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REGENERATING THE BRAIN:
STEM CELL DIFFERENTIATION AND CHOLINERGIC DYSFUNCTION IN ALZHEIMER DISEASE

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Cover illustration: Neurons (left panel) and astrocytes (right panel) derived from human embryonic stem cells.

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In loving memory of my grandmother Helene Utas.

“Well, the going rate for change is not cheap.
Big ideas are expensive.”

Bono

REGENERATING THE BRAIN: STEM CELL DIFFERENTIATION AND CHOLINERGIC DYSFUNCTION IN ALZHEIMER DISEASE

THESIS FOR DOCTORAL DEGREE (Ph.D.)

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ABSTRACT

Hippocampal neurogenesis in the adult brain is important for learning and memory processes, which are heavily affected in Alzheimer disease (AD). Thus, targeting processes that could stimulate neurogenesis is a logical step in the search for strategies offering neuronal renewal and brain repair. However, the pathological lesions in the AD brain start to accumulate decades before the onset of clinical symptoms and neuronal plasticity is likely to be compromised by the pathological burden. The aim of this thesis was to provide a deeper understanding of how pathological mechanisms in AD affect stem cell differentiation and cholinergic signaling mechanisms, with implications for future regenerative therapies.

The progressive loss of cholinergic neurons in AD may be a consequence of accumulation of β-amyloid (Aβ) in the brain. In ***paper I***, there were prevailing differences in Aβ assemblies between early onset AD and late onset AD and that reduced cholinergic activity correlated with distinct Aβ oligomers. In ***paper II***, the impact of nerve growth factor (NGF) and Aβ treatment on the development of cholinergic neurons from human embryonic stem (hES) cells was investigated. NGF treatment increased differentiation into functional cholinergic neurons, oligomeric Aβ treatment decreased the number of functional neurons, and fibrillar Aβ promoted glial differentiation. In ***paper III***, fibrillar Aβ treatment altered the secretion of cholinergic enzymes from hES cells, resulting in low levels of acetylcholine. These changes were linked with an altered secretion pattern for cytokines, reduced neuronal differentiation and increased gliogenesis. In ***paper IV***, the effects of hippocampal human neural stem cell transplantation alone, or in combination and modulation of Aβ levels with (+)-phenserine or the partial α7 nicotinic acetylcholine receptor (nAChR) agonist JN403 on neurogenesis, graft survival, astrocytosis and cognitive performance in young Tg2576 mice (representing the early stages of AD) was studied. Neural stem cell transplantation increased endogenous neurogenesis and reduced memory impairment in AD mice not receiving the drugs but not in those receiving the drugs. JN403 decreased the number of α7 nAChR-expressing astrocytes, which correlated with reduced neurogenesis. We thus hypothesize that α7 nAChR-expressing astrocytes are involved in neurogenic processes during the development of neuropathology.

It is hoped that the findings presented in this thesis will provide novel targets for further studies, with potential for stimulating neuronal regeneration in AD.

LIST OF SCIENTIFIC PAPERS

I. Fuxiang Bao*, **Linn Wicklund***, Pascale N. Lacor, William L. Klein, Agneta Nordberg and Amelia Marutle*. * Contributed equally*
Different β-amyloid oligomer assemblies in Alzheimer brains correlate with age of disease onset and impaired cholinergic activity.
Neurobiol Aging (2012) 33(4):825.e1-13

II. **Linn Wicklund**, Richardson N. Leão, Anne-Marie Strömberg, Malahat Mousavi, Outi Hovatta, Agneta Nordberg and Amelia Marutle.
β-Amyloid 1-42 Oligomers Impair Function of Human Embryonic Stem Cell-Derived Forebrain Cholinergic Neurons.
PLoS ONE (2010) 5(12): e15600

III. **Linn Malmsten**, Swetha Vijayaraghavan, Outi Hovatta, Amelia Marutle and Taher Darreh-Shori.
Fibrillar β-amyloid 1-42 alters cytokine secretion, cholinergic signaling and neuronal differentiation.
Accepted for publication in J Cell Mol Med.

IV. Anna M. Lilja, **Linn Malmsten**, Jennie Röjdner, Larysa Voytenlo, Alexei Verkhratsky, Sven Ove Ögren, Agneta Nordberg and Amelia Marutle.
Stem cell transplant-induced neurogenesis and cognition in Alzheimer Tg2576 mice.
Submitted manuscript.

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LIST OF ABBREVIATIONS

ACh	acetylcholine
AChE	acetylcholinesterase
AD	Alzheimer disease
AICD	APP intracellular domain
ANOVA	analysis of variance
APOE	apolipoprotein E
APP	amyloid precursor protein
Aβ	β-amyloid
BDNF	brain-derived neurotrophic factor
BFCN	basal forebrain cholinergic neurons
BLBP	brain-lipid binding protein
BrdU	bromodeoxyuridine
BuChE	buturylcholinesterase
ChAT	choline acetyltransferase
ChEI	cholinesterase inhibitor
CREB	cAMP response element-binding protein
CSF	cerebrospinal fluid
DCX	doublecortin
DED	deuterium-L-deprenyl
DG	dentate gyrus
DMSO	dimethylsulfoxide
ELISA	enzyme-linked immunosorbent assay
EOAD	early onset Alzheimer disease
FAD	familial Alzheimer disease
^{18}F-FDG	^{18}F-fluorodeoxyglucose
GABA	γ-aminobutyric acid
GFAP	glial fibrillary acidic protein
hES	human embryonic stem
HFIP	hexafluoroisopropanol
hNSC	human neural stem cells
i.p.	intraperitoneal

ICM	inner cell mass
IGF	insulin-like growth factor
IL	Interleukin
INFγ	interferon-γ
iNOS	inducible nitric oxide synthase
iPS	induced pluripotent stem
LOAD	Late-onset Alzheimer disease
LTP	long-term potentiation
mAChR	muscarinic acetylcholine receptor
MAP	microtubule-associated protein
MAPK	mitogen-activated protein kinase
MCI	mild cognitive impairment
MSD	Meso Scale Discovery
MWM	Morris water maze
nAChR	nicotinic acetylcholine receptor
NE	neuroepithelial
NFT	neurofibrillary tangle
NGF	nerve growth factor
NMDA	N-methyl-D-aspartate
PET	positron emission tomography
PIB	Pittsburg compound B
PSEN	presenilin
qPCR	quantitative polymerase chain reaction
RAGE	receptor for advanced glycation products
RG	radial glia
SEM	standard error of the mean
SVZ	subventricular zone
TGFβ	transforming growth factor β
ThT	Thioflavine T
TNFα	Tumor necrosis factor α

INTRODUCTION

In Greek mythology, the Titan Prometheus stole fire from the Olympic gods and gave it to mankind, resulting in the development of all art and science. For his crime, Prometheus was doomed to eternal torment, and each day an eagle was sent to feast on his liver, which then regenerated to be eaten again the next day (Figure 1). However, little did Prometheus or the Olympic gods know that the ever-lasting, regenerating liver was a result of the unlimited regrowth capacity of stem cells.

There has been great progress in stem cell biology and regenerative medicine over the last two decades, with resultant potential for a variety of therapeutic healthcare strategies to augment, repair, replace or regenerate organs and tissues.

Figure 1. Prometheus was doomed to eternal torment, and each day an eagle was sent to feast on his liver, which then regenerated to be eaten again the next day. The legend captures the body's remarkable ability to regenerate itself. Illustration: Oscar Utas Hornegård.

Until 25 years ago, the central dogma was that new neurons were not formed in the brain after adolescence, and that there was limited capacity for the structure of neurons to change or be rearranged in the existing circuitry of the brain in response to new experiences. However, in the years since, neurological studies have established that the brain retains its early capacity for plasticity, or the capacity to be reshaped, throughout the human lifetime. Nonetheless, there are many hurdles to be overcome before stem cell therapy can become a viable clinical option in the brain. It is imperative that we learn more about stem cell biology in order to understand how proliferation, migration and differentiation are regulated in the context of various pathological stimuli. Furthermore, it must be proved that stem cell therapy is safe and adds complementary benefit to existing therapies.

The studies in this thesis highlight the potential of stem cells derived from the embryo or fetal brain and their ability to regenerate new neurons and glial cells in a microenvironment mimicking Alzheimer disease (AD).

PLASTIC FANTASTIC – THE REGENERATIVE POTENTIAL OF STEM CELLS

Stem cells are defined by their unlimited capacity for self-renewal and differentiation into more than one cell type. Stem cells can be classified by their developmental potential: totipotency, pluripotency, and multipotency. Totipotent stem cells, derived from the zygote and the unspecialized cells of the 8-cell morula, possess the potential to produce both intra- and extra-embryonic tissue. The morula continues to divide to form the blastocyst, a hollow structure made up of an outer layer of trophoblast cells and an inner cell mass (ICM) within the cavity. The trophoblast develops into extra-embryonic tissue, such as the placenta. The ICM, a cluster of pluripotent stem cells, forms the three germ layers (endoderm, mesoderm and ectoderm) of the embryo and ultimately develops into the entire fetus (Figure 2). Multipotent stem cells, with the capacity to differentiate into several cell types in a single germ layer, emerge during development of the embryo.

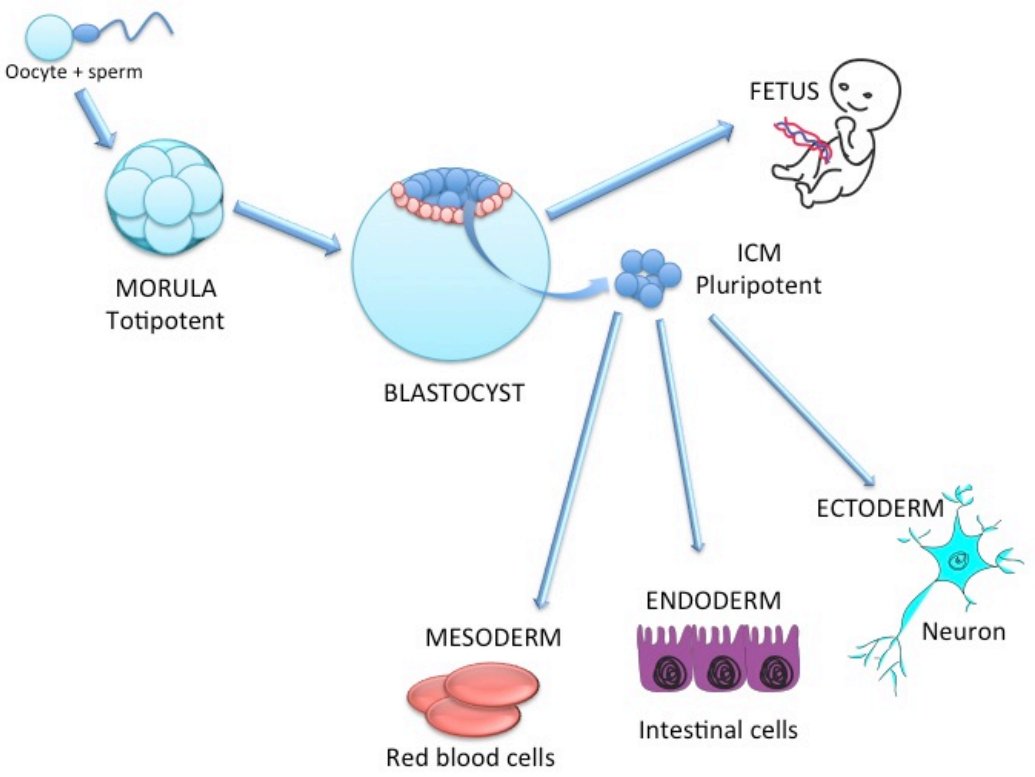


Figure 2. Pluripotent human embryonic stem cells originate from the inner cell mass (ICM) of the blastocyst. These cells can differentiate into any cell type in the body from the three different germ layers; mesoderm, endoderm and ectoderm.

Human embryonic stem cells

Human embryonic stem (hES) cells are derived from the ICM of the blastocyst and thus have the capacity to differentiate into any cell type in the body. Permanent hES cell lines were first derived in 1998 from surplus embryos from *in vitro* fertilization (IVF) or from embryos found to have genetic defects by pre-implantation genetic diagnosis (Thomson et al., 1998). However, the use of non-human materials bears the associated risk of transmitting pathogens, thus placing limitations on their use in pharmaceutical and clinical therapeutic applications. Improvements in the derivation process and the quality of hES cell lines that have been made in recent years include the development of xeno-free culture systems using human skin fibroblasts instead of mouse embryonic fibroblasts as feeder cells (Hovatta et al., 2003; Strom et al., 2010), and recombinant laminin matrixes (Rodin et al., 2014; Rodin et al., 2010). hES cell cultures can be expanded indefinitely *in vitro* and have provided an invaluable model system for studying

developmental biology, and may provide a source for generating cell types that can be used for cell replacement paradigms.

Neural stem cells in the fetal brain

The mammalian brain develops from the embryonic neuroectoderm, and gives rise to the cells of the entire nervous system. Neuroepithelial (NE) cells are primordial neural stem cells that are derived from the neuroectoderm that forms the neural tube. Their differentiation is determined by the concentration gradient of various morphogens, such as retinoic acid, bone morphogenetic protein, and sonic hedgehog protein (Bally-Cuif and Hammerschmidt, 2003; Briscoe and Ericson, 2001; Pierani et al., 1999). NE cells can be regarded as "true" neural stem cells that can differentiate into either neurons or glial cells with equal probability. NE cells can transform into neural crest cells and radial glial (RG) cells. RG cells divide asymmetrically, and subsequently give rise to neurons and intermediate progenitor cells of neuronal, astroglial and oligodendrocytic lineage, as reviewed by Cameron and Rakic (Cameron and Rakic, 1991; Rakic, 2003). It is thought that the expression of the intermediate filament protein nestin, which is expressed in both NE and RG cells, distinguishes progenitor cells from more differentiated cells (Lendahl et al., 1990).

Neural stem/progenitor cells can be derived from human or other mammalian fetal brain tissue. Neural stem cells are multipotent and can differentiate into astrocytes, oligodendrocytes and neurons, thus enabling *in vitro* modeling of nervous system development and diseases.

Neural progenitor cells in the adult brain

The mammalian brain continues to generate neurons, astrocytes, and oligodendrocytes after reaching adulthood. Neural progenitor cells are confined to specific brain regions, such as the subventricular zone of the lateral ventricle, and the granular layers of the hippocampus, cortex, cerebellum, spinal cord, striatum and olfactory bulb (Davis and Temple, 1994; Dore-Duffy et al., 2006; Goritz et al., 2011; Hartfuss et al., 2001; Johansson et al., 1999; Reynolds and Weiss, 1992; Sabelstrom et al., 2013). However, neural progenitor cells are difficult to identify

because of their heterogeneity, and several different progenitors for the same lineage can coexist within a tissue (Goritz and Frisen, 2012). RG-like cells persist postnatally in several brain regions (Malatesta et al., 2000). In fact, the description of stem cells as RG-like or astrocyte-like has generated confusion, and their exact relationship is hard to decipher. Nonetheless, mounting evidence suggests that glial cells are a substantial source of new neurons in the adult brain, as reviewed recently (Goritz and Frisen, 2012; Morrens et al., 2012).

Adult neural progenitor cells are usually isolated from rodent brains, followed by subsequent expansion of the cultures. They can also be isolated from postmortem human brain tissue or human brain biopsies (Leonard et al., 2009; van Strien et al., 2014), but this procedure is usually more difficult. These cells can then be used to study the behavior of human adult neural progenitor cells *in vitro*, in healthy individuals, or in patients with neurodegenerative diseases.

Induced pluripotent stem cells

The discovery of induced pluripotent stem (iPS) cells has revolutionized the field of stem cell research and regenerative medicine; the Nobel Prize in Physiology or Medicine 2012 was awarded to Sir John B. Gurdon and Shinya Yamanaka *"for the discovery that mature cells can be reprogrammed to become pluripotent"*. Gurdon and colleagues showed in 2003 that the nuclei of adult mammalian somatic cells can be reprogrammed to express the pluripotency marker Oct-4, when transferred into amphibian oocytes (Byrne et al., 2003). A few years later, Yamanaka and colleagues showed that reprograming mouse and human fibroblasts was possible by introducing the transcription factors Oct-4, Sox-2, Klf4 and c-Myc (the Yamanaka factors) into the cells (Takahashi et al., 2007; Takahashi and Yamanaka, 2006). These cells represent an invaluable tool for studying the mechanisms underlying both normal and pathogenic human tissue formation and regeneration. iPS cells also provide a source of patient-specific stem cells with potential for therapeutic use in a variety of disorders. However, despite their immense potential, concerns have been raised regarding the safety of the procedure, since both the use of viral vectors and the insertion of these transgenes into human cells has been implicated in tumorigenesis (Okita et al., 2007).

NEW NEURONS IN OLD BRAINS

In the adult mammalian brain, neurogenesis occurs mainly in two brain regions: the dentate gyrus of the hippocampal formation and the subventricular zone (SVZ) of the lateral ventricle (Altman and Das, 1965; Cameron and Gould, 1994; Doetsch et al., 1997; Kempermann et al., 1998b; Kuhn et al., 1996; Lois and Alvarez-Buylla, 1994; Luskin, 1993). Neurons born in the SVZ migrate through the rostral migratory stream and are incorporated into the olfactory bulb to become interneurons (Lois and Alvarez-Buylla, 1994). Eriksson and colleagues investigated whether neurogenesis persists in the adult human brain by injecting the thymidine analog bromodeoxyuridine (BrdU) into terminally ill cancer patients, which enabled postmortem identification of the progenitor cells that had committed to neuronal differentiation (Eriksson et al., 1998). The authors concluded that the human hippocampus retained the ability to generate neurons throughout life, although it seemed that the SVZ lacked this possibility. Since then, a new sophisticated technique that offers unique possibilities to estimate the age of neurons in various regions in the human brain has been developed, which is taking advantage of the integration of ^{14}C in human DNA (generated by the nuclear bomb testing during the Cold War) (Spalding et al., 2005). Recent studies have supported the findings that neurogenesis persists in the human hippocampus (Spalding et al., 2013) but seems to be very limited or doesn't exist in the neocortex and the olfactory bulb (Bergmann et al., 2012; Bhardwaj et al., 2006). Interestingly, it was recently discovered that new neurons also integrate into the striatum, which is adjacent to the SVZ (Ernst et al., 2014). The findings suggest that neurogenesis in the human brain may differ from that in other mammals, and this needs to be taken into consideration when studying these processes in animal models of disease.

The role of hippocampal neurogenesis in learning and memory

The hippocampus is essential for learning and memory, which depend on functional and structural changes such as long-term potentiation (LTP) and synaptic remodeling (Bliss and Lomo, 1973; Matsuzaki et al., 2004). A number of studies in rodents have shown that adult neurogenesis contributes to normal

cognitive function and memory formation (Rola et al., 2004; Shors et al., 2001; Shors et al., 2002; Trouche et al., 2009).

Neurogenesis in the dentate gyrus (DG) of the hippocampus involves multiple developmental steps, in which resident neural stem cells and progenitor cells proliferate, differentiate and migrate to the granular cell layer, where the immature neurons integrate into existing networks and terminally differentiate into dentate granular cells (Ehninger and Kempermann, 2008), schematically illustrated in Figure 3.

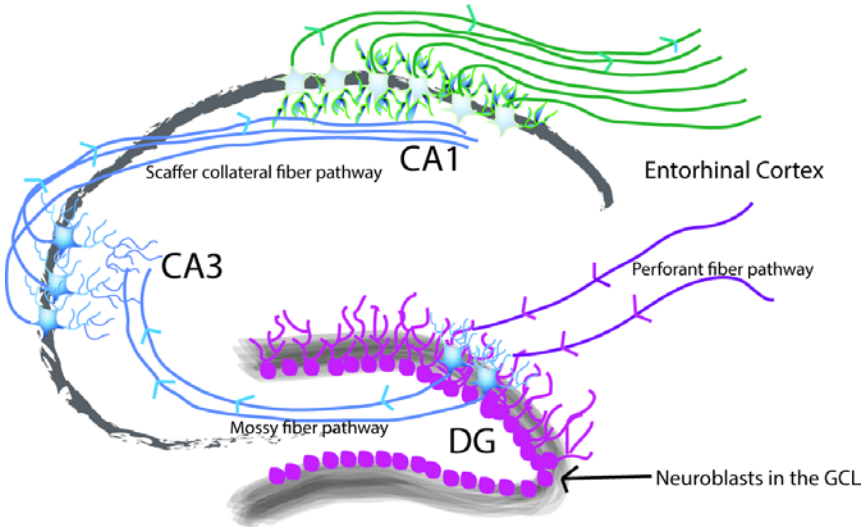


Figure 3. Schematic illustration of hippocampal neurogenesis. Abbreviations: DG – dentate gyrus, GCL – granular cell layer.

In detail, RG-like progenitor cells (type 1 cells), which have radial processes that span the entire granular cell layer of the DG (Kempermann et al., 2004), are generally identified by their expression of markers such as nestin and glial fibrillary acidic protein (GFAP) (Fukuda et al., 2003). RG-like cells can self-amplify and give rise to intermediate type 2a and type 2b cells, which express nestin and later doublecortin (DCX) but not GFAP (Kempermann et al., 2004). The type 2 cells give rise to type 3 cells, which are DCX-expressing neuroblasts that proliferate and terminally differentiate into dentate granular cells that integrate into existing networks (Figure 4) (Benarroch, 2013; Ming and Song, 2011).

The heterogeneous nature of the precursor cell populations in the hippocampus generates several questions that have implications for future studies

of neurogenesis. First, it is important to understand the inter-relationships between the different precursor cells; i.e. can a type 3 cell revert back to a type 2 or even a type 1 cell under the right conditions? At what point do the precursor cells become lineage-restricted? Second, are type 1 cells multipotent *in vivo* and how does this relate to gliogenesis in the hippocampus? Third, does the course of neuronal development change in response to pathological stimuli such as those encountered in neurodegenerative diseases?

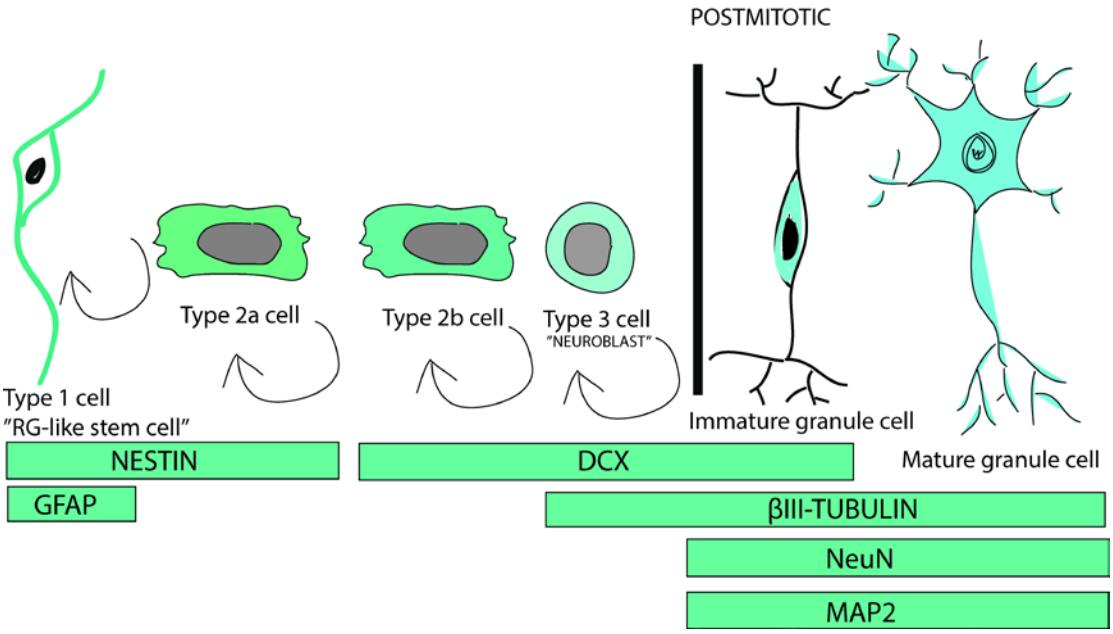


Figure 4. Sequence of cell types in hippocampal neurogenesis and the markers they express. Abbreviations: RG – radial glia, DCX – doublecortin.

Regulation of adult hippocampal neurogenesis

The neurogenic niche, which consists of endothelial cells, astrocytes, microglia, and mature neurons, regulates the permissiveness of neuronal development from progenitor cells (Ming and Song, 2011; Morrens et al., 2012). The molecular regulation of adult neurogenesis is highly dependent on extrinsic signals as well as intrinsic cellular factors. Extracellular signals include morphogens, growth factors such as brain-derived neurotrophic factor (BDNF), vascular endothelial growth factor, and insulin-like growth factor (IGF)-1, and neurotransmitters such as GABA, serotonin and glutamate, as reviewed by several authors (Benarroch, 2013;

Kempermann, 2011; Kuhn et al., 2001). Furthermore, external factors such as physical activity and environmental enrichment can effectively induce hippocampal neurogenesis (Brandt et al., 2010; Kempermann et al., 1998a; Kempermann et al., 1998b; Wolf et al., 2011). However, environmental cues can also induce opposite and negative effects; for example, stress-induced responses have been shown to inhibit neurogenesis (Gould et al., 1997; Gould et al., 1998). Hippocampal neurogenesis occurs throughout life but decreases with advancing age in rodents (Kuhn et al., 1996), implying very limited regenerative potential in the senescent brain. However, it seems that the aging rodent brain may still be responsive to therapeutic interventions that enhance neurogenesis (Jin et al., 2003). A recent study suggests that neurogenesis does not decline with advancing age in humans (Spalding et al., 2013), which raises hopes of stimulating regenerative mechanisms in the normal aging brain.

Further, it appears that neural progenitor cells can remain dormant under physiological conditions, but possess the capacity to respond to insult or injury to the brain. For example, neurogenesis is generally increased after acute seizures in animal models of temporal lobe epilepsy (Gray and Sundstrom, 1998), and cortical neurogenesis has been observed in a rat model of ischemic stroke (Gu et al., 2000). These observations imply that the brains' regenerative capacity is increased in response to insult, which could prove useful in therapeutic approaches against neurodegenerative diseases, such as AD.

ALZHEIMER DISEASE

AD, a lethal and progressive neurodegenerative disorder affecting over 30 million people worldwide, is the most common form of dementia. The clinical course of the disease starts with subtle changes in episodic memory, which later spread to other cognitive domains such as language, orientation and behavior. As the disease progresses, the patient's cognitive and functional abilities relentlessly decline. In advanced AD, patients need help with the basic activities of daily living such as eating, dressing, and sanitary needs. This devastating disease robs a person of his/her identity, leaving an empty shell, one who has lost the ability to communicate with and recognize loved ones.

The prevalence of AD increases exponentially with age; only 1 % of people aged 60-64 are afflicted but, amongst the oldest-old (age 85 and over) approximately 30 % suffer from the disease (Hebert et al., 2013; Prince et al., 2013; Thies and Bleiler, 2013). With longer life expectancies, the prevalence of AD is expected to triple by the year 2050 (Hebert et al., 2013; Prince et al., 2013). The sinister nature of the progression of the disease, the increasing number of patients, and the long duration of the illness contribute significantly to the high socioeconomic cost of this rapidly growing epidemic (Wimo et al., 2013).

The German physician Alois Alzheimer described the main pathological hallmarks of AD in 1906 (Alzheimer et al., 1995). Extensive research in recent decades has provided useful information on the underlying disease mechanisms and disease progression. However, the etiology of AD is still not completely understood and the numerous investigative studies and clinical trials carried out over the past decades have not yet resulted in effective therapies that can halt or change the course of the disease.

Pathological changes in the Alzheimer brain

The characteristic neuropathological lesions in the AD brain are β-amyloid (Aβ) plaques and neurofibrillary tangles (NFTs). Postmortem studies have enabled the staging of these and have demonstrated distinct spatio-temporal distributions. NFTs are first observed in the temporal lobe, and spread from there to the entorhinal region and the hippocampus, and then to the neocortex (Braak and Braak, 1995; Braak and Braak, 1997). Amyloid pathology first initiates in the neocortex, in particular in the basal portions of the frontal, temporal and occipital lobes, but by the end stages of the disease deposits can be observed in all neocortical areas (Braak and Braak, 1997). The hippocampus is, however, only mildly affected by amyloid pathology, even in the end stages (Braak and Braak, 1991).

β-amyloid

According to the amyloid cascade hypothesis, accumulation of Aβ in the brain causes a series of pathological events, such as inflammatory processes, oxidative stress, and NFT formation, which subsequently lead to synaptic dysfunction and

neuronal cell death (Hardy and Selkoe, 2002; Hardy and Higgins, 1992; Mattson, 2004).

The Aβ peptide is derived from the amyloid precursor protein (APP) by proteolytic cleavage, and abnormal processing, decreased degradation or clearance of the APP leads to the gradual accumulation of Aβ. APP is widely expressed in the nervous system and has been attributed with a number of physiological functions such as neuronal survival, neuritic outgrowths, and synapse formation (De Strooper and Annaert, 2000; Mattson, 1997; Tyan et al., 2012). APP is processed by two physiological pathways: the non-amyloidogenic pathway and the amyloidogenic pathway (Haass et al., 1993). In the non-amyloidogenic pathway, α-secretase cleaves the APP within the Aβ domain, releasing sAPPα and the C83 fragment, whereupon γ-secretase cleaves the C83 fragment into the p3 fragment and the APP intracellular domain (AICD). The physiological functions of these cleavage products are not entirely understood, although the AICD is thought to regulate the transcription of several genes, and to modulate calcium homeostasis and ATP content (Cao and Sudhof, 2004; Hamid et al., 2007; Pardossi-Piquard et al., 2005), whereas sAPPα is believed to modulate neuronal excitability, synaptic plasticity, and cell survival (Mattson, 1997). In the amyloidogenic pathway, β-secretase cleaves APP, releasing a soluble sAPPβ fragment, which is subsequently cleaved by γ-secretase, resulting in the formation of the Aβ peptide and the AICD (Haass et al., 1993). Proteolytic cleavage by γ-secretase generates peptides of different lengths: Aβ forms that are 1-40 and 1-42 amino acids long are the most prominent forms in the AD brain. Aβ 1-42 is more liable to aggregate and is therefore one of the main constituents of Aβ plaques (De Strooper and Annaert, 2010; Selkoe, 2001).

Initially, it was believed that fibrillar Aβ present in the plaques was mediating the neurotoxic effects. However, the Aβ plaque burden correlates poorly with the severity of dementia, and emerging evidence suggests that Aβ oligomers, rather than Aβ fibrils, may be the fundamental molecular pathogens that trigger synaptic dysfunction and the memory deterioration observed in the disease (Lambert et al., 1998; Selkoe, 2008; Walsh et al., 2002). Aβ can aggregate

in vitro to different sized aggregation forms (Chromy et al., 2003); various structurally distinct forms, including dimers (Walsh et al., 2002), globulomers (Barghorn et al., 2005), protofibrils (Harper et al., 1997), and ring structures (Chromy et al., 2003), have been identified (Figure 5). There is strong evidence of the neurotoxic properties of Aβ oligomers *in vitro* (Jang et al., 2007; Lacor et al., 2004; Lacor et al., 2007; Urbanc et al., 2010) but, to date, only a few studies have explored the occurrence of different oligomeric Aβ species in autopsy brain tissue from healthy subjects and AD patients (Lesne et al., 2013; Mc Donald et al., 2010; Shankar et al., 2008). Furthermore, it has been hypothesized that large, insoluble deposits of Aβ might serve as reservoirs of bioactive synaptotoxic Aβ oligomers (Haass and Selkoe, 2007). However, the nature of the relationships between different Aβ species regarding their length, aggregation form, and propensity for inducing neurotoxicity *in vivo* remains elusive.

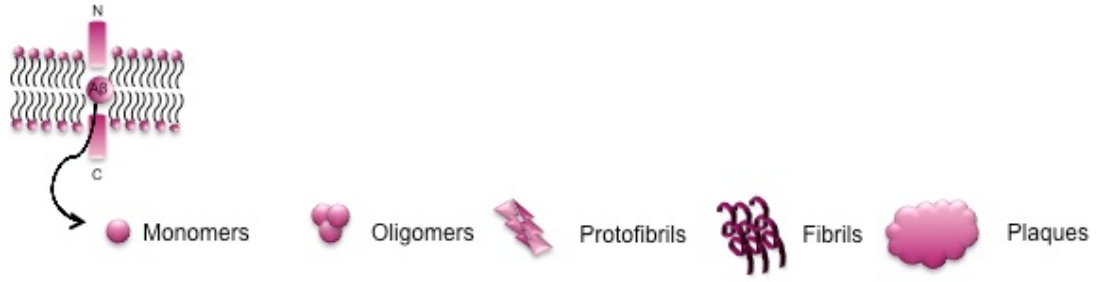


Figure 5. Schematic illustration of Aβ aggregation from soluble Aβ monomers to Aβ oligomers and further maturation to Aβ fibrils.

Tau

Tau is a microtubule-associated protein (MAP), which stabilizes the microtubules and regulates axonal transport within neurons. Abnormal hyperphosphorylation of tau and its aggregation into NFTs are pathological hallmarks of AD (Alzheimer et al., 1995; Grundke-Iqbal et al., 1986). The non-fibrillized hyperphosphorylated tau sequesters the normal tau, MAP1, and MAP2, and disrupts the microtubule, which leads to slow progressive retrograde degeneration and thus loss of synapses in the affected neurons, as reviewed by Alonso and Iqbal (Alonso et al., 1994; Iqbal et al., 2009). In contrast to the number of Aβ plaques, the number of NFTs correlates well with the severity of AD (Arriagada et al., 1992a). NFTs seem to be required for clinical manifestation of AD, since Aβ pathology alone does not

produce cognitive symptoms (Iqbal et al., 2009; Jack et al., 2010). In related tauopathies that are clinically characterized by dementia, such as fronto-temporal dementia with tau mutations, progressive supranuclear palsy, and Pick's disease, neurofibrillary degeneration occurs without an Aβ plaque burden, as reviewed by Iqbal (Iqbal et al., 2009). NFTs can be present in cognitively normal individuals as well, but the lesions are then confined to the entorhinal cortex, Braak stage I-II (Price and Morris, 1999). The true nature of the relationship between tau and Aβ is currently under investigation, and the ongoing studies will hopefully increase our understanding of the interplay between these molecules in AD (Giacobini and Gold, 2013).

Cholinergic dysfunction in AD

Basal forebrain cholinergic neurons (BFCNs) are important for memory function, learning, and behavior, and are progressively lost in AD pathogenesis (Mesulam et al., 1983a; Mesulam et al., 1983b; Mufson et al., 2003). Relevant structures in the basal forebrain include the septal area (Ch1), the vertical and horizontal limbs of the diagonal band of Broca (Ch2 and Ch3), and the nucleus basalis of Meynert (Ch4) (Mesulam et al., 1983a; Mesulam et al., 1983b) (Figure 6). The BFCNs provide the main cholinergic innervation of the hippocampus (Ch1-2), the olfactory system (Ch3), and the amygdala and cortex (Ch4) (Mesulam and Geula, 1988; Mesulam et al., 1983a; Mesulam et al., 1983b; Selden et al., 1998).

A number of studies have demonstrated a reduction in BFCNs (Davies and Maloney, 1976; Whitehouse et al., 1981; Whitehouse et al., 1982) and in cortical and hippocampal choline acetyltransferase (ChAT) activity in AD brains (Candy et al., 1983; DeKosky et al., 1992; DeKosky et al., 2002; Perry et al., 1978), which correlates with cognitive decline in the disease (DeKosky et al., 1992; Perry et al., 1978). Intriguingly, the loss of synapses correlates well with the cognitive decline observed in AD, and has been suggested to precede the loss of BFCNs (Terry et al., 1991). Thus, the relationship between neuroplasticity and cognition in the brain seems far more complex than if restricted to a certain neurotransmitter system or neuronal subtype.

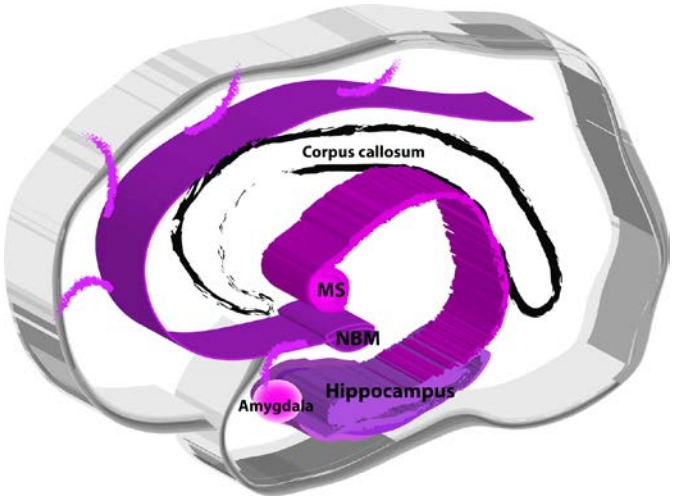


Figure 6. Projections of basal forebrain cholinergic neurons in the brain. Abbreviations: MS – medial septal nuclei, NBM – nucleus basalis of Meynert.

Nicotinic acetylcholine receptors

Several lines of evidence point to a link between nAChRs and the development of AD, as reviewed by Paterson and Nordberg (Paterson and Nordberg, 2000). Neuronal nAChRs belong to a gene superfamily of ligand-gated ion channels. The subunits can be classified as α (α2-10) or β (β2-4) subfamilies, with either a homomeric or heteromeric pentameric subunit arrangement. Neuronal nAChRs are expressed on axons, as well as on presynaptic and postsynaptic terminals; activation of the receptor modulates the release of various transmitters, including acetylcholine (ACh), noradrenaline, dopamine, glutamate and GABA (Alkondon et al., 1997; Marshall et al., 1997; Paterson and Nordberg, 2000; Wonnacott, 1997).

Studies in AD autopsy brain tissue have shown loss of nicotinic receptor binding sites as well as reduced protein levels (Guan et al., 2000; Nordberg and Winblad, 1986). The α7 nAChR subtype, which is involved in neuroprotection and plasticity (Kihara et al., 1997; Liu and Zhao, 2004), is highly expressed on hippocampal neurons, and may thus be involved in the integration of synaptic functions in the hippocampus (Albuquerque et al., 1997). Although the expression of α7 nAChRs on neurons is significantly lower in postmortem AD brains than in healthy control brains, the expression of α7 nAChRs on reactive astrocytes is increased; these astrocytes seem to accumulate in the vicinity of the Aβ neuritic plaques (Marutle et al., 2013; Yu et al., 2005), suggesting that they are involved in

inflammatory processes in the brain.

Impaired neurotrophin signaling

Neurotrophin signaling is impaired in AD and levels of the neurotrophins nerve growth factor (NGF) and BDNF decline during progression of the disease as a result of dysmetabolism and impaired axonal transport (Bruno et al., 2009; Capsoni and Cattaneo, 2006; Laske et al., 2006; Michalski and Fahnestock, 2003; Peng et al., 2005). The actions of NGF on its high affinity receptor neurotrophic tyrosine kinase receptor A are important for the proliferation, differentiation and survival of BFCNs ,(Heese et al., 2006; Schindowski et al., 2008). BDNF, which regulates synaptic plasticity and is involved in regulating memory formation (Laske and Eschweiler, 2006), is decreased in both the brain and cerebrospinal fluid (CSF) in AD (Laske et al., 2006; Michalski and Fahnestock, 2003; Peng et al., 2005), correlating with cognitive decline (Laske et al., 2006). Impaired balance between levels of mature NGF and the NGF precursor, shifting the balance in favor of the precursor, has been suggested to underlie the cholinergic dysfunction observed in AD (Cuello et al., 2010). In light of this, it can be speculated that the decreased levels of NGF and BDNF, as well as the impaired signaling of these neurotrophins, could contribute to the progression of AD.

Inflammation in AD

The AD brain is characterized by low levels of systemic inflammation, with increased reactive microgliosis and astrocytosis in the vicinity of the Aβ plaques, as has been reviewed by several authors (Akiyama et al., 2000; Wyss-Coray and Mucke, 2002; Yu et al., 2005). Reactive astrocytes are also located in the hippocampus, where the fibrillar Aβ burden is usually low (Marutle et al., 2013). However, it has not been established whether inflammation is a cause, a contributor, or a consequence of AD.

Microglia, the residing brain macrophages, are usually maintained in a quiescent state in the brain. However, when activated, they can display a plethora of functions such as recognition of pathogens, phagocytic properties, antigen presentation for activation of T lymphocytes, production of cytokines, chemokines,

and proteases, and participation in the regulation of stem cell differentiation, as reviewed by Boche (Boche et al., 2013). Macrophages can be classified as classically activated (M1) or alternatively activated (M2) phenotypes, each of which are capable of performing a subset of functions (Gordon, 2003). The M1 activation state is defined by an enhanced microbicidal capacity, and production of high levels of pro-inflammatory cytokines, which could exacerbate neurodegeneration (Combs et al., 2001). The M2 phenotype can be regulatory macrophages or can have functions associated with wound healing. Regulatory macrophages produce the anti-inflammatory cytokines IL-10 and transforming growth factor β (Fadok et al., 1998; Martinez et al., 2008). Wound-healing macrophages produce extracellular matrix components, as reviewed in (Kreider et al., 2007; Mosser and Edwards, 2008). It has been suggested that the same macrophage can adapt to either the M1 or M2 phenotype, depending on the type of stimulus (e.g. chronic disease or injury) or the cell status before the stimulus (activated or not) (Mosser and Edwards, 2008). Inflammation in the AD brain has been associated with the M1 activation of microglia, which are surrounding the neuritic plaques (Griffin et al., 1989).

Astrocytes have multiple functions in the adult brain, including brain homeostasis, provision of structural and metabolic support to neurons, and involvement in the regulation of neurogenesis and modulation of synaptic activity (Parpura et al., 2012). Furthermore, astrocytes are essential components of the neurovasculature and regulate the properties of the blood-brain barrier. Increased levels of the Aβ peptide activate astrocytes, with subsequent release of pro- and anti-inflammatory mediators in the brain (Heneka et al., 2010; Parpura et al., 2012). Glial-derived inflammatory molecules can suppress LTP, which is a crucial factor for memory formation and consolidation in the hippocampus (Murray and Lynch, 1998; Tancredi et al., 1990). Moreover, chronic inflammation may disrupt the normal production and secretion of glial-derived growth factors supporting the surrounding neurons (Nagatsu and Sawada, 2005), suggesting that several mechanisms may contribute to cognitive dysfunction in AD.

It has been hypothesized that pro-inflammatory stimuli caused by high Aβ levels in the brain can create a self-propagating cycle that can lead to increased

APP processing, further Aβ accumulation and inflammation in patients with AD (Heneka et al., 2010; Heneka et al., 2005). The proposed mechanisms by which Aβ activates microglia and astrocytes involve Aβ binding to the receptor for advanced glycation end products (RAGE) and to other scavenger receptors (Jones et al., 2013; Paresce et al., 1996; Yan et al., 1998), followed by the release of inflammatory mediators such as cytokines and nitric oxide (Heneka et al., 2010; Jana et al., 2008; Lindberg et al., 2005). Activated microglia and astrocytes are also implicated in the phagocytosis of Aβ, as a means of counterbalancing the increased Aβ load (Bolmont et al., 2008; Hjorth et al., 2010; Nagele et al., 2003). Studies using postmortem brain tissue have revealed the presence of senescent and dystrophic microglia in the aged and AD brain. It is suggested that these microglia are unable to clear the amyloid load, and it is therefore possible that both deficient and excessive inflammatory responses result in pathological conditions (Streit, 2005; Streit et al., 2004).

Cholinergic regulation of inflammation

In the periphery, ACh signaling can inhibit cytokine release by acting on α7 nAChRs on macrophages, thus exerting an anti-inflammatory effect. This is a physiological pathway in which the autonomic nervous system, via the vagus nerve, can modulate cytokine production, a phenomenon that has been termed the "cholinergic anti-inflammatory reflex" (Czura et al., 2003; Czura and Tracey, 2005). It has been hypothesized that Aβ accumulation increases the activity of the ACh hydrolyzing enzyme butyrylcholinesterase (BuChE), which could contribute to the early cholinergic deficit observed in AD (Darreh-Shori et al., 2011a; Darreh-Shori et al., 2011b; Darreh-Shori et al., 2009a; Darreh-Shori et al., 2009b). Thus, reduced cholinergic signaling could disrupt the normal regulation of inflammatory cascades, leading to over-activation of the immune responses in the brain. A link between inflammation and diminished neurogenesis has been proposed (Ekdahl et al., 2003; Monje et al., 2003). However, whether Aβ-induced inflammatory events exert similar effects on stem/progenitor cell populations in the brain remains to be determined.

Metabolic changes

A growing body of evidence suggests that metabolic disturbances in the brain, such as insulin-resistance, impaired glucose utilization, and mitochondrial dysfunction, could contribute to the pathological changes observed in AD (De Felice et al., 2014; de la Monte and Tong, 2014; Ferreira et al., 2014; Riemer and Kins, 2013).

Insulin and IGF have potent effects in the brain, stimulating both synaptogenesis and synaptic re-modeling (Abbott et al., 1999). Dysregulated insulin signaling is associated with impaired neurogenesis and inflammation (Craft and Watson, 2004). The term insulin resistance refers to the reduced ability of insulin to act on target tissue expressing insulin receptors, and is linked to processes associated with cognitive decline, as well as increased intracellular Aβ deposits, reduced Aβ clearance, and increased tau phosphorylation (Cholerton et al., 2013). Type 2 diabetes confers an increased risk of AD and vascular dementia (Luchsinger, 2008; Strachan et al., 2008). Metabolic syndrome and type 2 diabetes have been linked with brain injury over time, and the vasculature damage and inflammation associated with these conditions can contribute to diffuse white matter loss and subsequent hippocampal atrophy (den Heijer et al., 2003).

There is also evidence that oxidative damage (Nunomura et al., 2001) and a reduced number of mitochondria (Hirai et al., 2001) occur early in the disease process. Mitochondria are the main source and target of oxidative stress, and damage to mitochondria in neurons could be detrimental because of the limited capacity of neurogenesis to affect the adult human brain.

Collectively, these findings indicate that disturbances in insulin signaling and related metabolic changes could be part of the underlying molecular mechanisms contributing to the cognitive decline and histopathological lesions observed in AD.

Genetics and risk factors

AD can be classified as sporadic or familial (FAD); FAD constitutes only 1 % of all diagnosed AD cases (Pastor and Goate, 2004). Genetic studies have revealed

autosomal-dominant mutations in three genes in FAD – the APP gene on chromosome 21, and the presenilin (*PSEN*) genes, *PSEN1* on chromosome 14 and *PSEN2* on chromosome 1, which are components of the γ-secretase complex (Goate et al., 1991; Levy-Lahad et al., 1995; Sherrington et al., 1995). To date, over 260 mutations in these three genes have been discovered (www.molgen.ua.ac.be/ADMutations). Transgenic mouse models that harbor some of these mutations have been developed; these are valuable *in vivo* model systems for studying disease pathogenesis (Ashe and Zahs, 2010; Lithner et al., 2011).

The main genetic risk factor for developing AD is the apolipoprotein E (APOE) ε4 allele (Corder et al., 1993); heterozygotes have a 4-fold increased risk and homozygotes have a 15-fold increased risk (Ashford, 2004). The APOE gene is located on chromosome 19 and is polymorphic (ε2, ε3, ε4), thus enabling six different genotypes. An estimated 20-30 % of the US population carries one or two copies of APOE ε4, whereas only about 2 % carries two copies (Farrer et al., 1997). Further, it is estimated that 40-65 % of patients diagnosed with AD are ε4 carriers (Thies and Bleiler, 2013). Although carrying one or two APOE ε4 alleles is associated with an increased risk of developing AD, it has a low predictive value for discovering who will develop AD (Patterson et al., 2008).

Advancing age is regarded as the most prominent risk factor for developing AD (Kawas, 2003; Nussbaum and Ellis, 2003). However, sporadic AD is considered a multifactorial disease and there are several risk factors implicated in its etiology. Cardiovascular disease and factors that are associated with cardiovascular disease, such as diabetes mellitus (Reitz et al., 2011), high blood glucose, smoking (Anstey et al., 2007; Rusanen et al., 2011), obesity (Kivipelto et al., 2005; Whitmer et al., 2008), hypertension, and high blood cholesterol in midlife (Anstey et al., 2008; Kivipelto et al., 2005), increase the risk of developing AD. Conversely, factors that protect the heart, such as physical activity, could therefore also protect the brain and reduce the risk of developing AD and other dementias (Reitz et al., 2011). Engaging in social and cognitive activities could also support brain health (Hall et al., 2009), and it has been hypothesized that education, occupational attainment, and leisure activities in later life increase neuronal connections in the brain, which

can compensate for the early brain changes of AD (Evans et al., 1997; Stern et al., 1994). This concept, named the *cognitive reserve*, could account for the individual variations in susceptibility to age-related brain changes (Stern, 2012).

Brain imaging and CSF biomarkers

The rapid development of new molecular imaging techniques has enabled longitudinal studies of pathological changes in living patients, which it is hoped will help the evaluation of treatment strategies as well as the early diagnosis of AD. Early detection or prediction of AD could enable intervention strategies with disease-modifying effects. Amyloid positron emission tomography (PET) tracers, such as ^{11}C-Pittsburgh compound B (PIB), have enabled investigation of Aβ deposition *in vivo* in AD patients (Klunk et al., 2004; Nordberg, 2004; Nordberg et al., 2010). Longitudinal PIB-PET imaging studies of patients with MCI or AD have revealed that Aβ levels reach a plateau early in the disease progression (Engler et al., 2006; Forsberg et al., 2010; Forsberg et al., 2008; Kadir et al., 2010; Scheinin et al., 2009). Interestingly, a widespread Aβ plaque burden can be present in cognitively normal individuals (Aizenstein et al., 2008; Mintun et al., 2006), but these plaques seem to lack dystrophic neurites (Arriagada et al., 1992a; Arriagada et al., 1992b; Katzman et al., 1988). Since the development of PIB-PET, other amyloid PET tracers have been developed and approved by the U.S. Food and Drug Administration and the European Medicines Agency for use in clinical practice. Several tau tracers are also in development for use in *in vivo* PET imaging for AD (Maruyama et al., 2013; Okamura et al., 2013; Villemagne et al., 2014; Zhang et al., 2012). The expectation is that these tau tracers will enable detection of specific AD forms of tau inclusion as well as increasing understanding of how tau pathology spreads in living patients. In addition, longitudinal imaging of tau and Aβ in patients may provide new insights into the relationships among the different pathophysiological processes in AD, while also offering a powerful tool for the evaluation of anti-tau treatment (Giacobini and Gold, 2013).

Glucose metabolism deteriorates in both MCI and AD patients (Mosconi et al., 2005); monitoring this deterioration using the glucose analog ^{18}F-fluoro-deoxy-D-glucose (^{18}F-FDG) is an established method in clinical practice.

FDG-PET measurements in patients with FAD and a PSEN1 mutation indicate that glucose hypometabolism can be detected years before cognitive symptoms arise (Scholl et al., 2011). In AD patients, glucose metabolism continues to deteriorate even after the amyloid load has reached its plateau (Kadir et al., 2012).

Nicotine binding using ^{11}C-nicotine-PET has demonstrated regional decreases in the number of binding sites in AD patients (Nordberg et al., 1990; Nordberg et al., 1995). Cortical ^{11}C-nicotine binding also correlates with attention in patients with mild AD (Kadir et al., 2006; Nordberg et al., 1995).

The PET tracer ^{11}C-deprenyl binds to monoamine oxidase type B, an enzyme located in the outer mitochondrial membrane, predominantly on reactive astrocytes (Fowler 2005). A pioneering multitracer PET study by Carter and colleagues suggested that astrocytosis may be an early phenomenon in AD, since higher astrocytosis was seen in a cohort of MCI patients compared with a cohort of AD patients (Carter et al., 2012). Thus, like the accumulation of amyloid, astrocytosis could be regarded as an early event in the disease pathogenesis process, with an onset many years before the clinical symptoms occur. However, the spatial distribution pattern for astrocytosis seems to be different from that for amyloid deposition, as shown in both PET studies in living patients and binding studies in postmortem AD brains (Carter et al., 2012; Kadir et al., 2011; Marutle et al., 2013). It has also been shown that microglia activation, measured using (R)-[^{11}C]-PK11195, is increased in both MCI and AD patients (Schuitemaker et al., 2013; Yokokura et al., 2011). These early inflammatory events may be driving the pathogenesis of the disease, but could also be a protective mechanism initiated to circumvent the pathological burden of amyloid accumulation, oxidative stress and metabolic disturbances in the brain.

Three established CSF biomarkers are currently used to diagnose AD in clinical practice. These are used to measure the total amount of tau, which reflects the intensity of neuronal degeneration; the amount of phosphorylated-tau, which is believed to correlate with tangle pathology; and the amount of Aβ42, which is inversely correlated with the amyloid plaque burden (Blennow et al., 2010). The combination of these biomarkers can distinguish AD patients from controls with a

sensitivity and specificity of over 80 % (Blennow and Hampel, 2003). Clinical research studies employing molecular imaging techniques and CSF biomarkers have shown that the pathological processes associated with AD precede the onset of clinical symptoms by approximately 10-20 years (Hardy and Selkoe, 2002; Jack and Holtzman, 2013; Jack et al., 2010; Nordberg et al., 2010; Villemagne et al., 2013) (Figure 7).

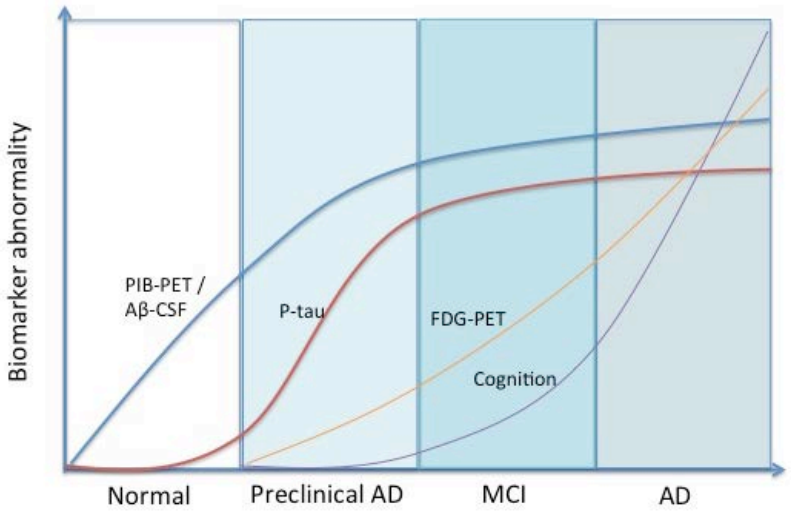


Figure 7. Temporal changes of biomarkers in preclinical Alzheimer's Disease (AD), mild cognitive impairment (MCI), and AD (Jack and Holtzman, 2013; Jack et al., 2010; Nordberg et al., 2010). Abbreviations: FDG – fluoro-deoxy-D-glucose, PET – positron emission tomography, PIB – Pittsburgh compound B.

Diagnostic criteria for AD

A definite AD diagnosis is verified postmortem by histopathological examination of the brain for the presence of amyloid plaque and NFTs. However, AD can be diagnosed clinically in living patients. In Sweden, dementia is commonly classified according to the Diagnostic and Statistical Manual of Mental Disorders, fourth edition (DSM IV). The criteria for diagnosing dementia of the Alzheimer type are memory impairment and one or more of the following: aphasia (language problems), agnosia (failure to recognize objects), apraxia (impaired motor ability), or deterioration in executive function. This should also be accompanied by loss of independence.

Two sets of criteria for the clinical diagnosis of AD have been published;

one was developed by the International Working Group (Dubois et al., 2010; Dubois et al., 2007) and the other by the Alzheimer's Association and the National Institute of Aging (Albert et al., 2011; Sperling et al., 2011). Three stages of AD are identified. In the *preclinical AD* stage, the patient has pathological changes in biomarkers in the CSF and/or plasma, measurable changes in the brain, but no cognitive symptoms. The second stage is *mild cognitive impairment* (MCI), also referred to as *prodromal AD*, where the patient has biomarker evidence from CSF or imaging that support the presence of AD pathological changes, accompanied by subjective or objective memory impairment, although their ability to carry out everyday activities is not affected. The last stage, *AD dementia,* is characterized by biomarker evidence of Alzheimer pathology and specific memory changes that impair the patient's ability to function in daily life. Although both criteria recognize the use of biomarkers in the diagnostic guidelines, these are not required by the Alzheimer's Association and the National Institute of Aging. In addition, the two sets of criteria have different views on what is meant by disease (Morris et al., 2014).

NEUROGENESIS IN AD

It has been hypothesized that memory deficits seen during normal and pathological aging may be linked to alterations in hippocampal neurogenesis (Lazarov and Marr, 2010; Mu and Gage, 2011; Shruster et al., 2010). Investigation of postmortem AD brains has indicated that neurogenesis appears to increase in the hippocampus during the disease process (Jin et al., 2004b), which may reflect compensatory mechanisms initiated to circumvent pathological conditions. In contrast, however, impaired hippocampal neurogenesis has also been reported (Crews et al., 2010). Furthermore, another study demonstrated a decrease in the number of stem cells (type 1 cells) and increased proliferation of neuronal precursor cells (type 2b cells) in the AD brain, although differentiation into mature neurons remained virtually unchanged (Perry et al., 2012). This study also indicated that cholinergic pathology correlated with the reduced number of stem cells. It has been suggested that amyloid pathology could transiently promote the generation of immature non-functional neurons that are unable to integrate into existing networks (Shruster et al., 2010; Waldau and Shetty, 2008). Boekhoorn

and colleagues demonstrated that increased cell proliferation in the hippocampus of AD subjects reflects glial and vasculature-associated changes, rather than indications for altered neurogenesis (Boekhoorn et al., 2006). These studies indicate that the pathophysiological environment in AD could affect neurogenesis, although decreased neurogenesis has not been confirmed.

Investigations into hippocampal neurogenesis in animal models of AD have also provided conflicting results; both impaired neurogenesis (Haughey et al., 2002a; Haughey et al., 2002b; Rodriguez et al., 2008; Wang et al., 2004; Zhang et al., 2007) and increased proliferation and neuronal differentiation have been reported (Jin et al., 2004a; Lopez-Toledano and Shelanski, 2007). Such discrepancies may be a consequence of different animal models used, differences in the age of the animals, which would influence their pathological burden, or differences in the markers used to study neurogenesis.

TREATMENT STRATEGIES FOR AD

A moment of relief – current symptomatic treatment

Current treatment options, cholinesterase inhibitors (ChEIs) and/or the N-methyl-D-aspartate (NMDA) receptor antagonist memantine, give symptomatic relief to AD patients. The ChEIs donepezil and galantamine target the enzyme AChE, whereas rivastigmine targets both AChE and BuChE (Darreh-Shori et al., 2002). Since both enzymes hydrolyze ACh, ChEIs are designed to reduce the activity of either AChE or BuChE in the brain, thereby prolonging the neurotransmitter signal. Memantine, on the other hand, inhibits the NMDA receptor, thus protecting the neurons from glutamate-induced neurotoxicity.

PET studies have reported both increased regional cerebral glucose metabolism and stabilization of glucose metabolism in AD patients following treatment with galantamine, rivastigmine and donepezil (Keller et al., 2010; Stefanova et al., 2006; Teipel et al., 2006). Rivastigmine has also been shown to increase ^{11}C-nicotine binding, as measured by PET in the brains of AD patients (Kadir et al., 2007). Further, the increased regional cerebral blood flow following galantamine treatment positively correlates with the density of nAChRs and cognition (Keller et al., 2010), indicating that nAChRs may be a potential drug

target. Although these drugs can reduce the symptoms of the disease, they cannot ameliorate decreased cognitive function or reverse ongoing neurodegeneration, which highlights the need for disease-modifying interventions early in the disease progression process.

Current development of therapeutics for AD

Current research efforts are directed towards identifying disease-modifying therapies. There are several candidate drugs in various phases of development, and ongoing clinical trials are investigating therapeutic strategies for inhibiting Aβ production and aggregation, and the administration of NGF, anti-inflammatory drugs, and drugs targeting tau phosphorylation (Mangialasche et al., 2010). One clinical trial investigating the **AChEI** and amyloid-lowering drug (-)-phenserine demonstrated enhanced glucose metabolism and cognition as well as a reduced Aβ plaque load, as measured with ^{11C}PIB-PET (Kadir et al., 2008), which lends support to the view that reducing the brain amyloid load could improve cognition in AD patients. (-)-Phenserine reached phase III clinical trials, which showed beneficial results in AD patients (Winblad et al., 2010). However, in another of the phase III trials, (-)-phenserine failed to show any efficacy and further development of the drug was abandoned. It has since been argued that this trial had some methodological lapses, and (-)-phenserine may thus have been prematurely dropped (Becker and Greig, 2012; Becker and Greig, 2013).

Other clinical trials aiming at reducing Aβ production with **γ-secretase inhibitors** have unfortunately been terminated because of cognitive worsening and the development of adverse side effects. This highlights the importance of developing more selective inhibitors or modulators that have minor effects on other γ-secretase substrates (De Strooper and Annaert, 2010).

Vaccines against Aβ have been tested in clinical trials since 2001. One trial was aborted because of adverse side effects such as encephalitis and increased loss of brain volume (Gilman et al., 2005; Orgogozo et al., 2003). At present, a variety of Aβ antibody therapies are being tested in clinical trials. These include both active and passive immunization with antibodies that recognize different conformations of the Aβ peptide (Gandy and DeKosky, 2013; Grill and Cummings,

2010; Karran and Hardy, 2014; Lemere and Masliah, 2010; Morgan, 2006). The first study to show target engagement demonstrated a 15-25 % reduction in amyloid but no cognitive benefits in patients with mild to moderate AD (Rinne et al., 2010). In addition, two recent phase III clinical trials were unable to show cognitive benefits in mild to moderate AD (Doody et al., 2014; Salloway et al., 2014). There are several possible explanations for these failures. For example, a greater reduction in amyloid may be required to show functional benefit. Or it could be that primary prevention of amyloid pathology is required to improve cognition and the therapy was given to patients who already had advanced synaptic and neuronal loss. Or cognitive impairment in AD may be a consequence of the accumulation of Aβ oligomers rather than amyloid plaques and, because amyloid imaging cannot visualize Aβ oligomers, the response to anti-amyloid therapy was not documented (Gandy and DeKosky, 2013). To date, clinical trials aiming at reducing the brain amyloid load have not been able to show robust proof of concept with cognitive benefits to patients, as reviewed by (Gandy and DeKosky, 2013; Karran and Hardy, 2014). It could simply be that reduction of the amyloid load is not a suitable treatment strategy for AD and that the amyloid cascade hypothesis is erroneous.

The development of novel **nAChR agonists** is an important strategy; these molecules could protect neurons against Aβ-induced toxicity and improve cholinergic neurotransmission (Toyohara and Hashimoto, 2010). Thus, cholinergic drugs with nAChR agonist activity are currently being evaluated in ongoing clinical trials (Dunbar et al., 2007). One of these studies, which used a selective agonist for α4β2 and α2β2 nicotinic receptors over a period of 12 weeks, failed to show proof of concept according to the AD Assessment Scale-Cognitive Subscale (Frolich et al., 2011). However, long-term studies might be warranted in order to fully evaluate the therapeutic potential of nicotinic drugs and to target other nicotinic subtypes.

The first clinical trial of **growth factor treatment** used an intracerebroventricular infusion of NGF in one AD patient; the results showed increased glucose metabolism and nicotine binding, measured using PET (Olson et al., 1992). Unfortunately, there were adverse side effects such as pain, weight lost and the appearance of a herpes zoster infection. While a second study with a

reduced dose of NGF showed increased nicotine binding, this was not as prominent as in the first study (Eriksdotter Jonhagen et al., 1998) and some of the patients still suffered from pain. A different approach using genetically modified fibroblast-expressing NGF inserted into the forebrains of AD patients improved cognition and cerebral blood flow (Tuszynski et al., 2005). In a similar approach utilized by Eriksdotter-Jonhagen and colleagues, in continuation of their pioneering studies with NGF, NGF-secreting capsules were implanted into the basal forebrains of six AD patients. The surgical implantations were well tolerated, with positive findings in cognition and nicotinic receptor binding in two patients (Eriksdotter-Jonhagen et al., 2012; Wahlberg et al., 2012).

Future AD therapy will probably be dependent on early diagnosis and subsequent early treatment. The recent progress in molecular imaging and CSF biomarkers could help the early detection and diagnosis of the disease (Hampel et al., 2010; Nordberg et al., 2010). This is important for evaluating both the short- and long-term effects of drug treatment, which would help establish the most effective time for administering treatment, with subsequent improvements to the efficacy of both current and future drugs.

Stimulating regeneration in the AD brain

Neurogenic mechanisms in the brain are novel therapeutic targets for preventing deterioration of neurological function. Compromised neurogenesis has been demonstrated with increasing pathology in animal models (Donovan et al., 2006; Lilja et al., 2013b; Rodriguez et al., 2008); these studies have also indicated that the type and magnitude of the pathology could influence the efficacy of the regenerative treatment regimens. There is evidence from studies in AD Tg2576 mice that stimulation of endogenous regenerative mechanisms may be possible by reducing the pathological burden through pharmacological treatment with the APP-lowering neurotrophic drug (+)-phenserine (Lilja et al., 2013a; Lilja et al., 2013b). Moreover, the neurosteroid allopregnanolone appears to induce endogenous neurogenesis and restore memory function, as well as reducing Aβ burden and inflammation, in the 3xTg-AD mouse model (Chen et al., 2011; Singh et al., 2012). Altering the microenvironment in the brain by reducing the pathological

burden, in combination with application of drugs with neurotrophic mechanisms of action, may thus be a viable approach for stimulating regeneration in the AD brain.

Other strategies for enhancing endogenous neurogenesis in AD include environmental enrichment and physical activity. Both enrichment and physical activity reduced the Aβ1-42/Aβ1-40 ratio and increased the number of newborn neurons in the APP23 mouse model of AD (Mirochnic et al., 2009; Wolf et al., 2006).

Stem cell transplantation

It has long been argued that stem cell replacement therapy could be an attractive therapeutic method of compensating for lost or damaged neuronal cells, thus potentially restoring functionality to AD brains. However, questions have also been raised as to whether neuronal replacement can ever be a viable strategy for AD because of its multifactorial etiology and the loss of multiple neuronal subtypes in several brain regions (Lindvall and Kokaia, 2010).

A recent study has shown that hippocampal transplantation of murine NSCs improved cognitive deficits in the 3xTg-AD mouse model through a BDNF-secreting mechanism, without affecting the pathology (Blurton-Jones et al., 2009). Moreover, improved endogenous neurogenesis and memory has been demonstrated following intra-hippocampal transplantation of murine neural precursor cells over-expressing the IL-1 receptor antagonist in Tg2576 mice (Ben Menachem-Zidon et al., 2014). Several studies have demonstrated improved spatial memory following murine or human NSC transplantation in rats with cholinergic lesions, although these animals did not have amyloid or tau pathology (Moghadam et al., 2009; Park et al., 2012a; Park et al., 2012b).

However, we need to better understand the mechanisms of the changes following stem cell transplantation before these findings can be translated into therapeutic approach in clinical practice. Thus, it was considered important to investigate in experimental studies whether modulation of the AD brain microenvironment could benefit effective regeneration to support cognitive functions.

AIMS

The main aim of this thesis was to investigate how the pathophysiological microenvironment in the AD brain affects stem cell neuronal differentiation and cholinergic signaling mechanisms, with implications for future regenerative therapies (Figure 8).

The specific objectives were the following:

Paper I To characterize the presence of different Aβ oligomer assemblies in postmortem brains of AD patients and to examine the relationship between these and the age of disease onset and cholinergic synaptic function.

Paper II To investigate the effects of NGF, and fibrillar and oligomeric Aβ on human embryonic stem cell-derived cholinergic neuronal development.

Paper III To study how Aβ-evoked inflammatory responses influence human embryonic stem cell differentiation and cholinergic signaling.

Paper IV To investigate how hippocampal stem cell transplantation in combination with the amyloid-modulatory drug (+)-phenserine or the α7 nAChR agonist JN403 affect endogenous neurogenesis and cognition in AD Tg2576 mice.

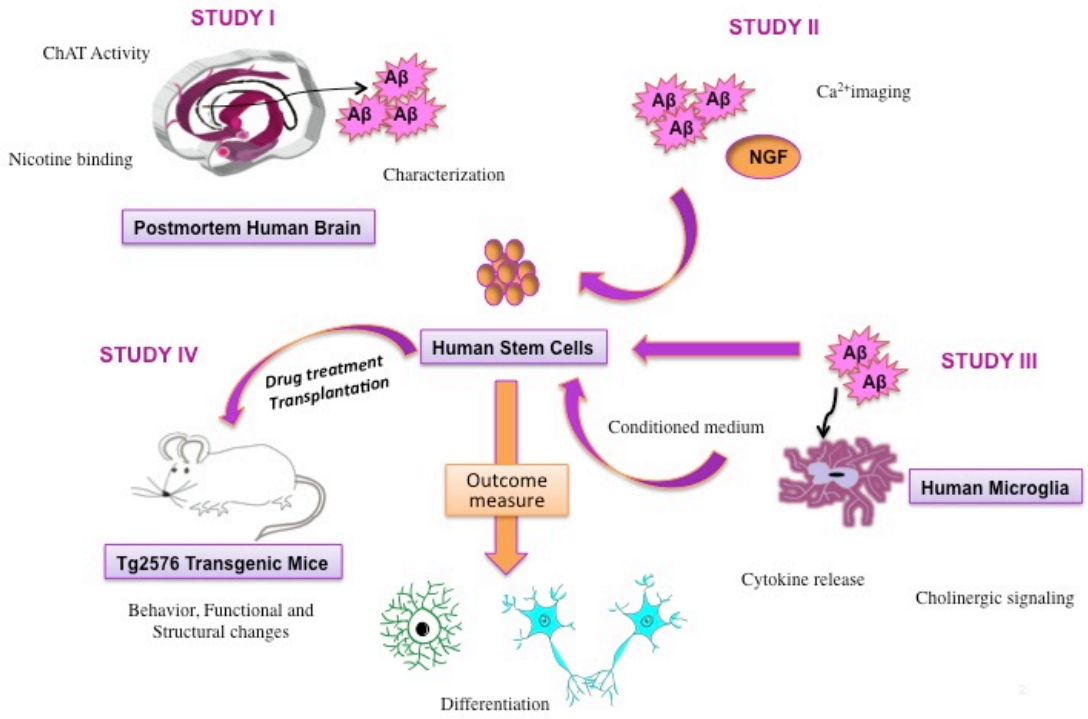


Figure 8. Schematic overview of the projects presented in this thesis. The pathophysiological environment in the Alzheimer disease (AD) brain may limit the use of therapies intended to stimulate neurogenesis. Taken together, these studies will improve understanding of how neuroplasticity and regenerative mechanisms can be stimulated in order to develop novel, effective treatment strategies for AD.

METHODOLOGICAL CONSIDERATIONS

This section contains a general discussion of the model systems and methods used in this thesis. Detailed descriptions of each experimental procedure are provided in the respective papers.

ETHICAL CONSIDERATIONS

Human brain tissue was obtained from the Brain Bank at Karolinska Institutet and the Netherlands Brain Bank, and permission to use autopsy brain material in experimental procedures was granted by the Regional Human Ethics committee in Stockholm and the Swedish Ministry of Health (S024/01).

The human embryonic stem cell lines HS293 and HS346 were derived from fresh, poor quality embryos that had been donated to the Fertility Unit of the Karolinska University Hospital, Huddinge, Sweden, for research. Informed consent was given by both partners after receiving oral and written descriptions of the study. The Ethics Board of Karolinska Institutet approved the derivation and research use of these lines (S454/02).

All experimental animal procedures were carried out in accordance with the guidelines provided by the Swedish National Board for Laboratory Animals. The ethical applications were approved for drug treatment, human stem cell transplantation, and Morris water maze (MWM) tests using Tg2576 mice (S53/10, S54/10 and S172/11).

OF MICE AND MEN - MODEL SYSTEMS USED

Postmortem human brain tissue

Although postmortem brain tissue represents the end stage of the disease, it allows the underlying molecular disease processes to be studied in patients with AD and compared with healthy controls. The postmortem delay should be kept as short as possible to best preserve the tissue for subsequent morphometric and biochemical studies, and substantial differences in postmortem delay between subjects need to be taken into consideration when selecting the tissue. In **paper I**, because ^{3}H-nicotine binding (to assess the density of nAChRs) and ChAT activity

were measured, it was important to take into consideration whether the subjects were smokers or had received drug treatment, as this could have confounded the results.

Human embryonic stem cells

The hES cell lines HS293 and HS346 used in ***papers II-III*** were derived at the Fertility Unit at the Karolinska University Hospital, Huddinge, Sweden. Each hES cell line was derived from one donor and was developed under special conditions. These hES lines have remained chromosomally stable after many (>100) passages; the karyotype of HS293 is 46, XY and that of HS346 is 46, XX. Although in theory all hES cell lines are the same, their propensity for differentiation, behavior and surface markers can differ (Adewumi et al., 2007; Cahan and Daley, 2013). It is therefore imperative to compare different stem cell lines to obtain comprehensive results and allow general conclusions to be drawn.

In order to exclude animal components, the cells were grown on human foreskin fibroblasts and were cultured in a commercially available serum-replacement medium (Hovatta et al., 2003). For neural induction, the cells were removed from the feeder layer and cultures were expanded in serum-free medium. The cells were propagated as free-floating neurospheres and mechanically passaged every 2-3 weeks. At this stage, the neurospheres constitute a mixture of NE and RG cells expressing nestin, Pax6, brain lipid-binding protein (BLBP) and GFAP, but they do not express the primitive endodermal marker α-fetoprotein or the mesodermal marker brachyury (Nilbratt et al., 2010).

Human neural stem cells

The human neural stem/progenitor cells (hNSCs) transplanted into the hippocampi of Tg2576 mice in ***paper IV*** were originally isolated from 18- to 22-week-old human fetal cortical tissue and were purchased from Lonza Walkersville, Inc. These cells, which were expanded in serum-free culture medium, can be readily differentiated into neurons and glial cells both *in vitro* and *in vivo* (Kwak et al., 2010; Kwak et al., 2011; Marutle et al., 2007). The hNSCs were cultured as free-floating neurospheres; the neurospheres are easily expandable *in vitro* and are

multipotent and do not form teratomas *in vivo.* These attractive features explain why we transplanted hNSCs instead of the hES cells used in ***papers II-III***.

Microglia

Microglia are the macrophages of the CNS and ultimately have myeloid origin (Chan et al., 2007). The human primary microglia used in ***paper III*** were derived at the National Institutes of Health, USA, and are commercially available for research via 3H Biomedical AB, Uppsala, Sweden. The cells were derived from the whole brain at gestation weeks 15-20, and cultured in proprietary medium for 1-2 weeks to enable selection of microglia. However, human microglia are difficult to derive and are easily contaminated with fibroblasts, and it is therefore important to monitor the cultures carefully for both morphology and expression of microglia specific markers.

Although the literature indicates that murine microglia are more commonly used, their properties differ from those of human microglia. For example, inducible nitric oxide synthase (iNOS) expression and NO production have been well established in rodent microglia but iNOS expression appears to be restricted to astrocytes in the human brain (Zhao et al., 1998). Such discrepancies may reflect species-specific responses, highlighting the importance of using human cell lines. However, one of the challenges of primary cell cultures is that the *in vitro* conditions could stimulate the highly sensitive microglia to acquire amoeboid morphology (they become spherical in shape, lack processes, and contain numerous phagocytic vacuoles), which does not necessarily represent their *in vivo* status (Boche et al., 2013; Streit, 2004).

Tg2576 mice

Mice expressing the APP Swedish mutation (APPSWE2576Kha; Tg2576), which were used in ***paper IV,*** were bred at the Karolinska Institutet animal care facility by backcrossing with B6SJL (F1) females (Taconic). Their genotype was determined using polymerase chain reaction (PCR) technology, and wild-type littermates served as control animals. All animals were housed in enriched cages with 12-hour light-dark cycles and *ad libitum* access to food and water.

Tg2576 mice express high levels of soluble oligomers in the brain and, at around 10 months of age, start to deposit Aβ plaques (Lithner et al., 2011; Mustafiz et al., 2011). These mice also exhibit reduced levels of the synaptic marker synaptophysin from 1 month of age, impaired memory performance from 6 months of age, and reduced neuronal maturation at 15-18 months of age (Lesne et al., 2006; Lilja et al., 2013b; Unger et al., 2005). They do, however, lack tangle pathology and neuronal loss in the brain, which are prominent features in the human AD brain.

Although no transgenic mouse model replicates the full spectrum of AD changes, the models provide a valuable *in vivo* model system for studying molecular pathological changes and the effects of various interventions, which are not feasible in living patients (Lithner et al., 2011). Animal models rely on the genetic mutations associated with FAD, based on the rationale that the downstream events of the initial trigger share common features (LaFerla and Green, 2012). Because of the differences between the mouse models and AD patients, findings from such studies need to be interpreted with caution.

In ***paper IV***, Tg2576 mice aged 7-9 months were used as a model system of the early pathological changes in AD in order to study regenerative mechanisms in the brain. We were also able to assess how regenerative processes could be modulated through paradigms involving pharmacological intervention and hNSC transplantation.

EXPERIMENTAL PROCEDURES

Aβ preparation and characterization

In ***paper I***, Aβ oligomers were extracted from postmortem autopsy brain tissue from AD patients and healthy subjects using a modified protocol as described earlier (Shankar et al., 2008). Frozen tissue was first homogenized in tris-buffered saline (TBS) to obtain a water-soluble fraction. The resulting pellet was re-homogenized after centrifugation in TBS-T extraction buffer to obtain membrane-associated Aβ oligomers, and the remaining pellet was then homogenized in guanidine-HCl extraction buffer. The different Aβ assemblies were assessed using

western blotting and Aβ oligomer-specific antibodies (Lambert et al., 1998). The identification and derivation of soluble Aβ assemblies are difficult, mainly because soluble Aβ assemblies are sensitive to the solutions and detection conditions used. There is also a risk that *in vitro* artifacts will be formed when extracting different Aβ assemblies, especially when using harsh extraction buffers such as guanidine-HCl.

In ***papers II-III***, recombinant oligomeric Aβ species were prepared by dissolving 1,1,1,3,3,3-hexafluoro-2-propanol (HFIP)-pretreated $Aβ_{1-40}$ or $Aβ_{1-42}$ (rPeptides, Bogart, GA, USA) in DMSO, followed by sonication and filtration. This protocol yields a high proportion of monomers, which then aggregate into larger Aβ assemblies that were assessed using western blotting in ***paper I***. To verify that the oligomeric Aβ did not aggregate into fibrillar Aβ species, a thioflavine T (ThT) fluorescence assay was performed. The ThT fluorescence of the samples was measured at 37°C in neural proliferation medium over 72 h, i.e. the same conditions as used in the cell culture experiments. The ThT assay is a widely accepted assay that can detect protein fibrillization over a period of time by measuring the intensity of fluorescence emitted when ThT binds to β-plated sheets.

Different experimental conditions, including the different aggregation states of Aβ that are generated, could influence the differentiation of hES cells. Such discrepancies should be taken into consideration when comparing results from different studies using recombinant or synthetic Aβ.

Quantitative gene expression

Reverse transcription combined with real-time quantitative PCR (qPCR) is a powerful method of quantifying gene expression. In ***paper II***, qPCR was used to detect differences in gene expression between groups receiving Aβ or NGF treatment and an untreated control group. One of the most commonly used methods of analyzing data from real-time qPCR experiments is relative quantification, which relates the PCR signal of the target transcript to that of another sample, such as one from a control group. To avoid confounding factors

that could influence the PCR signal, such as RNA input errors or reverse transcription efficacy errors, the target transcript is normalized to that of an internal control. The internal control should have a constant level of expression, which should ideally not change with the treatment. In ***paper II***, the 2-ΔΔCt method was used to calculate the relative gene expression from the real-time qPCR experiments (Livak and Schmittgen, 2001).

It is also important to consider the primer and probe design and their efficacy. The amplicon should be unique to the gene of interest and should preferably span exon-to-exon boundaries to be mRNA-specific. The primers should have close to 100 % efficiency, which can be easily tested prior to experiments by making dilution series. Analyzing the qPCR data using the ΔΔCt method is based on the assumption that the primers have 100 % efficiency.

Quantitative protein measurements

In ***papers I-II***, western blotting was used to detect and quantify different oligomeric Aβ assemblies. Western blotting separates proteins according to size and enables detection using specific antibodies. The method also permits quantification, although it is considered to be a semi-quantitative method. However, in some experimental settings, western blotting is the only available technique for quantifying different protein assemblies. For instance, in ***paper I***, in order to detect and quantify different oligomeric Aβ assemblies it was imperative to separate the aggregates according to size.

In ***papers III-IV***, enzyme-linked immunosorbent assays (ELISAs) were used to quantify protein expression. The ELISA is a conventional method of quantifying protein expression that is based on the capture and detection of an antigen using epitope-specific antibodies. A similar method, Meso Scale Discovery (MSD) technology, was used in ***paper III*** to detect cytokine secretion in the cell medium. MSD technology uses electrochemiluminescence detection of the captured antigen. The ruthenium-conjugated detection antibodies yield a luminescent signal upon electrochemical stimulation of the electrode surface of the microplate, which makes it highly sensitive. In comparison, ELISAs use

enzyme-linked detection antibodies to emit fluorescence upon addition of a substrate.

In ***papers II-III***, fluorescent immunocytochemistry was used to detect and quantify the number of glial cells, neurons, and neuronal subtypes after various treatments. The number of immunoreactive cells was quantified by manually counting >600 cells that had migrated and differentiated away from the sphere. In ***paper IV***, immunohistochemistry on mice coronal brain sections was employed to study the regional distribution of astrocytes and to quantify the number of α7 nAChR-expressing astrocytes in the hippocampus. The extent of neurogenesis in the DG of the hippocampus was also quantified by counting the number of DCX-positive cells. Double labeling with a human nuclei-specific antibody and a marker for either neurons or astrocytes was used to determine the fate of the grafted cells. To ensure comparable results between the different animals, 3 coronal sections from approximately the same hippocampal region were chosen for the different staining experiments.

Drug treatment

In ***paper IV,*** the amyloid-lowering neurotrophic drug (+)-phenserine or the partial α7 nAChR agonist JN403 was administered to Tg2576 mice in combination with intrahippocampal hNSC transplantation. (+)-Phenserine (25 mg/kg) or JN403 (0.3 mg/kg) were administered by intraperitoneal (i.p.) injection once daily for 5 weeks. To monitor for potential adverse drug reactions, JN403 was initially administered at dosages of 0.01 mg/kg (days 1-2) and 0.1 mg/kg (days 3-4) before reaching the full dose from day 5.

(+)-Phenserine is an APP synthesis inhibitor, and thus lowers Aβ levels (Lahiri et al., 2007; Shaw et al., 2001). Furthermore, (+)-phenserine has neurotropic and neuroprotective actions both *in vitro* (Lilja et al., 2013a) and *in vivo* (Lilja et al., 2013b; Marutle et al., 2007).

JN403 is a selective α7 nAChR partial agonist which has a significantly lower affinity for other subtypes of human nAChRs. Furthermore, *in vivo* pharmacological characteristics show good systemic exposure and brain

penetration, and acute administration of JN403 facilitates learning and memory in the social recognition test in mice (Feuerbach et al., 2009).

I.p. injections are commonly used to administer drugs to small laboratory rodents, since intravenous access can be challenging. Absorption of the drug is slower than after intravenous injection, and the bioavailability is lower. The primary route of absorption is into the mesenteric veins, which drain into the portal vein, and consequently the substance may undergo hepatic metabolism (Turner et al., 2011). The pharmacokinetics of drugs administered by i.p. injection are thus similar to those after oral administration, although i.p. injection is considered to be a parenteral route. The advantage, however, is the ease of administration, even when larger volumes are required.

Stem cell transplantation

In ***paper IV***, Tg2576 mice received bilateral hippocampal injections of 25 000 hNSCs per hemisphere. The animals were anesthetized using a constant flow of 4% isoflurane and kept warm under a heating lamp throughout the transplantation procedure. The head of the mouse was fixed using ear and tooth bars before a skin incision was made, to facilitate the location of the following coordinates relative to the bregma: AP -2.06, ML ±1.75, DV -1.75. Prior to the transplantation procedure, methylene blue was injected to verify that the coordinates targeted the DG of the hippocampus. The animals were monitored daily after the transplantation procedure to ensure recovery after surgery.

Stem cells have been reported to exert immunomodulatory actions (Kokaia et al., 2012). Immunosuppressive drugs may prevent graft rejections but the use of such substances could be inappropriate in studies where immunomodulatory effects may be of interest. Previous studies in APP overexpressing transgenic mice have indicated that immunosuppressive drugs are not necessary to ensure proper integration of the graft in time frames similar to that used in ***paper IV*** (Kwak et al., 2011; Marutle et al., 2007).

The number of hNSCs transplanted could influence the results when comparing studies using transplantation paradigms. However, a study in stroke-

damaged rats has demonstrated that transplanting a greater number of stem cells does not result in a greater number of surviving cells or increased neuronal differentiation (Darsalia et al., 2011).

Morris water maze

In ***paper IV,*** the MWM test was used to assess hippocampal-dependent spatial learning and memory following transplantation of hNSCs in Tg2576 mice***.*** The mice were randomly placed in one of four fixed positions around the wall of a circular 1 meter diameter swimming pool. During the acquisition period, the mice learned the location of a hidden platform by using visual cues on the walls around the pool. In order to assess hippocampal-dependent memory, the animals were subjected to a probe trial 24 hours after the last acquisition trial, in which the platform was removed and the escape latency was measured. The baseline acquisition values for escape latency were subtracted from the follow-up probe values (Δ latency) to evaluate differences in learning and memory between the groups. However, in the probe trial the standard measurement of memory was the time spent in the target quadrant compared with that spent in the other quadrants, rather than the latency to reach the target quadrant. The mice in ***paper IV*** spent only a short time in the target quadrant, thus showing a low persistence, which is not unusual for mice.

Ataxia, poor vision and impaired motor behavior are parameters that could influence the outcome of the MWM test, and such attributes should be carefully monitored prior to testing. One of the advantages of the MWM test is that it is a well-recognized hippocampal-dependent memory test that has been extensively studied in Tg2576 mice.

Statistics

GraphPad Prism 5.0 or 6 (GraphPad Software, Inc., La Jolla, CA, USA) was used for all univariate statistical analyses in ***papers I-IV***. In ***papers I*** and ***IV***, the data were assumed to have a non-gaussian distribution and were thus analyzed using the Mann-Whitney test for comparing two groups or the Kruskal-Wallis 1-way analysis of variance (ANOVA) by rank, followed by Dunn's *post hoc* test for

comparison among more than two groups. The Spearman rank correlation was used in correlation analysis, which was visualized graphically using simple regression analysis.

In the *in vitro* experiments in ***papers II*** and ***III,*** the unpaired Student's **t**-test was used for comparing two groups. ANOVA followed by Dunnett's *post hoc* test was used to compare more than two groups. The Z-test was used for differences in proportion for data from the calcium imaging experiment in ***paper II***.

Orthogonal projections to latent structures (SIMCA-P software, Umetrics AB, Umeå, Sweden), a method of multivariate data analysis, were employed to confirm the parameters differentiating the groups in ***paper I*** from each other (Wold et al., 2001). All multivariate analyses were performed using mean centering and unit variance scaling. Values were also log-transformed in order to acquire a more normal distribution of the data. In all papers, the data are presented as means ± standard errors of the mean (SEM). P-values <0.05 were considered to be significant.

RESULTS AND DISCUSSION

This section summarizes and discusses the main findings of the thesis. An in-depth description of all results can be found in the respective papers.

DIFFERENT Aβ ASSEMBLIES IN EOAD AND LOAD

Age is the most significant risk factor for AD (Kawas, 2003; Nussbaum and Ellis, 2003) and the contribution of the different Aβ oligomer assemblies in normal aging and during disease progression is not well understood. The main difference between early-onset AD (EOAD; disease onset <65 years) and late-onset AD (LOAD; disease onset >65 years) is that EOAD is associated with a more clinically aggressive disease progression compared to LOAD. In ***paper I,*** we extracted Aβ oligomers from postmortem frontal cortex brain tissue from AD patients and control subjects, and the various Aβ assemblies were studied using oligomer-specific antibodies. This study revealed that both pentamers (5mers) and decamers (10mers) in the soluble fraction were elevated in AD patients compared with controls. Intriguingly, we also detected various oligomeric species in non-demented subjects, indicating a distinct physiological role for Aβ oligomers. Recently, Aβ oligomers have been detected in cognitively intact individuals ranging from young children to the elderly, which is supportive of our findings (Lesne et al., 2013).

When stratifying the AD patients as EOAD or LOAD, there were significant differences in the Aβ oligomer assemblies detected. The levels of dodecamers (12mers) in the soluble fraction and pentamers in the insoluble fraction were higher in EOAD than in LOAD. It was noteworthy that we did not find any differences in ChAT activity, nAChR binding density, ^{3}H-PIB binding levels, or total levels of $A\beta_{1-40}$ or $A\beta_{1-42}$ between EOAD and LOAD. In addition, a PET study in EOAD patients reported regional glucose hypometabolism in the brain compared with LOAD patients, while comparable high ^{11}C-PIB uptake was observed in both groups (Rabinovici et al., 2010). An improved understanding of the pathological differences associated with the Aβ assemblies during the progression of disease may have implications for the development of therapeutic strategies for AD. Our findings indicate that patients with EOAD and LOAD may

need tailored anti-amyloid therapy, and that they should be stratified accordingly to achieve cognitive benefits in clinical studies. It is also tempting to speculate that the differences in the Aβ assemblies between EOAD and LOAD could have implications with respect to the plasticity of the brain, which could explain why some people develop AD at an early stage and some seem to have a more resilient brain, and are thus better equipped to cope with the pathological burden.

Aβ OLIGOMERS CORRELATE WITH IMPAIRED CHOLINERGIC ACTIVITY

Both synaptic loss and cognitive impairment show strong correlations with levels of soluble Aβ in AD (Lue et al., 1999; McLean et al., 1999). However, which of the different Aβ assemblies contribute to neuronal dysfunction and memory loss in AD is under heated debate, and a more comprehensive understanding of the pathophysiological role of Aβ oligomers will have important implications for the development of new treatment strategies.

The significant decrease in ChAT activity measured in ***paper I*** correlated with increased levels of pentamers in the soluble fraction, which further supports an essential role for distinct Aβ oligomers in AD pathogenesis. It has previously been shown that cholinergic dysfunction causes a rapid increase in APP in both the cortex and the CSF, leading to an amyloidogenic metabolism that contributes to neuropathology and cognitive dysfunction (Giacobini, 2002). This is thus indicative of synergistic effects of Aβ accumulation and impaired cholinergic transmission.

The substantial loss of nAChRs in AD patients in ***paper I*** correlated with increased ^{3}H-PIB binding. This observation corroborates earlier *in vivo* findings where brain areas with high fibrillar Aβ burdens also showed decreased density of nAChR binding sites (Kadir et al., 2006). These findings suggest that both oligomeric and fibrillar forms of Aβ induce impairment of the cholinergic system in AD pathogenesis.

CHOLINERGIC DIFFERENTIATION OF STEM CELLS

Elucidation of the molecular signals governing cholinergic neuronal development and dysfunction is essential for the development of drugs that could stimulate

regeneration and neuroprotection in the AD brain. In ***paper II,*** we generated and characterized BFCNs derived from hES cells by stimulating the cells with NGF, as previously described (Nilbratt et al., 2010). The levels of NGF decline during AD and BFCNs are dependent on NGF for maintenance of their cholinergic phenotype and synaptic integrity (Capsoni and Cattaneo, 2006; Cuello et al., 2010). In ***paper II,*** differentiation to BFCNs was more extensive in hES cells treated with NGF than in untreated hES cells. Significantly more NGF-exposed BFCNs responded to both ACh and KCl, reflecting an increase in the proportion of neurons expressing cholinergic receptors as well as voltage-gated calcium channels. Since then, only two additional protocols have been developed for the generation of BFCNs using hES cells and iPS cells (Bissonnette et al., 2011; Crompton et al., 2013; Duan et al., 2014). Although these two protocols both appear to generate robust populations of BFCNs, the use of mouse embryonic fibroblasts as a feeder layer could introduce variability in the cultures and complicate further therapeutic use of the cells. In comparison, our protocol is entirely xeno-free, thus providing a reproducible environment that offers the possibility of moving towards translational and clinical research.

EFFECTS OF Aβ ON STEM CELL DIFFERENTIATION, INFLAMMATION AND CHOLINERGIC SIGNALING

Neurogenesis is affected by inflammatory processes (Ekdahl et al., 2003; Monje et al., 2003), which may be linked to altered cholinergic signaling. Our findings from ***paper I*** suggested that distinct Aβ assemblies could induce impairment of the cholinergic system, which prompted us to investigate the impact of fibrillar and oligomeric Aβ on cholinergic signaling, inflammatory processes and neuronal development of hES cells (***papers II*** and ***III***).

Oligomeric Aβ impairs the differentiation of cholinergic neurons

The quantification of immunocytochemical staining in ***paper II*** showed that, following oligomeric $A\beta_{1\text{-}42}$ exposure, the proportion of βIII-tubulin-positive cells was similar to that in untreated hES cells. However, the number of MAP2-positive cells was decreased by oligomeric $A\beta_{1\text{-}42}$ exposure, indicating that the capacity of these cells to differentiate into mature neurons was reduced. Furthermore,

oligomeric $A\beta_{1-42}$ inhibited differentiation into BFCNs and significantly decreased the number of functional neurons, as deduced by intracellular Ca^{2+}-imaging. Exposure to oligomeric $A\beta_{1-40}$ impaired cholinergic differentiation, although the functional properties of the hES cell-derived neurons were improved compared to untreated cells. These findings suggest a physiological function for $A\beta_{1-40}$ regarding neuronal development, and indicate that the mechanism of action of $A\beta_{1-40}$ on stem cell differentiation may be different from that of $A\beta_{1-42}$. The findings from ***paper II*** also suggest that factors governing neurogenesis in the AD brain depend on the state of aggregation of Aβ. In combination, these findings suggest that oligomeric $A\beta_{1-42}$ could disrupt the maturation and function of newly formed neurons, and that progenitor cells existing in neurogenic regions in the brain may have a diminished capacity for regeneration in AD pathogenesis. Therefore, targeting the microenvironment with growth factor support or pharmacological treatment could promote survival and maturation of stem cell-derived neurons. However, anti-amyloid therapy targeting both $A\beta_{1-40}$ and $A\beta_{1-42}$ may not be a suitable strategy, given the possible supportive role for $A\beta_{1-40}$ in stem cell differentiation.

Fibrillar Aβ shifts the balance of ACh synthesis and degradation

It has been hypothesized that Aβ accumulation disrupts normal inflammatory regulation in the brain by increasing the activity of the ACh hydrolyzing enzyme BuChE (Darreh-Shori et al., 2011a; Darreh-Shori et al., 2011b; Darreh-Shori et al., 2009a; Darreh-Shori et al., 2009b).

In ***paper III***, fibrillar Aβ caused a time-dependent reduction in the release of ChAT, while the secreted levels of the ACh-degrading enzyme BuChE from differentiating neurospheres increased highly significantly. In contrast, oligomeric Aβ did not influence the secretion of these proteins. Thus, prolonged exposure to fibrillar Aβ caused a pronounced shift in the ACh regulatory machinery that favored maintenance of low levels of ACh. Furthermore, a comparison between the levels of functional BuChE and the levels of total BuChE protein suggested that fibrillar Aβ induced a hyperactive phenotype of BuChE, a phenomenon that has also been reported in the CSF of AD patients (Darreh-Shori et al., 2011a; Darreh-

Shori et al., 2011b; Darreh-Shori et al., 2012). Given that ACh is expected to exert suppressive, anti-inflammatory effects on immunogenic cells such as the cholinoceptive astroglial cells, altered levels of these cholinergic enzymes could promote an environment favoring increased secretion of inflammatory mediators, with repercussions for neurogenesis.

Fibrillar Aβ promotes glial differentiation and inflammatory mechanisms

In ***paper III***, we found that neurospheres derived from hES cells secreted the pro-inflammatory cytokines IL-1β, TNFα, INFγ, IL-6 , IL-2 and IL-10. A transient increase in IL-2 and IL-1β levels was observed after 20 days of differentiation following fibrillar Aβ treatment, indicating that these signaling molecules may have a role in the fate-commitment of the cells. After 29 days of differentiation, IL-6 levels had increased over 20-fold following fibrillar Aβ exposure. In contrast, oligomeric Aβ treatment did not affect the secretion of cytokines from neurospheres.

The increased secretion of cytokines coincided with reduced neuronal differentiation of the neurospheres, and increased numbers of stem cell-derived glial cells. In ***paper II,*** an increase in the gene expression of the glial marker GFAP was demonstrated following fibrillar Aβ exposure. Furthermore, in ***papers II*** and ***III,*** immunocytochemical and morphological examinations of the differentiating neurospheres showed an increased number of cells expressing GFAP and a reduced number of βIII-tubulin-positive cells. In ***paper III***, we also demonstrated a transient increase in the levels of S100B, an astrocytic secretory protein with cytokine-like properties, while the levels of GFAP steadily increased throughout the course of differentiation following fibrillar Aβ exposure. These findings suggest either that the secreted cytokines drive the differentiation of neurospheres toward gliogenesis or that increased secretion of the cytokines is a consequence of the increased gliogenesis induced by fibrillar Aβ treatment.

Our observations lead us to propose a mechanism whereby fibrillar Aβ increases glial differentiation by promoting a microenvironment favoring hypo-cholinergic signaling and increased cytokine secretion.

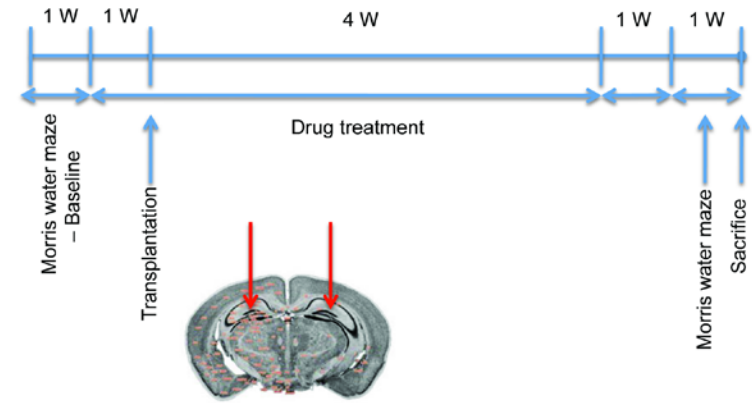
Fibrillar Aβ reduces cytokine secretion from microglia

Different aggregation forms of Aβ can activate microglia, with consequential neurodegenerative effects (Garcao et al., 2006; Maezawa et al., 2011). Microglia are also implicated in the regulation of postnatal neurogenesis (Walton et al., 2006). Thus, we were interested in whether Aβ altered innate inflammatory responses from human microglia. In ***paper III***, examination of the cytokine secretion profile revealed that exposure of oligomeric Aβ to microglia reduced the secretion of IL-1β, although levels of the other cytokines assessed (including IFNγ, TNFα, IL-2, IL-10, and IL-6) remained unchanged. Fibrillar Aβ reduced the secretion of TNFα, IL-1β, IL-2, and IL-10 after 48 hours exposure.

Exposure of the differentiating neurospheres to conditioned medium from microglia treated with fibrillar Aβ promoted differentiation of glial cells, while simultaneously impairing maturation of neurons. These findings suggest that suppressing the basal physiological secretion of cytokines from microglia could alter neuronal differentiation in favor of gliogenesis, possibly reflecting the dual function of the innate immune system in modulating cell genesis and repair. Alternatively or additionally, these findings may point to a yet unidentified factor(s) in the conditioned medium that compromises the neuronal differentiation of neurospheres.

COMBINING DRUG TREATMENT AND STEM CELL TRANSPLANTATION

The findings presented so far clearly indicate that the pathophysiological environment affects neurogenesis. A recent study has indicated that there is a regenerative window in Tg2576 mice and that endogenous neurogenesis can be enhanced by treatment with the amyloid-modulatory neurotrophic drug (+)-phenserine (Lilja et al., 2013b). In ***paper IV,*** the aim was to investigate whether altering the brain microenvironment using (+)-phenserine or the partial α7 nAChR agonist JN403, in combination with hippocampal stem cell transplantation, could improve neurogenesis and cognition in young Tg2576 mice (representing the early stages of AD) (Figure 9).



Treatment groups
Vehicle injected + saline (SHAM + SAL) n = 9
Human neural stem cells + saline (HNSC + SAL) n = 8
Human neural stem cells + JN403 (HNSC + JN) n = 5
Human neural stem cells + (+)-phenserine (HNSC + PHEN) n= 6

Figure 9. Schematic overview of the study design in ***paper IV***.

Hippocampal stem cell transplantation improves neurogenesis and cognition

Bilateral hippocampal transplantation of hNSCs in 6- to 9-month-old Tg2576 mice increased the number of DCX-positive cells in the DG. The DCX-positive cells were found exclusively in the subgranular layer, whereas hNuclei-positive cells, a marker for the grafted hNSCs, were found in the polymorph layer of the DG, indicating that the hNuclei-positive cells were derived from the transplanted cells, whereas the DCX-positive cells were mainly derived from the endogenous stem cell pool. Furthermore, the increase in endogenous neurogenesis was associated with improved hippocampal-dependent memory, as assessed by the MWM. These findings are in line with other studies, which have reported memory improvement following transplantation of murine stem cells (Ben Menachem Zidon et al., 2013; Blurton-Jones et al., 2009).

The amyloid-lowering drug (+)-phenserine interferes with stem cell transplantation-induced neurogenesis and cognition

Surprisingly, we found that administration of (+)-phenserine prevented the hNSC-induced increase in endogenous neurogenesis and memory improvement in Tg2576 mice. In an earlier study, (+)-phenserine induced increased neuronal differentiation of transplanted hNSCs in the brains of AD APP23 transgenic mice

(Marutle et al., 2007), although no functional outcome was assessed in that study. Intriguingly, in ***paper IV,*** the grafted cells survived for longer and neuronal differentiation increased in Tg2576 mice following (+)-phenserine treatment (Figure 10), but this failed to translate into cognitive benefits.

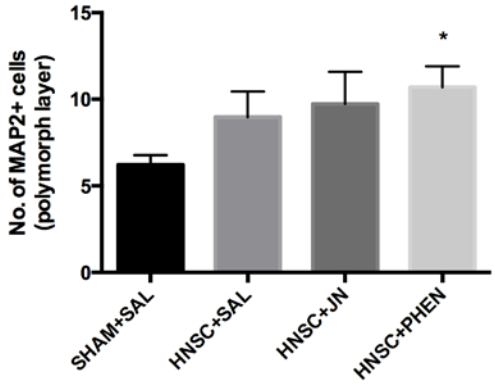


Figure 10. The total number of MAP2-positive cells co-located with hNuclei-positive cells, following transplantation of hNSCs into the dentate gyrus of Tg2576 mice. For abbreviations, see Figure 9.

In vitro, (+)-phenserine exerts prosurvival effects on progenitor cell populations; both the mitogen-activated protein kinase (MAPK) and phosphokinase C signaling pathways are reportedly mediators of these neurotrophic actions (Lilja et al., 2013a). MAPK and the downstream transcription factor cAMP response element-binding protein (CREB) are important regulators of BDNF (Autry and Monteggia, 2012; Lu et al., 2008) and other key regulators of adult neurogenesis (Faigle and Song, 2013). It is plausible that hNSCs exert their beneficial effects on neurogenesis and memory through signaling cascades regulating trophic support, which has been shown previously following murine stem cell transplantation (Blurton-Jones et al., 2009). Thus, (+)-phenserine co-administration could interfere with hNSC signaling cascades, inhibiting the neurotrophic effects of these cells on the brain environment that supports neurogenesis in Tg2576 mice. However, further studies are needed to support this hypothesis.

The α7 nAChR agonist JN403 impairs neurogenesis by down-regulating α7 nAChR-expressing astrocytes

The α7 nAChRs are involved in both neuroprotective (Kihara et al., 1997; Liu and Zhao, 2004) and inflammatory processes (Shytle et al., 2004; Tracey, 2002; Tracey, 2009). Although the α7 nAChRs are widely distributed in the hippocampus (Court et al., 1997), it is not clear whether the beneficial responses of agonist stimulation are mediated by the α7 nAChRs expressed on astrocytes, the α7 nAChRs expressed on neurons, or a combination of the two. In ***paper IV***, we observed a high number of α7 nAChR-expressing astrocytes surrounding the injection site in the DG of Tg2576 mice. In addition, a positive correlation was found between the numbers of α7 nAChR-expressing astrocytes and the numbers of DCX-positive neurons in the DG. These findings indicate that this subclass of astrocytes is involved in repair processes and tissue remodeling in the brain.

In ***paper IV,*** JN403 prevented the beneficial effects of hNSC transplantation on neurogenesis and memory. In fact, JN403 actually reduced the number of DCX-positive neurons compared with the group receiving hNCS transplantation only. Furthermore, JN403 reduced the number of α7 nAChR-expressing astrocytes in the DG, possibly suppressing their normal physiological and neuroprotective functions. It should be mentioned, however, that α7 nAChR expression on the neurons was not quantified in this study and therefore it cannot be excluded that JN403 exerted antagonistic functions through mechanisms involving neuronal α7 nAChRs. Since α7 nAChRs are easily desensitized, the dosing regimen or the long treatment period in ***paper IV*** could possibly have had a desensitizing effect, thus becoming contra-therapeutic in this particular setting. It is therefore possible that a different dosing regimen and/or treatment period could have had the opposite effect to that shown in this study, which would be interesting to investigate in the future. In addition, α7 nAChRs are expressed on human stem cells (as shown in ***paper II***), where they probably have effects on cholinergic signaling, which may affect stem cell survival and differentiation.

In conclusion, our findings indicate that α7 nAChR-expressing astrocytes may be an important component of the neurogenic niche in the brain, and should be considered further in the development of therapeutic strategies for AD.

CONCLUDING REMARKS AND FUTURE PERSPECTIVE

The main focus of this thesis was the study of the influence of the pathological microenvironment in the AD brain on stem cell regeneration and plasticity, with associated implications for finding targets for novel intervention strategies.

STEM CELL DIFFERENTIATION IS AFFECTED BY AD PATHOLOGY

The findings presented in this thesis clearly show that different aggregation forms of Aβ can induce impairments in both cholinergic activity and cholinergic neuronal differentiation. The hippocampus receives robust cholinergic innervations and stem cells express cholinergic receptors and enzymes, indicating that cholinergic signaling can regulate adult neurogenesis. Given that the accumulation of Aβ occurs between 10 and 20 years before the onset of clinical AD symptoms, subtle changes in the plasticity of the cholinergic system could thus occur early in the disease process, which could affect regenerative processes that then diminish with increasing pathology. Furthermore, hNSC transplantation stimulates endogenous neurogenesis in young AD Tg2576 mice (when the pathological burden in the brain is low). This may imply that regenerative processes could be induced in preclinical AD or in MCI patients, by applying disease-modifying strategies (those that include regenerative approaches) early in the AD pathological process (Figure 11).

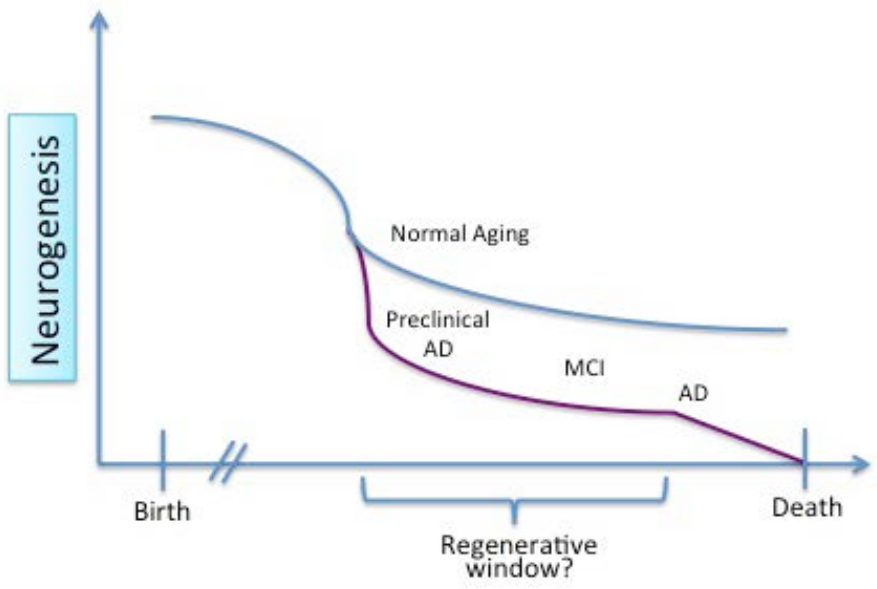


Figure 11. Tentative time-line for establishing regenerative therapies for AD.

TARGETS FOR REGENERATIVE THERAPIES IN AD

NGF treatment enhances cholinergic neuronal differentiation and may thus enhance plasticity in the cholinergic system in the AD brain. However, since oligomeric Aβ impairs the function of new neurons and fibrillar Aβ alters inflammatory processes and stimulates glial differentiation, further studies are warranted to investigate whether NGF treatment can sufficiently enhance cholinergic regeneration and plasticity mechanisms in the presence of AD pathology.

My findings also indicate that suppressing the basal physiological secretion of cytokines could impair neuronal differentiation. In addition, it was found that α7 nAChR-expressing astrocytes may represent an inflammatory component that promotes neurogenesis through their involvement in processes that support tissue remodeling and repair. Thus, broad anti-inflammatory therapies may reduce unwanted inflammation but may also be contra-therapeutic by impeding the beneficial functions of inflammation.

It is thus proposed that regenerative therapies for AD must also target the pathological burden of the disease for there to be long-term benefits in cognitive functioning. The results presented in this thesis indicate that targeting growth factor depletion, decreasing $A\beta_{1\text{-}42}$ load, and stimulating α7 nAChR-expressing astrocytes could lead to disease-modifying therapies that will enhance regeneration in the brain (Figure 12). However, such putative approaches need to be further explored in future studies.

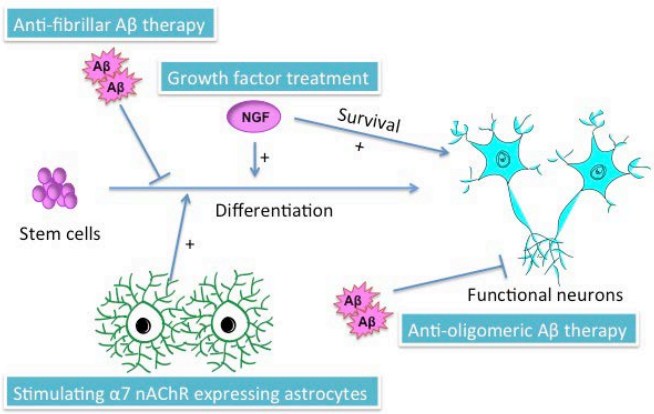


Figure 12. Regenerative targets for disease-modifying therapies in AD.

POTENTIAL AND PITFALLS - REGENERATIVE STUDIES IN AD

The use of human stem cells for studying neurogenesis in AD presents several challenges and limitations. Mimicking AD pathology *in vitro* only replicates some aspects of the microenvironment in the AD brain. The discovery of iPS cells has revolutionized the field of regenerative medicine and has generated unique cellular model systems obtained from living FAD and sporadic AD patients that enable detailed study of neurogenesis in relation to patient-specific disease mechanisms. Initial studies have focused on FAD-iPS cells, especially regarding APP and tau metabolism and related signaling pathways (Doege and Abeliovich, 2014). The derivation of neurons using iPS cell reprograming technologies could provide a source of neuronal cells for replacement therapies in those afflicted with or at risk of developing AD, and for testing novel drugs with potential for disease-modifying effects, which is likely to accelerate new drug discovery.

Although such model systems will play a central role in regenerative medicine, several questions remain to be addressed in future studies. The foremost of these questions is: do iPS cells from FAD and sporadic AD patients show a deviant pattern of differentiation or is it the local microenvironment in the AD brain, generated by a plethora of cell types, that affects neurogenesis? If the latter is true, it implies that hES cells or hNSCs will be equally good models for regenerative studies in AD. In fact, hES cells have been extensively studied and defined xeno-free and feeder-free culture systems have been developed, offering phenotype stability (Rodin et al., 2014). A recent report has also shown efficient derivation of iPS cells under xeno-free and feeder-free conditions (Nakagawa et al., 2014). However, the use of the reprograming factors still leads to some safety concerns, especially with regard to long-term epigenetic and genetic stability, as well as the high tumorogenic potential of these cells. These could all have implications for cell replacement therapies in clinical practice (Forsberg and Hovatta, 2012). Before such safety issues can be adequately addressed, hES cells are likely to continue to play an important role in regenerative studies in AD, especially with regard to the evolvement of cell transplantation paradigms.

FROM BENCH TO BEDSIDE – ADVANCING STEM CELL THERAPIES TO THE CLINIC

A translational approach using hES cells, AD-iPS cells, and animal models of AD could aid the search for novel drug targets that stimulate regeneration in the AD brain. However, the current limited knowledge regarding *in vivo* neurogenesis during the disease course highlights the need to develop biomarkers that can effectively measure and evaluate neurogenic processes in the brain and the efficacy of regenerative therapies.

This thesis shows that grafted hNSCs interact with the local microenvironment and exert beneficial effects through stimulating endogenous neurogenesis, which holds great therapeutic promise. Although safety issues regarding stem cell transplantations remain to be resolved, the question remains: which patients would benefit most from such therapies? If regenerative therapies must be introduced early for long-term beneficial effects, we need to effectively predict which patients will develop AD. Thus, carriers of known FAD mutations may be a candidate group for proof-of-concept regenerative studies.

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