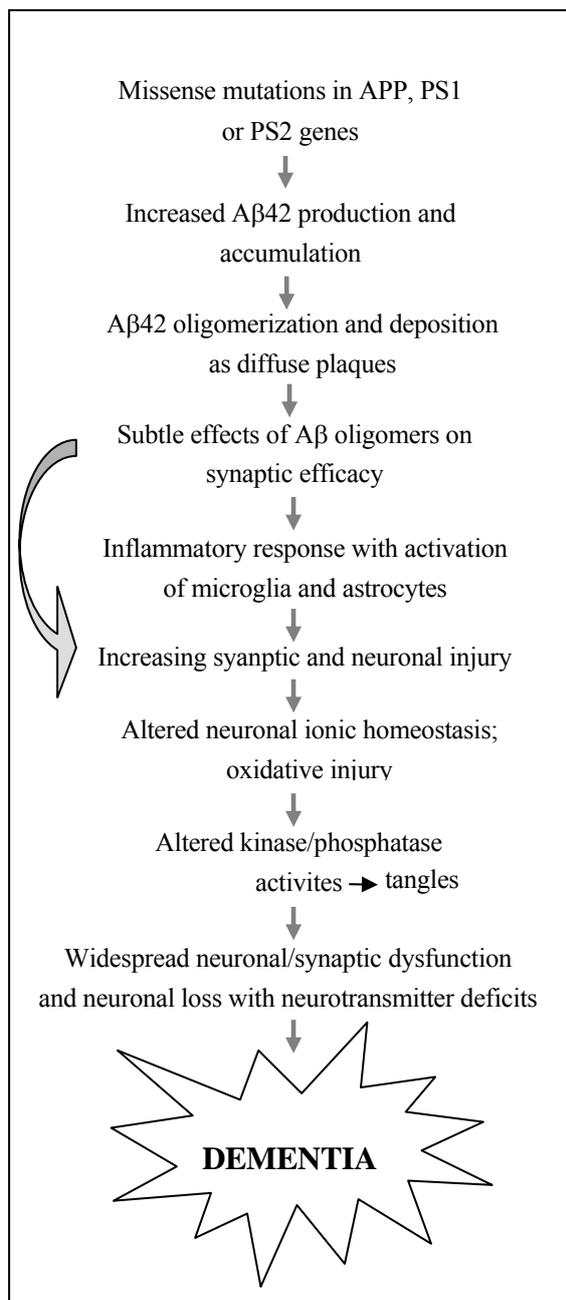


Figure 4. The amyloid cascade hypothesis as suggested by Hardy and Selkoe, 2002. The large arrow indicates that A β oligomers may directly injure the synapses and neurites of brain neurons.

Like all hypotheses, the amyloid cascade also carries limitations. One argument against the hypothesis is that there is no correlation between the increase in A β 1-42 production in patients with APP mutations and age of onset of the disease. Another complicating finding is that transgenic mice expressing APP or PS1 mutations show plaque pathology, but no NFTs (Janus et al., 2000). Also, transgenic mice displaying progressive A β deposition fail to show neuronal loss (Games et al., 1995; Hsiao et al., 1996; Irizarry et al., 1997). However, recently a triple transgenic mouse was developed that convincingly shows that plaque depositions precedes tau pathology, and with age these mice display synaptic dysfunction (Oddo et al., 2003).

The issues concerning the amyloid cascade hypothesis are discussed further in the section “Modelling of Alzheimer’s disease”.

Neurodegeneration in AD

Both apoptotic and necrotic processes have been implicated in the neuronal loss associated with AD (for review see Wolozin and Behl, 2000a; Wolozin and Behl, 2000b; Behl C, 2000).

Apoptosis

Apoptosis is a highly regulated physiological process in which cells undergo a series of genetically programmed events that eventually lead to cell death (Kerr et al., 1972). Apoptosis plays an important role in embryogenesis, fetal development and immune cell maturation, making apoptosis an essential physiological mechanism that controls the number of cells in various tissues during development and in the adult organism. Apoptosis is an active process, controlled by genes that can be activated by environmental stimuli, including DNA damage, oxidative stress and exposure to drugs, toxins, hormones and virus. Upon initiation of apoptosis, a cascade of cellular reactions follows, among them the activation of caspases. Caspase-3 is a main player in apoptotic cell death that also is implicated in neuronal cell death and degeneration. A dramatic hypertrophy of the brain of caspase-3 knockout mice illustrates the central role of this particular caspase in apoptosis of nerve cells (Kuida et al., 1996). Also, p53 is an important transcription factor involved in regulating apoptotic processes. Normally it is maintained at low levels and becomes activated after DNA-damage (Evan and Littlewood, 1998). Morphologically, apoptosis is characterized by chromatin condensation, DNA fragmentation, and plasma membrane blebbing and cell shrinkage. In the end-stage, the dying cell disintegrates to form apoptotic bodies that are phagocytosed by nearby cells, thus preventing a local secondary inflammation. Apoptotic cells are recognized microscopically as eosinophilic bodies.

Signs of apoptosis in AD brains include increased DNA damage, increased caspase activity and altered expression of other apoptosis-related genes (Su et al., 1994; Masliah et al., 1998). In the search for other markers of apoptosis, DNA fragmentation has been found to co-localize with the expression of c-Jun protein, an immediate early-gene that is required for apoptosis in neurons (Anderson et al., 1995). In some cases, a co-localization with the pro-apoptotic Bax protein has been found (Su et al., 1997). Many *in vitro* studies have tried to resolve the question of apoptosis or necrosis in AD by initiating nerve cell death by AD-relevant toxins, such as A β . For instance, in cultured

primary neurons, addition of A β induced apoptotic features (Loo et al., 1993; Millet et al., 2005). A β -induced apoptosis has been shown to involve altered intracellular Ca²⁺ production (Mattson and Chan, 2001). Other in vitro studies indicate that the mechanism of apoptosis in AD is associated with increased oxidative stress, perturbed calcium homeostasis, mitochondrial dysfunction and caspase activation (Mattson, 2000; Behl, 2000). In the AD brain, the density of TUNEL-stained neuronal nuclei show direct correlation with that of AT8-, AT180- and Tau2-positive neurons in entorhinal cortex, cornu ammonis (CA) and the parietal cortex, indicating that phosphorylation of tau is associated with neuronal apoptosis (Kobayashi K et al., 2003).

Necrosis

Necrosis is a passive pathological event, that is not developmentally programmed, and which arises from insult or trauma to cells. It is characterized by cellular and mitochondrial swelling, followed by lysis of the cell causing a release of the cellular content to the surrounding area. This leads to a massive inflammatory response and phagocytosis by macrophages. A β has been described to mediate necrotic processes by the generation of reactive oxygen species (ROS) and oxidative stress-induced damage is thought to induce rapid necrotic events (Behl et al., 1994; Harris et al., 1995; Behl, 1999). Also, addition of A β to clonal or primary cultures has been reported to induce neither DNA laddering nor nuclear condensation, but rather a rapid disintegration of plasma membranes and neurite filament breakdown (Behl et al., 1994). The contrasting findings of divergent effects of A β may be explained by an ability of A β to induce a rapid response in nerve cells, representing necrosis, followed by a long term apoptosis. It also appears that apoptosis and necrosis overlap in causing neuronal cell death, since cell death that starts as apoptosis may finally lead to necrosis (Ankarcrona et al., 1995).

Neuronal cell loss in Alzheimer's disease hippocampus

Neuronal loss occurs selectively in vulnerable areas of AD brains when compared with age-matched controls (Terry et al., 1981). Terry reported using, semiautomatic image analysis, a specific neuronal loss up to 25% in temporal and frontal cortices of AD subjects. The major proportion of cell loss was found in large-sized pyramidal neurons. A selective neuronal loss in different hippocampal subfields has also been found to occur in the normal aging brain and in AD (West et al., 1994; Šimić et al., 1997). Pyramidal neurons of the hippocampus are particularly prone to form NFTs. Entorhinal layer II, V, CA1 subfield and subiculum are the regions most affected by NFT formation in AD, while spiny stellate cells and other interneurons seem to be resistant. Hippocampal atrophy is a common feature of advanced AD (Tomlinson et al., 1970) and is considered as a good marker of the disease. For example, in one large study hippocampal atrophy was observed in 87% of all AD cases studied (De Leon et al., 1989). In another study, AD patients showed a mean reduction of 32% in the total volume of the subdivisions of the hippocampal formation (HF) as compared to controls (Šimić et al., 1997).

West and collaborators have shown that the mean number of neurons per hippocampus in hilus, CA1, and the subiculum is significantly smaller in AD patients, with the most pronounced neuronal loss in CA1 (68% neuronal loss), compared to the age matched control group (West, 1993; West et al., 1994). This loss of neurons is disease-specific and not related to aging, per se. Šimić's study differs from the study by West and co-workers; their conclusion being that neuron loss occurs in CA1 during normal aging and

that it is the atrophy in CA1, not neuron loss, that distinguishes AD brains from controls (Šimić G et al et al., 1997). In AD, the mean total estimated number of neurons is markedly reduced compared with control, and both the hippocampal volume and brain volume decreases significantly with disease progression. A linear correlation between CA1 neuron number and hippocampal volume further indicates that hippocampal atrophy in AD occurs as a result of neuron loss (Kril et al., 2004).

In preclinical AD, the earliest definable stage of AD, no difference could be observed in the number of neurons in any subdivision of hippocampus, suggesting that early AD is not characterized by a significant neuronal loss in hippocampus (West et al., 2004). The absence of differences in neuronal number between the preclinical stage of AD and the control state implies that the deposition of A β and the development of senile plaques precedes the neuronal death in hippocampus. In another study, Price et al showed that there is little, or no, neuronal loss in preclinical AD, while there is a substantial loss in very mild AD (Price et al., 2001).

Neuroinflammatory processes in AD

General

Inflammatory processes, with microglia and astrocytes as the main players, are also involved in the pathogenesis of AD. Inflammation is a defense reaction against insults, but it can also be harmful and promote neurodegenerative processes (Wyss-Coray and Mucke, 2002). Microglia and astrocytes are activated in response to inflammation and provide more fuel to the reaction by producing new mediators (Eddelston and Mucke, 1993; Aldskogius et al., 1999; Benveniste et al., 2001). A local up-regulation of cytokines, acute phase reactants and other inflammatory mediators follows the activation. In AD, microglia seem to play a central role, becoming activated in the presence of A β plaques, leading to a chronic inflammation in the CNS (Cotman et al., 1996). A β can attract and activate microglia leading to clustering of microglia around A β deposits in the brain. Also, astrocytes have been shown to cluster around senile plaques in AD and secrete many proinflammatory cytokines similar to that of microglia. Deficits in the clearance of A β by astrocytes may also be part of the pathology of AD. Increased levels of cytokines such as interleukin-1 β (IL-1 β) (Blum-Degen et al., 1995) and tumor necrosis factor- α (TNF- α) (Tarkowski et al., 1999) have been demonstrated in CSF samples from AD patients, further indicating that an inflammatory reaction is ongoing.

Interleukin-1 (IL-1) system and AD

Interleukin (IL)-1, originally discovered as an endogenous pyrogen, is an important pro-inflammatory cytokine that occurs in neuroinflammation (Feuerstein et al., 1998; Rothwell, 1991; Rothwell and Luheshi, 2000). Two IL-1 receptors exist, type I (IL-1RI) and type II (IL-1RII), both binding to the agonists IL-1 α and IL-1 β , as well as to the endogenous antagonist IL-1ra (Rothwell and Luheshi, 2000). Increases in IL-1, in particular the IL-1 β subtype, have been observed following various experimental brain lesions, including neurotoxicity induced by methamphetamine (Yamaguchi et al., 1991), kainic acid (Minami et al., 1991; Vezzani et al., 1999) and trimethyltin (Brucoleri et al., 1998; Nilsberth et al 2002) as well as following brain ischemia (Minami et al., 1992;

Touzani et al., 1999) and in transgenic mice with cerebral amyloid deposits (Apelt and Schliebs, 2001).

Both *in vivo* and *in vitro* studies have provided data suggesting that IL-1 β may exacerbate neuronal damage induced by hypoxic, ischemic, excitotoxic or traumatic injury (Pringle et al., 2001; Rothwell and Luheshi, 2000). In contrast, higher concentrations of IL-1 β (10-500 ng/ml) have been reported to be neuroprotective *in vitro* against neurodegeneration induced by oxygen/glucose deprivation (Pringle et al., 2001) as well as following excitotoxic insults (Strijbos and Rothwell, 1995). In AD, IL-1 is up regulated and seems to be overexpressed early in the plaque formation process, possibly by activated microglia (Griffin et al., 1995). IL-1 promotes synthesis and processing of APP and may directly stimulate further A β production and deposition in plaques (Buxbaum et al., 1992; Mackenzie, 2000). IL-1 β has also been shown to be elevated in the CSF in AD patients (Tarkowski et al., 2003). In AD brain, affected regions containing many neuritic plaques display increased numbers of glia immunoreactive for cytokines, including IL-1 (Akiyama et al., 2000; Sheng et al., 1998).

Treatment strategies

At present there is no disease modifying treatment of AD, despite several years of intense research efforts. Early drug discovery research focused on symptomatic treatments (Gallagher and Colombo, 1995; Weinstock, 1995) and available treatments today are typically given after the degenerative processes have progressed to the point of diagnostic certainty. Therefore, by the time drug treatment is initiated, the neural damage is so extensive that therapeutic possibilities are severely reduced. Acetylcholine esterase inhibitors (AChE-Is) and a glutamate NMDA-receptor antagonist are the most used specific treatments for AD (Scarpini et al., 2003). Acetylcholine (ACh) is an important neurotransmitter involved in cognition and cholinergic neurons are extremely vulnerable and become dysfunctional in AD (Davies and Maloney, 1976). AChE-Is; tacrine, donepezil, rivastigmine and galantamine, block the enzyme responsible for the breakdown of ACh, acetylcholine esterase (AChE), and thereby increase synaptic levels of this neurotransmitter. The increased acetylcholine obtained by inhibiting AChE results in activation of both muscarinic and nicotinic receptors. Memantine, a glutamate non-competitive NMDA receptor antagonist, represents the first member of a new class of medications suggesting clinical benefit in patients with moderate to severe AD (Reisberg et al., 2003). In patients receiving donepezil, memantine treatment resulted in significantly better outcomes than placebo (no memantine treatment), when measuring cognition, activities of daily living and behavior (Tariot et al., 2004).

Inhibitors of BACE and γ -secretase are potential therapeutic targets in disease modifying therapies. The optimal way to prevent the production of A β appears to be inhibition of BACE, rather than γ -secretase, because it can produce toxic fragments even without γ -secretase. In particular BACE1, the major neural β -secretase, provides an attractive drug target since BACE1 $-/-$ transgenic animals are viable and show decreased A β generation (Cai et al., 2001; Luo et al., 2001; Luo et al., 2003). Two inhibitors of BACE, OM99-1 and OM99-2, were recently synthesized (Ghosh et al., 2000). These potent inhibitors may constitute interesting leads to identify compounds with reduced molecular size and polarity, allowing enhanced blood brain barrier (BBB) penetration. More difficulties

have emerged in the search for γ -secretase inhibitors. γ -Secretase is an important enzyme in the Notch pathway, involved in the regulation of neuronal differentiation and spermatogenesis. Notch signaling may also affect bone marrow. Hence, γ -secretase inhibitors are prone to cause severe side effects, although studies suggest that it may be possible to develop γ -secretase inhibitors that do not affect Notch cleavage (Petit et al., 2001).

The clinical immunization studies performed by Wyeth /Elan have addressed another attractive approach for slowing/preventing AD. Pre-clinical studies in transgenic mice immunized with the A β 1-42 peptide have been shown to have reduce plaque burden and eventually a complete clearance of plaques (Schenk et al., 1999). In 2001, trials were initiated on 375 patients of whom 300 were immunized with A β . However, shortly after the start of this trial, 6% of those vaccinated with A β developed symptoms consistent with meningoencephalitis (Orgogozo M et al., 2003) and the trial was stopped. The patients continued to be monitored for both health and cognitive status and in late 2002 it was reported that many of the patients that were part of the Wyeth/Elan trial had developed anti-A β antibodies (Hock C et al., 2002). Also, the patients with brain reactive anti-A β antibodies had a significantly slower rate of cognitive decline than in those patients that did not have brain reactive anti-A β antibodies (Hock C et al., 2003). There is also one case report from the trial, suggesting a partial clearance of A β in the cortex (Nicoll et al., 2003). Despite the setbacks, this approach warrants further investigations, since it is clear that many patients developed sufficient antibody titers. Another set of experiments have shown that passive immunization with peripheral administration of antibodies against A β is sufficient to reduce amyloid burden in mice brains (Bard F et al., 2000).

Anti-inflammatory therapy is another approach in the search for drugs against AD. A number of epidemiological studies have demonstrated a reduced risk for AD in populations with long-term use of non-steroidal anti-inflammatory drugs (NSAIDs) (McGeer et al., 1996; In t'Veld et al., 1998; Akiyama et al., 2000). NSAIDs, the most thoroughly documented COX inhibitors, are widely used as treatments for a variety of inflammatory conditions, particularly arthritis (Wolfe et al., 2000). While epidemiological evidence supports the general neuroprotective nature of NSAIDs (In t'Veld et al., 2001), prospective studies have provided conflicting results. Ibuprofen and some other NSAIDs may have efficacy in AD, but inconsistent results between NSAIDs suggest that the action of COX enzymes in AD may not be as significant as previously predicted or that the possible disease modifying effects may be the result of other mechanisms. *In vitro*, various NSAIDs have been shown to dose-dependently inhibit the formation of fibrillar A β 1-40/1-42 (Hirohata et al., 2005). Another group of drugs that have a potential to be used in the treatment of AD are the statins (Wolozin et al., 2000). While these drugs are generally used to treat hypercholesterolemia (Jick et al., 2000), preclinical studies have shown that a decrease in cholesterol levels reduces A β production and plaque formation (Simons M et al., 1998; Fassbender K et al., 2001).

Modelling of Alzheimer's disease

In vivo

Different approaches have been used to create models of AD. *In vivo* models have been developed that aim to mirror AD symptoms, or model certain aspects of the pathophysiology, such as neurodegeneration or amyloid deposition. Several transgenic

mouse models engineered to express wild-type APP, fragments of APP, A β alone, familial AD-linked mutant APP and /or PS1 have been developed (for review see Wong et al., 2002). These transgenes produce severe amyloid pathology, but no neurodegeneration or NFT formation. The most promising mouse transgene seems to be a triple transgenic model (3xTg-AD) developed by Oddo et al (Oddo et al. 2003), that harbors PS1_{M146V}, APP (Swe) and tau_{P301L} transgenes. The 3xTg-AD mice develop plaques and tangles progressively as well as exhibit deficits in synaptic plasticity, including LTP. Studies in this model suggest that A β pathology precedes tau pathology by several months, a finding that is consistent with the amyloid cascade hypothesis.

Another approach to develop models of AD is to produce lesions of specific brain regions. These models are usually based on administration of neurotoxins. A key advantage with neurotoxin-based models is that specific regional neurodegeneration can be obtained. However, there are also major limitations, since only one component of the disease, neurodegeneration, can be studied. For example, trimethyltin (TMT) induces neuronal loss in the hippocampal formation and other limbic structures and can therefore mimic some of the changes seen in the hippocampus of AD patients. Following TMT exposure an extensive loss of CA3 pyramidal neurons as well as alterations in APP and IL-1 β have been found (Nilsberth C et al., 2002). Lesioning of the nucleus basalis of Meynert (NBM) causes cholinergic cell loss in the basal forebrain and permanent cholinergic hypoactivity (Dunnett et al., 1987; Olton, 1990). Degeneration of neurons in NBM, the origin of the major cholinergic projections to the neocortex, occurs early in AD and is correlated with the cognitive decline. Acute excitotoxic or immunotoxic lesions in NBM in rodents have revealed a role of the basal-cortical system in attention, learning and memory. Using this type of model, the therapeutic rationale of cholinergic enhancement strategies was established as well as beneficial effects shown with neurotrophic factor administration and transplantation of cholinergic-enriched fetal grafts (see Winkler et al., 1998).

In vitro

While all *in vitro* models constitute simple approximations of a disease, they possess certain advantages over *in vivo* systems. For example, *in vitro* studies allow pathological factors to be characterized in more detail, at a cellular level and temporal processes can be substantially compressed, while maintaining controlled conditions. Various *in vitro* systems, such as cell lines (Misonou et al., 2000), primary cultures (Zheng et al., 2002) and organotypic slice cultures (Harrigan et al., 1995), have been used to study pathophysiological mechanisms believed to contribute to the development of AD. Neuronal or non-neuronal cell-lines, such as neuroblastoma cells (for example SH-SY5Y) or human embryonic kidney cells (HEK 293), can be stably or transiently transfected with different genes, for example tau, APP and PS. SH-SY5Y cells transfected with human tau, with or without pathogenic mutations, have been used in studies to reproduce the formation of PHF tau in tissue cultures (Ferrari et al., 2003). Non-transfected cell lines such as differentiated PC12 cells exposed to A β 1-42, have been used to study A β -induced neurotoxicity (Bergamaschini et al., 2002). Also, more complex primary cultures, for example primary hippocampal cultures or mixed cortical neuron/glia co-cultures, have been used as *in vitro* models of AD. In primary hippocampal cultures, exposure to A β 25-35 induces activation of GSK3 β and tau phosphorylation (Takashima et al., 1998), and in mixed cortical neuron/glia co-cultures exposure to N-methyl-D-aspartate (NMDA) induces increased expression and release of

ApoE (Petegnief et al., 2001). Primary cultures of fibroblasts from humans with familial AD with mutated APP or PS can also be cultured.

In vitro modelling of the disease primarily involves studies on neurodegeneration, often induced by toxic insults such as A β exposure. The toxic effects of A β in various forms can be investigated in cell cultures: 1) preparations of synthetic A β peptides, 2) preparations of synthetic A β separated into different fractions (monomers, oligomers/protofibrils and fibrils) or 3) conditioned media obtained from A β -secreting cells.

Bacterial lipopolysaccharide (LPS) endotoxins are cell surface proteoglycans of gram-negative bacteria that function as general activators of immune cells (Rietschel et al., 1994). In nervous tissue, LPS has been found to bind to microglial cells and lead to their activation (Kloss et al., 2001). The family of LPS receptors includes CD14, CD11/CD18, and Toll-like receptors (Lien et al., 1999). In particular CD14, a 55kd glycoprotein, functions as a common receptor for LPS (Wright et al., 1990). When LPS binds to CD14 expressed by monocytes or neutrophils the cells become activated, release cytokines, such as IL-1 β and TNF α , and up-regulate cell surface molecules, including adhesion molecules (Shapira et al., 1994). In mixed neuron-glia cultures, exposure to LPS leads to microglial activation and subsequent production of pro-inflammatory and cytotoxic factors (Jeohn et al., 1998). LPS exposed *in vitro* cultures constitute a useful tool to study the possible link between neuroinflammation and neurodegeneration, since LPS may activate inflammatory processes in brain tissue. Indeed, pathways implicated in LPS cytotoxicity may share commonalities with inflammatory processes in neurodegenerative conditions such as AD. For example, fibrillar A β 1-42 has been shown to bind to CD14 receptors on microglia, leading to cytokine release and neurotoxicity (Fassbender et al., 2004).

Organotypic cultures

Slices of developing, postnatal brain tissue can be grown *in vitro* for weeks, or sometimes even months (Gähwiler, 1984; Thiebaud et al., 1997). Such organotypic cultures are more advanced than cell lines and primary cultures. The long-term culturing of organotypic brain cultures permits recovery from dissection trauma and allows adaptation to the *in vitro* environment. The basic cellular and connective organization of the donor brain regions are well preserved, making the slice cultures suitable experimental models for studies on toxic, degenerative or developmental changes in the brain. To be able to culture slices of CNS tissue for prolonged time periods, a few essential conditions are necessary: strict sterile conditions and culture temperatures above 30°C. The tissue needs to be attached to a substrate and supplied with growth medium and sufficient oxygenation (Gähwiler et al., 1997). Organotypic cultures have been applied to study synaptic circuits with electrophysiology (Streit P et al., 1989) as well as in studies on neurogenesis and cell proliferation (Haydar et al., 1999). This culture technique has also been applied in studies on perinatal asphyxia (Morales et al., 2005) and on neurotoxic factors (Luthman J et al., 1998). Organotypic cultures possess advantages over both *in vivo* studies and dissociated primary cultures. There is an easy access to the tissue and a better control of experimental conditions, in combination with a preserved basic cellular composition and synaptic circuitry. Different brain regions can be maintained in organotypic cultures, such as striatum, cortex, and hippocampus. In some cases, co-cultures of more than one brain region are used. For example, co-cultures composed of tissue derived from cortex and striatum have been used to investigate neural dynamics that emerge from cortico-corticostriatal interactions (Plenz and Aertsen, 1996). This model was further developed by Herrera-Marschitz et al, who added a third brain region, the substantia nigra (Herrera-Marschitz et al., 2000).

Since organotypic slice cultures retain important anatomical features and advanced synaptic organization (Gähwiler, 1981) this technique is useful to characterize complex neuropathological processes. There are different approaches for culturing organotypic slices, which differ with respect to how the tissue slices are embedded and maintained.

Roller-tube cultures

In roller-tube cultures, the tissue is embedded in a plasma clot on glass coverslips that thereafter undergoes continuous slow rotation resulting in a continuous movement of the liquid–gas interface (Fig. 5). This leads to attrition of the tissue resulting in a pseudo-monolayer of cells.

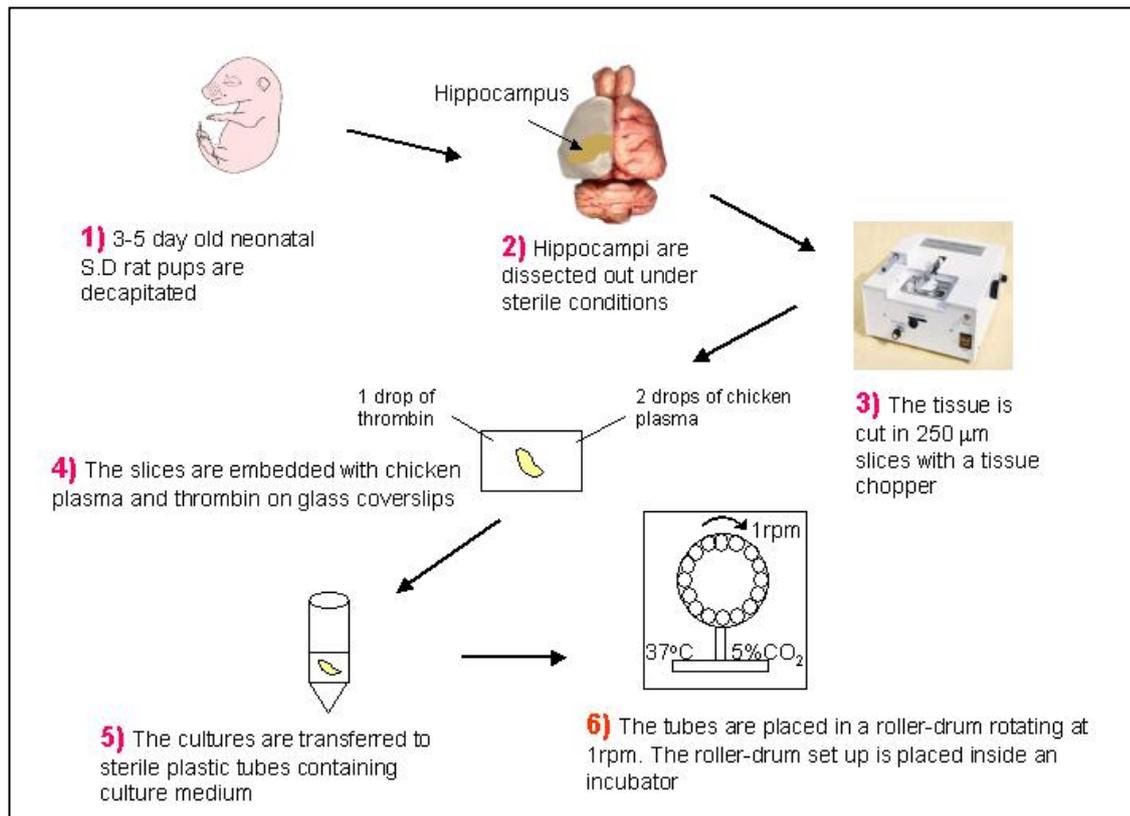


Figure 5. Scheme showing the different steps in the preparation of roller-drum cultures.

In roller-drum cultures individual cells can be viewed with a phase-contrast, or fluorescence microscope, without the need for sectioning. Roller-drum cultures are also easily manipulated, and diffusion barriers for exogenously applied substances are reduced. At the same time, roller-drum slice cultures show limitations. For example, since the cultures are grown in a test tube it is not possible to perform repeated morphological observations during culturing.

Membrane cultures

In membrane cultures of the Stoppini type (Fig. 6) (Stoppini et al., 1991), the slices are placed at the air–medium interface on semiporous membranes and kept stationary during the entire culturing process. They obtain oxygen from above and medium from below. Membrane cultures are suitable for techniques such as western and northern blotting, where more tissue is needed to perform the analysis.

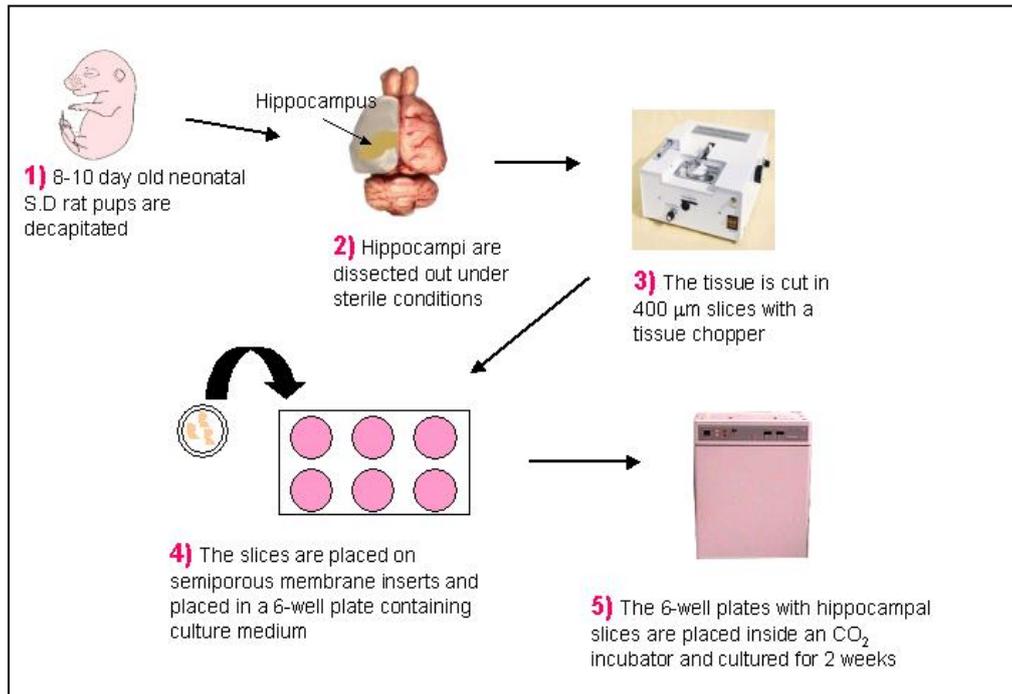


Figure 6. Scheme showing the different steps in the preparation of membrane cultures.

Table 1 Advantages/disadvantages with the different organotypic culturing techniques.

Roller drum culture	Membrane cultures
Advantages	Advantages
<ul style="list-style-type: none"> • Neurons can be visualized without the need for sectioning • Diffusion barriers of exogenously substances added are limited • Suitable for electrophysiological studies 	<ul style="list-style-type: none"> • Suitable for western and northern blotting, where more tissue is needed. • Morphology better preserved • Repeated morphological observations are possible • Suitable for electrophysiological studies
Disadvantages	Disadvantages
<ul style="list-style-type: none"> • Repeated morphological observations during culturing are not possible to perform 	<ul style="list-style-type: none"> • Thick tissue, have to section the tissue before in order to get a good staining • Applied substances have to pass the membrane to reach the culture

Organotypic cultures as drug screening tools

During the last years there has been an increasing interest in developing functional bioassays using tissue culture systems, often referred to as “high-content” or high-information-content” systems (HCS). These systems rely on freshly isolated cell cultures, or cell lines grown in multi-well plates, and the development of fluorescence imaging to enable automated functional screening. The complexity of the HCCs is advancing and bioassays based on organ systems are emerging. Over the last years *in vitro* models of acute and chronic neurodegenerative disorders have in particular been developed in hippocampal slice cultures using the Stoppini type of cultures. These cultures have allowed Sundström and others to develop functional screens in which the outcome measure is closely related to the disease state. These organotypic-based screens have a low throughput (10 compounds a week), but have certain advantages over other HCS. For instance, test compounds can be studied in a model system that reflects the complex environment *in vivo*. Also, evaluation of drugs can be performed in an assay that more closely resembles animal models, thereby allowing qualitative selection of compounds for *in vivo* testing (Sundström et al., 2005). Hence, organotypic cultures can be viewed as a bridge between *in vitro* and *in vivo* systems.

AIMS OF THE STUDY

The neurodegenerative processes observed in AD are believed to be caused by a number of interrelated pathophysiological events, in which A β peptide plays a key role. The general aim of this thesis was to evaluate the usefulness of organotypic hippocampal slice cultures in modelling components implicated in neurodegenerative and neuroinflammatory processes in AD.

The specific aims for each study were to:

Paper I.

- Investigate the ability of the synthetic A β peptide, A β 25-35, to form fibrils *in vitro*.
- Characterize the aggregations occurring after A β 25-35 exposure in organotypic roller-drum cultures.
- Evaluate the toxic effect of A β 25-35 exposure on different nerve cell populations (pyramidal cells, interneurons and neurons immunoreactive for phosphorylated tau) and on glial cells.

Paper II.

- Investigate the neurotoxic effect of IL-1 β in organotypic roller-drum cultures.
- Evaluate the neuroprotective actions of trolox, MK-801 and clomethiazole against the IL-1 β induced neurotoxicity.

Paper III.

- Characterize the neurodegenerative effects induced by LPS exposure on different nerve cell populations in organotypic roller-drum cultures.
- Investigate the ability of LPS to induce inflammation, i.e. microglia activation.

Paper IV.

- Perform a quantitative characterization of A β 25-35 induced tau phosphorylation and to study the link between A β -exposure, neurodegeneration and tau phosphorylation in organotypic membrane cultures.
- Evaluate the participation of GSK3 β and Cdk5 in A β -induced tau-phosphorylation.

METHODOLOGICAL CONSIDERATIONS

Most of the methods have been described in detail in Papers I-IV. The following section lists some of the methods used together with some general considerations.

Experimental animals (Paper I-IV)

Pregnant Sprague Dawley rats (B & K, Sollentuna, Sweden) and the delivered pups were housed together in a cage at 22°C with alternating 12 h light-dark cycles at the animal department, AstraZeneca, Södertälje. Food and water were available ad libitum. All studies were approved by the South Stockholm Committee for Ethical Experiments on Laboratory Animals (Dnr S28.01 and S212.03) and followed the provisions and general recommendations of Swedish animal protection legislation.

Organotypic roller-drum cultures (Paper I-III)

The experiments in paper I-II were based upon organotypic roller-drum cultures (Gähwiler 1981), in order to investigate specific mechanisms involved in neurodegeneration and neuroinflammation. Three to five day old postnatal Sprague Dawley rat pups were sacrificed by decapitation and their brains removed under sterile conditions. The hippocampus was dissected out, placed in Gey's balanced salt solution (GBSS) and thereafter cut into 250 µm thick slices with a tissue chopper. The slices were transferred to a petri dish containing GBSS and only intact hippocampal slices containing the CA1-CA3 and dentate gyrus were selected under a light microscope. The tissues were then attached to sterile 12 x 24 mm glass cover slips with a mixture of reconstituted chicken plasma and thrombin (1000 NIH units) in the proportions 1:2; thrombin:chicken plasma. After coagulation for 30 min at room temperature, the cover slips were transferred to sterile plastic tubes containing 1.0 mL of culture medium. The medium (100 mL) consist of 56.0 mL Dulbecco's modified Eagle's medium with glutamine (Gibco), 32.5 mL Hank's balanced salt solution (Gibco), 0.66 mL of a 45% glucose solution (Sigma), 1.0 mL of a 1 M HEPES solution (Gibco) and 10 mL heat-inactivated fetal bovine serum (HyClone). 1% antibiotic-antimycotic solution (Sigma) containing penicillin, streptomycin and amphotericin B was added to the medium until the first medium change. The cultures were grown for three weeks in a roller-drum placed inside an incubator (37°C, 5% CO₂) before initiation of any experiment. The roller-drum was tilted at 5° to the horizontal axis and rotated at 60 revolutions per hour exposing the cultures to gaseous or water phases every min.

At 21 days *in vitro* (DIV) Aβ₂₅₋₃₅, IL-1β or LPS, was added at different concentrations and time-points as indicated in paper I, II and III respectively. The roller-drum culture technique permits an easy access of the different stimulus to the cells in the culture, since the medium containing the stimulus is in direct contact with the culture every minute.

Organotypic membrane cultures (Paper IV)

In paper IV, a quantitative study on the relationship between A β (25-35) and tau phosphorylation was investigated. For this purpose we used organotypic membrane cultures. Sprague Dawley rat pups (postnatal day 7-9) were decapitated and their brains removed and placed in Gey's balanced salt solution (GBSS, Gibco). The hippocampus was dissected out and cut in 400 μ m slices with a tissue chopper (McIlwain, USA). Under light microscope, hippocampal slices containing CA1-CA3 and dentate gyrus (DG) were selected out and placed on a Millicell culture-insert (30 mm, 0.4 μ m, Millipore). The inserts were placed inside 6-well tissue culture-plates containing 1.1 ml of culture medium, allowing the slices to be exposed to oxygen from above and medium from below. The culture medium consisted of BME (44%, Sigma), EBSS (23%, Gibco), horse serum (23%, Sigma), supplemented with (final concentration) glucose (40 mM); L-glutamine (3 mM); HEPES (20 mM); NaCl (136 mM); CaCl₂ (2 mM); NaHCO₃ (5 mM); MgSO₄ (2.5 mM); insulin (1 mg/l); ascorbic acid (0.5 mM) and antibiotic/antimycotic (0.05%). The slices were cultured (37°C and 5% CO₂) for 2 weeks before initiation of any experiment.

At 14 DIV, the membrane cultures were exposed to A β as described in paper IV. The exposure of membrane cultures is somewhat different from the roller-drum cultures, since the medium is not in direct contact with the culture. Instead, the medium containing A β is lying under the membrane. Every day during the exposure period, medium taken from underneath the membrane was pipetted on top of the membrane to ensure a direct contact with the culture.

Synthetic A β peptides (Paper I and IV)

A β (25-35) and A β (35-25) were purchased from Bachem, B \ddot{u} bendorf, Switzerland (Sigma no H-1192 and H-2964). The peptides were initially dispersed in dH₂O at a concentration of 1 mg/ml and thereafter stored in -20°C until used. Further dilutions of the peptides were performed in the culture medium.

Electron microscopy (Paper I)

To investigate the possibility of A β ₂₅₋₃₅ to form fibrillar aggregates were performed by electron microscopy (EM) studies. A β ₂₅₋₃₅ was dispersed in distilled water at a concentration of 1 mg/mL and centrifuged at 1000 g for 2 min to remove possible non-dissolved material (stock solution). Further dilution was then made in culture medium to a final concentration of 50 μ M. Samples were taken for electron microscopy (EM) after incubation for either 2 or 48 h at 37°C in a humidified atmosphere of 5% CO₂ in air (tissue culture conditions). The incubated solutions were centrifuged at 20,000 g for 20 min to sediment aggregated material. This material was then suspended in 100 μ L of water by gentle sonication and 5 μ L aliquots were placed on grids covered by a carbon-stabilized formvar film. After 2 min, excess fluid was removed with a filter paper, and

the grids were negatively stained with 2% uranyl acetate in water. The specimens were finally examined and photographed in a Jeol EM 100CX at 60 kV.

Immunohistochemistry (Paper I-IV)

Analysis of the A β -, IL-1 β - and LPS-induced toxicity on different neuronal subtypes, phospho-tau immunoreactive neurons, astrocytes and microglial cells, was performed by immunohistochemistry. The primary antibodies used in the immunohistochemical studies in this thesis are listed in Table 1.

Table 2. Primary antibodies

Antibody	Strain	Dilution	Sources	Papers
NMDA-R1	mouse	1:500, 1:600	A	I-IV
GAD-65	mouse	1:200	B	I
GABA	rabbit	1:5000, 1:10 000	B	I, III
pS396	rabbit	1:250	C	I, IV
AD2	mouse	1:5000	D	I
AT8	mouse	1:250	E	I
GFAP	mouse	1:100	B	I
ED1	mouse	1:1000	F	I
CD11b, clone OX42	mouse	1:200, 1:500	F	I
CD11b, clone ED7	mouse	1:250	F	III
p53	mouse	1:1100	G	III

A; PharMingen, B; Sigma, C; Biosource, D; Bio-Rad, E; Innogenetics, F; Serotec, G; AMS Biotechnology

Roller-drum cultures on coverslips were fixed in 0.4% paraformaldehyde (PFA) and 0.125% glutaraldehyde in PBS for 30 min, while membrane cultures were fixed in 4% PFA at +4°C for at least 4h. Following several washes in PBS the cultures were incubated with a 10% methanol solution containing 3% hydrogen peroxide to quench endogenous peroxidase activity. Cultures were blocked for 1 h in 1% BSA, washed in PBS, and incubated with the appropriate primary antibody, diluted in PBS containing 0.3% Triton X-100 and 0.5% normal serum, for 72 h at +4°C. Membrane cultures were carefully scraped of the membranes and processed for immunohistochemistry as free-floating sections. Hippocampal neurons were identified with antibodies against the NMDA receptor glutamate ion channel receptor subunit, NMDA-R1, glutamic acid decarboxylase 65, GAD65 and gamma aminobutyric acid, GABA. The different phospho-tau antibodies (pS396, AD2 and AT8) detected phospho-tau immunoreactive neurons and antibodies against glial fibrillary acidic protein, GFAP and the antibodies ED1 and CD11b stained astrocytes and microglial cells/macrophages. In paper III, double-staining of cultures with p53 and NMDA-R1 was performed. After the incubation with primary antibodies, cultures were washed and biotinylated secondary antibodies were used in order to visualize the immunoreactivity. After incubation with the avidin-biotin complex and the subsequent DAB reaction, the cultures were dehydrated, cleared in xylene, and mounted on glass slides. In paper I, co-staining of GABA-stained cultures with GFAP was used to secure that the GABA-positive astrocytes that we observed really were astrocytic. Binding of the GFAP antibody was visualized using a nickel-intensified DAB protocol, leading to a black/violet staining of astrocytic processes.

Unspecific staining was blocked by incubation with normal serum. The omittance of primary antibodies served as a general control of unspecific immunoreactivity due to unspecific binding of secondary antibodies (Paper I and IV).

Cell counts (Paper I-III)

The cells were counted under light microscopy at a 10-20x magnification. The cell counting was performed using an ocular grid, which guided the experimenter in the cell counting, starting from one corner of the tissue, ending in the opposite, diagonal, corner. All stained cells within the culture tissue, showing phenotypic characteristics of the cell type analyzed (neuronal processes and intact cell soma and nucleus) were counted, with the experimenter blinded to the treatment protocol.

TUNEL labeling and Fluoro-Jade B staining of neurons undergoing neurodegeneration (Paper I, III, IV)

To detect degenerating neurons, TUNEL labeling or Fluoro-Jade B staining of the cultures was performed. In the Fluoro-Jade B staining procedure, the fixed cultures were first immersed in a solution containing 1% sodium hydroxide in 80% ethanol, followed by 70% ethanol and dH₂O. The cultures were then transferred to a solution of potassium permanganate and after rinsing in dH₂O the cultures were placed in the Fluoro-Jade B staining solution for 20 min. After rinsing in dH₂O the cultures were dried, cleared in xylene, mounted and examined with a fluorescence microscope using an FITC filter.

Western blot

The western immunoblotting technique was used to quantitatively study the effect of A β 25-35 on protein levels of different phospho-tau epitopes and the kinases GSK3 β and Cdk5. The slices were lysed in a 1% triton-lysis buffer. Following sonication (4 pulses x 2) cell lysates were centrifuged at 14 000 rpm for 15 minutes at +4°C and supernatants were collected and stored at -80°C until analysis. The protein content in the supernatants was measured using the BCA Protein Assay kit. Samples containing 35-50 μ g protein were separated on 10% NuPage[®]Bis-Tris gels and proteins transferred to Hybond nitrocellulose membranes (Amersham Biosciences). Membranes were blocked in PBS containing 0.05% Tween and 5% non-fat dry milk prior to the incubations with various primary antibodies diluted in either 5% BSA or 5% milk (4°C, overnight). A panel of phospho-tau specific antibodies (Tau[pS¹⁹⁹], Tau[pS²⁰²], Tau[pT²⁰⁵], Tau[pS³⁹⁶] and Tau[pS⁴⁰⁴]) and antibodies against GSK3 β and GSK3 α/β [pY^{279/216}], p35/p25, Cdk5 were used. Primary antibodies were used at the following dilutions: Tau-5 (RDI) 1:200, Tau[pS¹⁹⁹] 1:500, Tau[pS²⁰²] 1:500, Tau[pT²⁰⁵] 1:500, Tau[pS³⁹⁶] 1:500 and Tau[pS⁴⁰⁴] 1:500, GSK3 α/β [pY^{279/216}] (Biosource International) 1:500, GSK3 β (Santa Cruz, H-76) 1:500, Cdk5 (Santa Cruz, C-8) 1:500, p35/p25 (Sigma) 1:500 and β -actin (Sigma) 1:15 000. All anti-phospho-tau antibodies were from Biosource International. Blots were incubated with horseradish peroxidase (HRP)-conjugated anti-mouse or anti-rabbit

antibodies (Amersham Biosciences) diluted 1:5000 and developed by the enhanced chemiluminescence (ECL) western blotting detection system. Average densities of the bands were measured in Fluor-STM MultiImager using Quantity One software. The optical densities for phospho-tau at different residues were normalized to total tau levels, as detected with the phosphorylation-independent Tau-5 antibody. The optical densities for activation-associated GSK3 β [pY²¹⁶] signals were normalized to levels of total GSK3 β (H-76) and total GSK3 β signals were normalized to β -actin.

RESULTS AND DISCUSSION

A β 25-35-induced neurodegeneration, tau phosphorylation and kinase activation (Paper I and IV)

Addition of A β to cell lines or dissociated cultures of nerve cells has been shown to induce tau phosphorylation (Busciglio et al., 1995; Takashima et al., 1998). In more complex systems, such as organotypic cultures, A β has been shown to induce neurodegeneration (Allen YS et al., 1995; Harrigan MR et al., 1995). It was therefore considered of interest to characterize the effect of A β -exposure, using the synthetic A β 25-35 peptide, on different nerve cell populations, on phospho-tau immunoreactive neurons and on glial cells, with immunohistochemical staining of roller-drum hippocampal cultures prepared from neonatal rats (**Paper I**). The link between A β , neurodegeneration and tau phosphorylation was further characterized using membrane hippocampal cultures, allowing a quantitative investigation with western immunoblotting (**Paper IV**).

Rat hippocampal roller-drum cultures were exposed to the neurotoxic A β ₂₅₋₃₅ fragment of A β (Yankner et al. 1990), and examined if neuronal and glial cells were changed either morphologically or in number. After mixing the A β ₂₅₋₃₅ peptide in culture medium, highly fibrillar networks developed within 2 days as demonstrated with EM, indicating that this peptide possessed characteristics similar to full length A β , such as the ability to form fibrils. Previous studies have shown that both A β ₂₅₋₃₅ and A β ₁₋₄₂ form stable aggregates, even when diluted in medium (Pike et al. 1991). Indeed the A β ₂₅₋₃₅ treated cultures showed deposits that were positive for Thioflavine T and Congo red and expressed the classical green birefringence under polarized light, suggesting β -pleated sheet formation (**Paper I**, Fig. 7). These results showed convincingly that A β 25-35 can be used as a substitute for full-length A β in *in vitro* studies of A β -toxicity.

In addition, A β 25-35 was able to induce degeneration of different nerve cell populations (NMDA-R1 immunoreactive pyramidal cells and GABA-ergic interneurons) and phospho-tau immunoreactive neurons. The pyramidal neurons were severely affected by the A β exposure and the cell loss appeared to be both time- and concentration-dependent, causing a 60% cell loss following 96 h exposure to 50 μ M A β 25-35 (**Paper I**, Fig. 8). The finding of an increased Fluoro-Jade B staining, concomitant with the degeneration of pyramidal cells, implies that the A β ₂₅₋₃₅-exposure induced neurodegeneration in the roller-drum cultures, although it cannot be said which type of cell death was involved.

Figure 7.

Fibrillar aggregates formed by A β_{25-35} . (A) Amyloid aggregates stained with Thioflavine T. (B) Control culture stained with Congo red. (C and D) A β_{25-35} exposed cultures stained with Congo red (arrows). (E) Congo red stained A β_{25-35} aggregates expressing green birefringence under polarized light. Scale bar B-D = 50 μ m, E = 100 μ m.

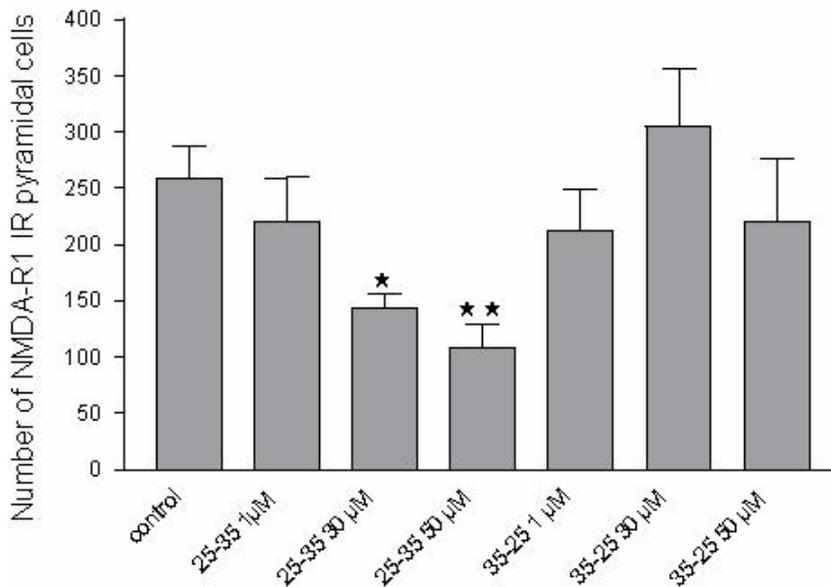
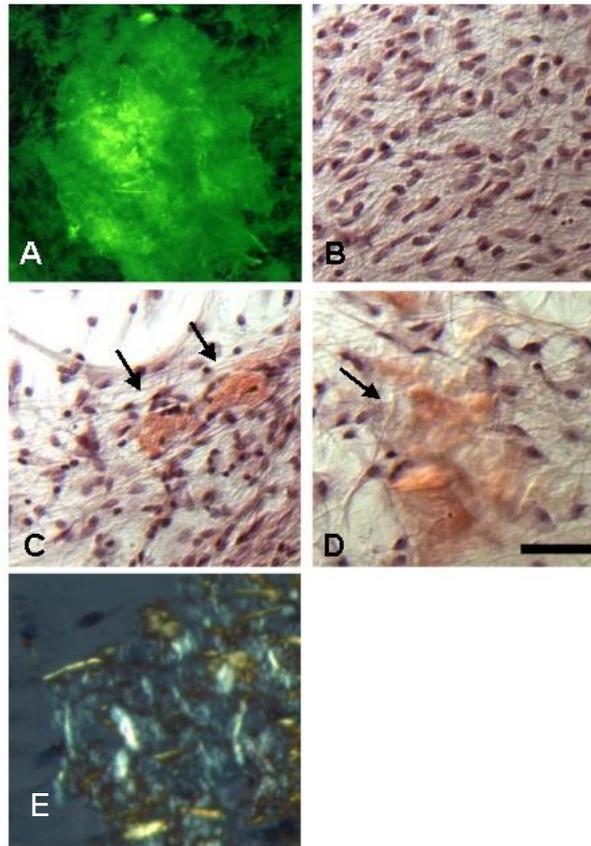


Figure 8. Concentration-dependent effects of A β_{25-35} on the pyramidal cell loss.

Neurons immunoreactive for S202/T205, S396 and S396/S404 phospho-tau showed major morphological alterations after the A β_{25-35} exposure. Their neuronal processes were bent, curly and fragmented and many neurons had lost their processes entirely (**Paper I**).

A β_{25-35} induces tau phosphorylation on epitopes S199 and S396 (**Paper IV**)

The ability of A β_{25-35} to induce tau phosphorylation in another organotypic model, membrane hippocampal cultures, was further examined. A quantitative investigation using western immunoblotting was performed showing increased protein levels of pS396 (**Paper IV**, Fig. 9) and pS199, concomitant with an increased level of GSK3 β following A β_{25-35} -exposure (96 h, 50 μ M).

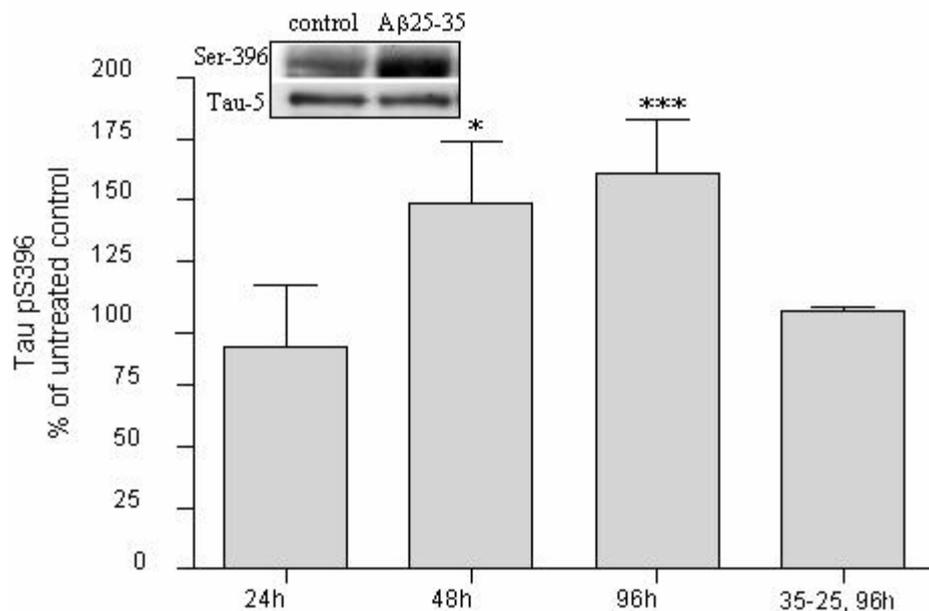


Figure 9. Time-dependent effects of A $\beta_{(25-35)}$ exposure on protein levels of Tau[pS³⁹⁶] in rat hippocampal membrane cultures. The inserted picture shows a representative western immunoblot of pS³⁹⁶ on cell lysates from control and A $\beta_{(25-35)}$ exposed cultures (50 μ M, 96 h).

Other phospho-tau epitopes investigated (S202, T205 and S404) and p35/p25 levels remained unchanged following the A β exposure. These results imply that in organotypic cultures, GSK3 β represents the major contributor to A β -induced phosphorylation of tau at S396 and S199, while Cdk5 is not involved.

Effect of A β_{25-35} on GABA-ergic interneurons, astrocytes and glial cells (**Paper I**)

GAD 65 and GABA immunoreactive neurons were detected in the roller-drum cultures, both markers known to identify GABA-ergic interneurons. The GAD 65-positive cells

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were severely affected following 96 h exposure to A β (25-35), with only a few small and condensed GAD 65-positive cell bodies remaining. In contrast, GABA immunostaining showed neurons with intact processes after the A β exposure. Thus, the GABA-producing enzyme GAD seems to be more sensitive to A β -exposure, while GABA itself is not affected in the same way. Therefore, GAD 65 and GABA-immunoreactive neurons may constitute two subtypes of GABAergic interneurons, with distinct properties and sensitivities for A β .

GFAP-positive astrocytes turned into a more reactive phenotype and showed a changed morphology, including an expansion of their processes, after the A β (25-35) exposure. A large network of astrocytic processes developed that were spread out over the culture, making it difficult to distinguish the cell bodies from the network of processes. In contrast, no obvious effect on microglial cells and macrophages was induced by A β (25-35). In the cultures, microglia and macrophages were identified with the OX42 and ED1 antibodies. Although a few affected immunoreactive cells could be seen, most cells seemed unaffected following A β ₂₅₋₃₅ exposure. This finding is in line with previous *in vitro* studies, where no or very small microglial activation has been demonstrated after exposure to A β ₂₅₋₃₅. Specific domains of A β , including the non-toxic 10-16 region, have been proposed to be necessary for microglial activation (Giulian et al 1996). This means that the lack of any obvious morphological changes in labeled cells following the A β ₂₅₋₃₅ exposure may be due to an inability of this A β -peptide to activate microglia.

In vitro modeling of inflammation using IL-1 β and LPS (Paper II and III)

IL-1 β -induced neurodegeneration and protective roles of Trolox and MK-801 (Paper II)

According to the amyloid cascade hypothesis, inflammation and oxidative stress are two processes implicated in the pathophysiology of AD. Since the aim of this thesis was to model AD-pathology in organotypic cultures, roller-drum cultures were applied as a model system for studying inflammation-induced neurodegeneration and the eventual protective roles of the free radical scavenger trolox and the non-competitive NMDA-receptor antagonist MK-801. Several pieces of evidence support the involvement of cytokines in AD, including the findings of increased CSF levels of IL-1 β and IL-6 as well as an increased number of glial cells immunoreactive for IL-1 in association with neuritic plaques (Sheng JG et al., 1998). Exposure of roller-drum cultures to rat recombinant IL-1 β for 96 h at a concentration of 1, 3 or 10 ng/ml induced a loss of both pyramidal and granular NMDA-R1 immunoreactive cells. The loss was associated with the occurrence of swollen cells and the presence of extensive extracellular debris. The loss of NMDA-R1 immunoreactive cells was found to be dependent on the concentration of IL-1 β . Also, mouse recombinant IL-1 β was able to induce a decreased number of NMDA-R1 immunoreactive pyramidal cells, but not to the same extent as seen with rat IL-1 β . The vitamin E analog trolox (30 μ M) provided a full protection against rat IL-1 β (10 ng/ml)-induced loss of NMDA-R1-immunoreactive pyramidal neurons. A protective effect was also observed with 30 μ M MK-801, while no protection was observed with the GABA_A mimetic clomethiazole, CMZ (30 μ M) (**Paper II**, Fig. 10).

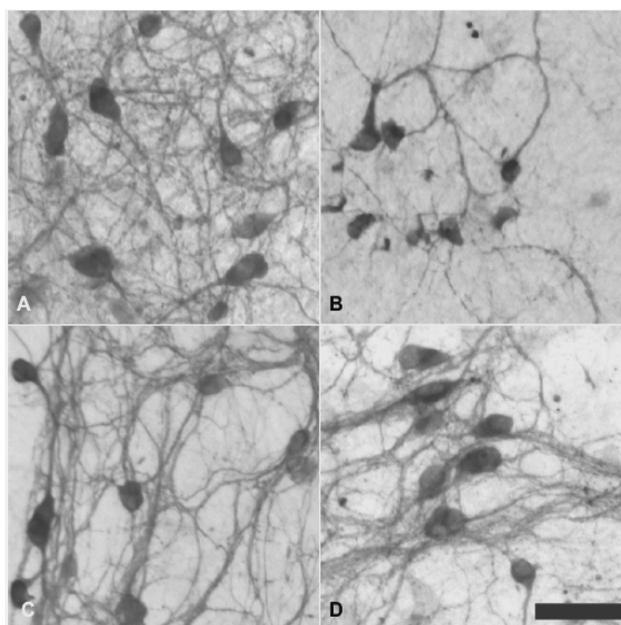


Figure 10. Effects of trolox or MK-801 on IL-1 β -induced loss of NMDA-R1 immunoreactive pyramidal neurons in roller-drum cultures. (A) control culture. (B) IL-1 β exposed culture. (C) Culture after exposure to a combination of IL-1 β and trolox, or (D) MK-801. Trolox and MK-801 were present at during the entire exposure to IL-1 β . Scale bar 50 μ m.

The data obtained with MK-801 and trolox suggest that neurotoxic action of IL-1 β in hippocampal slice cultures involves NMDA receptor mediated processes concomitant with the formation of free radicals, while the toxicity appears to be unaffected by inhibitory GABA_A receptors. IL-1 β has been shown to increase the activity of superoxide dismutase and cause accumulation of reactive oxygen species (ROS) in hippocampus (Verkerk et al., 2001). Moreover, IL-1 enhances hypoxia-induced neurodegeneration (Pringle et al., 2001) and may mediate cell death by directly activating apoptotic cascades (Becher et al., 1998). These findings provide further support for the involvement of excitotoxic processes in IL-1 β mediated neurotoxicity, and point to the likelihood that down stream events of the NMDA receptors include oxidative stress processes. This *in vitro* model, with IL-1 β -induced neurotoxicity in hippocampus, may serve as a useful system for elucidating cellular populations and mechanisms involved in cytokine-induced neurodegeneration.

LPS induced microglial cell activation and neurodegeneration (Paper III)

LPS is a major component of the cell wall of gram-negative bacteria and a strong stimulus of immune and inflammatory responses (Rietschel et al., 1994). By the binding to specific receptors expressed by monocytes and neutrophils, LPS lead to their activation (Kloss et al., 2001). LPS activates inflammatory processes in brain tissue and thereby constitutes a useful tool to study the possible link between neuroinflammation and neurodegeneration. Pathways implicated in LPS cytotoxicity may share commonalities with inflammatory processes in neurodegenerative conditions such as AD. For example, fibrillar A β (1-42) has been shown to bind to CD14 (an LPS receptor), leading to cytokine release and neurotoxicity (Fassbender et al., 2004). In paper III, LPS derived from *Salmonella abortus equi* bacteria was used as a general inflammation-inducer and the effects on neurons and microglial activation investigated. Once again organotypic roller-drum cultures from

hippocampus were used as model system for studies of inflammation and its contribution to neurodegeneration. LPS (100 ng/ml) was able to increase the density of CD11b positive cells, most likely representing microglia and macrophages in the cultures (**Paper III**, Fig. 11).

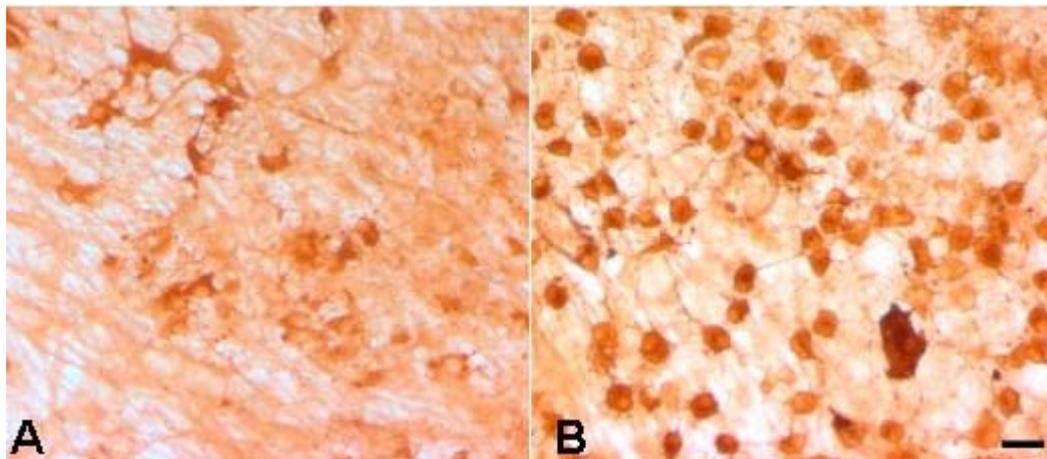


Figure 11. Effect of LPS on CD11b immunopositive cells in roller-drum cultures. Both control and LPS-exposed cultures contained CD11b-immunoreactive cells. (A) Control culture. (B) LPS-exposed culture. The CD11b immunopositive cells occur either as small and round macrophage-like cells, without any apparent processes, or as small microglia-like ramified cells. Scale bar: 50 μ m.

Several studies have demonstrated the potency of LPS as a microglia activator *in vitro* (Nakamura et al., 1999; Lee et al. 2002). Moreover, the LPS-exposure also affected neurons in the cultures. The neurotoxic effect of LPS was similar to that of A β (Paper I) and IL-1 β (Paper II) with regard to degeneration of NMDA-R1 pyramidal cells. A rapid loss of pyramidal cells was found following exposure to LPS. This cell loss was both concentration- and time-dependent (**Paper III**, Fig. 12) and did not only affect the pyramidal cells. A similar effect on GABA-ergic interneurons was found, meaning that the neurotoxic action of LPS was non-selective to specific neuronal cell populations in the cultures.

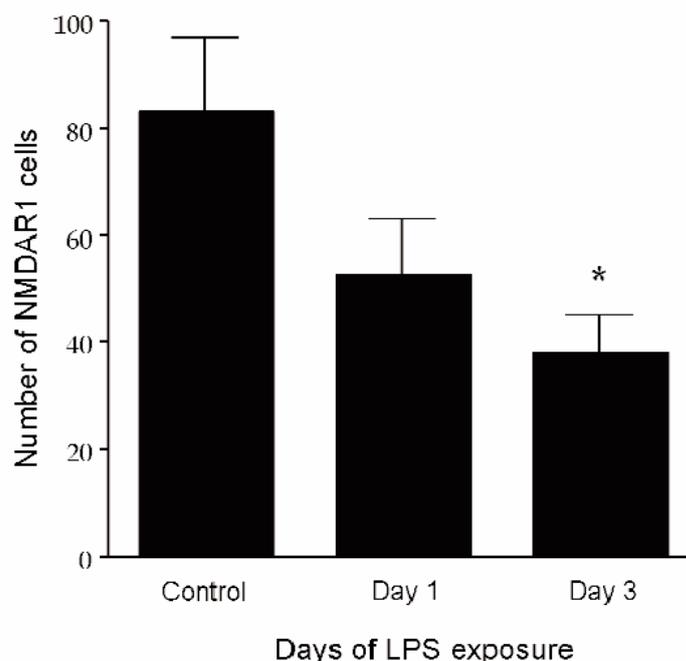


Figure 12. Time-dependent effects of Salmonella LPS (100 ng/ml) on the number of NMDA-R1 immunoreactive neurons in rat hippocampal slice cultures. The number of NMDA-R1 immunoreactive cells decreased to 64% of control after 24 h of LPS exposure and continued to decrease to 46% of control after 72 h.

The rapid loss of nerve cells that followed the LPS exposure indicates that LPS primarily induced a necrotic-like cell death. However, the fact that we saw an increased number of neurons double-labelled for p53/NMDA-R1 and TUNEL/NMDA-R1 also suggests that the LPS-induced neurodegeneration included apoptotic processes. Taken together, these data show that both necrosis and apoptosis appear to be involved in LPS induced cell death.

The present work shows that LPS can act as a potent neurotoxin in hippocampus even though in most *in vitro* or *in vivo* systems a combination of LPS and other pro-inflammatory factors, primarily interferon- γ (IFN) has been used to induce robust neurotoxicity (de Bock et al., 1998; Matsuoka et al., 1999). The present finding of LPS as a potent inducer of apoptosis is supported by an *in vivo* study where peripheral LPS-injection in rats caused a significant increase in TUNEL staining and an increased caspase-3 activation in cortical and hippocampal tissue (Nolan et al., 2003). LPS and its possible significance for studies on AD has been evaluated, showing an increased expression of IL-1 and IL-6, followed by an altered expression of brain APP isoforms after peripheral injection of LPS in mouse (Brugg et al., 1995). In addition to increased levels of APP, significant increases in A β 1-40 and A β 1-42 were seen in brain tissue from APPSwe transgenic mice after LPS treatment (Sheng et al., 2003), providing further support for the hypothesis that neuroinflammation, APP processing and A β neurotoxicity are related.

CONCLUSIONS AND FUTURE PERSPECTIVES

When the work with this thesis was started it was decided to examine different components of AD-pathogenesis in a complex *in vitro* model. An *in vitro* approach was used since this allows for controlled conditions of exposure to different agents, including neurotoxic factors implicated in the pathophysiology of AD. Also, time sequences can generally be shortened using *in vitro* systems, for example by maintaining constantly higher concentrations of different agents. In addition, the subsequent analyses are generally less laborious and less time consuming with tissue obtained for *in vitro* cultures. Organotypic cultures were chosen for this aim, since they mimic the *in vivo* tissue more closely and present several advantages in comparison with other *in vitro* model systems, such as cell lines and primary cultures. For example, organotypic cultures contain all the cell types from the tissue it derives from, allowing for careful investigations on the interplay between different cell populations. Moreover, the cultures can be cultured over long periods of time, permitting longitudinal studies. Also, since these cultures closely mimic an *in vivo* situation, but still are *in vitro*, different pathological processes implicated in AD can be coupled together. At the same time there are some disadvantages. When studying tau phosphorylation for instance, high levels of phosphorylated tau can be detected in non-treated control cultures. This may be due to the fact that the tissue is of fetal origin or possibly that the cultures are maintained under stress conditions. A comparison between *in vitro* and *in vivo* model systems is shown below (Table 3).

Table 3. A comparison between different *in vitro* and *in vivo* systems.

	<i>In vitro</i> cell-lines	<i>In vitro</i> primary cultures	<i>In vitro</i> organotypic cultures	<i>In vivo</i> brain specific lesion	<i>In vivo</i> transgenic animals
<i>Benefits</i>	Controlled cell system, only containing one cell-type	Contains cells from dissociated <i>in vivo</i> tissue	Mimics the structure of the <i>in vivo</i> brain	Specific brain regions can be studied	Possible to induce mechanisms of diseases
<i>Drawbacks</i>	Simple. Only one cell type	Natural contacts between cells are lost	Technical difficulties	Restricted components of the disease studied	Long time before pathological alterations appear
<i>Association with AD</i>	Distant from pathophysiology	May be used for some pathophysiology understanding	Can be used for pathophysiology understanding	Mimics restricted parts of symptomatology	Closely related to several key components of the pathophysiology
<i>Usefulness for therapy evaluation</i>	Fast screening	Low through-put screening and some target evaluation of disease modifiers	Relatively high capacity testing of disease modifiers, possible. Allows for systematic target evaluation	Low through-put evaluation of symptomatic therapies	Very low through-put testing of disease modifiers and possibly some symptomatic therapies

The neurodegenerative processes observed in AD are believed to be initiated by pathological actions of the A β peptide. Even though A β is undoubtedly a key player in the pathophysiology of AD, there are other components involved, such as tau phosphorylation, NFT formation and inflammation. According to the amyloid cascade hypothesis, both NFTs and inflammation are thought to be secondary events to A β deposition. This thesis was performed to evaluate the usefulness of organotypic cultures in detailed investigations on the role of certain pathophysiological components of the disease, and how they may interrelate.

In paper I the properties of A β (25-35) were investigated and used as a substitute for full length A β . A β (25-35), the neurotoxic fragment of A β (Yankner et al. 1990; Pike et al. 1993) was shown to possess similar properties to full length A β in terms of fibril formation, causing neurodegeneration and inducing tau phosphorylation. These findings imply that organotypic cultures exposed to the synthetic A β (25-35) peptide can be used in detailed morphological studies on the relation between A β -exposure, neurodegeneration, tau phosphorylation and glial activation.

Inflammatory processes and their contribution to neurodegeneration was the topic for the two next studies. In paper II IL-1 β was used as a potent inducer of inflammation and neurodegeneration. After exposure we found a massive loss of NMDA-R1 immunoreactive pyramidal cells. This neurodegenerative effect of IL-1 β could be inhibited by trolox and MK-801. These findings suggest that NMDA receptor-mediated processes concomitant with formation of free radicals are involved in IL-1 β -induced neurotoxicity. The developed model of neuroinflammation constitutes a useful tool to examine some of the inflammatory processes involved in the pathophysiology of AD. This is of particular relevance since IL-1 β , a factor known to be induced in the AD pathology, was shown to cause pyramidal cell loss. In addition, the effects of different neuroprotective drugs can be evaluated in this model system.

Paper III also investigated inflammation-mediated neurotoxic processes. Here, LPS was used to induce a general inflammation in roller-drum cultures. LPS was shown to induce an activation of microglia/macrophages, concomitant with a concentration-dependent loss of NMDA-R1 immunoreactive pyramidal cells. The neurodegeneration appeared to mostly be of a necrotic character, although increased expression of p53 in the NMDA-R1 cells indicated an involvement of apoptotic events as well. Using LPS as an inflammation-inducer, a simplified model of inflammation can be established, which can mirror some mechanisms implicated in several neurodegenerative disorders. Since LPS may activate inflammatory processes in brain tissue it also constitutes a useful tool to study the possible link between neuroinflammation and neurodegeneration.

In studies on pathogenic events leading to AD primarily there is a focus on the neurotoxicity of A β -peptide and its role in inducing tau phosphorylation and the subsequent formation of NFTs. Paper IV, which was aimed to serve as a follow-up study connected to paper I, investigated the relationship between A β and tau phosphorylation. For this purpose we chose organotypic membrane cultures from hippocampus, since the effect of A β (25-35) on protein levels of phosphorylated tau and GSK3 β was evaluated using western immunoblotting. Increased levels of Tau [pS396] and [pS199] could be seen, concomitant with increased protein levels of active GSK3 β ([pY216]) following the A β exposure. Hence in paper IV an organotypic model was used that present with other possibilities than the models described in paper I-III.

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Alzheimer's disease

This thesis evaluated the effects of A β , IL-1 β and LPS on neurodegeneration, tau phosphorylation and microglia activation, in four separated studies. However, the link between the different neurotoxins and their interrelationship remains to be investigated. For example, studies evaluating the effect of A β on IL-1 β by immunohistochemistry and western blotting/ELISA analyses and the effect of IL-1 β on tau phosphorylation using the same techniques would be of interest. This would connect study I and II, and explore the differences and similarities between A β and IL-1 β exposure and their effects on tau phosphorylation. Also, since a relationship between the LPS receptor CD14 and A β 1-42 have been demonstrated, it would be of interest to also investigate the effect A β on CD14 immunoreactive cells by immunohistochemistry in roller-drum cultures. The effect of LPS-exposure on phospho-tau and IL-1 β immunoreactive neurons could also be investigated, which would be a follow up of studies I and III. Also, in cell medium from membrane cultures exposed to LPS, alterations in IL-1 β levels released into the medium can be studied by an ELISA-based system, which would connect studies II and III.

Below is a list of potential future experiments based on findings from this thesis:

- Investigations of the effect of A β on IL-1 β and other possible neurotoxic cytokines, by immunohistochemistry and western blotting/ELISA analyses.
- Investigations of the effect of IL-1 β on tau phosphorylation by immunohistochemistry and western blotting/ELISA analyses.
- Investigate the effect of A β on CD14 by immunohistochemistry, which would explore similarities, if any, between A β and LPS exposure.
- Study the effect of LPS on tau phosphorylation and on IL-1 β by immunohistochemistry and western blotting/ELISA.
- Expose membrane cultures to LPS and investigate the effect on IL-1 β released into the medium with an ELISA-based system.

Much AD research is focusing on the identification of molecular targets that may be useful for the development of novel treatment strategies. However, a major challenge for drug discovery for AD has been to translate encouraging *in vitro* findings to relevant actions *in vivo*. Organotypic cultures may therefore present opportunities to serve as an advanced *in vitro* platform in the drug discovery process, as a step between simplified *in vitro* systems, such as cell lines and primary cultures, and *in vivo* testing in different disease models. Since key disease processes of AD can be modeled in organotypic cultures, these cultures may be particularly useful for advanced *in vitro* evaluation of disease modifying therapies targeting A β deposition, tau phosphorylation, or neurodegenerative and neuroinflammatory events. Of particular interest in this regard are the possibilities to culture brain slices from transgenic animals. If the pathophysiological characteristics of such animals can be replicated *in vitro* using organotypic culturing of tissue from the same strain, unique possibilities are presented to reduce barriers between *in vitro* and *in vivo*. Such bridging studies would also allow for a more stringent testing of identified compounds, with the possibilities to better separate challenges dependent on pharmacodynamic versus pharmacokinetic properties. Hence organotypic cultures have great potential for studies on both understanding further the pathophysiology of AD and in the identification and evaluation of future treatments.

POPULÄRVETENSKAPLIG SAMMANFATTNING

Alzheimers sjukdom är den vanligaste formen av demens hos äldre och karakteriseras av en gradvis nedsättning av intellektuell funktion. Dessa symptom beror på att kontakterna mellan nervceller i framförallt hippocampus och andra delar av cortex cerebri bryts, vilket leder till att cellerna förtvinar och slutligen dör. De hjärndelar som är mest påverkade vid Alzheimer's sjukdom är hippocampus, entorhinal cortex, amygdala, cerebral cortex och vissa basala nuclei. Den huvudsakliga mikroskopiska förändringen i Alzheimer hjärnan är uppkomsten av senila plack och neurofibrillära tangles, neuronbortfall och synaptisk degeneration. Även oxidativ stress och neuroinflammation är associerade med sjukdomen. En central faktor i sjukdomsprocessen har visats vara klyvningen av amyloid precursor protein (APP) till amyloid- β -peptid ($A\beta$), ett fragment bestående av 40-42 aminosyror. Histopatologiskt har det visat sig att $A\beta$ klumpar ihop sig och lagras extracellulärt (utanför cellerna) i hjärn parenchyma och i blodkärl. Intracellulärt (inuti cellerna) aggregerar det mikrotubuli-associerade proteinet tau i s.k. neurofibrillära tangles, NFTs. NFTs består i huvudsak av paired helical filaments, PHF, (tvinnade strukturer) av hyperfosforylerat tau, vilka också inducerar nervcells degeneration. Tau fungerar som en klinisk biokemisk markör för neurofibrillär degeneration då olika antikroppar såsom AT8, AD2 och PHF-1 används för att märka in patologisk tau i post-mortem (efter döden) human hjärna. $A\beta$ har visats inducera tau fosforylering vilket leder till dissociation av tau från mikrotubuli och aggregering i olösliga PHFs. Vidare har flera studier visat att inhibering av tau-fosforylering reducerar $A\beta$ -inducerad celldöd. Olika protein kinaser, t.ex. GSK3 β , tros spela en huvudroll i regleringen av tau fosforylering både i fysiologiska och patologiska tillstånd.

En annan viktig komponent vid Alzheimers sjukdom är neuroinflammation. Den ständiga närvaron av $A\beta$ i Alzheimer hjärnan håller mikroglia celler (en icke-neuronal cell typ i centrala nervsystemet, CNS) ständigt aktiverade vilket leder till en kronisk inflammation i CNS. Likaså tyder ökade nivåer av cytokiner (små lösliga proteiner) t.ex. IL-1 β , i CSF-prover (cerebral spinal fluid) från Alzheimer patienter, på att en inflammatorisk process är pågående.

Målet med den här avhandlingen var att utvärdera en avancerad *in vitro* modell (provrörmodell) i vilken olika patologiska processer vid Alzheimer sjukdom kan modelleras. Tekniken att odla organotypiska kulturer gör det möjligt att *in vitro* komma så nära det går en *in vivo* situation och innebär att en del av en vävnadsbit (skiva) odlas. Skivkulturer från hjärnvävnad kan hållas i kultur under långa perioder och innehåller en neurokemisk och strukturell organisation som vanligtvis har många likheter med hjärnregionen den härstammar från. Vi har valt att odla skivkulturer från hippocampus då detta är en hjärnregion som tidigt i sjukdomen drabbas av plack- och NFT-bildning, samt nervcellsöd. Organotypiska kulturer odlas framförallt på två olika sätt: i provrör i en snurrande trumma (roller-drum) eller på membran i 6-håls platta. I roller-drum kulturer bäddas vävnaden in i ett plasma/trombin-clot på täckglas och läggs i provrör innehållande medium (närlösning). Att odla kulturerna på membran innebär att kulturerna är vilande och hålls vid gränssnittet mellan luft och cellodlingsmediet. Båda odlingssätten ger kulturer med en väl bevarad organotypisk organisation. Då olika toxiska ämnen involverade i Alzheimer hjärnan såsom $A\beta$ och IL-1 β , har visats vara direkt toxiska i neuronala cell kulturer, gör det möjligt att utveckla en *in vitro* modell av Alzheimers sjukdom baserad på $A\beta$ /IL-1 β inducerad neurodegeneration. I artikel 1

studerades effekten av A β -fragmentet A β 25-35 på olika celltyper i roller-drum kulturer från hippocampus. A β 25-35 visade sig kunna aggregera i plack-formationer i kulturerna och var framförallt toxisk mot pyramidceller, celler med fosforylerat tau och astrocyter vilka fick förkortade och trubbiga utskott som följd, medan interneuron och mikroglia verkade vara mindre påverkade.

I artikel 2-3 har den inflammatoriska processen vid Alzheimers sjukdom speglats. Också här användes roller-drum kulturer och genom att tillsätta IL-1 β (artikel 2) och LPS (artikel 3) startades en inflammatorisk reaktion som inducerade neurodegeneration. I artikel 2 kunde den IL-1 β inducerade neurodegenerationen inhiberas av Vitamin E analogen Trolox och NMDA-receptor antagonisten MK-801, vilket tyder på att fria radikaler och NMDA-receptor medierade processer är involverade i IL-1 β inducerad celledöd. I artikel 3 användes LPS för att inducera en mer generell inflammatorisk reaktion som egentligen inte bara är kopplad till Alzheimers sjukdom, utan kan användas för att spegla inflammatoriska sjukdomar generellt. Både pyramidceller och GABA-interneuron påverkades av LPS och minskade kraftigt i antal efter exponeringen. Celldödsprocessen studerades och både apoptotiska och nekrotiska processer visade sig vara involverade i LPS-inducerad neurodegeneration.

Artikel 4 kan ses som en naturlig följd av studie 1. Här gjordes en mer detaljerad studie av relationen mellan A β och tau fosforylering i membrankultur systemet. I dessa kulturer kunde en kvantitativ undersökning av förmågan av A β att inducera tau fosforylering göras. Kulturerna behandlades med A β 25-35 och med tekniken western blot, kunde effekten på tau fosforylering undersökas på proteinnivå. Även involveringen av kinaserna GSK3 och Cdk5 i A β -inducerad tau fosforylering undersöktes och det visade sig att GSK3 β , men inte Cdk5, var involverad.

Sammanfattningsvis, baserat på ovanstående resultat, har dessa studier visat att organotypiska kulturer utgör ett avancerat modellsystem av *in vivo* hjärnan i vilka flera aspekter av Alzheimers sjukdom kan studeras. Kulturerna är mer komplexa än både cell-linjer och primär kulturer och speglar på så vis en *in vivo* situation bättre. Modellen skulle i framtiden kunna användas som ett led i testningen av läkemedel mot Alzheimers sjukdom och andra neurodegenerativa sjukdomar.

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