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STIMULATING NEUROPROTECTIVE AND REGENERATIVE MECHANISMS IN ALZHEIMER DISEASE

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Cover illustration: Neurogenesis in the dentate gyrus of the hippocampus (courtesy of Katherine Taylor).

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In loving memory of my grandparents Brita and Carl-Erik

"The inner fire is the most important thing mankind possesses"
-Edith Södergran

ABSTRACT

The processes involved in neuroprotection and brain repair are an important aspect of the preservation and restoration of neuronal functions affected by pathological lesions. Mechanisms that stimulate, manage and regulate these processes thus hold potential for the development of treatment strategies for Alzheimer disease (AD). The aim of this thesis was to increase our understanding of the stimulation of neuroprotective and regenerative mechanisms, in particular with respect to amyloid-β (Aβ) accumulation and other pathological processes associated with AD.

Mounting evidence suggests that the continuous loss of cholinergic neurons and nicotinic receptors (nAChRs) in the hippocampus and cerebral cortex could be mediated through an interaction between α7 nAChRs and Aβ species. In ***paper I***, we investigated interaction of α7 nAChRs with different forms of Aβ, and the functional consequences of these interactions. We found that α7 nAChRs play an important role in mediating neuroprotective actions against Aβ-induced neurotoxicity, and that the assembly form of Aβ is important for the interaction with α7 nAChRs and the downstream effects in neuronal cells. Fibrillar Aβ appears to cause cytotoxic effects by blocking α7 nAChRs, whereas oligomeric Aβ seems to activate α7 nAChRs to modulate calcium-dependent synaptic function.

In ***paper II***, we characterized the neuroprotective and neurotrophic actions of amyloid-modulatory candidate drugs (–)- and (+)-phenserine and its primary metabolites, and investigated the primary signaling pathways responsible for mediating these effects. (+)-Phenserine increased the proliferation of mouse neural progenitor cells in culture via activation of MAPK signaling pathways, including elevated cortical levels of brain-derived neurotrophic factor in mouse brain. In ***paper III***, we investigated the modulating effects of (+)-phenserine on the changes in brain synaptic function, hippocampal neurogenesis, and inflammatory cells at different stages of amyloid pathology. (+)-Phenserine increased proliferation of neural progenitor cells, and increased the maturation of newborn neurons in the hippocampi of young adult Tg2576 mice but not in older mice with advanced Aβ plaque pathology.

In ***paper IV***, we investigated the effects of stem cell transplantation and modulation of Aβ and α7 nAChRs on endogenous neurogenesis and astrocytosis, graft survival, and cognition. Intrahippocampi transplantation of human neural stem cells (hNSCs) improved spatial memory in young adult Tg2576 mice, and increased endogenous hippocampal neurogenesis. (+)-Phenserine increased graft survival but blocked the hNSC transplant-mediated increase in endogenous neurogenesis, indicative of interfering mechanisms of action. We found that α7 nAChR-expressing astrocytes accumulated along the needle track after transplantation, and that the numbers of these astrocytes correlated with the degree of endogenous hippocampal neurogenesis. Hence, we

postulate a hitherto unexplored role for α7 nAChR-expressing astrocytes in neurogenesis and tissue remodeling.

The clinical implications of stimulation of neuroprotection and brain repair in the course of AD are currently under investigation. However, it is my hope that the cumulative findings presented in this thesis will provide a better understanding of the possibilities and limitations of these therapeutic strategies that aim to change or halt the clinical progression of AD.

SAMMANFATTNING PÅ SVENSKA

Idag är över 35 miljoner individer i världen drabbade av demens. I takt med att andelen gamla i befolkningen stiger, beräknar man att antalet patienter kommer att fördubblas vart tjugonde år, vilket utgör ett allt större medicinskt, ekonomiskt och socialt problem.

Den vanligaste demensformen är Alzheimers sjukdom (AD), som kännetecknas av patologiska förändringar i hjärnan i form av extracellulära depåer av amyloida plack av proteinet amyloid-β (Aβ) och intracellulära neurofibrillära nystan av tau-protein. En annan viktig konsekvens vid AD är en drastisk förlust av framförallt kolinerga nervceller i basala framhjärnan och dessa cellers projektionsområden i cortex och hippocampus. Denna förlust är kopplad till minnesstörningar som framträder i patienter under sjukdomens förlopp. Behandling med olika typer av kolinesterashämmare är idag den mest vanliga behandlingsformen och verkar genom att stimulera frisättning av signalsubstansen acetylkolin i kvarvarande kolinerga neuron.

Idag tros Aβ aktivera olika sjukdomsprocesser, som tillsammans leder till försämrad signalering mellan nervceller och till nedsättning av de kognitiva funktionerna, som är karaktäristiska vid AD. Huvudfokus i min avhandling är att undersöka hur vi kan stimulera skyddande, (neuroprotektiva) och återuppbyggande (regenerativa) processer i hjärnan, med implikation för utvecklingen av nya behandlingsstrategier vid AD. I ett translationellt tillvägagångssätt har jag studerat dessa processer i modellsystem med neuronala celler och stamceller, kombinerat med läkemedelsbehandling och transplantationsstudier i AD transgena möss.

I ***studie I*** undersökte vi i) hur stimulering av α7 nikotinreceptorer, som är viktiga för minne och inlärning, verkar skyddande mot Aβ-medierad toxicitet, samt ii) hur olika former av Aβ interagerar med nikotinreceptorer. Aggregationsformen av Aβ visade sig ha stor betydelse för interaktionen, där mindre och lösliga, oligomera former binder till α7 nikotinreceptorer för att modulera synaptisk aktivitet, medan de stora, fibrillära formerna, tycks blockera dessa nikotinreceptorer för att orsaka neurotoxicitet.

Studie II syftade till att karaktärisera neuroprotektiva och regenerativa, samt neurotrofiska egenskaper hos den Aβ-sänkande läkemedelskandidaten fenserin och dess metaboliter, samt att undersöka vilka mekanismer som medierar dessa effekter. Substanserna uppvisade neurotrofiska såväl som neuroprotektiva effekter i olika cellulära modellsystem, som delvis var medierade via proteinkinas C och MAPK-signalering. Potentiell translationell relevans av fynden undersöktes med hjälp av 4-6 månader gamla Tg2576 transgena möss där fenserin ökade uttrycket av en neurogenes-markören doublecortin, samt ökade nivåer av den neurotrofiska faktorn BDNF.

Fortsättningsvis utvärderade vi i ***studie III*** hur en sänkning av Aβ nivåer påverkar neurotrofiska och patologiska processer i hjärnan, samt när under sjukdomsförloppet det är möjligt att stimulera regenerativa effekter. Studien visar att fenserin sänker nivåer av de vanligaste amyloidformerna Aβ1-40 och Aβ1-42 i 4-6 och 15-18 månader gamla Tg2576 transgena möss. Behandlingen gav även en ökad cellproliferation i hippocampus hos såväl unga som äldre djur, och en ökad förgrening av nybildade neuron i hippocampus hos de unga djuren, men inte hos de ändre djuren med framträdande amyloid-patologi.

Baserat på fynden i de tidigare studierna, ville vi i ***studie IV*** undersöka hur regenerativa processer och minnesfunktioner kan stimuleras *in vivo*, genom att kombinera transplantation av humana stamceller och farmakologisk behandling med läkemedel som angriper amyloidproduktion och stimulerar α7 nikotinreceptorer i Tg2576 transgena möss. Stamcellstransplantation orsakade en minnesförbättring hos mössen, som var associerat till en ökad nybildning av nerveller (neurogenes) i hippocampus. Samtidig behandling med fenserin ökade överlevnaden av transplanterade celler men motverkade de stamcells-medierade effekterna på kognition och neurogenes. Fynden indikerar att fenserin verkar antagonistiskt istället för additivt via liknande neurotrofiska mekanismer som de transplanterade stamcellerna. Kombinationsbehandling med α7 nikotinagonisten JN403 visade att det föreligger ett samband mellan antalet α7 nikotinreceptoruttryckande astrocyter och graden av neurogenes i hippocampus. Vi postulerar att förekomsten av denna population av astrocyter i hippocampus kan spela en viktig roll vid regenerativa processer i hjärnan.

Vi vet idag att ansamlingen av amyloid sker tidigt under sjukdomsförloppet vid AD och troligen måste vi därför introducera effektiv behandling i ett tidigt skede av sjukdomen. Resultaten i min avhandling visar att möjligheten att stimulera neuroprotektion och regeneration är möjlig vid en ålder där patologin ännu inte är så utbredd. Tillsammans ämnar studierna att hjälpa till i utvecklingen av läkemedel som skyddar mot Aβ-inducerad toxicitet, samt att öka förståelsen för möjligheter och begränsningar med att stimulera neurotrofiska och regenerativa processer i hjärnan hos AD patienter. Den kliniska tillämpningen av studier som syftar till att stimulera neuroprotektion och neurogenes återstår att utreda, och kommer förhoppningsvis bidra till terapeutiska strategier som kan modulera eller bromsa det kliniska förloppet vid AD.

LIST OF PUBLICATIONS

This thesis is based on the following papers:

Paper I

Anna M. Lilja, Omar Porras, Elisa Storelli, Agneta Nordberg and Amelia Marutle.

Functional interactions of fibrillar and oligomeric amyloid-β with alpha7 nicotinic receptors in Alzheimer's disease.

J Alzheimers Dis (2011) 23(2), 335-47

Paper II

Anna M. Lilja, Yu Luo, Qian-sheng Yu, Jennie Röjdner, Yazhou Li, Ann M. Marini, Amelia Marutle, Agneta Nordberg and Nigel H. Greig

Neurotrophic and neuroprotective actions of (–)- and (+)-phenserine, candidate drugs for Alzheimer's disease.

PloS ONE (2013) 8, e54887

Paper III

Anna M. Lilja, Jennie Röjdner, Tamanna Mustafiz, Carina M. Thomé, Elisa Storelli, Daniel Gonzalez, Christina Unger-Lithner, Nigel H. Greig, Agneta Nordberg and Amelia Marutle.

Age-dependent neuroplasticity mechanisms in Alzheimer Tg2576 mice following modulation of brain amyloid-β levels.

PLoS ONE (2013) 8, e58752

Paper IV

Anna M. Lilja, Linn Malmsten, Jennie Röjdner, Larysa Voytenko, Alexei Verkhratsky, Sven Ove Ögren, Agneta Nordberg and Amelia Marutle.

The amyloid-modulatory and neurotrophic drug (+)-phenserine and α7 nicotinic agonist JN403 interfere with stem cell-induced endogenous neurogenesis and cognition in transplanted Tg2576 mice

Submitted manuscript

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LIST OF ABBREVIATIONS

AD	Alzheimer Disease
MCI	Mild cognitive impairment
Aβ	Amyloid-β
NFT	Neurofibrillary tangle
APP	Amyloid precursor protein
ADDL	Aβ-derived diffusible ligand
ChAT	Choline acetyltransferase
IL-1β	Interleukin-1β
TNFα	Tumor necrosis factor-α
MCP-1	Monocyte chemo-attractant protein-1
FAD	Familial Alzheimer's Disease
PS	Presenilin
CSF	Cerebrospinal fluid
ACh	Acetylcholine
nAChR	Neuronal nicotinic acetylcholine receptor
mAChR	Neuronal muscarinic acetylcholine receptor
AChE	Acetylcholinesterase
GABA	γ-aminobutyric acid
MAPK	Mitogen-activated protein kinase
CREB	cAMP response element-binding protein
NPC	Neural precursor cell
BrdU	Bromodeoxyuridine
SVZ	Subventricular zone
SGZ	Subgranular zone
MWM	Morris water maze
MRI	Magnetic resonance imaging
PET	Positron emission tomography
BDNF	Brain-derived neurotrophic factor
^{11}C-DED	^{11}C-L-deuterodeprenyl
FDG	Fluorodeoxyglucose
AChEI	Acetylcholinesterase inhibitor
NMDA	N-methyl-D-aspartate
NGF	Nerve growth factor
NSC	Neural stem cell
$[Ca^{2+}]_i$	Intracellular calcium levels
DCX	Doublecortin
hNSC	Human neural stem cell
HFIP	1,1,1,3,3,3-hexafluoro-2-propanol
LDH	Lactate dehydrogenase
ELISA	Enzyme-linked immunosorbent assay
MSD	Meso Scale Discovery
ThT	Thioflavin T
DG	dentate gyrus
PI3K	Phosphatidyl inositol-3 kinase
JAK	Janus kinase-2
STAT	Signal transducer and activator of transcription-3
^{11}C-PIB	^{11}C-Pittsburgh compound-B
i.p.	Intraperitoneal
PKC	Protein kinase C
iPSC	Induced pluripotent stem cell

1 INTRODUCTION

1.1 ALZHEIMER DISEASE

More than 35 million people worldwide are currently afflicted by dementia, and this number is expected to double every 20 years. The prevalence of dementia increases dramatically with age and, as life expectancy continues to increase, the population above 60 years of age is expected to increase by 1.25 billion by 2050. This increase is equivalent to 22% of the current world population, with the most rapid increase in the proportion of elderly expected in China, India and Latin America (Chan et al., 2013; Prince et al., 2013). This expectation of a dramatic increase in dementia cases places an enormous burden on caregivers and relatives. The worldwide cost of dementia to society has been estimated as 604 billion USD, equivalent to 1% of the world's gross domestic product (Wimo et al., 2013).

Fifty to 70 % of all patients with dementia have Alzheimer disease (AD), which is manifested clinically by a progressive decline in cognitive function, starting with subtle impairments to episodic memory, moving on to alterations in language and changes in behavior and personality, and ending with the need for total care. The earliest clinical features of a heterogeneous group of cognitive disorders such as AD can be described as mild cognitive impairment (MCI). Some patients with MCI will go on to develop AD, some will develop other types of dementia, and some will remain cognitively stable or will revert to normal. MCI may be the result of neuronal degeneration, but can also be caused by depression, trauma, ischemia, metabolic disturbances, or other conditions (Petersen et al., 2009; Winblad et al., 2004).

The German physician Alois Alzheimer first described the pathological features of AD in 1906 (Alzheimer et al., 1995) and extensive research over the last two decades has finally provided information on the underlying pathogenesis and disease progression. The development of biomarkers for early diagnosis and evaluation of novel treatments in recent years was groundbreaking, but this branch of investigation is as yet in its infancy. Despite the large numbers of investigative studies and clinical trials over the last 30 years, the challenge to develop treatment strategies that can effectively prevent, halt or delay the onset and progression of AD remains.

1.1.1 Pathogenesis

The characteristic histopathological features of the AD brain include extracellular depositions of amyloid-β (Aβ) plaques and neurofibrillary tangles (NFTs) consisting of hyper-phosphorylated tau. The development of Aβ and tau pathogenesis typically follows distinct patterns that have been classified in stages (A–C and I–VI, respectively) according to the brain regions affected. In stage A, amyloid progression exclusively involves neocortical regions, whereas in stage B, Aβ has progressed to isocortical areas including small amounts of Aβ deposition in the hippocampus and in some cases also in the entorhinal cortex. In stage C, Aβ deposits are present throughout all isocortical areas including the sensory and motor cortex, and subcortical regions. Progression of tau pathology follows a different pattern, typically progressing from the transentorhinal region (transentorhinal stages I–II), and spreading to the hippocampus (limbic stages III–IV), and then to the isocortical regions (isocortical stages V–VI) (Braak and Braak, 1991; Braak and Braak, 1997; Thal et al., 2002).

1.1.1.1 The Aβ cascade theory and beyond

The amyloid casacade theory (Hardy and Higgins, 1992) postulates that accumulation of Aβ in the brain is a primary event that triggers other secondary pathological events, such as inflammatory processes, altered protein kinase signaling and oxidative stress, resulting in neuronal and synaptic dysfunction and eventually cell death. The hypothesis that Aβ is the main cause for AD pathogenesis still has strong support, although a growing body of evidence suggests that, because of the multifactorial nature of the disease, AD is unlikely to be caused solely by the accumulation of Aβ. Interestingly, NFTs but not Aβ plaques are associated with cognitive decline (Arriagada et al., 1992). Furthermore, there is a strong correlation with synaptic loss in AD (Terry et al., 1991). Studies indicating that AD-related genes, including familial AD (FAD) mutations (see section *1.1.4 Risk factors and genetics*), cause synaptic dysfunction and neurodegeneration without the involvement of Aβ have led to the identification of amyloid-independent pathological pathways for the disease (Chetelat, 2013; Pimplikar et al., 2010). Hence, the fact that both amyloid-dependent and amyloid-independent mechanisms contribute to AD pathology through parallel pathways should be taken into consideration in the development of effective treatment strategies, as discussed further in section *1.4 Development of treatment strategies*.

1.1.1.2 Aβ processing and deposition

The membrane-bound amyloid precursor protein (APP) can be processed along either the non-amyloidogenic or the amyloidogenic pathway. In the non-amyloidogenic pathway, APP is cleaved within the Aβ sequence by α-secretase to form soluble sAPPα and is then further cleaved by γ-secretase to yield a p3 fragment. Proteolytic cleavage of APP in the amyloidogenic pathway by β-secretase releases a soluble sAPPβ fragment, and subsequent cleavage by γ-secretase results in the formation of Aβ peptides. The most abundant Aβ peptides in the brain are 40 and 42 amino acids long; Aβ1-42 is more hydrophobic and prone to aggregate than the shorter Aβ fragments (De Strooper et al., 2010; Selkoe, 2001). It has been suggested that the shorter Aβ fragments Aβ1-14, Aβ1-15 and Aβ1-16 are formed via concerted cleavage by α- and β-secretase and do not contribute to Aβ aggregation, whereas Aβ1-17 and longer fragments are formed through an amyloidogenic, γ-secretase-dependent pathway (Portelius et al., 2009). Aβ peptides aggegate in a multistep process into various assemblies ranging from small oligomers to protofibrils, which later form Aβ plaques (Rochet and Lansbury, 2000) (figure 1). The degradation of Aβ by enzymes such as neprilysin decreases with age (Hellstrom-Lindahl et al., 2008), and in AD (Miners et al., 2008).

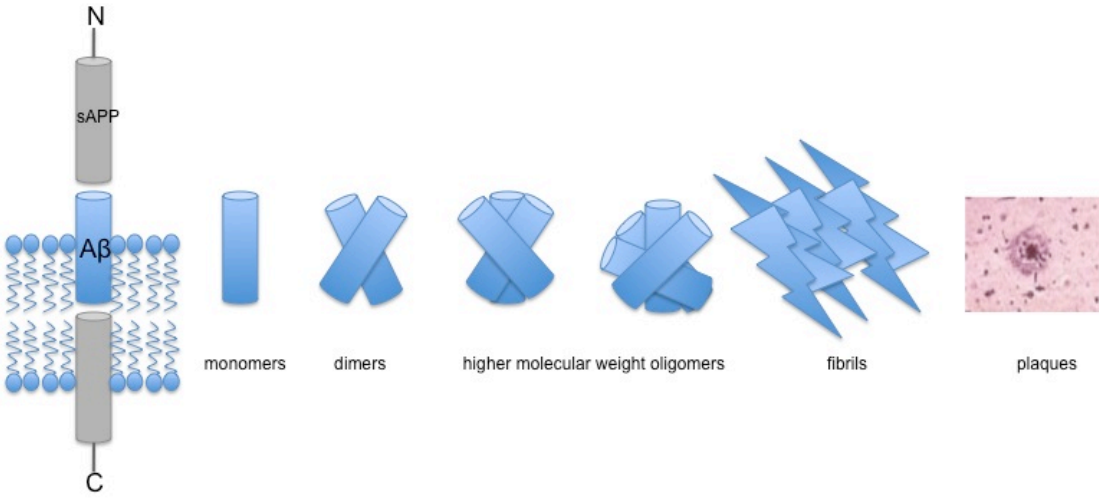

Figure 1. Schematic outline of Aβ aggregation from monomers to plaques.

One of the important goals of AD research is to elucidate which aggregated forms of Aβ are involved in mediating the impaired cellular functions in the brain. An increasing number of studies have indicated that Aβ oligomers may be the main contributors to cognitive decline in AD (Lambert et al., 1998; Walsh and Selkoe, 2007), and that the

presence of these correlates with cognitive decline better than the presence of fibrillar Aβ (Naslund et al., 2000). One of the main focuses of current AD research is to understand the actions of the various Aβ assemblies on their cellular targets. To date, *in vitro* studies have mainly used recombinant or synthetic Aβ. Isolation of Aβ species from AD postmortem brains is laborious, mostly because the oligomers are sensitive to the reagents used in experimental protocols and can be difficult to detect. The specificity of antibodies targeting oligomeric Aβ is another important issue. Recent findings suggest that Aβ dimers isolated from AD autopsied brain tissue impair synaptic function and are associated with Alzheimer-type dementia (Mc Donald et al., 2010; Shankar et al., 2008). Other studies suggest that Aβ-derived diffusible ligands (ADDLs) (Gong et al., 2003; Lambert et al., 1998) could be important components of AD pathology. Characterization of Aβ assemblies in postmortem human brain tissue has revealed that higher molecular weight oligomers, including dodecamers (Gong et al., 2003), decamers and pentamers, seem to be more prevalent than others in the AD brain, and that pentamers are correlated with reductions in choline acetyltransferase (ChAT) levels in the frontal cortices of AD patients (Bao et al., 2012). Other species thought to play a role in AD pathogenesis are 56 kDa assemblies (referred to as Aβ*56) (Lesne et al., 2006), globulomers (Gellermann et al., 2008), and protofibrils (Harper et al., 1997; Walsh et al., 1997).

Aβ probably also plays a physiological role in the healthy brain. In fact, various Aβ oligomer assemblies have been found and characterized in the brains of cognitively normal control subjects (Bao et al., 2012; Lesne et al., 2013). Furthermore, monomeric Aβ protects rat cortical neurons against trophic deprivation and excitotoxicity via the phosphatidyl inositol-3 kinase (PI3K) pathway (Giuffrida et al., 2009).

1.1.1.3 Tau

Tau is a microtubulin-associated protein that is abundant in neurons in the CNS, mainly in the neuron axons but also in the dendrites (Grundke-Iqbal et al., 1986; Ittner et al., 2010). Tau stabilizes the microtubule structure of the neuron and regulates axonal transport (Ittner and Gotz, 2011). When tau is hyperphosphorylated, it dissociates from the microtubules and assembles into paired filaments, which then aggregate to form NFTs. Dissociation from the microtubules results in changes to the axonal transport system, and subsequent synaptic loss (Iqbal and Grundke-Iqbal, 2005).

Synaptic dysfunction and neuronal degeneration parallel the formation of NFTs, but the causal link between these events is as yet unclear (Serrano-Pozo et al., 2011).

1.1.1.4 Inflammatory changes

The classical neuropathological features, Aβ plaques and NFTs, are accompanied by hallmark increases in activated microglia and reactive astrocytes in the brains of AD patients (Beach et al., 1989; Itagaki et al., 1989; Masliah et al., 1991). The inflammatory responses could have a beneficial function; glial cells clear Aβ and debris through phagocytic mechanisms and play an important role in tissue repair and remodeling. However, uncontrolled inflammation with excess production of neurotoxic species can further potentiate pathological processes in AD (Glass et al., 2010). Recent data suggest that microglia and astrocytes play important roles in regulating and maintaining neuronal activity, which can be adversely influenced by elevated Aβ levels (Graeber, 2010). Aβ in turn increases the synthesis of microglia and reactive astrocytes, and the release of pro-inflammatory cytokines such as interleukin-1β (IL-1β) and tumor necrosis factor-α (TNFα), and chemokines such as monocyte chemo-attractant protein-1 (MCP-1) (Combs et al., 2001; Lindberg et al., 2005; Meda et al., 1995). MCP-1 also contributes to the recruitment of astrocytes around Aβ plaques (Wyss-Coray et al., 2003). Aβ-mediated activation of microglia stimulates the production of reactive oxygen species, which in turn leads to oxidative stress and mitochondrial dysfunction (Baloyannis et al., 2004; Butterfield et al., 2001; Sas et al., 2007). The increased production of cytokines and reactive oxygen species results in targeting of cholinergic neurons and activation of astrocytes, which further amplifies the inflammatory signals (Glass et al., 2010). Whether inflammation in AD is a cause or consequence of the disease, is as yet unknown.

1.1.2 The cholinergic system and nicotinic receptors in AD

The cholinergic innervation system in the brain consists of basal forebrain cholinergic neurons that project to the hippocampus, amygdala, and cerebral cortex. Cholinergic neurotransmission is mediated by the release of acetylcholine (ACh), which is synthesized by ChAT and upon release interacts with neuronal nicotinic and muscarinic ACh receptors (nAChRs and mAChRs, respectively). ACh is inactivated through hydrolysis by acetylcholinesterase (AChE) in the synaptic cleft (Paterson and Nordberg, 2000; Schliebs and Arendt, 2006).

The nAChRs play an important role in regulating cognitive functions such as learning, memory and attention. They are located pre- and postsynaptically, as well as peri- and extra-synaptically, and modulate the release not only of ACh but also of other neurotransmitters such as dopamine, noradrenaline, serotonin, γ-aminobutyric acid (GABA), and glutamate (Paterson and Nordberg, 2000; Wonnacott, 1997). The nAChRs are ion channels composed of either α subunits (α2-10) or a combination of α and β subunits (β2-4). These combinations give rise to receptors with distinct physiological and pharmacological properties. The most common nAChR subunits in the mammalian brain are α3, α4, α7 and β2 (Gotti and Clementi, 2004; Paterson and Nordberg, 2000). This thesis focuses mainly on the α7 nAChRs, which are expressed throughout the human brain with the highest levels in the hippocampus, caudate nucleus, thalamic nuclei, geniculate bodies, diagonal band of broca and nucleus basalis of meynert (Paterson and Nordberg, 2000; Rubboli et al., 1994).

Although several other neurotransmitter systems are affected in AD, the reduction in synthesis of ACh (the so-called cholinergic deficit) is the most severe effect and this correlates well with cognitive decline (Kadir et al., 2006; Nordberg et al., 1995). Progressive degeneration of cholinergic neurons occurs in AD, accompanied by reductions in the levels of ChAT (Davies and Maloney, 1976; Perry et al., 1977) and also in nAChRs, mostly affecting levels of neuronal α3, α4 and α7 nAChR subunits in the brain (Nordberg, 2001; Paterson and Nordberg, 2000). Although one study showed that levels of α4β2 nAChRs had already been reduced by the time the MCI stage was reached (Kendziorra et al., 2011), another found that, when measured using [^{3}H]epibatidine binding, the loss in nAChRs occurred after the transition from MCI to AD (Sabbagh et al., 2006). Interestingly, the number of α7 nAChRs is reduced on neurons but is up-regulated on astrocytes surrounding Aβ plaques in AD postmortem brains (Yu et al., 2005). Regional distribution of mRNA levels for α3 and α4 nAChRs are not altered, whereas mRNA levels for α7 nAChRs are elevated in the hippocampi of AD patients (Hellstrom-Lindahl et al., 1999). These findings suggest that the altered nAChR levels in AD occur mainly after transcription.

1.1.3 Amyloid-β interactions with nicotinic receptors

It is possible that Aβ-mediated neurotoxicity is the result of an interaction between Aβ and nAChRs in the brain; Aβ has been shown to interact with nAChRs on neurons with resultant impairment of synaptic function (Pettit et al., 2001; Wang et al., 2000a;

Wang et al., 2000b). One suggested mechanism is that Aβ/α7 nAChR complexes on glutaminergic neurons are internalized and then contribute to intracellular accumulation of Aβ, endocytosis of N-methyl-D-aspartate (NMDA) receptors, and impaired synaptic function (Snyder et al., 2005). In support of this, increased accumulations of Aβ have been found in cholinergic neurons with high expression of α7 nAChRs in the AD brain (Nagele et al., 2002), which could cause these neurons to be particularly vulnerable in AD (D'Andrea and Nagele, 2006). Several studies have indicated that nAChRs might be involved in promoting neuroprotective mechanisms in the brain (Buckingham et al., 2009; Kihara et al., 1997; Liu and Zhao, 2004; Picciotto and Zoli, 2008), which has led to an interest in developing drugs that activate nAChRs. Several workers have shown that nAChR agonists protect neurons against Aβ-induced toxicity (Kihara et al., 1997; Liu and Zhao, 2004). Both α4β2 and α7 nAchRs have been implicated in neuroprotection against Aβ-induced toxicity (Kihara et al., 1998; Takada et al., 2003), although α7 nAChRs are considered the primary mediator.

A number of mechanistic explanations for these effects have been proposed. A study that tested the neuroprotective effects of various nicotinic agonists showed that the extent of protection was associated with the extent of upregulation of α7 nAChRs (Jonnala and Buccafusco, 2001), suggesting that this type of positive feedback loop could be important in potentiating the neuroprotective effect. But what downstream signalling pathways are involved in nAChR-mediated neuroprotection? The well known anti-apoptotic PI3K/v-akt murine thymoma viral oncogene homolog (PI3K-AKT) pathway has been identified as an important component, possibly through the up-regulation of the anti-apoptotic protein BCL 2 (Arias et al., 2004). The Janus kinase-2/signal transducer and activator of transcription-3 (JAK/STAT) pathway is also activated through stimulation of α7 nAChRs, where JAK may link the PI3K pathway with the neuroprotective STAT signaling pathway (Shaw et al., 2002). However, it is not known whether this pathway is essential for neuroprotection. Another pathway that has been identified as an important mediator of α7 nAChR-induced neuroprotection, is the mitogen-activated protein kinase (MAPK)/ERK pathway. α7 nAChR agonists have been shown to promote neuronal survival via activation of ERK1/2, which is upstream of the transcription factor c-Myc (which provides anti-apoptotic effects) and the cAMP response element-binding protein (CREB; which is important for a variety of functions, including memory formation) (Bitner et al., 2007; Dajas-Bailador et al., 2002b; Ren et al., 2005). The α7 nAChR-mediated increase in

intracellular levels of calcium ions, both via the receptor but also through the activation of intracellular stores, is also thought to be important for neuroprotection (Dajas-Bailador et al., 2002a; Ren et al., 2005)

Paradoxically, Aβ also activates signalling pathways such as MAPK-CREB mentioned above, and studies have indicated that the concentrations and time course of Aβ exposure determine which pathways are activated (Bell et al., 2004; Dineley et al., 2001). This suggests that it might be possible to pharmacologically intervene in downstream processes to shift the actions of Aβ towards a pro-survival route.

In addition to their association with neuroprotection, a recent study reported that α7 nAChRs also play an important role in the integration and maturation of newborn hippocampal neurons (Campbell et al., 2010). These findings suggest that α7 nAChRs are important in mediating both neuroprotective and neurotrophic effects.

1.1.4 Risk factors and genetics

Several risk factors are considered to be important in the etiology of AD (Reitz et al., 2011). Advancing age is the greatest risk factor but lower levels of formal education have also been implicated (Stern, 2012; Stern et al., 1994), possibly as a result of a smaller *cognitive reserve* as compensation for increasing pathological changes in the brain. The term cognitive reserve, which has emerged from epidemiological studies, is associated with the theory that the brain possesses an intrinsic ability to cope with pathology through cognitive processing and compensatory mechanisms, which can help to delay the cognitive decline in AD (Stern, 2012). The level of cognitive and social engagement could also be important for brain function and the risk of dementia (Fratiglioni et al., 2004). Many risk factors for cardiovascular diseases have also been shown to increase the risk of developing AD and other dementias. These riskfactors include high blood glucose and diabetes mellitus (Ahtiluoto et al., 2010), hypertension, obesity in midlife, and high cholesterol levels (Kivipelto et al., 2005). Traumatic brain injury is another important factor which can increase the risk of AD by a factor of approximately 4.5 (Plassman et al., 2000).

Alzheimer's disease can be classified as sporadic or hereditary (FAD), the latter representing 5-10% of all diagnosed AD cases. Early and late onset AD are differentiated depending on when the first symptoms appear: before or after the age of 65 years. To date, genetic studies have revealed nearly 260 mutations (http://www.molgen.ua.ac.be/ADMutations/) in three genes associated with familial

autosomal-dominant AD: *APP* on chromosome 21, including the Swedish double mutation 670/671, and the genes encoding the two presenilin (PS) proteins that are components of the γ-secretase complex, *PSEN1* on chromosome 14 and *PSEN2* on chromosome 1 (Bertram and Tanzi, 2008; Mullan et al., 1992; St George-Hyslop, 2000). Generally, these mutations give rise to increased production of Aβ. Mutations such as the Swedish mutation that are situated close to the β-secretase cleavage site on the *APP* gene result in increased production of both Aβ1-40 and Aβ1-42 (Citron et al., 1992). Mutations close to the γ-secretase cleavage site on the APP gene and those on the *PSEN* genes selectively increase the formation of Aβ1-42 (Goate et al., 1991; Kumar-Singh et al., 2006). A recent study of AD patients with the arctic APP mutation showed low levels of fibrillar Aβ in the brain but pathological levels of Aβ and tau in the cerebrospinal fluid (CSF) (Scholl et al., 2012), indicative of oligomeric rather than fibrillar Aβ in the brains of these patients. Several other susceptible genes have also been identified as risk factors for AD. The most common of these, *ApoE*, encodes for apolipoprotein E, which is involved in cholesterol transport and metabolism and exists in the three isoforms ε2, ε3, and ε4. The ε4 allele is known to increase the risk of AD and to result in earlier onset of the disease. The risk is increased three-fold in ε4 heterozygotes and 15-fold in homozygotes (Ashford, 2004).

In contrast to most other mutations, a rare gene variant with a mutation close to the β-secretase cleaving site on the *APP* gene lowers the production of Aβ and is neuroprotective. Individuals with this mutation perform better than control subjects in cognitive tests, which raises interesting questions on whether the improved cognition is linked to the Aβ effects (Jonsson et al., 2012).

1.2 TRANSGENIC MOUSE MODELS OF AD

Several transgenic mouse models expressing human FAD mutations have been developed; these are used as *in vivo* model systems in research to study the pathological processes of AD and to test the effects of potential therapeutic interventions (Ashe and Zahs, 2010; Hall and Roberson, 2012; Philipson et al., 2010). Although these mouse models have provided critical knowledge regarding the mechanisms of AD, they do not capture its complete pathogenesis. Because of differences between mouse strains (Table 1) and between experimental animals and AD patients, findings from these mice have to be interpreted with caution. Transgenic mice harboring mutations in genes coding

for APP, PS1 and tau (referred to as 3xTg-AD) exhibit Aβ pathology, NFTs and impaired synaptic plasticity, mirroring AD pathology to a large extent (Oddo et al., 2003a; Oddo et al., 2003b). New transgenic rodent models, such as the transgenic rat TgF344-A, which develops amyloid pathology, NFTs, and substantial neuronal loss (Cohen et al., 2013), are also under development.

Table 1. Main features and characteristics of commonly used transgenic mouse models in AD research.

Strain	FAD mutation	Neuropathology	Behaviour	Reference
Tg2576 (APPswe)	APP695 (K670N/M671L)	Aβ deposition at 9 months, no neuronal loss but reduced spine density	Spatial learning deficits at 5–6 months	(Hsiao et al., 1996; Lesne et al., 2006; Perez-Cruz et al., 2011; Stewart et al., 2011)
APP23	APP571 (K670N/M671L)	Aβ deposition at 6 months, some neuronal loss	Impairment in passive avoidance tests, spatial memory.	(Calhoun et al., 1998; Lalonde et al., 2002; Sturchler-Pierrat et al., 1997)
APP/PS1	APP571 (K670N/M671L, PS1 (A246E)	Aβ deposition at 3–4 months, minor neuronal loss	Cognitive impairment at 4 months	(Borchelt et al., 1997; Holcomb et al., 1998)
3xTg-AD	APP695 (K670N/M671L, PS1(M146V), tau (P301L)	Aβ deposition at 6 months, NFTs at 15 months, impaired synaptic plasticity	Retention/ retrieval deficits at 4–5 months	(Billings et al., 2005; Oddo et al., 2003a; Oddo et al., 2003b)
5xTg-AD	APP695 (K670N/M671L, Florida (I716V) and London (V717I), PS1 (M146L and L286V)	Aβ deposition at 2 months, neuronal loss	Spatial learning deficits at 4–6 months	(Oakley et al., 2006; Ohno, 2009)

1.3 NEW NEURONS IN ADULT BRAINS – A PARADIGM SHIFT

Neural precursor cells (NPCs) in the CNS are multipotent cells that can mature into neurons, astrocytes or oligodendrocytes (Palmer et al., 1999; Palmer et al., 1995). Neurogenesis is generally defined as the generation of functional neurons from these precursor cells, thus including every step from cell proliferation to the integration of the newborn neurons into functional neural circuits. These processes were initially thought

to occur only during the embryonic and fetal stages of development. The first evidence that neurogenesis occurs postnatally in the hippocampus and in the olfactory bulb was demonstrated in the 1960s by injecting rodents with [^{3}H]-thymidine to label dividing cells and then morphologically studying their fate (Altman and Das, 1965; Altman and Das, 1967; Caviness, 1973). However, newborn neurons in the adult brain were not proven functional until an important study in songbirds showed functional integration of newborn neurons in the CNS (Paton and Nottebohm, 1984). A series of papers later showed that newly generated neurons survived for long periods, had extending axons, and could receive synaptic input (Kaplan and Bell, 1983; Kaplan and Hinds, 1977; Stanfield and Trice, 1988). Later, hippocampal neurogenesis was also confirmed in adult animals (Kempermann et al., 1998; Kuhn et al., 1996). Despite the results of numerous studies in rodents, it was thought for a long time that neurogenesis did not occur in the adult human brain. New neurons in the brains of adult humans were first discovered through the pioneering work of Eriksson and colleagues in 1998 (Eriksson et al., 1998). In this study, five terminally ill patients (average age 64 years) received injections of the thymidine analog bromodeoxyuridine (BrdU) before death, which subsequently enabled postmortem analysis and the identification of neural precursors that had undergone neuronal differentiation in the brain (Eriksson et al., 1998). It later became evident that the date of cell birth could be estimated in older adult populations by measuring ^{14}C levels in genomic DNA, since the levels of ^{14}C increased after atomic bomb testing during the cold war (1955–1963) (Spalding et al., 2005).

Hence, it is now well known that neurogenesis continues throughout adulthood, mainly in two regions of the brain: i) the subventricular zone (SVZ) lining the lateral ventricles, from where new neurons migrate along the rostral migratory stream to the olfactory bulb where they mature into interneurons, and ii) the subgranular zone (SGZ) of the dentate gyrus (DG) in the hippocampus, as reviewed in a number of publications (Gage et al., 1998; Ming and Song, 2005; Ming and Song, 2011; Suh et al., 2009). Neurogenesis in the DG involves multiple developmental steps, in which NPCs in the subgranular layer undergo cell proliferation, neuronal differentiation, migration to the molecular granular layer of the DG, molecular and axonal targeting of the newborn neuron, and functional integration into existing neuronal networks (Ehninger and Kempermann, 2008), as schematically illustrated in figure 2. Spalding and colleagues estimate that about 700 new neurons are born each day in the human hippocampus (Spalding et al., 2013). In contrast, neurogenesis in the olfactory bulb is sparse, or may not occur at all, in humans (Bergmann et al., 2012). This is not surprising, considering

the important role that the hippocampus plays in cognitive function in humans, while the olfactory bulb is less developed in humans than in rodents.

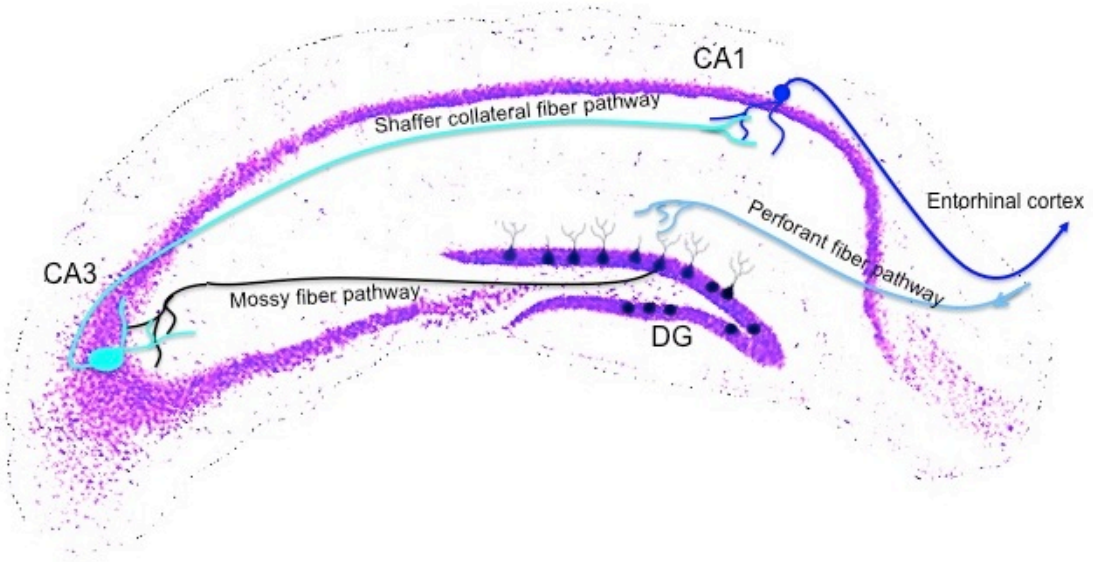

Figure 2. Schematic illustration of hippocampal neurogenesis.

1.3.1 Adult neurogenesis is regulated by a myriad of factors

Neurogenesis is intricately regulated by a large number of intrinsic and extrinsic factors. Proliferating NPCs are usually found in the vicinity of the vasculature of the brain, where vasculature-derived neurotrophic factors such as vascular endothelial growth factor stimulate neurogenesis (Jin et al., 2002; Schanzer et al., 2004). Astrocytes in the vicinity of the neurogenic zones in the brain are known to specifically regulate neurogenesis (Song et al., 2002) via astrocyte-secreted factors such as Wnt3a (Barkho et al., 2006; Lie et al., 2005), and membrane-bound factor Ephrin-B (Ashton et al., 2012). Astrocytes are associated with neuronal development, neurotransmission, synaptic plasticity and maintenance of brain homeostasis, and a number of studies have shown that they provide trophic, structural, and metabolic support to neurons (Nedergaard and Verkhratsky, 2012; Parpura and Verkhratsky, 2012; Ullian et al., 2004).

Extrinsic factors such as physical activity and an enriched environment are also potent regulators of neurogenesis. Physical activity stimulates the proliferation of NPCs, neuronal maturation and synaptogenesis (Ho et al., 2009; Kempermann et al., 1998; Snyder et al., 2009), and an enriched environment enhances hippocampal neurogenesis (Brown et al., 2003; Kempermann et al., 1998).

1.3.2 Does neurogenesis play a significant role in brain function and memory?

The functional relevance of adult hippocampal neurogenesis on behavioral traits and on learning and memory has so far mainly been studied in rodents, using hippocampus-dependent spatial memory tests such as the Morris water maze (MWM). A few studies have demonstrated a correlation between hippocampal neurogenesis and spatial learning and memory (Drapeau et al., 2003; Kempermann and Gage, 2002), but others have shown conflicting results (Gould and Tanapat, 1999; van Praag et al., 1999), probably because of confounding factors such as physical activity and stress, which can also affect neurogenesis. Although research since the first studies by Altman and colleagues some 50 years ago has provided us with a vast amount of knowledge in the field of adult neurogenesis, a number of questions remain. Nonetheless, given the rapid development of powerful tools, markers and model systems, there is reason to hope that current and future research will further improve our molecular understanding of neurogenesis and the intrinsic mechanisms behind neurogenesis during life, along with the contribution of neurogenesis to cognitive function.

Although postmortem studies have provided much information, non-invasive *in vivo* studies carried out over time are pivotal in order to understand the function of neurogenesis in physiological and pathological conditions, as reviewed by Ho et al. (Ho et al., 2013). These *in vivo* techniques include brain imaging with magnetic resonance imaging (MRI), involving scanners which can offer close to single-cell resolution and the possibility of measuring cerebral blood volume and blood flow. Interestingly, labeling transplanted stem cells with ^{19}F enables *in vivo* tracking of the graft by MRI (Boehm-Sturm et al., 2011). Imaging with positron emission tomography (PET) tracers that specifically label markers for regenerative processes in the brain is also a promising approach for studying neurogenesis *in viv*o. Nevertheless, to date no study has demonstrated a relationship between neurogenesis and alterations in hippocampal volume or function.

1.3.3 Neurogenesis in AD

The hippocampus is one of the earliest affected brain regions in AD (Braak et al., 1993). It is tempting to speculate that the mechanisms associated with cognitive reserve are also associated with increased neurogenesis, although this remains to be proven.

Expression of neuronal markers is increased in hippocampal regions of autopsied brains from AD patients (Jin et al., 2004b), suggesting that neurogenesis may be a natural defense strategy against neurodegeneration in AD. However, a recent study by Perry and colleagues showed that increased proliferation of NPCs in the hippocampus of AD patients does not result in increased numbers of matured neurons (Perry et al., 2012).

Investigations into hippocampal neurogenesis in mouse models of AD have provided conflicting findings. Most studies report compromised neurogenesis (Demars et al., 2010; Haughey et al., 2002; Zhang et al., 2007), but some have described increased neurogenesis (Jin et al., 2004a; Lopez-Toledano and Shelanski, 2007). These contradictory findings may be due to differences in the transgenic models used in the studies, the age of the mice, or the markers used to detect and quantify proliferating and differentiating NPCs.

Brain-derived neurotrophic factor (BDNF) plays an essential role in neuronal development; it is involved in cell proliferation, neuronal differentiation, integration into neuronal circuits, and synaptic plasticity in the brain (Autry and Monteggia, 2012; Ming and Song, 2005; Ming and Song, 2011). In AD, levels of BDNF are decreased in the entorhinal cortex and the hippocampus (Connor et al., 1997; Hock et al., 2000; Narisawa-Saito et al., 1996). Aβ oligomers impair BDNF axonal retrograde signalling *in vitro* (Poon et al., 2011), suggesting a possible mechanism for impaired synaptic function early in AD.

1.4 DEVELOPMENT OF TREATMENT STRATEGIES

A vast array of treatment strategies for AD is currently being developed or tested in clinical trials. These strategies include the use of anti-inflammatory drugs, antioxidants, serotonin receptor modulators, drugs targeting tau phosphorylation and aggregation, and anti-amyloid drugs (the latter is discussed further in section *1.4.3 Targeting Aβ*). The drugs under investigation have been reviewed elsewhere (Mangialasche et al., 2010; Misra and Medhi, 2013) or can be seen at www.clinicaltrials.gov. An outline of the various treatment strategies for AD is given in figure 3.

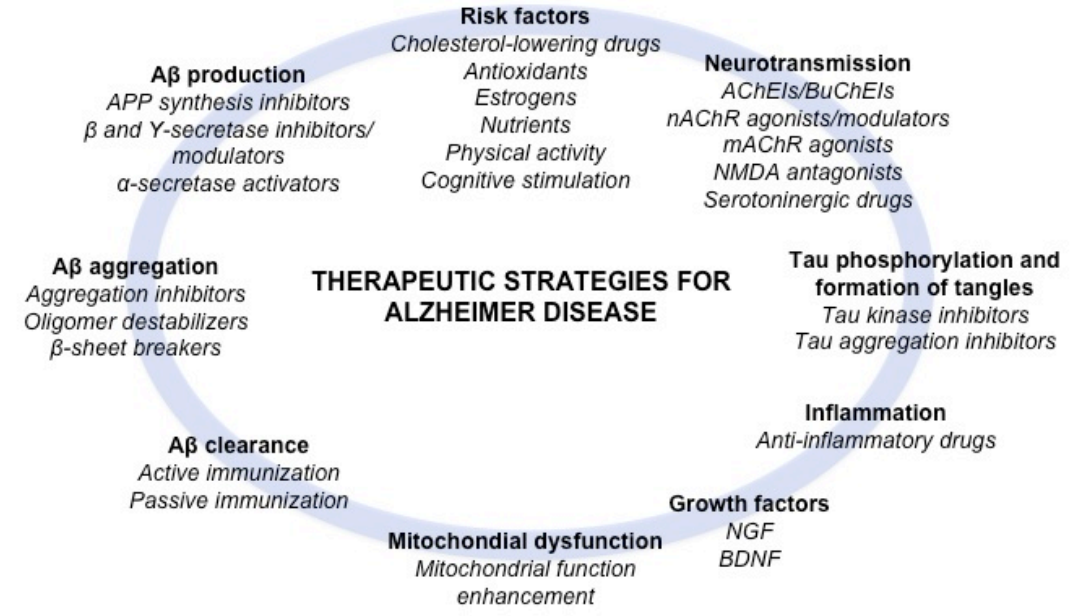

Figure 3. Outline of therapeutic strategies for AD.

1.4.1 Use of brain imaging and CSF biomarkers to evaluate treatment effects over time

The rapid development of molecular imaging techniques using selective radiotracers has provided new means of studying pathological changes and treatment effects in living patients. Together with development of CSF biomarkers, these techniques have enabled longitudinal monitoring of Aβ levels, tau levels, inflammatory changes, metabolic and structural alterations, and changes in neurotransmission. Recent assessment of various biomarkers in patients with FAD suggests that pathological changes in the brain start decades before the onset of cognitive symptoms (Bateman et al., 2012; Scholl et al., 2011). Consequently, early detection and prediction of AD could facilitate the evaluation of early intervention strategies.

The PET tracer ^{11}C-Pittsburgh compound-B (PIB), the most widely used amyloid tracer, has allowed visualization of the deposition of fibrillar Aβ very early in the course of AD, and has also facilitated investigation of Aβ progression in living patients (Nordberg, 2004; Nordberg et al., 2010). High PIB retention has been observed in cortical brain regions in patients with AD (Klunk et al., 2004) and those with MCI who later converted to AD (Forsberg et al., 2008; Kemppainen et al., 2007). Astrogliosis can also be seen *in vivo* using the PET tracer ^{11}C-deprenyl, which binds to monoamine oxidase type B predominantly localised to the outer mitochondria membrane of reactive astrocytes (Fowler et al., 2005). Recent data indicate that binding of ^{11}C-L-

deuterodeprenyl (^{11}C-DED) in the frontal and parietal cortices is higher in patients with MCI than in those with AD or control subjects (Carter et al., 2012). Furthermore, in a recent study in autopsied AD brain tissue, there were no correlations between ^{3}H-deprenyl binding and ^{3}H-PIB binding (Kadir et al., 2011), and each of these ligands showed different laminar distributions in the brain (Marutle et al., 2013), suggesting that the time course of the inflammatory process is different from that of Aß pathology. Kadir et al. also found a negative correlation between fibrillar Aβ levels and the number of nAChRs in the AD brain, as measured with ^{3}H-PIB and ^{3}H-nicotine binding, respectively (Kadir et al., 2011), which substantiates the possibility that nAChRs are involved in Aβ pathology. Cell function can be assessed by measuring glucose consumption and metabolism in the tissue with the glucose analog 2-^{18}F-fluoro-2-deoxy-D-glucose (FDG). FDG PET has shown that glucose metabolism decreases in the posterior singulate cortices, the temporal lobe including the hippocampus, and the entorhinal cortex in MCI and AD patients (Mosconi, 2005). FDG PET measurement in FAD patients with a *PSEN1* mutation suggests that aberrant glucose metabolism can be detected long before the onset of cognitive symptoms (Scholl et al., 2011). Furthermore, there is a strong association between decreased glucose consumption and cognitive decline (Landau et al., 2011).

The three most established and validated CSF biomarkers reflecting AD pathology are the levels of Aβ1-42, and total and phosphorylated tau. CSF Aβ1-42 levels are decreased in AD, possibly reflecting the increase in Aβ plaques in the brain, whereas CSF tau levels are elevated (Hansson et al., 2006; Mattsson et al., 2009).

A model involving the temporal patterns of five established biomarkers in AD, which was developed by Jack and colleagues in 2010, has recently been updated and modified. A tentative, hypothetical summary of these markers in combination with those previously discussed is illustrated in figure 4.

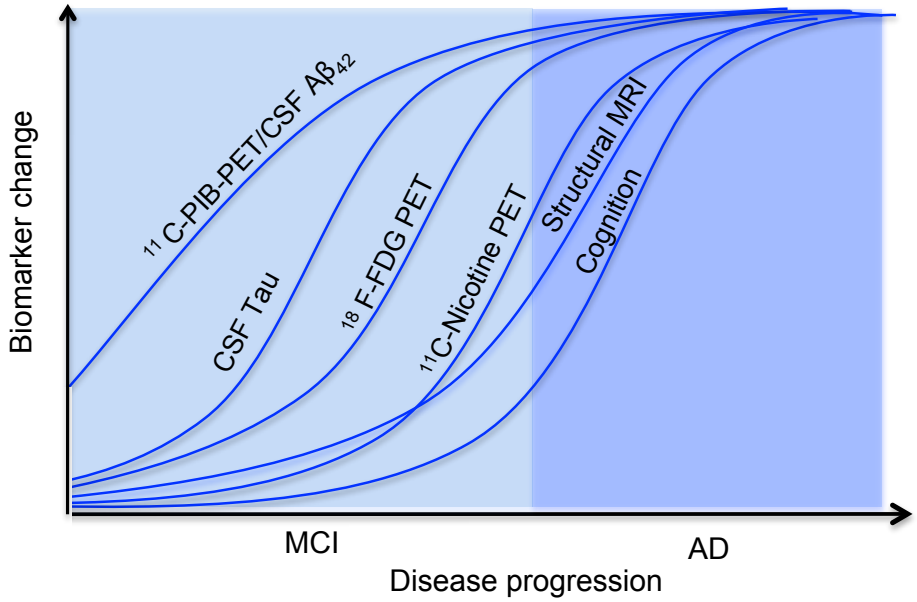

Figure 4. Pathological changes and the tentative time course of these changes in biomarkers used in studies of MCI and AD (Jack et al., 2013; Kadir et al., 2010; Nordberg et al., 2010).

1.4.2 Importance and caveats of current treatment

Current treatment for AD consists of the AChE inhibitors (AChEIs) donepezil, galantamine and rivastigmine, which have been approved for mild to moderate AD, and the NMDA receptor antagonist memantine, which has been approved for moderate to severe AD. The AChEIs were designed to reduce the activity of AChE, and BuChE in the case of rivastigmine, and thus prolong the effect of ACh in the synaptic cleft, whereas memantine inhibits NMDA receptors to prevent glutamate-mediated neurotoxicity. Improved cerebral glucose metabolism has also been observed in AD patients treated with rivastigmine, galantamine or donepezil (Keller et al., 2010; Mega et al., 2005; Stefanova et al., 2006; Teipel et al., 2006). Rivastigmine increased ^{11}C-nicotine binding in the brains of AD patients after 3 months' treatment, and a positive correlation between ^{11}C-nicotine and cognition was found in patients treated with galantamine or rivastigmine for 12 months (Kadir et al., 2008b; Kadir et al., 2007). However, despite the various positive effects that current treatment offers, there is an urgent need for novel, effective disease-modifying drugs.

1.4.3 Targeting Aβ

Of all the AD drugs currently in clinical trials, most target aspects of Aβ pathology. Some target Aβ production by inhibiting β- or γ-secretases, and some prevent Aβ aggregation and thus the formation of amyloid plaques. γ-Secretase inhibitors are currently being tested in clinical trials, and some studies have reported reduced Aβ levels but have also reported adverse effects. It is hoped that strategies to develop inhibitors that are more APP-selective, with fewer effects on other γ-secretase substrates (De Strooper et al., 2010; Imbimbo, 2008), and further evaluation of the inhibitors currently under development will reveal positive effects on cognition.

Immunization therapy that increases the removal of Aβ in the brain is also under evaluation. Aβ vaccines have been tested in clinical trials of AD patients since 2001. The first clinical trial was halted because of adverse drug reactions including encephalitis and increased loss of brain volume, and because no significant effects on cognition were observed (Fox et al., 2005; Gilman et al., 2005; Orgogozo et al., 2003). However, reductions in fibrillar Aβ have been reported in subsequent trials; at least 20 Aβ vaccines are currently in clinical trials, and trials of passive immunization in conjunction with administration of antibodies that recognize different parts of the Aβ peptides are also underway (Lemere and Masliah, 2010; Mangialasche et al., 2010). In 2010, the monoclonal antibody bapineuzumab was reported to significantly reduce fibrillar amyloid levels in a subgroup of patients after 78 weeks of treatment, as measured with PIB PET (Rinne et al., 2010). However, despite the reductions in Aβ, no effect on cognition was observed. Another monoclonal antibody, solanezumab, was recently studied in two 18-month trials. When the data from the two trials were combined, a trend towards a cognitive effect was shown (Gandy and DeKosky, 2013).

Two experimental AD drugs, the AChEI (–)-phenserine, and its cholinergically inert enantiomer (+)-phenserine, are both APP-synthesis inhibitors and thus lower Aβ levels (Greig et al., 2005; Lahiri et al., 2007; Mikkilineni et al., 2012; Shaw et al., 2001). A clinical study of (–)-phenserine treatment in patients with mild AD showed that the decreased amyloid load in the brain, as measured with PIB PET, correlated with increased levels of Aβ1-40 in the CSF, together with improvement in cognition after 3 months (Kadir et al., 2008a). (–)-Phenserine reached phase 3 clinical trials (Winblad et al., 2010), and is currently being reformulated to optimize its pharmacological actions (Becker and Greig, 2012). (+)-Phenserine has recently undergone phase 1 tolerability and target engagement trials, which reported lowered CSF levels of APP metabolites,

Aβ, tau and inflammatory markers in subjects with MCI (Maccecchini et al., 2012).

1.4.4 In search of prevention or disease modification – What can we learn from recent preclinical and clinical trials in AD?

The recent failed Aβ clinical trials in mild to moderate AD, with various drugs decreasing Aβ levels in the brain but showing no effects on cognitive function, suggest that earlier therapeutic interventions may be necessary (Selkoe, 2012). Current research is focusing on the development of treatments that target the underlying pathology and the administration of these in the early preclinical stages of AD (figure 5). In addition, several preventive clinical studies are planned in asymptomatic members of families at high risk of developing AD because of a genetic predisposition (Aisen et al., 2013). Current thinking is that future therapy will be dependent on early diagnosis and the ability to identify the right time for treatment during disease progression. Successful treatment of AD depends heavily on future advances in the identification of biomarkers, including structural, pathological and functional imaging as well as CSF markers, for early diagnosis and evaluation of the effects of new drugs (Hampel et al., 2010; Nordberg, 2011).

To date, a large number of novel treatment strategies have been successful in animal models, only to fail in subsequent clinical trials. Despite the great disappointment associated with these failures, the trials have provided important information that can be used to revise future pre-clinical and clinical trials. Factors that critically determine the outcomes of clinical trials include the cohort size, the length of the trial, and the choice of endpoints. The enormous cost associated with large trials is certainly a limiting factor for study design. It is also valid to question whether inadequacies in the interpretation and extrapolation of animal data could explain the lack of robust effects observed with some drugs once they have advanced to clinical trials. First, the endpoints in preclinical studies must be carefully selected and validated in order to answer the questions needed for advancement to clinical studies. The choice of animal model should be based on the required endpoints, and the age of the animals included in the studies should reflect the stage of the disease at which treatment is intended to start.

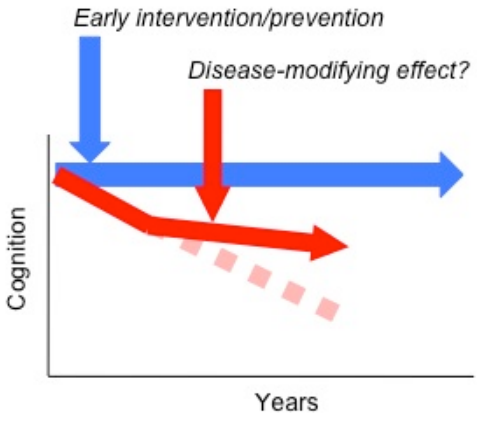

Figure 5. Schematic illustration of the concepts of prevention or disease-modifying intervention in AD.

1.4.5 Targeting nicotinic receptors

Ongoing trials are using cholinergic drugs with nAChR agonist activity, with the intention of enhancing cognition and stimulating neuroprotection. Treatment of mild to moderate AD with nicotinic agonists selective for α4β2 nAChRs has resulted in some effects on cognition (Dunbar et al., 2007). However, recent clinical trials have been terminated because of the poor recruitment status of the patients. α7 nAChR agonists are also currently being tested. The partial α7 nAChR agonist EVP-6124 was well tolerated in preclinical trials and a phase II clinical trial (Misra and Medhi, 2013). Results from the phase II 6-month trial in subjects with mild to moderate AD indicate promising benefits, as measured with a battery of cognitive tests (Hilt et al., 2012), but await publication. So far, preclinical studies of α7 nAChR agonists have demonstrated improvements in long-term memory, but subsequent clinical trials have only shown attention benefits (Thomsen et al., 2010). The possible explanations for this discrepancy are currently under debate. Unlike ACh, α7 nAChR agonists are not degraded but constantly activate and desensitize the receptor. This results in an inverse U-shaped dose-response curve, which makes drug administration challenging (Geerts, 2012). Furthermore, treatment duration has typically been short, perhaps too short to observe any potential α7 nAChR-mediated neuroprotective actions.

1.4.6 Stimulating regeneration as a potential treatment strategy for AD

The stimulation of neurotrophic processes and repair mechanisms in the brain is a novel and promising approach to the treatment of AD. The term regeneration refers to

the repair of tissue through either stimulation of intrinsic repair mechanisms or the transplantation of exogenous stem or progenitor cells. There is a great need for a deeper understanding of the balance between neurodegeneration and brain repair, and of the optimal timing for such treatments.

Stimulating endogenenous neuroregeneration

One of the advantages associated with strategies focused on enhancing the brain's intrinsic regeneration capacity is that they enable non-invasive approaches without the risk of an immune response to grafted cells. Recent increases in our understanding of molecular mechanisms and other factors associated with the stimulation of endogenous neurogenesis (see *section 1.3.3*) have enabled the identification of new drug targets and the development of new therapeutic interventions. Growth factors such as BDNF and nerve growth factor (NGF) are potent stimulators of endogenous neurogenesis, and are regarded as promising in this respect. In the first study of its kind, intraventricular injection of NGF into three AD patients has demonstrated increased ^{11}C-nicotine retention and increased glucose metabolism (Eriksdotter Jonhagen et al., 1998; Olson et al., 1992). However, the route of administration had to be reconsidered because of the development of spinal pain in the recipients. In a later study, genetically modified fibroblasts secreting NGF implanted into the forebrains of eight AD patients were shown to be safe after 22 months' follow-up (Tuszynski et al., 2005). In a more recent study, six AD patients underwent basal forebrain transplantation of bio-vehicles containing NGF-secreting fibroblasts. This procedure was deemed safe and well tolerated (Eriksdotter-Jonhagen et al., 2012). The effects on cognition, however, have yet to be reported.

Drugs such as antidepressants or atypical antipsychotics are reported to enhance neurogenesis in the brains of both rodents and humans (Nasrallah et al., 2010; Newton and Duman, 2007; Sahay and Hen, 2007; Santarelli et al., 2003). Preclinical data from studies in rodents suggest that endogenous factors such as estrogens may also stimulate neurogenesis (Tanapat et al., 1999). Extrinsic factors such as physical activity (Ho et al., 2009; Kempermann et al., 1998; Snyder et al., 2009) and an enriched environment (Brown et al., 2003; Kempermann et al., 1998) that have been shown to stimulate neurogenesis and synaptic plasticity in rodents could also provide important therapeutic strategy implications for AD.

Transplantation of stem cells or fetal grafts

Generally, exogenous cell replacement strategies have several advantages over other approaches. Large numbers of cells can be implanted and the source of the cells can be selected and optimized *in vitro* prior to grafting. However, given the widespread pathology and neurodegeneration resulting from these procedures, cell replacement strategies for AD have been considered unrealistic in comparison with their use in other diseases involving neurodegeneration in limited areas of the brain, such as the loss of dopaminergic neurons in the substantia nigra in Parkinson's disease (Lindvall et al., 1988; Morizane et al., 2008). To date, only a limited number of studies in animal models of AD have explored the outcomes of stem cell transplantation. For example, improved cognition and enhanced synaptic density were observed following hippocampal transplantation of mouse neural stem cells (NSCs) in 3xTg-AD mice, and these positive effects were associated with increased BDNF secretion (Blurton-Jones et al., 2009). Moreover, improved spatial memory following NSC transplantation has been observed in rats with cholinergic lesions (Moghadam et al., 2009; Park et al., 2012a; Park et al., 2012b).

Viewing stem cell therapy for AD solely as cell replacement poses great challenges, since this requires extensive migration of the graft to degenerated areas in the brain, and subsequent differentiation and integration into functional networks. Recent work, however, suggests that stem cell transplantation could also generate trophic support for endogenous progenitor cells and neurons in the brain (Blurton-Jones et al., 2009; Einstein and Ben-Hur, 2008). Thus, stem cells could be regarded as delivery vehicles for providing the brain with a myriad of neurogenic factors. NSC transplantation into the brains of animal stroke models has been shown to stimulate endogenous neurogenesis (Jin et al., 2011), but this has not yet been studied in AD animal models.

2 AIMS OF THE THESIS

The main aim of this thesis was to investigate neuroprotective and regenerative processes in the brain, along with implications arising from this for the development of novel treatment strategies for AD. *In vitro* cellular model systems, postmortem human brain tissue studies, and *in vivo* studies in an AD transgenic mouse model were used to investigate neuroprotective and regenerative mechanisms in relation to the pathological processes associated with AD (figure 6).

The specific objectives were the following:

Paper I To investigate the neuroprotective role of α7 nAChRs against Aβ-mediated neurotoxicity and the roles of different forms of Aβ in the interactions with nAChRs.

Paper II To characterize the neuroprotective and neurotrophic actions of amyloid-modulatory candidate drugs (–)- and (+)-phenserine.

Paper III To investigate the effects of modulating brain Aβ levels at different stages of amyloid pathology on synaptic function, hippocampal neurogenesis, and inflammatory cell changes.

Paper IV To investigate the effects of stem cell transplantation and modulation of Aβ and α7 nAChRs on endogenous neurogenesis, graft survival, and cognition.

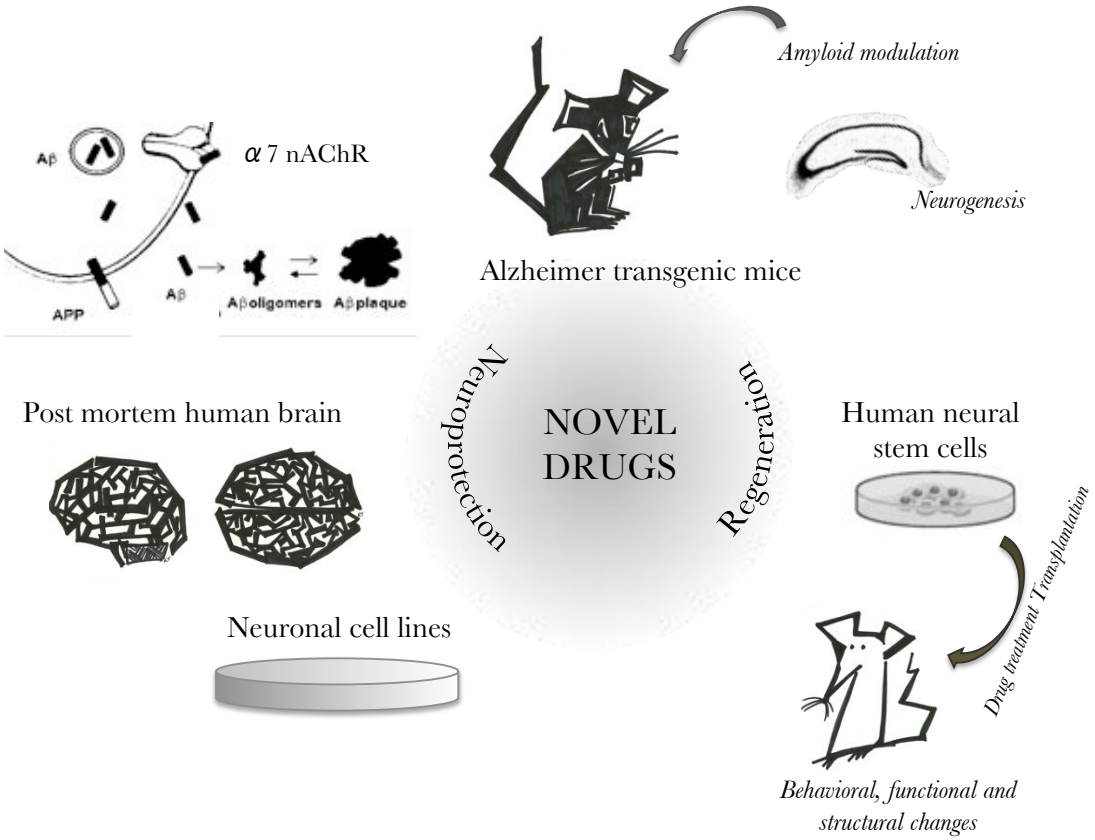

Figure 6. Schematic outline of the work undertaken in the thesis.

3 METHODOLOGY

In the following section, model systems and methods used in ***papers I–IV*** and in previously unpublished ***pilot studies*** are discussed in relation to their advantages and limitations. Some methodology is summarized here, but detailed descriptions of experimental procedures are provided in the respective papers.

3.1 ETHICAL CONSIDERATIONS

Human autopsied brain tissue was obtained from the Netherlands brain bank; permission to use this tissue in experimental procedures was granted by the Ethics Committee Review Board at Karolinska Institutet and the Swedish Ministry of Health (S024/01). All materials and data collected by the Netherlands brain bank were obtained on the basis of written informed consent. All animal experiments were carried out in accordance with the guidelines published by the Swedish National Board for Laboratory Animals. Ethical applications were approved for the drug treatment and isolation of progenitor cells and primary neurons from Tg2576 mice (S43/07, S53/10), and for human (h)NSC transplantation and MWM tests using Tg2576 mice (S54/10 and S172/11).

3.2 COMMENTS ON MODEL SYSTEMS USED

3.2.1 Cell cultures

The advantage of using immortal cell lines is that they are easy to expand in sufficient quantities for viability assays and receptor-binding experiments. Moreover, immortal cell lines have been well characterized and are widely used in *in vitro* model systems for molecular and mechanistic studies. The neuronal cell lines used in ***paper I*** were selected for their expression of α7 nAChRs. Pheochromocytoma PC12 cells undergo neuronal differentiation with neurite outgrowth after exposure to NGF. These cells were used to study α7 nAChR-mediated neuroprotection, while human neuroblastoma SH-SY5Y cells were used to assess intracellular calcium levels ($[Ca^{2+}]_i$) following α7 nAChR agonist and Aβ exposure. Wild-type and APPswe-transfected SH-SY5Y cells were also used in ***paper II*** to assess neuroprotective and neurotrophic drug actions.

The advantages of SH-SY5Y cells are that they are of human origin and they are adherent in comparison with the semi-adherent PC12 cells, which makes them more suitable on a practical level for calcium measurements in single cells using confocal microscopy. However, these tumor cell lines are limited as model systems for AD since they do not reflect the nature of neurons in the brain and are relatively insensitive to the physiological concentrations of Aβ found in AD. Therefore, primary progenitor cells and neurons were used in the relevant *in vitro* studies in ***paper II*** and in the pilot studies for ***paper IV***.

In ***paper II***, primary progenitor cells were isolated from the SVZ of Tg2576 mouse embryos, cultured in neurospheres, and used in *in vitro* studies of cell survival and growth. The translational relevance of these cells was assessed by measuring the early neuronal marker doublecortin (DCX) in the SVZ of adult Tg2576 mice.

An *in vitro* ***pilot study*** before the studies reported in ***paper IV*** investigated the effects of several classes of drugs with different mechanisms of action including the amyloid-modulatory drug (+)-phenserine and the α7 nicotinic agonist JN403, on the proliferation and neuronal and glial differentiation of hNSCs derived from fetal brains. Cortical primary Tg2576 neurons in culture were also investigated in this pilot study to assess the effects of the drugs on neuronal maturation. hNSCs transplanted into the hippocampi of Tg2576 mice either alone or in combination with drug treatment were subsequently assessed *in vivo* in ***paper IV***.

3.2.2 Postmortem human brain tissue

Although human autopsied brain tissue reflects the end stage of the disease, it is an irreplaceable *ex vivo* tool for studying disease processes in humans. The postmortem delay should be kept as short as possible, and limitations such as the sometimes substantial differences between individual subjects should be carefully considered when designing experiments. Another issue that must be taken into consideration is that some subjects will have been treated with drugs that could enhance neurogenesis in the brain, as reviewed in *section 1.3.3*. Autopsied human brain tissue extracts from 5 AD subjects (mean age 70 y; mean postmortem delay 7 h) and 5 control subjects (mean age 67 y; mean postmortem delay 9 h) were used in ***paper I*** for receptor binding assays and in ***pilot studies*** carried out prior to ***paper IV*** to assess the viability and differentiation of hNSCs in culture. Extracts containing as low as picomolar concentrations of Aβ were sufficient to significantly decrease the viability of primary cells, which is much more

relevant for mirroring an AD-like environment *in vitro* than the recombinant Aβ peptides used in ***paper I***.

3.2.3 Tg2576 mice

Despite the limitations associated with using transgenic mice in translational AD research studies, these mice are valuable *in vivo* models for studying the evolution from birth of molecular pathological changes since it is not possible to study these changes in living patients. Mice expressing the APP Swedish mutation (APPSWE2576Kha; Tg2576) show high levels of soluble oligomeric Aβ in the brain, up to the age of approximately 10 months, before the Aβ plaques begin depositing. Tg2576 mice also show reduced levels of the synaptic marker synaptophysin and memory impairment (Lithner et al., 2011; Mustafiz et al., 2011; Stewart et al., 2011; Unger et al., 2006). In ***papers II–IV***, Tg2576 mice were used to model the relationship between Aβ pathogenesis and regenerative processes in the brain, and the ways in which these processes can be modulated through pharmacological treatment or by stem cell transplantation. The mice were bred in the Karolinska Institutet animal care facility by backcrossing with B6SJL (F1) females (Taconic). Wild-type littermates served as control animals. All mice were housed in enriched cages with a 12-hr light-dark cycle and *ad libitum* access to food and water.

3.3 EXPERIMENTAL PROCEDURES

3.3.1 Receptor-binding assays

Postmortem brain tissue from AD and control subjects was used to study the interaction between Aβ and nAChRs, using the radioligands [^{3}H]PIB (which binds selectively to fibrillar Aβ (Ni et al., 2013)), [^{125}I]Aβ1-40, and [^{3}H]epibatidine (an nAChR ligand) (***paper I***). For the [^{3}H]PIB binding assays, frontal corticex tissue was homogenized in PBS to yield a crude membrane fraction that included the extracellular matrix. For the [^{3}H]epibatidine and [^{125}I]Aβ1-40 binding assays, membrane (P2) fractions from AD and control autopsied frontal cortices, with the extracellular matrix removed from the fraction, were used as previously described (Marutle et al., 1998). The membrane fractions were incubated with the radioligand, the reactions were terminated by filtration, and the radioactivity was counted.

3.3.2 Aβ preparation and characterization

Recombinant Aβ was used in ***paper I***, wheras Aβ assemblies in TBS extract from human autopsied brain tissue were used for the *in vitro* ***pilot study*** prior to ***paper IV***. To obtain recombinant fibrillar Aβ aggregates, Aβ1-40 and Aβ1-42 (Sigma, St Louis, MO, USA) were dissolved in H_2O and DMSO, respectively, and incubated at 37°C with agitation for 48–72 h before use. Recombinant soluble Aβ oligomers were obtained by dissolving 1,1,1,3,3,3-hexafluoro-2-propanol (HFIP)-pre-treated Aβ1-40 and Aβ1-42 peptides (rPeptide, Bogart, GA, USA) in DMSO, and then sonicating and filtering them to yield a pure, homogeneous oligomeric fraction. Recombinant Aβ was characterized using western blotting to verify that oligomeric Aβ remained non-fibrillized throughout the experiments. The aggregation and fibrillization processes of HFIP-pretreated oligomeric Aβ in the different buffers were assessed using thioflavin T (ThT) fluorescence assays. ThT assays are widely used to profile protein fibrillization over time, by measuring the intensity of fluorescence emitted from ThT when it binds to fibrillizing protein. For the ***pilot study***, a water-soluble TBS fraction of Aβ oligomers was extracted from autopsied brain tissue from a patient with AD and a healthy control as described previously (Bao et al., 2012). Aβ assemblies in these fractions were characterized according to size using western blotting and ADDL-specific antibodies.

3.3.3 Viability assays

In MTT and MTS proliferation assays, a tetrazolium compound (MTT or MTS) is reduced to formazan by nicotinamide adenine dinucleotide phosphate (NADPH) or nicotinamide adenine dinucleotide (NADH) dehydrogenase enzymes in metabolically active cells. The amount of formazan product, measured by absorbance, is directly proportional to the number of living cells (***papers I*** and ***II).*** In ***paper II***, MTS reduction was also used as a measure of cell proliferation. In ***paper I***, MTT assays were complemented by measuring the release of lactate dehydrogenase (LDH) from cells with cell membrane leakage (CytoTox ONE Homogeneous Membrane Integrity Assay, Promega, Stockholm, Sweden). LDH release is a valuable complementary test for viability which measures the amount of non-viable cells in the samples. Formation of the fluorescent product resorufin is then proportional to the amount of LDH

released.

3.3.4 Intracellular calcium measurements

In ***paper I***, we monitored α7 nAChR activation and the subsequent influx of Ca^{2+} ions by measuring changes in $[Ca^{2+}]_i$ in SH-SY5Y cells, using the calcium indicator Fluo-3. Although Fluo-3 is a widely used dye, it is non-ratiomeric and therefore its use is limited compared to ratiomeric dyes that have dual emissions to enable the use of an internal reference. SH-SY5Y cells were loaded with Fluo-3 AM (Invitrogen), an ester conjugate to facilitate penetration of the cell membrane, and excited using an inverted Meta-Zeiss 510 LSM confocal microscope (Carl Zeiss, GmbH, Germany). Recordings were taken during 60 min of incubation at room temperature and the fluorescence intensity was measured continuously throughout the incubation period.

3.3.5 Detection and quantification of protein expression

Antibody detection-based methods of protein detection or quantification were used in all the papers. Western blotting was used to separate Aβ assemblies in Aβ preparations and autopsied human brain tissue extracts according to size (***papers I*** and in the ***pilot study*** prior to ***paper IV***) and to quantify synaptophysin protein levels (***paper III***). While western blotting is an excellent tool for protein detection and separation of proteins by size, it is considered a semi-quantitative method. Enzyme-linked immunosorbent assays (ELISA), a conventional quantitative method, were used in ***papers II–IV*** to measure protein levels in mouse brain tissue extracts. A similar quantitative method, using Meso Scale Discovery (MSD) technology, was used in ***paper III*** to measure cytokine and chemokine levels in mouse brain cortical tissue extract, and in the ***pilot study*** related to ***paper III*** for quantification of mouse Aβ CSF levels. MSD technology is based on the capture and detection of antibodies to detect epitope-specific antigens. In contrast to regular ELISA, which uses enzyme-linked detection antibodies to yield fluorescent or color signals upon addition of substrate, MSD technology uses ruthenium-conjugated detection antibodies that emit light upon electrochemical stimulation of the electrode surface in the microplate. Multiple excitation cycles enhance the chemoluminiscence signal and further improve the sensitivity of this technique.

For the ***pilot study*** for ***paper IV***, immunocytochemistry was used to detect, quantify and morphologically characterize glial and neuronal phenotypes of

differentiated hNSCs in culture. Immunohistochemistry was also used in ***paper IV*** to study the regional distribution of astrocytes in coronal brain sections, which allowed quantification of the number of α7 nAChR-expressing astrocytes specifically in the DG of the hippocampus, and characterization of their morphological phenotypes in different regions of the DG.

3.3.6 Drug treatment

The full α7 nAChR agonist and α4β2 partial agonist varenicline, and the partial α7 nAChR agonist JN403 (Coe et al., 2005; Feuerbach et al., 2007; Mihalak et al., 2006) were used as tools to study the interactions of Aβ with α7 nAChRs in neuronal cell lines and in postmortem brain tissue (***paper I***). As JN403 is a more selective α7 nAChR agonist than varenicline, this drug was also given by intraperitoneal (i.p.) injection (0.3 mg/kg) in ***paper IV*** to treat Tg2576 mice in combination with hNSC transplantation.

In ***paper II***, the AChEI (–)-phenserine, its cholinergically inert enantiomer (+)-phenserine, and the primary metabolites of (+)-phenserine (+)-N1-norphenserine, (+)-N8-norphenserine and (+)-N1,N8-bisnorphenserine were characterized *in vitro* with regard to their neuroprotective and neurotrophic properties. To avoid interference from cholinergic actions, (+)-phenserine was selected to study the effects on neurotrophic actions and on Aβ pathology in Tg2576 mice (i.p. injections of 25mg/kg (+)-phenserine once daily) (***papers II–IV***).

3.3.7 Transplantation and CSF collection

In **paper IV**, Tg2576 mice received bilateral hippocampal injections transplanting 25,000 hNSCs per hemisphere or vehicle (coordinates relative to bregma: AP -2.06, ML ±1.75, DV -1.75). The mice were anesthetized using a constant flow of 4% isoflurane throughout the procedure. The heads of the mice were fixed using ear and tooth bars before a skin incision into the skull bone was made to facilitate the location of the coordinates to target the DG of the hippocampus. Lidocaine was used for local anesthesia during the procedure and the animals were monitored daily for body weight and healing of the incision site after the surgery. While the use of immunosuppressants helps to avoid the risk of graft rejection after transplantation procedures, immunosuppressants can affect inflammatory processes and their use should be carefully considered in the study design. Stem cells could play an immunomodulatory role *per se* (Einstein and Ben-Hur, 2008), and previous studies have indicated that

transplantation of hNSCs into the brains of APP transgenic mice does not require the use of immunosuppressants (Marutle et al., 2007) for studies with a time-frame similar to that in ***paper IV***. Accordingly, no immunosuppressants were used, and no symptoms indicative of a reaction to the transplant were observed.

For the ***pilot study*** relavant to ***paper III***, CSF was collected from the cisterna magna of 4- to 6-month-old Tg2576 mice. The animals were anesthetized with a 1:1 mixture of ketamine (100 mg/kg) and xylazine (20 mg/kg), and a 26 gauge needle connected to a syringe by a P20 polyethylene tube was used to collect up to 7 µl CSF per mouse. The animals were euthanized by decapitation, and the CSF was frozen and stored at -80°C until used for Aβ measurements.

3.3.8 Behavioral tests

MWM tests were used to assess the hippocampal-dependent spatial memory of Tg2576 mice and their age-matched wild-type littermates, and of Tg2576 mice after hippocampal hNSC transplantation (***paper IV***). The mice were placed in water at random sites (four fixed positions) around the wall of a round swimming pool of 1 m diameter. During the acquisition phase, the mice learned the location of a platform hidden under the water, aided by visual cues on the walls around the pool. In order to assess retention of this spatial memory, a probe trial was performed 24 hours after the last acquisition trial; in this, the platform was removed and the mice were allowed to swim for 60 seconds. The behavior of the mice in the MWM task was recorded by an automated video-tracking system (Ethovision). To evaluate differences in learning and memory between groups, Δ-latency values were calculated (follow-up probe values minus baseline acquisition values). The advantage of using the MWM to assess learning and memory in these mice is that it is a well-recognized behavioral test that has been widely used to characterize cognitive deficits in Tg2576 mice. Since impaired motor behavior or poor vision could greatly influence the performance of the mice in the MWM, the mice should be carefully monitored prior to testing. The pool size should also be selected carefully; since the smaller the pool, the easier it is for the mice to find the hidden platform.

3.3.9 Statistics

GraphPad Prism 5.0 or 6 (GraphPad Software, Inc.) was used for all statistical analyses. In all papers, parametric tests were used to compare statistical differences between treatment groups in the *in vitro* studies using cell cultures or tissue homogenates, whereas non-parametric tests were used for analysis of data from Tg2576 mice.

One-way ANOVA followed by Bonnferroni's or Dunnet's *post-hoc* tests was used to compare statistical differences between treatment groups in ***papers I*** and ***II***, the non-parametric Mann-Whitney test or Student's t-test was used for comparison between two groups, and the non-parametric Kruskal-Wallis one-way ANOVA by ranks followed by Dunns or Dunnet's *post-hoc* test was used for comparison between multiple (>2) groups in ***papers III*** and ***IV***). Spearman's rank correlation was used as a non-parametric test for correlation analysis, which was visualized graphically using simple regression analysis (***papers III*** and ***IV***). In all papers, the data are presented as means ± standard error of the mean (SEM). *P*-values <0.05 were considered to be significant.

4 RESULTS AND DISCUSSION

This section summarizes and discusses the main findings of the thesis. A more detailed description of the results can be found in the respective papers.

4.1 INTERACTION OF FIBRILLAR AND OLIGOMERIC FORMS OF Aβ WITH α7 nAChRs – RELEVANCE FOR NEUROPROTECTION

Mounting evidence suggests that the continuous loss of nAChRs in the hippocampi and cerebral cortices of patients with AD could be mediated through an interaction between α7 nAChRs and Aβ species (Jonnala and Buccafusco, 2001; Kihara et al., 2001). An exciting field of research postulating the potential advantages of targeting α7 nAChRs to induce neuroprotective mechanisms against Aβ-induced toxicity has recently emerged. Comparative studies on the effects of Aβ1-40 versus Aβ1-42 are sparse but available studies indicate that Aβ1-40 interacts with α7 nAchRs in a reversible manner, whereas Aβ1-42 binds to the receptor irreversibly (Lee and Wang, 2003). The importance of the assembly form of Aβ on the interaction with α7 nAChRs is an important aspect that has not to our knowledge been investigated previously. The interactions between different aggregated forms of Aβ and α7 nAChRs were thus studied in ***paper I***, using neuronal cells in culture and postmortem human brain tissue from AD patients. This study indicated that recombinant fibrillar Aβ1-40 causes cytotoxic effects in PC12 cells, whereas fibrillar Aβ1-42 and oligomeric Aβ1-40 and 1-42, in the form of dimers, decamers, dodecamers and larger oligomers of approximately 100 kDa in size, did not significantly reduce cell viability at physiologically relevant concentrations (nanomolar range), as reflected in the brains of sporadic AD patients (Hashimoto et al., 2010).

It has been suggested that α7 nAChRs exert neuroprotective effects through downstream signalling pathways such as i) the MAPK/ERK signaling pathway and activation of the downstream transcription factor CREB, or ii) the PI3K/Akt pathway, both of which are important for neurotrophic actions and cell survival (Abbott et al., 2008; Bell et al., 2004; Dineley et al., 2002; Dougherty et al., 2003). For further details, the reader is referred to section *1.1.3 Amyloid-β interactions with nicotinic receptors.* ***Paper I*** showed that the partial α4β2 and full α7 nAChR agonist varenicline and the partial α7 nAChR agonist JN403 protected the cells against fibrillar Aβ1-40-

induced toxicity, further indicating that α7 nAChRs mediate neuroprotective effects. We then hypothesized that these effects could be mediated a) through the signalling mechanisms mentioned above, b) by preventing Aβ from binding to nAChRs, or c) by a combination of these.

Using a human postmortem frontal cortex tissue homogenate, we found that [^{125}I]Aβ1-40 bound to α7 nAChRs. To further investigate the interaction between fibrillar Aβ and α7 nAChRs, we studied the effects of varenicline and JN403 on [^{3}H]PIB binding to Aβ in AD frontal cortex autopsied brain tissue. ^{3}H-PIB binds selectively to, and correlates with levels of, fibrillar Aβ at autopsy (Kadir et al., 2011; Ni et al., 2013). [^{3}H]PIB binding increased after exposure to these two compounds, possibly reflecting the displacement of Aβ from α7 nAChRs by α7 nAChR agonists, thus making the "free" or non-complex-bound Aβ more accessible for binding to [^{3}H]PIB (figure 7). A recent study using drugs with affinity for different nAChR subtypes confirmed the specific binding to α7 nAChRs (Ni et al., 2012).

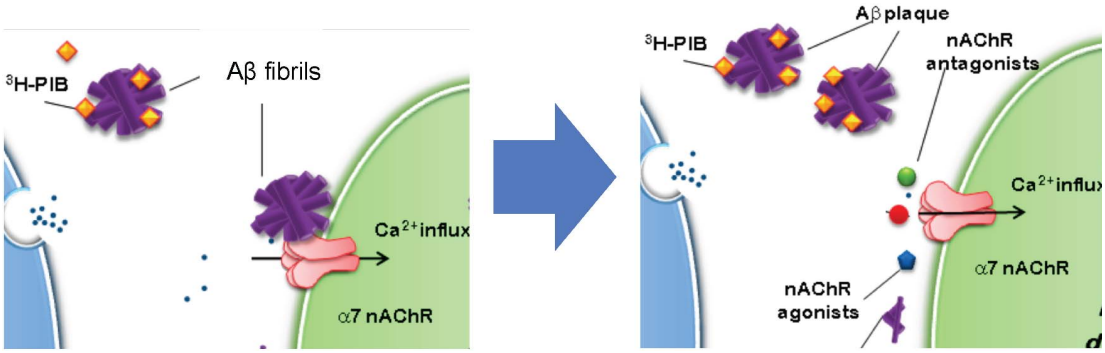

Figure 7. Illustration of a proposed interaction between fibrillar Aβ and α7 nAChRs in the presence of α7 nAChR ligands (modified illustration courtesy of Ruiqing Ni, Karolinska Institutet, Sweden).

The role of oligomeric Aβ in the interaction with α7 nAChRs was then tested by displacing the nAChR ligand [^{3}H]epibatidine with varenicline in the presence of oligomeric Aβ1-40 using a human postmortem frontal cortex tissue homogenate. Interestingly, the presence of 0.1 and 5 μM oligomeric Aβ1-40 resulted in a receptor occupancy of approximately 50 %. In addition, a shift in the affinity of varenicline to nAChRs from the pM to μM range was observed in the presence of 5μM Aβ1-40 (figure 8). This suggests that oligomeric Aβ modulates nAChRs allosterically and possibly changes the conformation of the receptor, which consequently alters the binding affinity of nAChR ligands. This finding, together with the observation of

increased [^{3}H]PIB binding, could shed light on current research aiming to develop α7 nAChR-positive allosteric modulators for AD treatment. At least one of these novel compounds, S24795, has been shown to prevent or reverse the binding of Aβ to α7 nAChRs (Wang et al., 2010; Wang et al., 2009), indicating that this type of drug could potentially preserve or potentiate the receptors' neuroprotective properties. It is suggested that varenicline and JN403 could display similar features.

To date, one study has modeled the interaction of Aβ with α7 nAChRs at the molecular level. Multiple binding sites were identified, and at least one Aβ-epitope was accessible for α7 nAChR binding in various Aβ species ranging from monomers to protofibrils. This epitope, named K28, binds to the same site as ACh, whereas other Aβ binding sites seem to be located on the periphery of α7 nAChRs and do not interfere with ACh binding (Maatuk and Samson, 2013). Hence, there is reason to expect that Aβ could modulate α7 nAChRs either allosterically or at the active site.

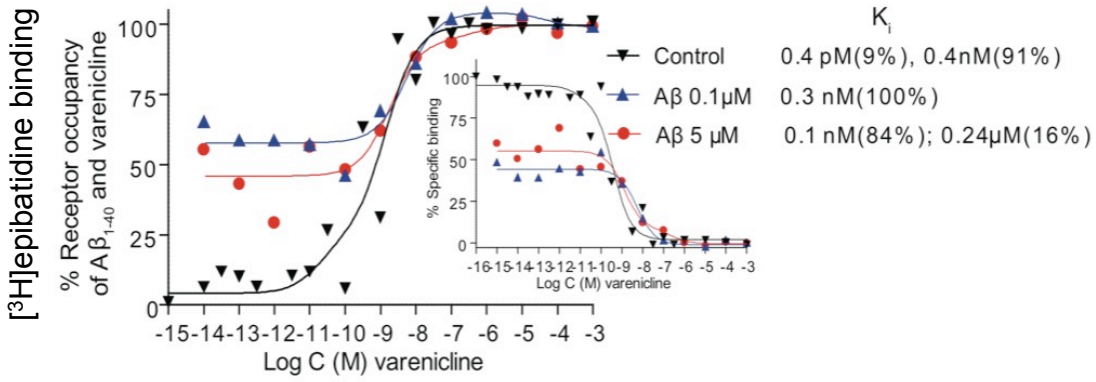

Figure 8. Oligomeric Aβ1-40 decreases the affinity of varenicline for nAChRs in the human frontal cortex. Reprinted from Lilja et al. (Lilja et al., 2011), with permission from IOS Press.

To further assess the functional effects of oligomeric Aβ on nAChRs, $[Ca2+]_i$ was measured in SH-SY5Y cells after exposure to Aβ1-40 and varenicline. Oligomeric but not fibrillar Aβ increased $[Ca2+]_i$, with a maximum response at 10 nM. This effect was attenuated by varenicline, suggesting that oligomeric Aβ1-40 activates α7 nAChRs to modulate Ca2+-dependent synaptic function. This is in line with previous findings showing that Aβ1-42 elevates Ca^{2+} levels through neuronal α7 nAChRs (Dougherty et al., 2003), and also indicates that varenicline prevents Aβ from binding to α7 nAChRs.

Taken together, these findings suggest that the α7 nAChRs are an important cellular target for Aβ, and that the aggregated form of Aβ is relevant to its effect on α7 nAChRs. It appears that some of the multiple Aβ binding sites characterized by Samson and Maatuk operate independently of the Aβ aggregation state, whereas other binding sites are specific for particular assembly forms and thus contribute to their unique properties. In the model systems studied in this thesis, fibrillar Aβ interacted with α7 nAChRs to induce neurotoxic effects, whereas oligomeric Aβ seemed to modulate synaptic function through the alteration of $[Ca^{2+}]_i$. Hence, it appears that targeting α7 nAChRs in order to stimulate neuroprotective actions against Aβ-induced toxicity will depend on the stage of amyloid pathogenesis at which the intervention is introduced.

4.2 STIMULATION OF REGENERATIVE PROCESSES AND THE IMPORTANCE OF Aβ MODULATION

Several studies, both *in vitro* (Haughey et al., 2002; Kwak et al., 2011; Wicklund et al., 2010) and *in vivo* (Zheng et al., 2013), have suggested that the pathophysiological environment in AD has adverse effects on stem cells and neurogenesis. In order to investigate the translation of *in vitro* results from cellular models of pharmacological modulation of Aβ on endogenous neurogenesis and synaptic function, *in vivo* studies using Tg2576 mice of different ages were carried out. The two experimental AD drugs (–)- and (+)-phenserine, both of which are APP synthesis inhibitors and thus lower Aβ levels (Greig et al., 2005; Lahiri et al., 2007; Mikkilineni et al., 2012; Shaw et al., 2001), were investigated.

4.2.1 (+)-Phenserine stimulates neuroprotective and neurotrophic processes via MAPK signaling and enhanced BDNF levels

The aims of ***Paper II*** were to characterize the neuroprotective and neurotrophic effects of (–)- and (+)-phenserine, and the primary metabolites of (+)-phenserine: (+)-N1-norphenserine, (+)-N8-norphenserine and (+)-N1,N8-bisnorphenserine, and also to investigate the primary signaling pathways responsible for mediating these effects. All the compounds lower APP levels through translational inhibition of the IL-1 response

element in the 5' untranslated region of the APP mRNA (Mikkilineni et al., 2012; Shaw et al., 2001; Yu et al., 2013) (figure 9).

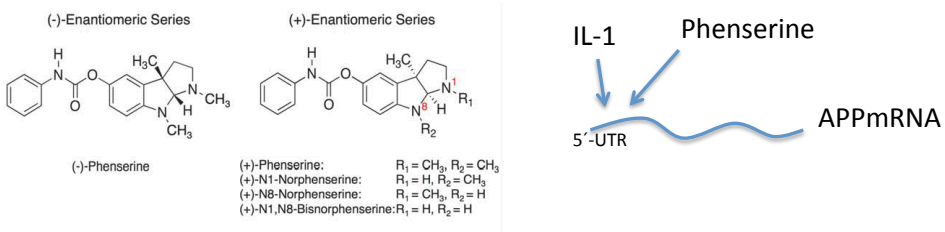

Figure 9. Chemical structures of phenserine and its primary metabolites, and schematic illustration of APP inhibition.

(+)-Phenserine, (−)-phenserine and (+)-N1-norphenserine increased the proliferation of SH-SY5Y cells. (+)-Phenserine had sustained effects on cell proliferation in the presence of sub-lethal levels of Aβ and H_2O_2, and displayed neuroprotective effects against H_2O_2- and glutamate-induced toxicity. (+)-Phenserine also enhanced cell proliferation and demonstrated pro-survival effects in primary Tg2576 progenitor cells in culture. Both the proliferative and neuroprotective actions were mediated, at least in part, through the protein kinase C (PKC) and MEK signaling pathways. MEK1 and MEK2 signaling are closely involved in the regulation of cell proliferation and cycle arrest, and MEK2 is especially known to promote cell survival. PKC acts upstream from MEK and is thus also closely involved in the regulation of these cellular processes (Skarpen et al., 2008; Ussar and Voss, 2004).

Merging evidence suggests that BDNF plays an important role in promoting neuroprotection in rodents and primates (Nagahara et al., 2009). It activates the MAPK/ERK signaling pathway, which in turn activates the downstream transcription factor CREB. CREB then promotes the expression of BDNF through a positive feed-back loop (Autry and Monteggia, 2012; Lu et al., 2008). Interestingly, we measured increased BDNF levels in the cerebral cortices of wild-type mice after (+)-phenserine treatment. Our findings thus indicate that (+)-phenserine exerts actions involving MAPK signaling pathways, including enhancement of BDNF levels.

4.2.2 Modulation of Aβ levels in the cerebral cortices and CSF of Tg2576 mice

The effects of (+)-phenserine on Aβ levels at different stages of amyloid pathology and the subsequent effects on synaptic function, hippocampal neurogenesis and inflammatory cell changes were investigated in ***paper III*** and its related ***pilot study***.

In ***paper III***, reductions in Aβ1-42 levels were observed in both 4- to 6-month-old and 15- to 18-month-old Tg2576 mice that received (+)-phenserine for 16 consecutive days. The effects of (+)-phenserine on Aβ levels in the CSF of 4- to 6-month-old APPswe transgenic mice were investigated in the ***pilot study***. The levels of Aβ1-42 were reduced (by 26 %) as was the Aβ42/40 ratio (by 21 %) in the (+)-phenserine-treated mice, but the difference did not reach statistical significance (figure 10). Clinical data have shown similar but more pronounced reductions in CSF Aβ1-42 levels after 10 days of (+)-phenserine administration to MCI patients (Maccecchini et al., 2012).

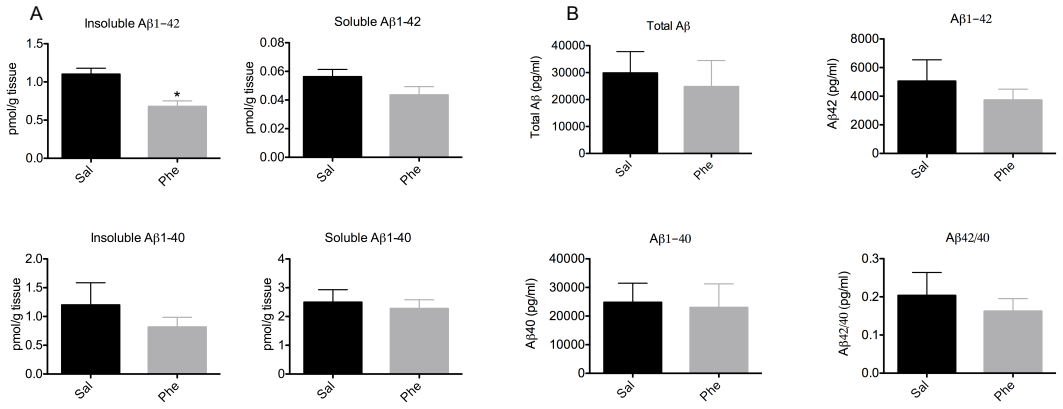

Figure 10. Aβ levels in (A) cerebral cortex and (B) CSF of 4- to 6-month-old Tg2576 transgenic mice treated with (+)-phenserine (Phe) or saline (Sal). Data are shown as mean values ±SEM.

4.2.3 Modulation of chemokine and cytokine levels in Tg2576 mouse brains

Because Aβ is known to stimulate the activation of microglia and astrocytes and the release of pro-inflammatory cytokines (Combs et al., 2001; Lindberg et al., 2005; Meda et al., 1995), we examined the effects of (+)-phenserine on the pro-inflammatory cytokines IL-1β and TNFα, and the chemokine MCP-1. Levels of IL-1β were elevated in Tg2576 mice compared to wild-type mice in both age groups; (+)-phenserine attenuated this increase in the older Tg2576 mice (15–18 months old). MCP-1 induces astrocyte chemotaxis and contributes to the recruitment of astrocytes around Aβ plaques (Wyss-Coray et al., 2003). Interestingly, we found age-dependent increases in cortical MCP-1, and an association between MCP-1 levels and lowered Aβ1-42 levels in the older Tg2576 mice. TNFα has been implicated in both the

pathogenesis of AD (Combs et al., 2001; Tarkowski et al., 2003a; Tarkowski et al., 2003b) and the mediation of neuroprotective effects through increased production of neurotrophic factors (Hattori et al., 1993; Sriram and O'Callaghan, 2007). In line with the latter, a trend towards increased TNFα levels was observed in (+)-phenserine-treated Tg2576 mice in both age groups.

4.2.4 Enhanced cell proliferation and DCX expression in the neurogenic zones of the brain

(+)-Phenserine treatment of the younger Tg2576 mice resulted in increased numbers of BrdU+ proliferating cells in the CA1 region of the hippocampus, a region especially vulnerable to Aβ (Burger, 2010), and also a trend towards increased numbers in the DG. A similar increase in BrdU incorporation was shown in the older Tg2576 mice after (+)-phenserine treatment. The increased cell proliferation in both age groups was associated with attenuated Aβ1-42 levels in the brains of these mice. Thus, reducing the Aβ load in the brains of older Tg2576 mice (15–18 months old) when Aβ plaque pathology is prominent could enhance cell proliferation in the hippocampus.

A significant reduction in hippocampal neurogenesis was observed in the older mice compared to the younger treatment groups, indicating that NPCs are fewer or more vulnerable in older animals. Previous work has indicated that the age-dependent decline in hippocampal neurogenesis occurs because of decreased neuronal maturation, decreased levels of neurotrophic factors, or aberrant vasculature in the vicinity of the neurogenic zone (Bernal and Peterson, 2004; Lugert et al., 2010; Shetty et al., 2005). Treatment of the younger (4- to 6-month-old) Tg2576 mice with (+)-phenserine stimulated the maturation and plasticity of newborn neurons in the hippocampal DG (***paper III***; figure 11), and increased the expression of the early neuronal marker DCX in the subventricular zone (***paper II***; figure 12). Regardless of the location of the neurogenic zone, NPCs follow similar general patterns including proliferation, migration, differentiation and integration into existing networks. Neuroblasts in the SVZ can be induced to migrate away from their usual route to the olfactory bulb towards a site of injury or neurodegeneration in the cerebral cortex or other brain areas, as reviewed by Christie and Turnley (Christie and Turnley, 2012). In the DG, however, there is little if any migration to other areas of the brain in response to injury or disease, although neurogenesis can be induced at the site of injury, with

improvement in memory functions (Christie and Turnley, 2012). Hence, pharmacological induction of neurogenesis in both the SVZ and the DG could have different, and probably positive, implications for the treatment of neurodegenerative diseases such as AD.

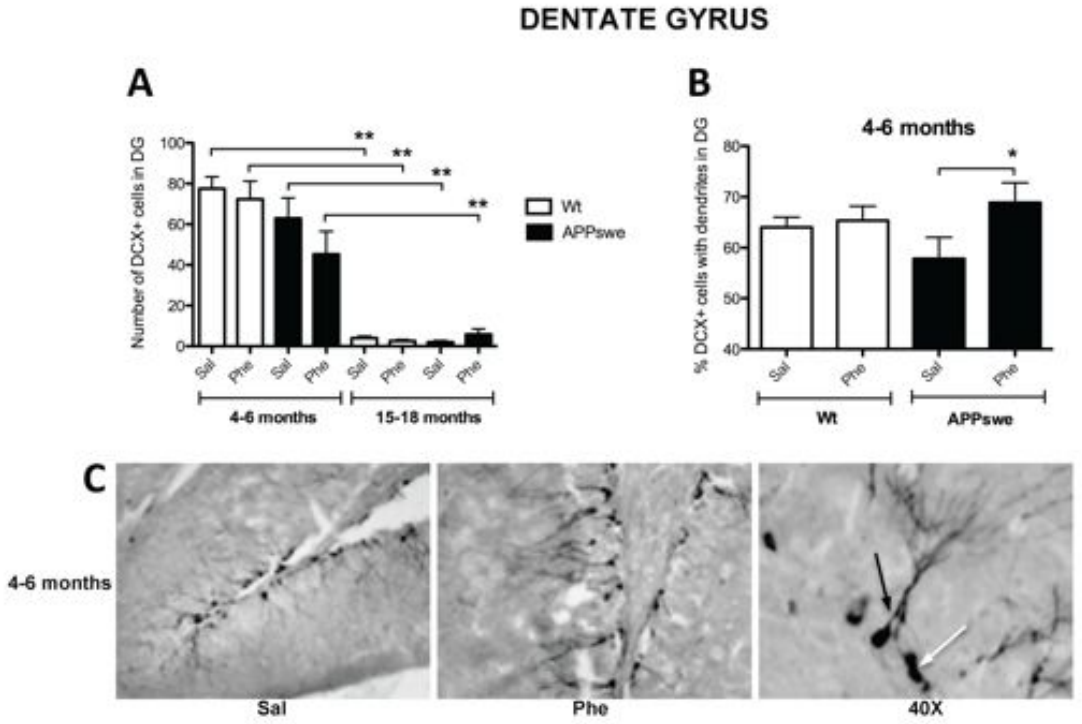

Figure 11. Increased dendritic arborization of newborn neurons in the DG of 4- to 6-month-old Tg2576 mice following (+)-phenserine treatment.

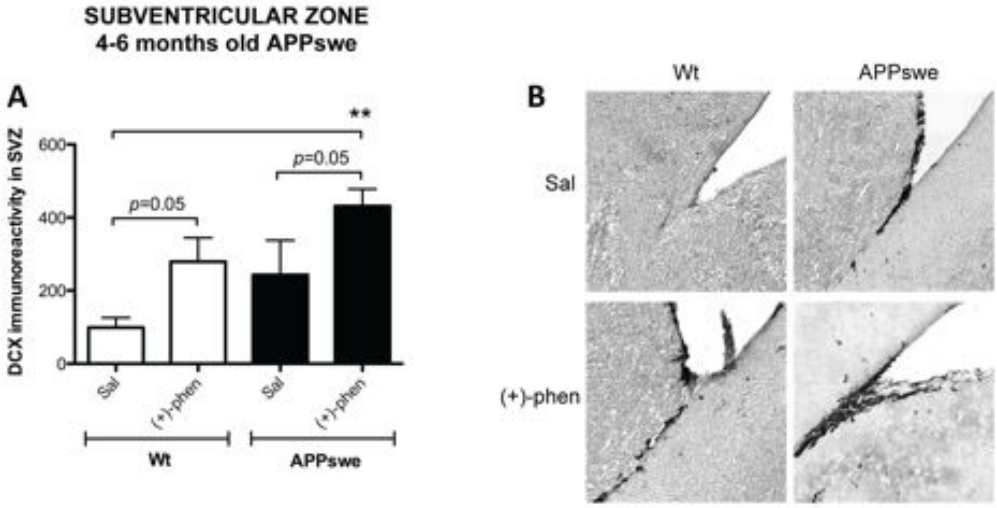

Figure 12. Increased DCX immunoreactivity in the SVZ of 4- to 6-month-old Tg2576 mice following (+)-phenserine treatment.

4.3 HUMAN NEURAL STEM CELL TRANSPLANTATION AND EFFECTS ON HIPPOCAMPAL NEUROGENESIS, SPATIAL MEMORY, AND α7 nAChR-EXPRESSING ASTROCYTES

The studies presented so far suggest that α7 nAChRs are important mediators of neuroprotective actions (***paper I***), that (+)-phenserine has neurotrophic actions in AD Tg2576 mice, and that early reduction of amyloid pathology could enhance endogenous neurogenesis (***papers II*** and ***III***). Although cell replacement therapies have in the past been regarded as highly challenging and, perhaps, not feasible for future treatment of patients with AD, recent experimental studies indicate that transplantation of stem cells into the brains of AD transgenic mice, in addition to increasing the levels of progenitor cells, could also supply trophic support to endogenous progenitor cells in neurogenic regions of the brain and to existing neurons (Blurton-Jones et al., 2009; Einstein and Ben-Hur, 2008). In ***paper IV***, we expanded our hypothesis to examine how the stimulation of regenerative mechanisms *in vivo* is related to cognitive status in younger Tg2576 mice (a model for early Aβ pathological changes in AD).

4.3.1 Pharmacological stimulation and neuronal induction in AD-like brain microenvironments

An *in vitro* ***pilot study*** was carried out before ***paper IV*** to select suitable drugs for pharmacological treatment. We investigated the neuroprotective and neurotrophic effects of various drugs: the α7 nAChR partial agonist JN403, the amyloid-modulatory drugs (–)-phenserine and (+)-phenserine, the antidepressant fluoxetine, the anti-inflammatory ibuprofen, and the AChE and α7 nAChR allosteric modulator galantamine. After 28 days of hNSC differentiation in culture, the number of cells positive to the neuronal marker βIII-tubulin was markedly reduced after exposure to a TBS extract from AD frontal cortex containing picomolar concentrations of Aβ, compared to untreated cells. Drug treatment with nanomolar concentrations of JN403 alleviated these effects to a large extent (figure 13A and B). When given in combination with similar concentrations of (+)-phenserine, JN403 also promoted neuronal maturation of Tg2576 primary corticex neurons (figure 13 C and D). The primary neurons were also stained for synaptophysin after JN403 exposure, but were not quantified due to the diffuse distribution of this marker (figure 14 A-C). JN403 and (+)-phenserine showed the most potent neurotrophic effects among the drugs studied,

and were selected for *in vivo* studies of hNSC transplantation of Tg2576 mice (***paper IV***), as described in the following section.

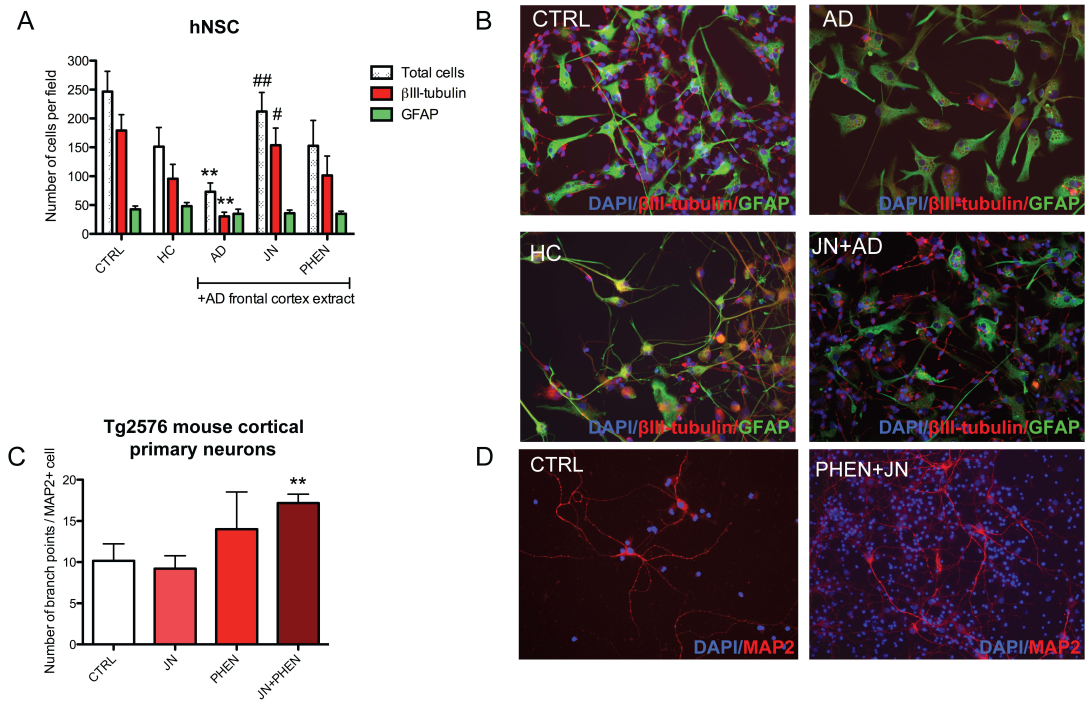

Figure 13. (A) Expression of neuronal marker βIII-tubulin (red) and glial marker GFAP (green) in hNSCs *in vitro* after 28 days' differentiation. The hNSCs were untreated (CTRL), or weekly exposed to brain extracts from a healthy control (HC), a patient with AD (AD), AD plus JN403 (JN), or AD plus (+)-phenserine (PHEN). (B) Representative images of CTRL, HC, AD, and AD+JN as outlined in (A). (C–D) Expression of the neuronal marker MAP2 (red) and the number of branch points in untreated Tg2576 transgenic mouse primary cortex neurons in culture (CTRL) and after weekly exposure to JN403 (JN), (+)-phenserine (PHEN), or JN403 plus (+)-phenserine (JN+PHEN) for 21 days. **p<0.01 compared with CTRL, #p<0.05, ##p<0.01 compared to AD. The data are expressed as means ± SEM.

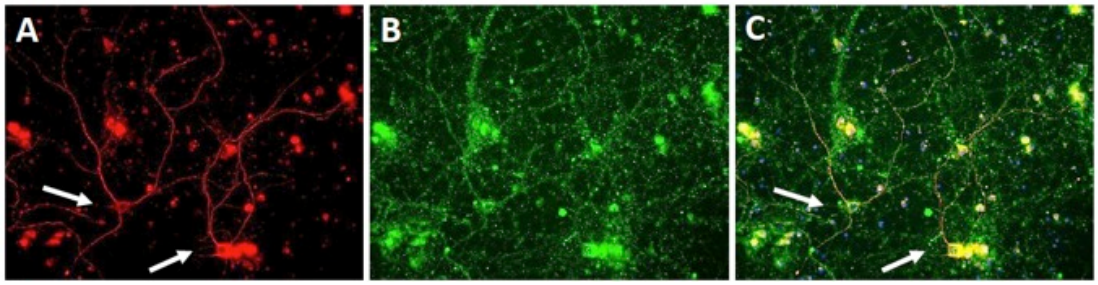

Figure 14. Representative images of mouse primary cortex neurons labeled for A) MAP2, B) synaptophysin, and C) overlay of MAP2 and synaptophysin. Arrows point to neuronal soma.

4.3.2 hNSC transplantation augments endogenous neurogenesis and improves cognitive function in Tg2576 mice

Intrahippocampal transplantation of hNSCs into 6- to 9-month-old Tg2576 mice was combined with 5 weeks' treatment with (+)-phenserine, JN403, or vehicle. The aims were to test spatial learning and memory prior to and after transplantation and drug treatment, and to investigate the effects of drug treatment on endogenous neurogenesis in the DG, hNSC transplant survival, and the number of α7 nAChR-expressing astrocytes in the neurogenic niche (for study design, see figure 15). Although Tg2576 mice of this age have elevated Aβ levels and increased astrocytosis, they also exhibit a degree of neuroplasticity, as shown in ***paper III***.

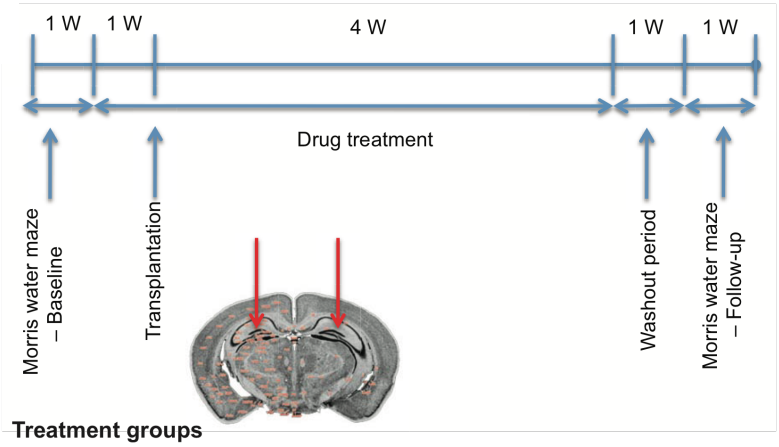

Figure 15. Experimental design as presented in ***paper IV***.

hNSC transplantation ameliorated the impaired hippocampal-dependent spatial memory in Tg2576 mice, at least in part by enhancing endogenous neurogenesis, as identified by increased numbers of DCX+ neurons in the DG. Thus, these findings support a direct connection between endogenous neurogenesis and cognitive function. They also suggest that transplantation of stem cells could offer valuable support for existing neurons and endogenous stem cell populations in the hippocampus. In line with these findings, a previous study has shown that hippocampal transplantation of murine NSCs improves cognition mediated by increased BDNF levels in aged 3xTg-

AD mice, despite heavy Aβ plaque and NFT pathology (Blurton-Jones et al., 2009). Thus, we hypothesize that impaired neurotrophic support in AD can result in impaired endogenous neurogenesis, which could be restored by hNSC grafts.

4.3.3 Distribution of α7 nAChR-expressing astrocytes in the hippocampal neurogenic niche

Although α7 nAChR-mediated neuroprotective effects have been confirmed in a number of experimental studies, including those in ***paper I***, little is known about whether the effects are the result of α7 nAChRs expressed on neurons or on astrocytes. Investigations into α7nAChRs on astrocytes are few, although these include reports on the presence of functional α7 nAChRs on astrocytes in rat hippocampus slices (Sharma and Vijayaraghavan, 2001; Shen and Yakel, 2012). The density of α7 nAChRs on neuronal cells was reduced and the number of α7 nAChR-expressing astrocytes surrounding Aβ plaques was increased in postmortem AD brains, indicating that this subset of astrocytes could play an important role in the inflammatory processes occurring in response to Aβ deposition (Yu et al., 2005). In the DG of hNSC-transplanted Tg2576 mice receiving JN403, the number of α7 nAChR- expressing astrocytes was significantly lowered versus those receiving saline. In addition to the effects on astrocytes, co-administration of hNSCs and JN403 inhibited the improved spatial memory and the induced endogenous neurogenesis observed after hNSC transplantation alone. In contrast to the *in vitro* findings where JN403 exerted neurotrophic actions, it seems that JN403 has antagonistic rather than agonistic effects in this *in vivo* model system.

Intriguingly, we found that α7 nAChR-expressing astrocytes accumulated along the needletrack, indicating that these cells are involved in modulating inflammation associated with tissue remodeling following injury. This led us to ask whether the numbers of α7 nChR-expressing astrocytes in the neurogenic niche could be linked with neurogenesis. In support of this theory, the increased number of α7 nAChR-expressing astrocytes was found to positively correlate with the number of DCX+ cells in the DG. Hence, we postulate for the first time that α7 nAChR-expressing astrocytes could be important in tissue remodeling and plasticity. These findings also raise the question of whether there are any functional differences between non-α7 nAChR-expressing astrocytes and α7 nAChR-expressing astrocytes in cell repair – and if so, what these differences are. Clearly, this requires further detailed

investigation.

4.3.4 (+)-Phenserine enhances graft survival but antagonizes hNSC-mediated effects on endogenous neurogenesis and cognition

Treatment with (+)-phenserine increased the survival of the grafted cells, in line with our previous findings that this drug exerts pro-survival effects on progenitor cells and hippocampus neurons (***paper II***). In contrast, (+)-phenserine prevented the increased neurogenesis and the improvements in hippocampus-dependent memory induced by hNSC transplantation. Based on the findings in ***paper II*****,** (+)-phenserine seems to exert neurotrophic effects through the MAPK and PKC signaling pathways, which are involved in a diverse repertoire of biological events including proliferation, differentiation, metabolism, motility, survival, and apoptosis. Consequently, this vast array of mechanisms could interfere with the effects of transplanted hNSCs on the brain microenvironment in order to support neurogenesis. Given that (+)-phenserine appears to enhance graft survival by acting directly on the implanted hNSCs, this effect would most likely not be antagonized.

Although hNSC transplantation holds great promise, impaired graft survival as a result of the increasing presence of pathological proteins in the AD brain should be considered. Hence, to sustain the efficacy of this intervention, we propose that combination treatment with drugs that target the different pathological processes without interfering with the stem cell-mediated neurogenic effects should be administered at specific disease stages. Current efforts to determine the time course of the pathological changes during the disease, using CSF and imaging biomarkers, are important in this respect as the results could indicate when different types of regenerative and neurotrophic therapies would be most beneficial for AD patients.

5 CONCLUDING REMARKS AND FUTURE OUTLOOK

The clinical implications of experimental studies investigating the stimulation of neuroprotection and brain repair are currently being explored. It is hoped that these studies will result in therapeutic strategies that will change or halt the clinical course of AD, in contrast to the currently available symptomatic treatment. In the translational approach presented in this thesis, the main aim was to investigate how neuroprotective and regenerative processes can be enhanced in a variety of experimental model systems relevant for AD, to define implications for the development of novel intervention strategies.

Do nAChRs have differential roles in neuroprotection and neurogenesis?

We found that α7 nAChRs play an important role in mediating neuroprotection against Aβ-induced neurotoxicity, and that the aggregated form of Aβ is important for the interaction with α7 nAChRs and the downstream effects in neuronal cells. This implies that the time of introduction of α7 nAChR-targeting interventions during the disease course determines the effects of the treatment. I postulate that α7 nAChR ligands that promote cell survival and neuroprotection through intracellular mechanisms, in combination with compounds that sterically occlude Aβ from binding to α7 nAChRs, would be promising treatment strategies for AD. These drugs could comprise positive allosteric modulators, which do not get desensitized as easily and are postulated to have good chances to be clinically successful.

In addition to the neuroprotective role of α7 nAChRs, we found that the numbers of α7 nAChR-expressing astrocytes were related to the degree of hippocampal endogenous neurogenesis of stem cell-transplanted Tg2576 mice. These findings prompted me to wonder what roles α7 nAChR-expressing astrocytes might play in the neurogenic niche and with respect to tissue remodelling and plasticity, and whether α7 nAChR-expressing astrocytes in the vicinity of neurogenic zones in the brain possess neurotrophic properties. These and other questions could be used in future studies on α7 nAChRs and elucidation of their role in regenerative processes.

Therapeutic window for enhancing endogenous neurogenesis

This thesis has also provided new insights into a number of different effects mediated by the amyloid modulatory drug (+)-phenserine, a current and promising candidate drug

for AD therapy. The drug exerted neuroprotective, pro-survival and cell proliferative effects of neuronal and progenitor cells in culture, which translated into neuronal maturation of the latter cells *in vivo* in AD transgenic mice. These effects on regeneration and plasticity in the brain were achievable only during the specific time period when neurogenesis in the brain was still measurable and inducible. Although some of the data indicate an association between the effects on Aβ pathogenesis with those on neurotrophic processes, this should be interpreted with caution, as a causal relationship remains to be proven. These findings gave rise to further questions that require investigation. For example, can regenerative therapies prevent the age-dependent decline in neurogenesis and preserve cognitive function, and at what stage during the disease course should Aβ-targeting therapies be introduced in order to halt or prevent amyloid pathogenesis? Given the age-dependent sigmoidal increase in Aβ levels (Karran et al., 2011) and previous indications that endogenous neurogenesis shows an exponential decline with age (Knoth et al., 2010; Lazic, 2011), interventions targeting Aβ or stimulating regenerative processes may be successful if introduced early enough during the disease course. Further investigation using (+)-phenserine to answer these questions would be on my wishlist. A recent research study posits that hippocampal neurogenesis in humans continues throughout life, with only minor decline associated with ageing (Spalding et al., 2013). These findings appear promising for the translational relevance of the results to date, and suggest that the therapeutic window for stimulating neurogenesis in humans is probably much broader than that in rodents.

Endogenous neurogenesis linked to cognition

We have shown for the first time that stem cell transplantation stimulates endogenous neurogenesis in an AD animal model. Enhanced neurogenesis was further associated with improved hippocampus-dependent memory in Tg2576 mice, indicating a link between endogenous neurogenesis and cognition. These findings indicate that hNSCs themselves possess an intricate innate signaling system, and secrete a plethora of neurogenic factors that could stimulate a number of processes during neurogenesis. Stem cell transplantation in AD patients could thus be superior in outcome to drugs acting at only one or even a few targets. Safety issues regarding the use of stem cell transplants, as well as the sustainability of the neurogenic effects observed after stem cell transplantation, however, needs to be studied in long-term trials.

In summary, this thesis indicates that the introduction of intervention strategies early in the disease course when i) amyloid burden is low, ii) neurogenesis is still substantial in the brain, and iii) memory deficits are mild, will enable detection of neuroprotective and neurotrophic effects.

Induced pluripotent stem cells (iPSCs), isolated from patient fibroblasts, are currently being developed to model diseases such as AD. It is hoped that this type of model system will contribute to the advancement of regenerative therapies to clinical stages. For continued studies on the aspects presented in this thesis, model systems such as iPSCs-derived neurons and glial cells would constitute unique tools for further investigating cellular processes as well as molecular targets for neuroprotective and neurotrophic drugs such as (+)-phenserine and α7 nAChR modulators. On a systems biology level, the development of biomarkers for *in vivo* non-invasive assessment of neurogenesis and pathological changes is essential for the evaluation of these types of interventions longitudinally, at both pre-clinical and potentially clinical levels.

As discussed throughout the thesis, a large number of factors and their interplay are important for the development of AD. Despite the occurrence of disease-specific pathological changes in the brain, some individuals do not develop AD. The factors behind this phenomenon may be many, including genetic, epigenetic and lifestyle factors, but I believe that the brain's ability to use intrinsic mechanisms to stimulate neuroprotection and brain repair to sustain cognitive functions, could be part of the answer.

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