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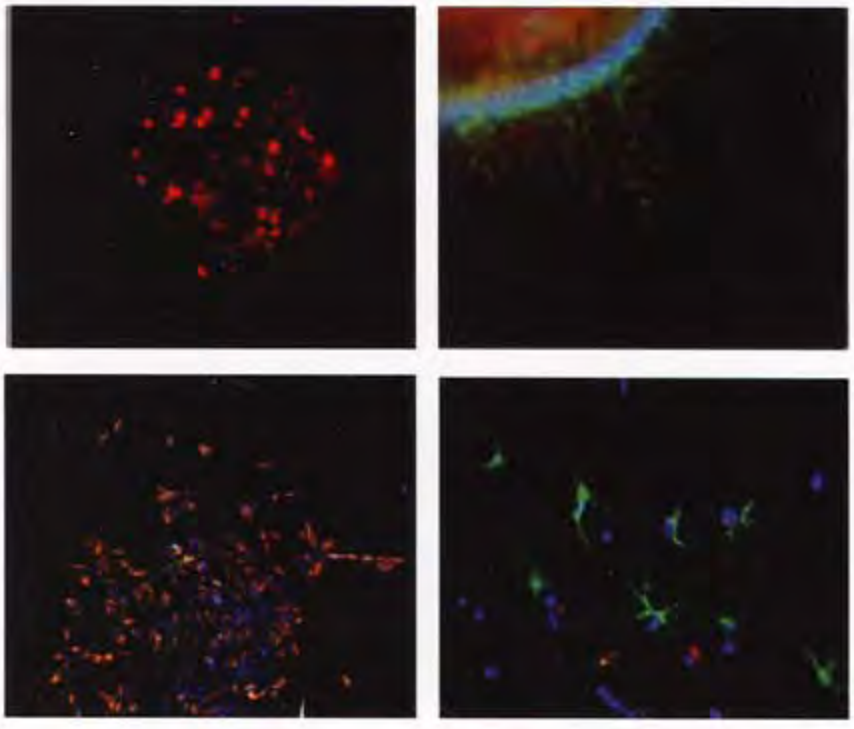
Stem Cell Differentiation, Plasticity and Regenerative Mechanisms in the Cholinergic System – Implications for Alzheimer´s Disease



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STEM CELL DIFFERENTIATION, PLASTICITY AND REGENERATIVE MECHANISMS IN THE CHOLINERGIC SYSTEM – IMPLICATIONS FOR ALZHEIMER´S DISEASE

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To my family for their love and support

ABSTRACT

Stem cells are immature cells with self-renewal capacity. They are able to differentiate into multiple lineages that may serve as a source of expandable cells for various applications. To generate cell populations of a specific lineage, it is crucial to understand the regulatory role of local environmental cues, intrinsic factors and signaling pathways that control their cellular phenotype.

The main purposes of the present work were to examine factors that act as induction signals and modulate neuronal differentiation and plasticity, especially as regards the cholinergic phenotype, and also to examine possible interactive mechanisms between neurotrophic factors, cholinergic receptors and the amyloid precursor protein (APP) in stem cell biology.

Human embryonic stem (hES) cells from six different lines were used to evaluate defined conditions for neural differentiation in adherent and suspension culture systems. In serum-free and feeder-free cultures, a dynamic transition into neuroepithelial and radial glial cells was observed, and these neurogenic cells finally gave rise to neurons. For these lines of hES cells, a similar progression of neural differentiation occurred, suggesting that hES cells may serve as an important model system to study early neuronal developmental processes.

Alzheimer´s disease (AD) is associated with amyloid plaques, neurofibrillary tangles, and a marked loss of basal forebrain cholinergic neurons and neuronal nicotinic acetylcholine receptors (nAChRs) expressed on these neurons.

Examination of the subregional lineage of hES cell-derived neurons revealed expression of transcription factors specifying telencephalic neuronal identity. Moreover, these cells also expressed α3, α4 and α7 nAChR subunits and M1, M2 and M3 muscarinic receptor subtypes. Upon stimulation with neurotrophic factors, including brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT3), ciliary neurotrophic factor (CNTF) and nerve growth factor (NGF), the expression of the cholinergic enzyme choline acetyltransferase was increased, which was also observed in the septal SN56 cell line following treatment with retinoic acid (RA). Additionally, RA treatment upregulated the expression of α3 but reduced the level of α4 nAChR subunits in SN56 cells.

Stimulation with neurotrophic factors also upregulated the α3 nAChR subunit and M3 mAChR subtype in hES cell-derived neurons, suggesting differential regulation of various AChR subtypes in both septal SN56 and hES cell-derived neurons. The expression of functional cholinergic receptors was demonstrated by a calcium increase evoked by acetylcholine (ACh), and the proportion of responding cells was dependent on the concentration of ACh, which suggests that multiple subtypes of cholinergic receptors were expressed in hES cell-derived neurons.

An understanding of stem cell biology during pathological conditions is crucial for future stem cell-based therapeutical approaches. As disturbances in the microenvironment due to brain injury or pathology could affect the microenvironmental equilibrium, the cells may be exposed to cues that are different from those in their normal conditions that may ultimately affect their phenotype and function. To study this, APP23 transgenic mice were treated with the novel drug (+)-phenserine, which reduced the expression of APP. This treatment also increased the neuronal differentiation of transplanted human neural precursor cells (NPCs), whereas astrocytic differentiation was reduced, indicating a role for APP as cell fate determinant of NPCs *in vivo.* These findings suggest that a combined treatment and transplantation approach may be necessary in order to further examine the potential of transplantable cells for developing future cell-based treatment strategies of neurodegenerative diseases, including AD.

In conclusion, the findings in the present study demonstrate that hES cells may serve as an important model system to study cellular mechanisms in early human neural development. Exploring the mechanisms of action of RA, BDNF, NT3, CNTF and NGF, which act as inductive signals for differentiation processes and plasticity of hES cells, NPCs and cholinergic neurons, may also lead to the development of novel strategies for future therapeutic interventions in AD.

LIST OF PUBLICATIONS

I. Nilbratt M., Friberg L., Mousavi M., Marutle A., Nordberg A.
Retinoic acid and nerve growth factor induce differential regulation of nicotinic acetylcholine receptor subunit expression in SN56 cells.
J Neurosci Res. (2007) 85:504-14.

II. Nat R., Nilbratt M., Narkilahti S., Winblad B., Hovatta O., Nordberg A.
Neurogenic neuroepithelial and radial glial cells generated from six human embryonic stem cell lines in serum-free suspension and adherent cultures.
Glia. (2007) 55:385-99.

III. Nilbratt M., Porras O., Marutle A., Hovatta O., Nordberg A.
Neurotrophic factors promote cholinergic differentiation in human embryonic stem cell-derived neurons.
Submitted manuscript. (2007).

IV. Marutle A., Ohmitsu M., Nilbratt M., Greig N.H., Nordberg A., Sugaya K.
Modulation of human neural stem cell differentiation in Alzheimer (APP23) transgenic mice by phenserine.
Proc Natl Acad Sci U S A. (2007). 104:12506-11.

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LIST OF ABBREVIATIONS

Aβ	Amyloid β-peptide
ACh	Acetylcholine
AChE	Acetylcholinesterase
AChEI	Acetylcholinesterase inhibitor
AD	Alzheimer´s disease
ApoE	Apolipoprotein E
APP	Amyloid precursor protein
BDNF	Brain-derived neurotrophic factor
bFGF	Basic fibroblast growth factor
BLBP	Brain lipid-binding protein
BMP	Bone morphogenetic protein
BrdU	Bromo-deoxyuridine
BTX	Bungarotoxin
BuChE	Butyrylcholinesterase
ChAT	Choline acetyltransferase
ChEI	Cholinesterase inhibitor
CNS	Central nervous system
CNTF	Ciliary neurotrophic factor
EGF	Epidermal growth factor
ERK	Extracellular signal-regulated kinase
FAD	Familiar Alzheimer´s disease
FBS	Fetal bovine serum
GABA	γ-aminobutyric acid
GFAP	Glial fibrillary acidic protein
GLAST	Astrocyte-specific glutamate transporter
hES	Human embryonic stem
ICM	Inner cell mass
IP_3	Inositol 1,4,5-triphosphate
LGE	Lateral ganglionic eminence
LIF	Leukemia inhibitory factor
LTP	Long-term potentiation
mAChR	Muscarinic acetylcholine receptor
MAP2	Microtubule-associated protein 2
MAPK	Mitogen-activated protein kinase
MGE	Medial ganglionic eminence
nAChR	Nicotinic acetylcholine receptor
NFT	Neurofibrillary tangle
NGF	Nerve growth factor
NMDA	N-methyl-d-aspartate
NPC	Neural precursor cell
NT	Neurotrophin
Oct-4	Octamer binding protein 4
$p75^{NTR}$	p75 neurotrophin receptor

PD	Parkinson´s disease
PET	Positron emission tomography
PI3K	Phosphatidylinositol-3 kinase
PS	Presenilin
RA	Retinoic acid
RAR	Retinoic acid receptor
RXR	Retinoid X receptor
Trk	Tyrosine receptor kinase

1 INTRODUCTION

1.1 THE CHOLINERGIC SYSTEM

The cholinergic system in the brain has a central role in cognitive functions such as attention, learning and memory. The group of cholinergic neurons in the basal forebrain extends from the medial septal region through the nucleus basalis of Meynert, and provides most of the cholinergic innervation to the hippocampus and neocortex[1].

In the central nervous system (CNS), the neurotransmitter acetylcholine (ACh) is synthesized by the enzyme choline acetyltransferase (ChAT) and interacts with the muscarinic acetylcholine receptor (mAChR) and the nicotinic acetylcholine receptor (nAChR) **(Fig. 1)**. Acetylcholine can also be hydrolyzed to choline and acetate in the synaptic cleft by cholinesterase. Two different forms of cholinesterase exist, acetylcholinesterase (AChE) and butyrylcholinesterase (BuChE), of which AChE is the more prominent esterase in the CNS. It is found in nervous tissue, plasma, blood cells and muscles, whereas BuChE, which originates from glial cells, is found in neurons and is also common in serum.

Several diseases in the CNS are associated with deficits in the cholinergic system such as Alzheimer´s disease (AD), Parkinson´s disease (PD), Tourett´s syndrome, epilepsy and schizophrenia[2].

1.1.1 Nicotinic acetylcholine receptors

Neuronal nicotinic acetylcholine receptors (nAChRs) are associated with various transmission activities and receptor activation modulates the release of several transmitters including ACh, glutamate, γ-aminobutyric acid (GABA), noradrenalin, and dopamine[3-5]. They belong to a gene superfamily of ligand-gated ion channels, which also includes glycine-, 5-hydroxytryptamine- and $GABA_A$ receptors[6]. All members of this superfamily have a pentameric subunit arrangement. The subunits of nAChRs are highly conserved during evolution[7] and the receptors have various locations on the neurons (axonal, preterminal, presynaptic and postsynaptic). Up to now, 12 genes encoding different nAChR subunits have been cloned[8]. The different subunits have been classified into two subfamilies of nine α (α2-α10) and three β subunits (β2-β4). The receptors are composed of five subunits, either as homomeric (α7-α9) or heteromeric receptors with distinct functional properties and tissue-

specific distributions[9, 10]. Depending on various subunit combinations, compounds bind to the receptors with different affinity. In autoradiographic studies, the distribution of nAChRs in the human brain has been mapped utilizing several radioligands including [^{125}I]α-bungarotoxin ([^{125}I]-αBTX), [^{3}H]-ACh, [^{3}H]-cytosine, [^{3}H]-epibatidine and [^{3}H]-nicotine[11]. Agonist potency varies with different compositions of receptors. In the brain the majority of high affinity nAChRs comprise the α4 (together with β2) and α3 subunits[12]. The other major nAChR subtype is the homomeric α7 receptor which has been examined by using [^{125}I]-αBTX. The α7 receptor has been detected in high densities in the substantia nigra and the hippocampus[13], whereas binding studies involving the use of [^{3}H]-epibatidine and [^{3}H]-nicotine, revealed that the density of α4β2 nAChR was highest in the cerebral cortex and thalamus[14, 15].

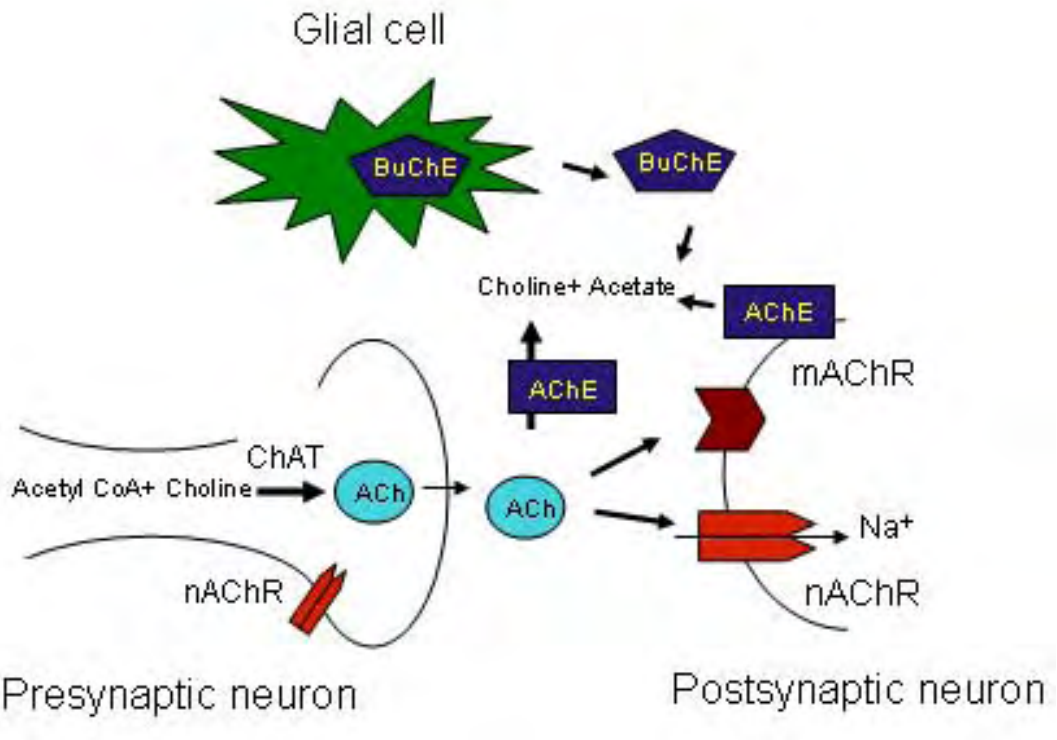

Fig. 1. Schematic drawing of the cholinergic synapse.

1.1.2 Muscarinic acetylcholine receptors

The muscarinic acetylcholine receptors (mAChRs) belong to the superfamily of G-protein-coupled receptors that include receptors for a variety of neurotransmitters. Five different subtypes of mAChR (M1-M5), each encoded by a different gene, have been identified in the CNS[16]

By interacting with heterotrimeric G-proteins, the mAChRs trigger a serie of cellular responses, leading to changes in second messenger levels due to activation of phospholipase C (PLC) and formation of inositol 1,4,5-triphosphate (IP_3), or through reduction of cAMP levels as a result of inhibition of adenylate cyclase, and changes in ionic conductances due to activation of K^+ channels.

The mAChR subtypes can be divided into two categories depending on whether they cause a stimulation of phosphoinositide hydrolysis (M1, M3 and M5) or inhibition of adenylate cyclase activity (M2 and M4)[16]. Muscarinic AChRs are widely distributed throughout the body and responsible for functions in the peripheral autonomic nervous system and brain. In the brain, the more prominent subtypes are the M1 and M4 subtypes, which have been detected in the cerebral cortex, striatum and hippocampus[17, 18]. Consistent with this distribution, mAChRs are implicated in learning and memory processes[19].

1.1.3 Cholinergic receptors in development and aging

In mouse cerebral cortex, functional nAChRs have been detected as early as embryonic day 10 (E10). In these cortical cells, α3, α4 and α7 nAChR subunits were found and it was shown that the nAChRs could mediate calcium signals[20]. In particular, the α7 nAChR subtype has high permeability to calcium[21], and since calcium signaling is known to affect a variety of developmental processes including cell proliferation, neurite outgrowth and retraction[22-25], these receptors may have important functions in development and plasticity[26]. In rats, an increased expression of α7 receptors conincides with synapse formations[27].

In human fetal brain, various types of nAChR are present during the first 8 weeks[28], suggesting important roles for nAChRs during early brain development. The distribution of subunits varies according to different brain regions and with age.

The number of [^{125}I]-αBTX binding sites and the mRNA level of the α7 nAChR subunit correlate with age in several brain regions during the development[29]. It has

also been found that the expression of α7 nAChR was significant higher in some regions in the fetal brain compared with aged brain[30]. Furthermore, age-dependent reduction in the population of [^{3}H]-epibatidine binding sites in the cerebellum, and frontal and temporal cortices has been observed[14].

Muscarinic AChRs are widely expressed in embryonic cells[31], and before gestational day 14 in fetal rat brain they appear to regulate neural cell proliferation and differentiation[32]. In neuronal progenitors, the expression of these receptors occurs prior to the onset of synaptogenesis and neurotransmission[33]. Stimulation with the mAChR agonist carbachol stimulated DNA synthesis by activation of extracellular-regulated kinases (ERK1/2) and phosphatidylinositol-3 kinase (PI3K) in bFGF-expanded neural progenitor cells dissociated from rat cortical neuroepithelium[34]. The activation of ERK1/2 is also correlated with long-term potentiation (LTP) and synaptic plasticity[35]. In human brain, age-correlated reduction of M1 and M2 mAChR subtypes has been found in the cerebral cortex, whereas there was an increase of these receptor subtypes in the thalamus, indicating an age-dependent alteration in the population of mAChRs[36].

1.2 ALZHEIMER´S DISEASE

Alzheimer´s disease is the most common neurodegenerative disorder and is associated with a gradual decline of several cognitive functions. The major risk factor for AD is age. The clinical diagnosis of AD is currently based on the outcome of several examinations including medical history, cognitive tests and neurological investigations.

1.2.1 Genetic risk factors of Alzheimer´s disease

The sporadic form of AD is the most common[37]. However, there are also familial forms of AD (FAD) that are inherited as autosomal dominant disorders with nearly 100% penetrance[38]. Certain mutations are associated with FAD, and they are located near or within the sequence of the APP gene which encodes Aβ on chromosome 21[39]. These point mutations cause different clinical phenotypes and a few of them are the "Dutch type"[40], the "Flemish type"[41], the "London type"[39], the "Arctic type"[42], the "Australian type"[43], the "Belgian type"[44], the "German type"[45] and the "Swedish type"[46] of FAD. Swedish FAD is caused by a double mutation (Lys to Asn at residue 595 plus Met to Leu at position 596), and is associated with elevated production of

APP[47], elevated levels of $A\beta_{1-40}$ and $A\beta_{1-42}$ and deficits in learning and memory functions[48].

In addition, gene defects on chromosomes 14 and 1 are also associated with early-onset FAD. On these chromosomes are the two genes that encode presenilin 1 (PS1) and presenilin 2 (PS2)[49, 50]. The two proteins are similar in size and both are located at the endoplasmic reticulum and the Golgi complex, but their normal biological roles are not fully known[51]. Point mutations in these genes cause elevated levels of $A\beta_{1-42}$ deposition[52, 53].

Late-onset FAD type occurs in association with the apolipoprotein E (apoE) gene. This protein is involved in the transport functions of lipids. There are three major protein isoforms of apoE (apoE2, apoE3, and apoE4), which are products of their alleles (ε2, ε3, and ε4), and harboring the apoE ε4 allele on chromosome 19 is associated with an increased risk of AD[54, 55].

1.2.2 Neuropathology of Alzheimer´s disease

The histopathological hallmarks of AD are intracellular neurofibrillary tangles (NFTs) and extracellular deposition of β-amyloid (Aβ) in neuritic plaques and diffuse deposits, atrophy, synaptic reduction and neuronal loss[56, 57].

The Aβ peptide consists of 38 to 42 amino acid residues and is a product generated from proteolytic processing **(Fig. 2.)** of the amyloid precursor protein (APP) by way of three different enzymatic cleavages during normal cellular metabolism[58]. The enzyme α-secretase cleaves APP, which results in release of the non-amyloidogenic secreted form of APP (sAPPα) from the cell surface[59]. In the amyloidogenic pathway, β-secretase cleaves APP at the N-terminal of Aβ and γ-secretase cleaves at its C-terminal. Active γ-secretase is a complex that is comprised of the four membrane proteins presenilin, nicastrin, Aph-1, and Pen-2[60], which not only cleaves APP but also Notch and other type I transmembrane receptors[61]. Gamma-secretase cleavage is variable and occurs after amino acids 38, 40 or 42. Although the form $A\beta_{1-40}$ is most common, only the $A\beta_{1-42}$ peptide has a strong tendency to self-aggregate and form long, insoluble amyloid fibrils, which accumulate and result in the formation of amyloid plaques[52, 62]. Reactive astrocytes and activated microglia are also often found in and around the plaques[63-65]. Activation of astrocyes and microglia also initiates inflammatory responses in the brain in cases of AD by secreting cytokines and chemokines, contributing to the degeneration of neurons[66-68].

Many studies have also shown that Aβ induces oxidative stress as manifested by lipid peroxidation, and DNA and protein oxidation, indicating that Aβ-induced oxidative stress is involved in the pathogenesis of AD[69, 70].

In recent years, imaging in order to detect Aβ deposition in AD patients by means of positron emission tomography (PET) has opened new windows to understand further the role of Aβ in AD[71-73].

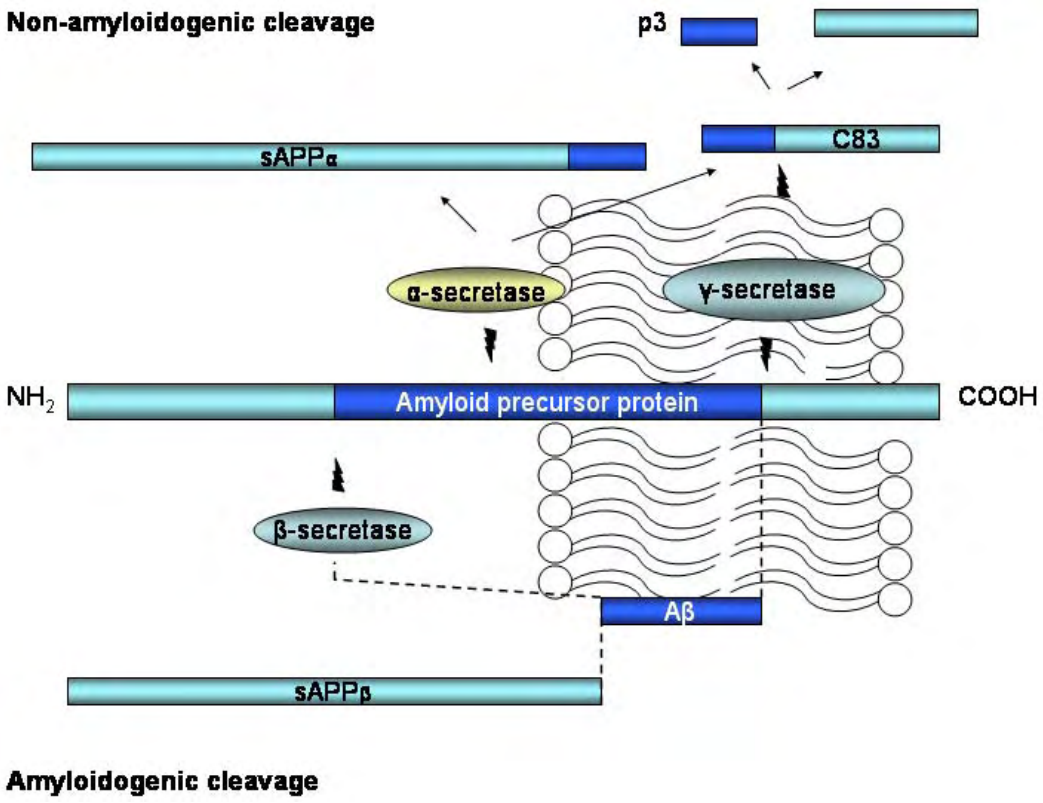

Fig. 2. Schematic illustration of the proteolytic cleavage of APP via amyloidogenic and non-amyloidogenic pathways.

The distribution pattern of amyloid deposits differs from that of NFTs[74]. Neurofibrillary tangles are intracellular lesions, found in the cytoplasm of degenerating neurons, which are composed of hyperphosphorylated aggregates of the microtubuli-associated tau protein. Tau protein plays a role in stabilizing the microtubules during their assembly and abnormal hyperphosphorylation of tau protein is believed to lead to destabilizing of microtubules, dysfunction in axonal transport and progressive neuronal loss[75, 76]. Selective reduction in the levels of ACh and ChAT activity, predominantly in the hippocampus and cerebral cortex, has also

been reported in the brains of AD patients[77, 78]. In severe AD, the level of AChE is reduced compared with that in normal subjects, whereas the level of BuChE is increased[79].

In addition, *in vivo* measurements by means of PET have provided data on early changes in the course of the disease, demonstrating that the cognitive decline in AD patients correlates with a loss of nAChRs[77, 80]. Autoradiographic studies in human *post mortem* brain tissues revealed a significant loss of nAChRs in AD patients compared with age-matched control subjects[81]. The protein levels of the α3 and α4 nAChR subunits have also been reported to be decreased in the hippocampus and temporal cortex, and the α7 subtype was reduced in the hippocampus of AD brain[82]. Consistently, reduction in the numbers of [^{3}H]-epibatidine and [^{3}H]-nicotine binding sites, which have high affinity for the α3 and α4 nAChR subunits, was also observed in cortical regions of AD patients who carried the APP_{swe} double mutation[83].

Most studies have revealed that mAChRs are preserved or increased in AD brains[77, 84, 85], although a decreased number of M2 mAChRs has been observed[86].

Degeneration of cholinergic neurons in the brain is a principal feature of AD and the populations of neurons in the nucleus basalis and the hippocampus are severely affected. These cholinergic deficits in AD are positively correlated with cognitive impairment[19, 87-89], as well as non-cognitive behavioral disturbances[90].

1.2.3 Transgenic mouse models

Various transgenic mouse models have been developed for AD research, carrying one or several forms of mutant human proteins (APP, PS and tau). Some of these transgenic mice models include Tg2576 ($APP_{K670N/M671L}$)[48], APP (V717F)[91], APP23 ($APP_{K670N/M671L}$)[92], PS1 (M146L and M146V)[93], Tg2576 and PS1 (A246E)[94], tau (P301L)[95], Tg2576/tau[96], hAChE-Tg[97], hAChE-Tg/Tg2576[98], and Tg2576/PS1/tau[99].

Many of the transgenic mice show an age-dependent formation of neuritic plaques consisting of Aβ. Variations in the location and rate of formation of Aβ deposits have been shown between different strains, with age of onset as regards the development of plaques varying from 6 to 15 months[100]. Transgenic mice also recapitulate other aspects of AD pathogenesis including age-related learning deficits, astrocytosis and microglial activation. However, single transgenic APP mice generally neither develop marked neuronal loss nor neurofibrillary tangles[101, 102], which are prominent

hallmarks of AD. Other transgenic mouse models coexpress human APP transgenes and other transgenes such as PS1 mutations that result in the development of tau pathology including neurofibrillary tangles[96, 103]. Although transgenic mice do not display all neuropathological features that are present in the brains of AD patients, they are still important model systems providing information about the molecular mechanisms and pathological processes that may be involved in the pathogenesis of the disease.

1.2.4 Therapeutic strategies in Alzheimer´s disease

1.2.4.1 Neurotransmitter treatments

Current treatment in AD is based on modulation of the availability of ACh at the synapse. In order to maintain the synaptic concentrations of ACh, treatment with acetylcholinesterase inhibitors (AChEIs) is used to interfere with the degrading activity of AChE. Currently, the three AChEIs donepezil, rivastigmine and galantamine are approved forms of medication for mild-to-moderate AD. Several clinical trials have demonstrated improved cognitive functions following the administration of these drugs[104-107]. PET studies have also demonstrated increases in cerebral blood flow, glucose metabolism and ^{11}C-nicotine binding sites in AD patients treated with AChEIs[108-110].

Besides the AChEIs, the non-competitive *N*-methyl-d-aspartate (NMDA) receptor antagonist memantine is also in clinical use, and is approved for moderate-to-severe AD[111, 112]. Memantine blocks the channels of the NMDA receptors, protecting cells against the neurodegenerative effects of the excitatory neurotransmitter glutamate. Memantine has been used in combination with an AChEI[113, 114].

1.2.4.2 New approaches in AD treatment

1.2.4.2.1 Nerve growth factor

Intraventricular administration of NGF to AD patients has earlier been shown to improve cerebral blood flow, glucose metabolism and the number of [^{11}C]-nicotine binding sites. Unfortunately, negative side effects were observed which limited the continued usage of NGF[115, 116]. A more recent approach, in which NGF was delivered to the brain using genetically modified fibroblasts, could increase glucose uptake and reduce the rate of cognitive decline in AD patients[117].

1.2.4.2.2 Cholinergic receptor agonists

The effect of stimulation of mAChRs and nAChRs by various agonists has also been studied. In several *in vitro* and *in vivo* models of neuronal death, nicotine and other agonists are neuroprotective against Aβ-induced neurotoxicity, and the attenuation of the neurotoxicity is believed to be mediated by α7 nAChRs[118-121]. In APP transgenic mice, nicotine treatment could ameliorate Aβ pathology[122, 123], and increased the levels of synaptophysin whereas a reduction in reactive astrocytes around the plaques was found[124]. Stimulation with the M1 receptor agonist talsaclidine increases proteolytic cleavage of APP, followed by an increase in the release of sAPP[125] which was also observed following stimulation by carbachol of the M1 and M3 receptors[126].

1.2.4.2.3 Anti-amyloid therapies

Since Aβ plays a causative role in the pathogenesis of AD, prevention of the production of Aβ from APP by inhibition of β- or γ-secretase has been suggested as a therapeutic strategy[127]. Hippocampal injections of β-secretase inhibitors in mice alter levels of Aβ[128], but β-secretase knockout mice have no clinical phenotype except for low concentrations of Aβ[129, 130]. Gamma-secretase mediated APP cleavage has similarities to the cleavage of Notch-1[131], a protein that controls cellular differentiation and is crucial for transcriptional regulation during development. Efforts have been made to produce γ-secretase inhibitors that reduce the Aβ level without affecting Notch signaling[132]. In animals, inhibition of γ-secretase reduces the level of Aβ in the brain[133], and several compounds have entered clinical trials.

There are also studies demonstrating strategies to enhance clearance of Aβ utilizing active or passive immunization. Following injection of human fibrillar Aβ into transgenic APP mice, reductions of cortical Aβ deposits, neuritic dystrophy and astrogliosis were reported[134]. Passive immunization with monoclonal and polyclonal antibodies against Aβ also reduces AD pathology in mouse models[135, 136], and cognitive and behavioral improvements after active and passive immunization against Aβ have been reported in mice[137, 138]. In a clinical trial it was found that Aβ immunization induced the production of antibodies against Aβ, at least in some AD patients.

Another anti-amyloid strategy involves the generation of small peptides having the capability to bind to Aβ and prevent the formation of β-sheets. Clinical studies are ongoing with several of these peptides[139].

1.2.4.2.4 Processing of the amyloid precursor protein

(–)-Phenserine, a novel AChEI, is currently in clinical trials for the treatment of AD. Experimental studies have demonstrated that phenserine, in addition to its AChE inhibitory effect, also regulates the expression and processing of APP. In cell cultures, phenserine treatment reduces the endogenous level and the secretion of sAPP, and it has also been shown that phenserine reduces the level of APP and improves cognitive performance in rodents[140]. This amyloid lowering effect appears to be independent of the AChEI activity of phenserine, since the stereoisomeric (+)-phenserine, which lacks AChEI activity, also reduces the protein level of APP in a manner similar to that of its potent (–)-enantiomeric form[141]. However, the dose of phenserine that is required to suppress APP production is far higher than that needed to elicit its AChEI activity, and it is this catalytic activity that is dose limiting *in vivo.* Administration of (+)-phenserine to mice also reduces β-secretase activity as well as $A\beta_{1-40}$ and $A\beta_{1-42}$ levels[142].

Recently, PET measurements have demonstrated that treatment with (–)-Phenserine affects Aβ and glucose metabolism in the brains of AD patients[143].

In addition, treatment with other AChEIs and BuChEI have been shown to modulate the release of sAPP and Aβ *in vitro*[144].

1.2.4.2.5 Inhibition of tau hyperphosphorylation

Other treatment strategies are associated with tau pathology. Since the formation of neurofibrillary tangles is caused by abnormal hyperphosphorylation of tau protein, inhibition of kinases that phosphorylate tau protein, including glycogen synthase kinase-3β and cyclin-dependent protein kinase 5, may be possible targets in AD treatment[145].

1.2.4.2.6 Preventive therapies

Epidemiological studies have also suggested that use of nonsteroidal anti-inflammatory drugs[146], estrogen[147], tocopherol (vitamin E)[148] and cholesterol-lowering drugs[149] may prevent AD.

1.3 STEM CELLS

Stem cells are defined as cells able to self-renew and differentiate. The potential of a stem cell to form various cell types is defined by their totipotency, pluripotency or multipotency. Totipotency is retained up to the eight-cell stage in the morula, whereas cells in the inner cell mass (ICM) of the blastocyst are pluripotent. At this stage, the stem cells can give rise to any mature type of cell. Multipotent stem cells are more committed to develop into specific cell types of a closely related family and can be isolated from fetal and adult tissue.

1.3.1 Embryonic stem cells

Embryonic stem (ES) cells can be isolated from the ICM and give rise to any of the three germ layers, endoderm, ectoderm and mesoderm. The majority of studies have taken place by using mouse ES cells or human ES (hES) cells. Although mES and hES cells have similar differentiation potential, their environmental requirements vary as regards maintaining the ES cells in an undifferentiated state. In 1994, the first study concerning culture of cells isolated from the human ICM was reported[150] and in 1998, Thomson and co-workers established the first permanent cell line of hES cells[151]. Subsequent studies of hES cells have reported their developmental capacity and ability to differentiate into multiple cell types including neurons, glial cells, cardiac muscle cells, endothelial cells, hematopoetic cells and skeletal myocytes[152, 153].

1.3.2 Neural precursor cells

Neural stem/precursor cells (NPCs) in the mammalian fetal CNS are concentrated in seven major regions: the ependymal zone of the lateral ventricles, the hippocampus, olfactory bulb, cerebral cortex, cerebellum, spinal cord and the subventricular zone[154-157]. During CNS development, NPCs give rise to neurons, astrocytes and oligodendrocytes in response to a variety of environmental signals[158]. These processes of differentiation into neurons and glial cells are related to the generation of neural progenitors, glioblasts and neuroblasts[155, 159]. Similar to ES cells, most of the current knowledge of these cells is derived from studies using rodent NPCs. Many of the *in vitro* experiments with NPCs involve the formation of round aggregates (neurospheres) of cells[156].

1.3.3 Neurogenic radial glial cells

The neural plate and neural tube are composed of a layer of neuroepithelial cells which forms the neuroepithelium. During neural development, the neuroepithelial cells give rise to radial glial cells which are more restricted in differentiation potential than the neuroepithelial cells[160]. From these radial glial cells, both neurons and astrocytes can be formed[161].

1.3.4 Mitogens and growth factors

Several signaling pathways are implicated in the control of self-renewal and commitment of stem cells and progenitors to a neuronal fate. These pathways include fibroblast growth factor (FGF), Wingless (Wnt) and Notch signaling pathways[162]. Moreover, several other soluble factors including epidermal growth factor (EGF), ciliary neurotrophic factor (CNTF) and leukemia inhibitory factor (LIF) have been shown to promote cell growth of NPCs *in vitro*[163-167].

During embryonic development, morphogens such as bone morphogenetic protein (BMP), sonic hedgehog, FGF and retinoic acid (RA), act as inductive signals for neuronal specification by interacting in a unique temporal order[168-170].

Retinoic acid, a natural derivative of vitamin A and a regulator of the vertebrate neural plate[171], has the ability to promote a neuronal phenotype in embryonic stem cells[172] and has been reported to enhance the number of neurons from hippocampal NPCs[173]. It also has regulatory functions as regards the transmitter phenotype[174, 175], especially of cholinergic functions. The effects of RA are mediated by retinoid receptors which are members of the nuclear receptor family. These nuclear receptors are ligand-induced transcription factors that respond to fatty acids, hormones and steroids. Retinoid receptors consist of RA receptors (RARα, β and γ) that can bind to both *all-trans* RA and 9-cis RA, and the retinoid X receptors (RXRα, β and γ) that only bind 9-cis RA. Both RARs and RXRs form hererodimers and promote transcription by binding to specific sequences of the DNA. Retinoic acid and its receptors are also critical components of synaptic plasticity in the hippocampus, a correlate of learning and memory[176, 177].

The neurotrophins are a family of growth factors that include nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT3) and neurotrophin-4 (NT4), which have central roles in the development of the nervous system and act by stimulating receptor tyrosine kinases (Trks) and the p75

neurotrophin receptor ($p75^{NTR}$)[178]. The neurotrophins and their receptors can be co-expressed by individual neurons of the CNS[179]. These factors mediate proliferation and differentiation of NPCs and can also regulate neurotransmitter release, LTP, axonal and dendritic growth and guidance, and synaptic plasticity. Studies have show that the expression of neurotrophins and their receptors increases transiently following brain insults, indicating a role in protection of damaged neurons and stimulation of synaptic reorganization[180, 181]. Furthermore, administration of neurotrophic factors to the adult forebrain subventricular zone *in vivo* increases the number of progenitor cells and newly generated neurons[182-184]. Although there are few reports about their effects on hES cells, there is one study showing that neurotrophic factors mediate hES cell survival[185] and another study demonstrating their capacity to modulate the fate of hES cells[186]. The prototype of neurotrophins, NGF, supports cholinergic functions both *in vitro*[187] and *in vivo*[188-190] and also increases the activity of AChE[191]. In addition, infusion of NGF in the adult brain reduces cholinergic degeneration in animals, thus improving cholinergic functions[192, 193].

1.3.5 Amyloid precursor protein in stem cell biology

Although many studies have been concerned with the processing of APP to yield Aβ as an important step in the amyloid cascade of AD pathogenesis, little is known about the physiological role of APP. In the mouse neural tube, APP mRNA is found at embryonic day 9.5 (E9.5) and APP expression is then increased during CNS development[194]. Studies involving the use of primary cultured neurons and NPCs have shown that treatment with sAPP promotes neurite outgrowth and cell proliferation[195, 196]. The mitogen-activated protein kinase (MAPK) pathway, which also stimulates neurotrophin responses and neurite outgrowth[178], is activated by sAPP[197]. Other studies have demonstrated that sAPP modulated the proliferation of neural precursors in the subventricular zone of adult mammalian brain and that over-expression of APP in the brain leads to predominantly glial differentiation of transplanted NPCs[198, 199]. Taken together, these findings support the hypothesis that APP not only has a key role in AD pathology but may also have regulatory functions in stem cell biology and neurogenesis.

1.3.6 Therapeutic potential of stem cells

Stem cells have been proposed as a unique source of transplantable cells advantageous for future neuroreplacement therapies in the treatment of several CNS disorders including AD, Huntingtons´s disease, multiple sclerosis, PD and spinal cord injury [199-203]. Crucial to the success of generating specialized cell populations, is an understanding of the mechanisms that influence the control of cell growth and differentiation by extrinsic and intrinsic factors.

A considerable number of studies have been performed to investigate whether transplantation of stem cells into the injured or diseased CNS has the potential to repair circuitries and thereby restore neurological function[204, 205]. The ideal donor cells for neural transplantation should be expandable *in vitro*, exhibit an immature but committed phenotype, and be able to migrate to desired regions of the brain or spinal cord upon transplantation.

Stem cell transplantation studies in animal models of neurodegeneration have indicated that these cells are able to migrate to sites of injury and differentiate into neurons and glial cells in the brain[206]. The microenvironments surrounding the implanted cells are populated by glial and endothelial cells which are important regulators of stem cell migration and differentiation. Disturbances in the microenvironment due to brain injury or pathology could affect the environmental equilibrium and expose the cells to factors that are different from those in their normal conditions.

Novel therapeutic strategies are being developed taking advantage of the ability of NPCs to proliferate *in vitro* and to survive following transplantation into the mammalian brain, where they may integrate and express foreign genes or repopulate the damaged or diseased CNS. Recent results indicate that human NPCs may hold great promise to replace degenerative cholinergic neurons, ameliorating the behavioral deficits of AD[207-209]. It has been demonstrated that human NPCs, injected into the lateral ventricle of 24-month old rats, improve cognition four weeks after transplantation[210]. These findings indicate that the aged rat brain is not only capable of providing the necessary environmental conditions for the neural precursors to retain their pluripotency, but also the potential for investigation of replacement therapies to improve features of memory and cognition in age-associated disorders.
Transplantation of neurons derived from hES cells has also been successfully integrated *in vivo*[211]. Together, these important findings indicate the great advantages

and opportunities that these cells may offer as a source of specialized human cells for biotechnological and future clinical therapeutic applications.

2 AIMS

The overall aim of the present work was to examine the regulation of extrinsic factors in the microenvironment on differentiation and plasticity of hES cells, neural precursor cells and cholinergic neurons and their implications for AD.

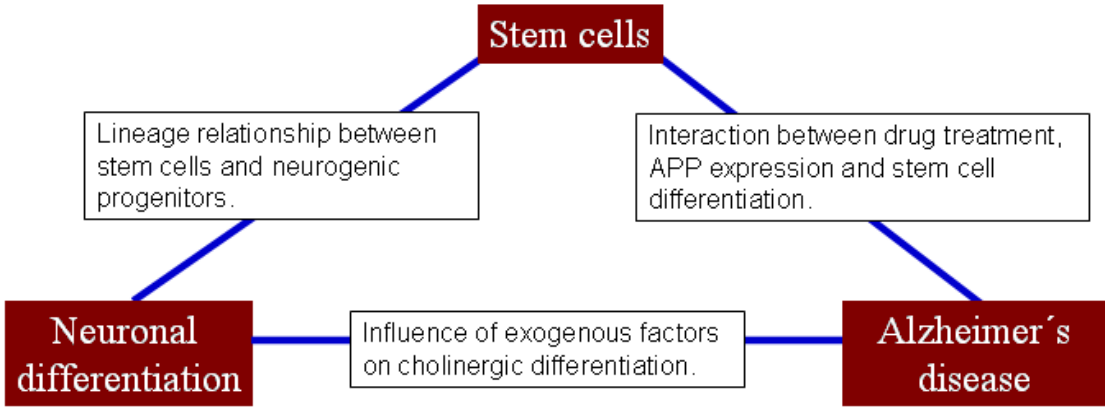

Fig. 3. The general focus of the thesis.

Specific aims were to:

- establish defined *in vitro* conditions that sustain the survival, proliferation and differentiation of hES cell-derived neurogenic progenitors, and to identify and characterize their lineage relationship in neurogenesis.

- examine the effects of regulatory exogenous factors on cholinergic characters, including ChAT and acetylcholine receptors, of hES cell-derived neuronal progenitors and neurons.

- investigate the influence of AD pathology on stem cell biology, and whether drug treatment affects the differentiation pattern of transplanted human NPCs.

3 MATERIALS AND METHODS

3.1 CELL CULTURE

3.1.1 Immortalized cell lines

SN56 cells were cultured in Dulbecco´s modified Eagle´s medium (DMEM) supplemented (10%) with fetal bovine serum (FBS) and gentamicin at 50 µg/ml. PC12 cells were cultured on rat tail collagen-coated plastic culture plates in RPMI 1640 medium supplemented (10%) with horse serum and FBS (5%) (Gibco). M10 cells, stably transfected with chicken α4 and β2 nAChR subunits, were cultured in DMEM medium containing FBS (10%), penicillin (100 units/mL) and streptomycin (100 µg/mL). To induce transcription of the α4β2 nAChR subtype expressed in these cells[212], 1 µM dexamethasone was added.

3.1.2 Human embryonic stem cells

In the present work, the six hES cell lines that were used were as follows: HS181, HS237, HS293, HS306, HS346 and HS382. All lines were derived at the Fertility Unit of Karolinska University Hospital Huddinge, Karolinska Institutet, Sweden. They were derived from the inner cell mass of 5/6-day-old pre-implantation blastocyst-stage embryos after *in vitro* fertilization. Only embryos that could not be used in infertility treatment were used, after informed consent from both partners of each couple. The Ethics Committee of Karolinska Institutet gave approval for the derivation, expansion and differentiation of the hES cell lines. All the lines were originally derived and cultured using postnatal human foreskin fibroblasts as feeder cells[213]. The HS293, HS306 HS346 and HS382 lines were derived using serum replacement (SR) instead of fetal calf serum[214] and since 2003 all lines have been cultured solely in SR-containing medium[215]. The hES cell colonies were cultured and passaged as previously described by Hovatta *et al.*[213]. Briefly, hES cell colonies were cultured in knock-out-DMEM medium (Gibco) supplemented with knock-out SR (15%), 2 mM L-glutamine, 0.1 mM MEM non-essential amino acids, penicillin (50 U/mL), streptomycin (50 µg/mL), basic FGF (bFGF, R&D; 8 ng/mL) at 37°C in 5% CO_2. The medium was changed every day. Colonies were passaged at 5- to 7-day intervals by mechanical dissection into small clusters (50-200 cells/cluster). Clusters of hES cells were replated on gamma-irradiated (35 Gyr) human foreskin fibroblasts.

3.1.2.1 Neural differentiation of human embryonic stem cells

For neural differentiation experiments we used the following passages: line HS181, passages 21 and 36; line HS237, passages 12, 49, 70, 72 and 73; line HS293, passages 50 and 75; HS306, passage 62; line HS346, passages 26, 27, 28; line H382, passage 10. We applied two neural differentiation protocols: differentiation in suspension and in adherent culture systems **(Paper II)** that were modified from previous work by Watanabe *et al.*[216] and Ying *et al.*[217].

The neural induction medium **(NIM)** contained DMEM/F12: Neurobasal (1:1) (Gibco-BRL) supplemented to give the indicated final concentrations: 1×N2 supplement (Gibco-BRL), 1 × B27 supplement (without vitamin A, Gibco-BRL), 2 mM Glutamax (Gibco-BRL).

The neural proliferation medium **(NPM)** contained DMEM/F12: Neurobasal (1:1) supplemented to give the indicated final concentrations: 0.5 × N2 supplement, 0.5 × B27 supplement, 2 mM Glutamax, bFGF at 20 ng/ml.

The neural differentiation medium **(NDM)** contained supplemented Neurobasal: 1 × B27, 2 mM Glutamax and BDNF at 10 ng/mL. In order to reduce the extensive cell death observed after direct transfer of hES cells to feeder- and serum-free conditions, the culture conditions for hES cells prior to passaging for neural induction were progressively changed. Cell colonies were passaged after culturing on a feeder layer with NIM for 1 day. Furthermore, hES cells were mechanically passaged as clusters (50-200 cells/cluster) rather than as single cells in order to improve their survival.

For neural differentiation in suspension culture, hES cell clusters were cultured on non-adherent Petri dishes (Falcon), 10-15 clusters/cm^2, in NIM. In these conditions they formed uniform aggregates in 2-4 days. Floating aggregates were transferred to NPM starting on day 5. To evaluate their differentiation, aggregates were plated on days 7, 14, 24 and 42 on polyornithine (15 µg/mL, Sigma)/human laminin (20 µg/mL, Sigma)-coated 4-well plates in NPM or NDM. Cell aggregates were attached and grew out over 2-5 days. In some experiments, suspension aggregates were dissociated by gentle trituration before plating. The medium was changed and dead cells were removed twice a week.

For neural differentiation in adherent culture, hES cell clusters (mechanically passaged after 1 day of pre-culture in NIM as described above) were plated on polyornithine/laminin-coated tissue culture plates (10-15 clusters/cm^2, Falcon) (day

1). The majority of the clusters adhered on the second day, and if they did not do so they were transferred to the suspension culture system or were allowed one more day to adhere. Adherent cultures were grown in NIM for the next 3 days and then in NPM for 3-20 days. For some experiments, cells were cultured in NDM for another 10 days. The medium was changed and detached or dead cells were removed every second day.

3.1.3 Culture of human neurospheres

Human NPCs (BioWhittaker, Walkersville, MD), originally isolated from 9-week-old fetal cortical tissue, were maintained as neurospheres in DMEM:F12 medium supplemented with 1 × B27, heparin, epidermal growth factor (EGF) + bFGF (each 20 ng/mL) and antibiotic-antimycotic mixture (1:100, Gibco). Prior to transplantation, the NPCs were incubated with 3 μM bromo-deoxyuridine (BrdU, Sigma) for 48 hr in order to distinguish them from the host cells.

To generate neurospheres from hES cells, cells from hES cell colonies were mechanically dissociated and cultured in the above medium. These spheres were routinely mechanically passaged every 2-3 weeks.

All cells were maintained in an atmosphere of 95% air, 5% CO_2 at 37˚C.

3.2 PROLIFERATION ASSAY

For proliferation assays **(Paper III)**, neurospheres derived from hES cells were dissociated with dispase (0.1 mg/mL) for 10 min at 37˚C. Cells were adjusted to 50 000 cells/ml and plated on culture dishes 24 hr prior to the administration of neurotrophic factor (50 ng/mL; Invitrogen). Measurement of cell proliferation using a BrdU incorporation assay was carried out according to the manufacturer´s instructions (Roche, Mannheim).

3.3 REVERSE TRANSCRIPTION PCR ANALYSIS

Total RNA was isolated from SN56 and PC12 cells using Micro-to-Midi purification kits **(Paper I)** or from human NPCs, hES cells and hippocampal and cortical tissues by Trizol reagent (Invitrogen) **(Papers II, III and IV)** according to the manufacturers´ instructions. Extracted RNA samples were treated with Rnase-free DNase at 37˚C for 20 min. Reverse transcription was performed using 1 μg total RNA in a 20 μL mixture consisting of 2.5 mM $MgCl_2$, 1 × PCR buffer II, 4 mM of a

mixture of all four deoxynucleotide triphosphates (dNTPs), 20 units Rnase inhibitor, random hexamers (2.5 μM) and 50 units M-MLV reverse transcriptase. The reaction **(Papers I, II, and III)** was performed in a reaction volume of 50 μL using a mixture containing 1.5 mM $MgCl_2$, PCR buffer, 0.4 mM dNTP mix, specific sense and antisense primers (0.5 μM each), 10 μL of RT reaction mixture (diluted 1:5) and 2.5 units of Taq DNA polymerase (Promega).

One μg of total RNA **(Paper IV)** was transcribed into cDNA with reagents from an iScript cDNA Synthesis Kit (Bio-Rad). Relative quantification via Real-Time PCR was determined using MyiQ Real-Time PCR Detection System Software (Bio-Rad), and reactions were performed in a thermal iCycler using Bio-Rad MyiQ IQ SYBR Green Supermix (Bio-Rad). A final dissociation step was performed at the end of each reaction to verify the presence of a single product.

The annealing temperature and the numbers of cycles used were dependent on the specific sequences of each set of oligonucleotide primers. PCR-products were amplified in the linear range. In all experiments, either cyclophilin or β-actin were used as house-keeping genes. A negative control in which reverse transcriptase was absent was run in parallel to all reactions to trace genomic DNA contamination. The PCR-products were examined by electrophoresis on 1.5% agarose gel followed by staining with ethidium bromide (0.5 μg/mL).

3.4 RECEPTOR BINDING ASSAY

For [^{3}H]-epibatidine binding assays **(Paper I)**, cell homogenates were prepared in assay buffer (50 mM Tris-HCl, pH 7.4). The density of [^{3}H]-epibatidine binding sites was measured by incubating (±)-[^{3}H]-epibatidine (specific activity 56.2 Ci/mmol; Du Pont/NEN) in the presence of cell suspension (0.2 mg protein/100 μL of homogenate) in a final volume of 1 mL homogenate buffer for 3 hr at 25˚C. Non-specific binding was determined in the presence of 1 mM (–)-nicotine. The reaction was terminated by filtration through Whatman GF/C glass filters, presoaked in 0.3% polyethylenimine solution for 3 hr. The samples were washed three times in assay buffer and the filters were transferred to vials containing 5 mL scintillation solution. Radioactivity was counted in a Wallac scintillation counter.

3.5 WESTERN BLOTTING

Cell homogenates of SN56 cells and PC12 cells **(Paper I)** were prepared in ice-cold 50 mM Tris buffer (pH 7.4) containing 1 mM phenyl-methylsufonyl fluoride. The homogenates were washed twice by centrifugation at 35,000 × g for 20 min at 4˚C and resuspended in Tris buffer containing 2% Triton X-100 and protease inhibitors. The suspension was centrifuged at 100,000 × g for 60 min at 4˚C.

Dissected tissues **(Paper IV)** from the cortex and the hippocampus of (+)-phenserine and saline treated animals were homogenized in ice-cold lysis buffer containing 1% NP40, 150 mM NaCl, 50 mM Tris (pH 8.0), and 1 × protease inhibitor cocktail (Roche Diagnostics, Indianapolis, IN). The homogenates were centrifuged and washed twice at 12,000 × g for 10 min at 4˚C.

Following centrifugation, the supernatants were used for protein quantification by the Bradford method using Bio-Rad Protein Assay Dye Reagent (Bio-Rad, Hercules, CA). Equal amounts of protein were separated by SDS-PAGE and blotted onto polyvinylidene difluoride membranes. For detection of α3, α4, α7 and β2, nAChR subunits the membranes were blocked in TBS-T (Tris buffer solution with 0.1% Tween 20, pH 7.6) with 5% non-fat dried milk and thereafter incubated with antibodies (0.2 µg/mL): of goat polyclonal anti-α3 (sc-1771), anti-α4 (sc-1772), anti-β2 (sc-1449), and rabbit polyclonal anti-α7 (sc-5544) (Santa Cruz Biotechnology Inc.). For detection of APP and GFAP, mouse monoclonal anti-Alzheimer precursor protein (22C11) A4 antibody (1:1000, Chemicon, Temecula, CA) and rabbit anti-GFAP antibody (1:1000, Promega, Madison, WI) were used.

After washing, the membranes were incubated with appropriate horseradish peroxidase (HRP)-conjugated anti-IgG and subsequently incubated with Enhanced Chemiluminescence reagents (Amersham) for 5 min. The signals were visualized by exposing the membranes to Hyper Performance Chemiluminescence film (Amersham). To standardize the amount of protein loaded onto each gel, anti-β-actin antibody was used. Films were scanned and the optical density of each specific band relative to that of ß-actin was analyzed by means of the NIH Image VDM software (version 1.55) or Image J software (version 1.33u).

3.6 ANALYSIS OF MITOGEN-ACTIVATED PROTEIN KINASE

For inhibition of Trk signaling, cells were exposed to the K252a inhibitor (200 nM; Biosource) 4 hr prior to growth factor stimulation. To remove cell debris, lysates

were centrifuged at 13,000 × g for 5 min at 4˚C and equal amount of proteins were used for Western blotting. The membranes were probed with phospho-ERK42/44 (1:1000, Cell Signaling) and the phosphorylated bands were visualized with anti-rabbit IgG conjugated to HRP.

3.7 IMMUNOSTAINING

SN56 cells and PC12 cells were cultured on 0.01% (w/v) poly-L-lysine-coated culture slides (BD Biosciences). The morphology of these cells was examined using an anti-neurofilament 200 antibody.

Cells derived from hES cells were cultured on poly-D-lysine and laminin coated glass coverslips. They were either fixed in methanol for 10 min at -20˚C or in 4% paraformaldehyde and permeabilized in blocking buffer (2% BSA and 5% goat serum) containing 0.05-0.1% triton x-100 at 4˚C. To characterize the hES cells at various stages of neuronal differentiation, a battery of markers was used. The cell surface marker SSEA4 and the transcription factors Nanog and Oct4 were used as markers for hES cells. Pax6, nestin, vimentin, glial fibrillary acidic protein (GFAP), glutamate astrocyte-specific transporter (GLAST) and brain lipid-binding protein (BLBP) served as markers for neuroepithelial cells and radial glial cells.

Free floating coronal brain sections (20 μm) were washed briefly in PBS **(Paper IV)**, denatured with 1 M HCl for 20 min and neutralized with PBS for 30 min at room temperature (RT). The sections were then blocked in PBS containing 0.25% Triton X-100 and 3% Normal Donkey Serum and incubated with sheep polyclonal anti-BrdU (1:1000, Abcam, Cambridge, MA), Mouse anti-Human Nuclei (1:100, Chemicon), mouse IgG2b anti-human βIII-Tubulin, clone SDL3D10 (1:2000, Sigma) and Mouse anti-NeuN (1:1000, Abcam), or rabbit IgG anti-human GFAP (1:500, Sigma). For apoptosis measurements, sections were incubated with affinity-purified rabbit anti-active Caspase-3 antibody (1:125, Promega, Madison, WI). Cells imaged by using a Leica DMRB fluorescent microscope **(Paper IV).**

The cells were then exposed to appropriate secondary antibodies conjugated to either Texas Red or fluorescein isothiocyanate (FITC) for 1 h at room temperature. α7 nAChRs were labeled by FITC-conjugated α-BTX (Molecular probes) **(Paper III)**.

To visualize the nuclei, cells were stained with Hoechst 33342 at 2-5 μg/mL or with propidium iodide (1 μg/mL; Sigma)**.**

3.8 TRANSGENIC MICE

APP23 transgenic mice (Novartis Pharma Ltd., Switzerland) were employed to breed a colony of experimental animals by backcrossing to C57/BL6 mice **(Paper IV)**. Mixed genotype groups were housed in standard mouse cages with access to food and water *ad libitum*, constant room temperature and humidity, and a 12/12 hr light/dark cycle. Genotypes were confirmed by PCR and wild-type littermates served as controls.

3.8.1 Treatment

Either (+)-phenserine (25 mg/kg/day, i.p.) or 0.9% saline (control) was administered for 14 consecutive days to a total of 55 age- and sex-matched APP23 (n=30) and wild-type (n=25) mice. The mice (aged from 4-7 months) were divided into two groups that were either sacrificed following 14 days of treatment (n=17 APP23 and n=13 wild-type, respectively) or received transplantation of human NPCs into the lateral ventricle (n=13 APP23 mice and n=12 wild-type, respectively). (+)-Phenserine or saline injection was continued once a day for 1 week after a 2-day recovery period from transplantation surgery. All animals were sacrificed after receiving a final dose of (+)-phenserine or 0.9% saline by giving an overdose of a 1:1 mixture of ketamine (100 mg/kg) and xylazine (20 mg/kg) and they were transcardially perfused with phosphate buffer.

3.8.2 Transplantation

For transplantation, mice were deeply anesthetized with a 1:1 mixture of ketamine (100 mg/kg) and xylazine (20 mg/kg) and mounted onto a stereotaxic apparatus (ASI Instrument, USA). Approximately 10^5 human NPCs (10 μL) were slowly injected into the right lateral ventricle of each mouse by way of a Hamilton gastight syringe (Hamilton, Reno, NV, USA) with a 22-gauge beveled needle attached. The tip of the syringe was held in place for 3 min during delivery and left in place for another 3 min after each injection. Coordinates (in reference to the bregma) for implantation were as follows: anterior posterior (A/P) –0.6 mm; medial lateral (M/L) +1.0 mm; dorsal/ventral (D/V) +2.4 mm. No immune suppression was utilized. All efforts were made to minimize the number of animals used and their suffering by observation of maintained body weight, and by monitoring incisions for swelling and proper healing of the incision site.

3.9 CALCIUM IMAGING

The cells were loaded with the two calcium sensitive dyes Fluo-3 AM and Fura Red-AM (1:3, Molecular Probes, Eugene, OR) for 30 min allowing semi-quantitative tracking of intracellular calcium. Cultures were excited at 488 nm. Fluo-3 was imaged at 505-550 nm emission and Fura-Red was imaged simultaneously at above 615 nm emission. The calcium measurements were conducted in DMEM:F12 (pH 7.4) or in Krebs-Ringer-Hepes buffer supplemented with glucose (20 mM), (KRH constituents in mM concentrations: NaCl 136; KCl 4.7; $CaCl_2$ 1.25; $MgSO_4$ 1.25 and HEPES 20; pH 7.4) at 25-27^{o}C. Calcium-free buffer was prepared without $CaCl_2$ and EGTA was added (500 μM). To verify the responsiveness of the dyes, ionomycin (2 μM, Sigma, St. Louis, MO) was applied at the end of the experiments. Cells were imaged using an inverted Meta-Zeiss 510 LSM confocal microscope with a × 40 [numerical aperture (NA), 1.3] objective. The pinhole was set to produce optical sections thinner than 4 μm.

3.10 STATISTICAL ANALYSIS

Data from saturation binding assays were analyzed by non-linear regression analysis (GraphPad PRISM 3.0) and the data were fitted to one or two site-binding model **(Paper I).** Differences between one treated group and the control group were analyzed with a two-tailed Student's *t*-test **(Paper I and IV).** Experimental groups that were significantly different from control groups were analyzed by ANOVA with Dunnett´s *posthoc* test (GraphPad PRISM 3.0) **(Paper III).**

4 RESULTS AND DISCUSSION

4.1 CHARACTERIZATION OF ACETYLCHOLINE RECEPTORS IN CHOLINERGIC CELLS

The cholinergic system is essential for many processes in the brain including cognitive functions. The loss of nAChRs in AD patients, measured *in vivo* by PET, is correlated with cognitive decline of these patients[80, 81]. As *postmortem* studies of AD brain have demonstrated reduction of various subunits of neuronal nAChRs[82], and increased expression of α7 nAChRs in astrocytes[218], the findings strongly underline the importance of understanding the regulatory mechanisms of individual subunits and their relationship to the synthesis of ACh.

The SN56 cell line, derived by fusion of mouse postnatal day 21 septal neurons with murine N18TG2 neuroblastoma cells[219], exhibits neuronal and cholinergic features including sodium-dependent high-affinity uptake of choline, depolarization-evoked release of ACh, and ChAT activity. These characteristics make it a valuable *in vitro* model of brain cholinergic neurons. Although this cell line has been used earlier to study cholinergic functions[219-226], so far there are no reported studies on the expression of nAChRs.

Saturation analysis of binding sites of the nAChR agonist [^{3}H]-epibatidine was performed to label α3 and α4 nAChR subunits. The saturation data best fit a two-site binding model, with two dissociation constants with a 30-fold difference in affinity. These dissociation constants were both in agreement with [^{3}H]-epibatidine binding data analyzed in rat brain[227]. Further characterization of the nAChRs in SN56 cells by RT-PCR analysis revealed transcripts encoding the α3, α4, α5, α7, β2 and β4 nAChR subunits **(Fig. 4A)**. Of these subunits, most neuronal nAChRs in the mammalian brain are composed of α4 (together with β2), α3 and α7 nAChR subunits[12].

4.2 RETINOIC ACID PROMOTES DIFFERENTIAL EXPRESSION OF NICOTINIC ACETYLCHOLINE RECEPTOR SUBUNITS

A variety of factors in the microenvironment stimulate the survival, proliferation and differentiation of ES cells, neuronal precursor cells and neurons and may also

influence the transmitter phenotype. Among these factors, RA is one of the most used epigenetic factors for triggering differentiation *in vitro* and it has been shown to direct cholinergic differentiation of human neuroblastoma cells[228, 229].

In SN56 cells, RA-induced differentiation promoted an increased density of [^{3}H]-epibatidine binding sites, whereas a significant reduction was observed in M10 cells **(Fig. 4B)**, which express only the α4β2 subtype of nAChRs[212]. In SN56 cells, the mRNA and protein levels of the α3 nAChR subunit were maximally elevated after 4 days, whereas α4 protein expression was reduced. Together, these data suggest that the extent and direction of regulation are dependent on the type of nAChR following RA-induced differentiation.

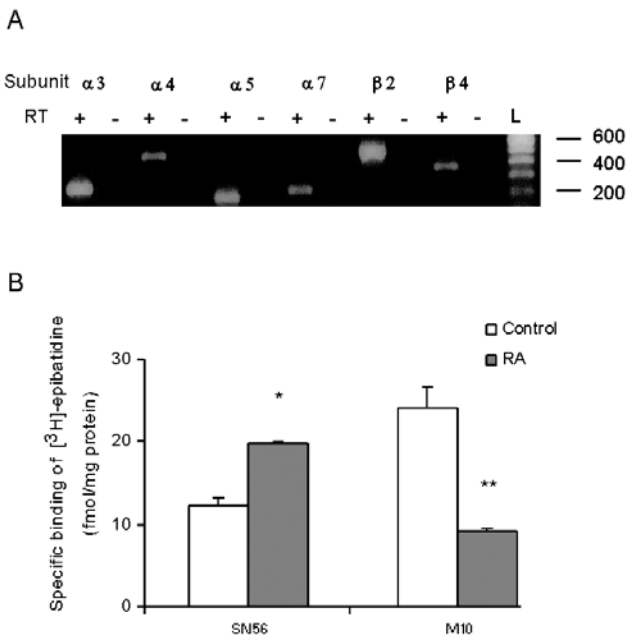

Fig. 4. Expression and modulation of nAChRs in SN56 cells. **(A)** RT-PCR analysis of the expression of nAChR subunits in SN56 cells. **(B)** Effects of RA treatment for 4 days on the density of [^{3}H]-epibatidine binding sites in SN56 and M10 cells. Values are shown as mean ± s.e.m. Significant difference from control: P * < 0.05, P ** < 0.01, Student's t-test.

Retinoic acid-induced expression of the α3 nAChR subunit was also observed in rat PC12 cells. Although RA induced neurite extensions in SN56 cells, similar morphological alterations were not observed in PC12 cells, which is consistent with earlier reports[230, 231]. These findings suggest that the regulation of morphological changes may vary in these two cell lines. Additionally, the availability of receptors or their coactivators that mediate morphological alterations, or the required doses for induction of neurite outgrowth, may differ in these two types of cells.

4.3 NERVE GROWTH FACTOR REGULATES EXPRESSION OF NICOTINIC ACETYLCHOLINE RECEPTORS

Nerve growth factor, the prototype growth factor of the neurotrophin family, is well known as an inducer of differentiation of PC12 cells and, consistent with our findings, promotes neurite outgrowth in these cells[232]. Furthermore, stimulation with nicotine increases the release of noradrenalin in NGF-treated PC12 cells[233], and cholinergic functions are supported by NGF, as has been reported in both *in vitro* and *in vivo* studies. Previously, the expression of nAChR subunits has been reported in PC12 cells, but there are conflicting reports concerning whether or not NGF may induce or reduce the expression of nAChR subunits. Consistent with earlier reports[234, 235], we found that the levels of mRNA encoding the α3, α7 and β2 nAChR subunits were up-regulated by NGF treatment. However, opposite findings have also been reported[236], which may be connected with a different subclone of PC12 cells. The regulation of various nAChR subunits was also examined in SN56 cells **(Fig. 5)**, with similar increases in mRNA levels for the α3 and β2 nAChR subunits, as observed in PC12 cells. Consistent with the decreased density of [^{3}H]-epibatidine binding sites in M10 cells following RA treatment, this treatment reduced the mRNA and protein expression of the α4 nAChR subunit.

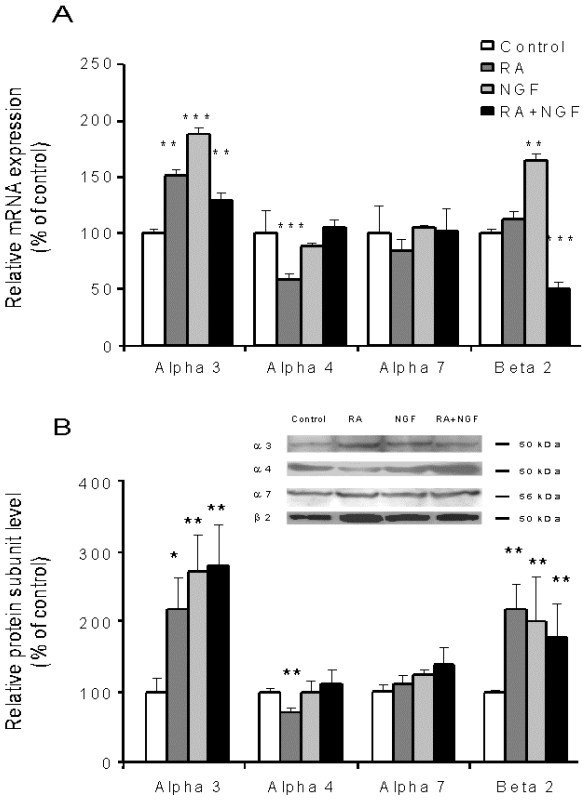

Fig. 5. Relative mRNA and protein expression of nAChR subunits in SN56 cells exposed to RA and NGF for 4 days. **(A)** Relative quantification of the mRNA levels of nAChR subunits normalized to cyclophilin. **(B)** Relative protein levels of nAChR subunits assessed by Western blot analysis. All values are shown as mean ± s.e.m. Significant difference from control: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

Discrepancies between the mRNA and protein expression of nAChR subunits may be a result of a post-transcriptional regulatory effect. It is plausible that the assembly of subunit proteins and the rate of protein receptor turnover may be affected by RA and NGF treatment. Furthermore, when RA was added together with NGF there were no additive effects on any of the subunit examined. Although it is possible that both factors may be acting at their individual saturating concentrations, the lack of synergy may also be caused by activation of the same pathways following RA and NGF treatment, by which the expression of a specific subunit may be modulated.

4.4 EFFECTS OF RA AND NGF ON THE EXPRESSION OF CHOLINE ACETYLTRANSFERASE

Differentiation induced by RA promoted neurite outgrowth in SN56 cells and the relative mRNA level of ChAT was gradually increased, with maximal elevation after 4 days **(Fig. 6)**. In contrast to RA treatment, no substantial alterations in the expression of ChAT or in cellular morphology were observed following exposure of SN56 cells to NGF. Thus, exposure to RA and NGF resulted in different responses, probably reflecting the cellular machinery that is activated by these factors. RARs and RXRs, which are ligand-activated transcription factors, mediate the cellular effects of retinoids. Previous reports have suggested that the induction of cholinergic properties in these cells is mediated by activation of RARα[221, 237]. In contrast, the effects of NGF are mediated by the TrkA and p75^{NTR}. However, NGF also activates the MAPK cascade, known to stimulate responses of neurotrophins and neurite outgrowth via p75^{NTR} in the absence of TrkA[238]. As these factors are potential modulators of the generation and expansion of neural stem and progenitor cells, it is crucial to understand their regulatory mechanisms in neuronal differentiation and cholinergic functions.

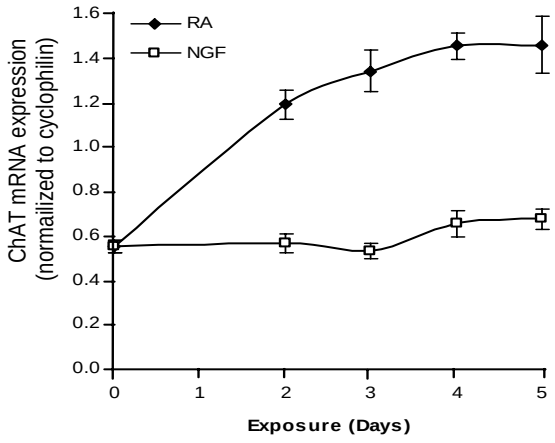

Fig 6. Time course of the effects of RA and NGF exposure on the relative mRNA expression of ChAT in SN56 cells. Values are shown as mean ± s.e.m.

4.5 NEURAL INDUCTION OF HUMAN ES CELLS

A major challenge in current research into hES cell biology is to define controlled culture conditions for the development of neural progenitors. Owing to differences between human and mouse ES cells[239], protocols established for *in vitro* differentiation of mouse ES cells cannot simply be applied to hES cells. In order to induce neural fate decisions in hES cells, various strategies including spontaneous differentiation in overgrowth of hES cells or in free-floating aggregates in the presence of fetal calf serum, stromal cells, RA and inhibitors of BMP have been reported[163, 240-242]. In the current work, neural differentiation was induced in hES using serum-free medium in the absence of inhibitors and RA. Prior to neural induction, hES cells were expanded on human fibroblasts as feeder cells in SR-medium and bFGF[213, 214] and expressed markers including Oct4, nanog and SSEA4, demonstrating an undifferentiated state **(Fig. 7)**.

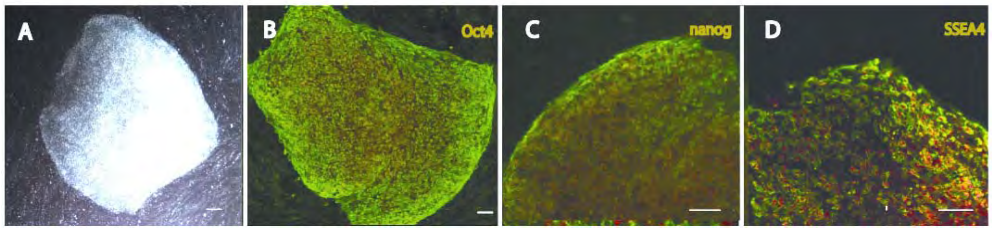

Fig. 7. Colony of hES cells cultured on human foreskin fibroblasts as feeder cells. **(A)** Undifferentiated colony of hES cells observed in dark-field microscopy. **(B-D)** Expression of markers of undifferentiated ES cells: Oct4, nanog and SSEA4. Scale bars are 50 μm.

In order to evaluate defined *in vitro* conditions for neural differentiation, both suspension and adherent culture systems were used for six hES cell lines (HS181, HS237, HS293, HS306, HS346 and HS382). As in the developing nervous system, the transcription factor Pax6 was present in the first stage in our culture system. Shortly after the induction of Pax6, the immature neuronal marker nestin was also expressed. As both of these markers are expressed in neuroepithelial cells, it was suggested that extensive neuronal differentiation occurred in hES cells in both adherent and suspension cultures, in a similar way as has been described *in vivo*[243]. Neuroepithelial cells also proliferate in the ventricular zone of the early neural tube in response to bFGF and other factors. This growth factor is also an essential survival

factor and is widely used for *in vitro* propagation of ES cells and NPCs originating from rodents and humans. Interestingly, it has been reported that co-administration of bFGF and NGF increased ChAT activity in cultured basal forebrain cells[167].

4.6 NEUROEPITHELIAL AND RADIAL GLIAL CELLS ARE NEUROGENIC

At the onset of neuronal induction, radial glial cells are appearing, which are the first cells that can be distinguished from neuroepithelial cells. Radial glial cells exhibit features of both neuroepithelial and glial cells[244], and have traditionally been considered to serve as scaffolding for migrating neurons and to give rise to astrocytes[245]. Interestingly, an increasing number of studies have demonstrated that radial glial cells also give rise to neurons[161, 246, 247]. Although there may be regional differences, the majority of cortical projection neurons originate from radial glial cells[246].

We observed a dynamic transition from neuroepithelial cells to radial glial cells in both adherent and suspension cultures. RT-PCR analysis of gene expression in various hES cell lines revealed a similar differentiation pattern in both adherent and suspension cultures after 14 days of differentiation **(Fig. 8)**. At this stage, neuronal markers were expressed, whereas expression of markers of astrocytes or oligodendrocytes was not detected. These findings suggest that similar neural induction occurred in all hES cell lines examined.

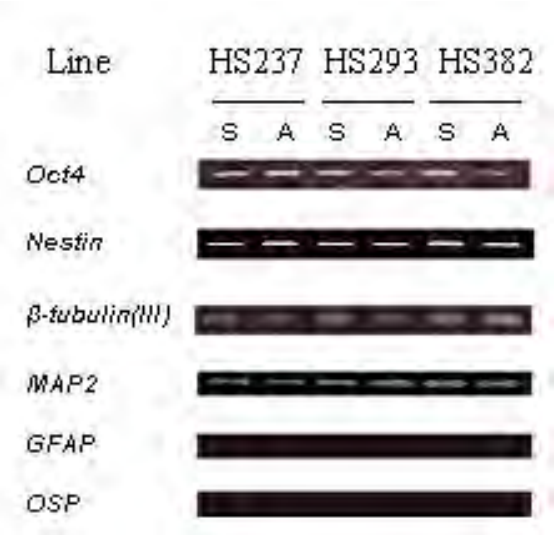

Fig. 8. RT-PCR analysis of gene expression in cells from three hES lines after 14 days of differentiation in adherent (A) and suspension (S) culture systems.

During neurogenesis from radial glial cells, both single stained βIII-tubulin-positive cells and double-labeled cells were observed (**Fig. 9)**, suggesting asymmetric division of the radial glial cells.

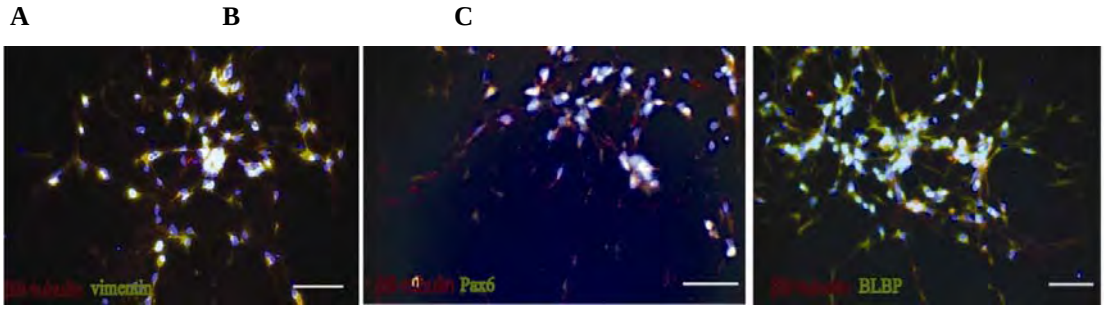

Fig. 9. Neurogenesis from radial glial cells. Both co-labeling and separate staining was observed in double labeling analysis of radial glial cell markers in relation to the neuronal marker βIII-tubulin. **(A)** Double labeling of vimentin and βIII-tubulin, **(B**) Pax6 and βIII-tubulin, and **(C)** BLBP and βIII-tubulin. Scale bars are 50 μm.

As neuroepithelial cells were the only neurogenic population in the cultures at a certain stage, it was concluded that these cells served as neural progenitors. The radial glial cells that were generated *in vitro* may not only provide support for the migration of neurons. It is also possible that these cells are involved in developmental patterning and regionalization of the CNS[244, 246].

In order to examine the differential potential of hES cell-derived radial glial cells, both adherent and suspension cultures were further differentiated. It was observed that nestin and MAP2-positive fibers were efficiently generated and organized in scaffolds in both culture systems (**Fig. 10**).

Thus, similar to neurogenesis *in vivo*, proliferating and differentiating radial glial cells gave rise to neurons in our cultures, suggesting that neural differentiation of hES cells is a valuable model to study early neural developmental processes. These findings are consistent with those in an earlier study carried out in mouse ES cells, in which there were similar characteristics of *in vitro*-generated radial glial cells[248].

Additionally, we also found that there were neurons in the vicinity of radial glial cells which expressed glutamate, suggesting that cortical projection neurons could be developed from hES cells[249].

Although RA has been widely used to induce differentiation of neuroblastoma cells, it was not necessary for induction of a radial glial phenotype in hES cells. However,

addition of RA to dividing mouse ES cells generated a population of progenitor cells that had characteristics of radial glial cells[250].

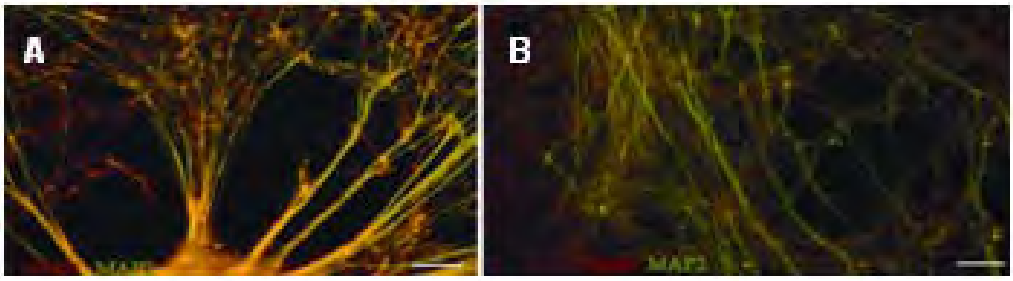

Fig. 10. Neurogenesis from human radial glial cells in both suspension and adherent cultures. **(A)** Generation of long filamentous nestin and MAP2-positive networks from plated suspension aggregates. **(B)** Similar filamentous structures were obtained from differentiating rosette-forming adherent cultures. Scale bars are 50 μm.

The early neuroepithelial cells derived from hES cells expressed Pax6 before Sox1, in contrast to the differentiation of mouse ES cells[250, 251]. As radial glial cells isolated from Pax6 mutant mice show lowered neurogenic potential[252], this transcription factor may also have a role as a fate determinant in hES cell-derived neural progenitors. In addition to Pax6, the transcription factor Mash1 was expressed early in the neurogenesis of hES cells which suggests a potential of hES cells to differentiate into neurons with a telencephalic identity, as both of these transcription factors are found in domains of the neuroepithelium of the forebrain[253, 254]. Moreover, in the developing telencephalon, all radial glial cells are dividing during the phase of neurogenesis[247, 255, 256], which is consistent with the proliferative staining of neurogenic cells that were derived from the hES cells.

Since GABA-positive cells have also been detected, it is plausible that a population of forebrain interneurons could be developed. Similar populations of neurons have also been reported in studies starting from both mouse and human ES cells[257, 258].

Human radial glial cells generated *in vitro* potentially offer a source of cells for pharmacological studies, owing to their neurogenic potential. In addition, this culture system may also serve as a platform for further directed differentiation of hES cells into neurons of a specific phenotype.

4.7 NEUROTROPHIC FACTORS AND HUMAN ES CELL-DERIVED NEURAL PRECURSORS

A variety of signaling pathways have been implicated in the control of self-renewal and commitment of stem cells and progenitors to a neuronal fate, including pathways involving FGF, Wnt and Notch signaling[162]. The neurotrophins are a family of growth factors that play essential roles in the development of the CNS. These factors can have multiple functions and they mediate proliferation, and regulate neurotransmitter release, LTP, axonal and dendritic growth and guidance, and synaptic plasticity[259]. Previously, it was reported that neurotrophins not only have the capacity to modulate the fate of hES cells but also to mediate their survival[185, 186]. Together, these important findings suggest that neurotrophic factors are suitable candidates involved in directed induction to a specific neuronal phenotype. It has also been shown that the mRNA levels of neurotrophic receptors (TrkB and TrkC) peak transiently between postnatal day 1 and P14 in rats, which is correlated to maximal neuronal growth, differentiation and synaptogenesis[260-262]. In an earlier study of hES cells, expression of TrkB, TrkC and $p75^{NTR}$ receptors was reported[185], which is consistent with our findings. As neurotrophins and their receptors are developmentally regulated, we hypothesized that neurogenic radial glial cells, derived from hES cells, serve as an attractive target of neurotrophic stimulation.

In hES cell-derived neuronal precursors, stimulation with neurotrophic factors increased the levels of phosphorylated ERK **(Fig. 11)**. The induction of pERK demonstrated activation of the MAPK pathway which is known to stimulate neurotrophin responses and neurite outgrowth[178], and is correlated with LTP, neuronal plasticity[35] and processing of APP[263]. This induction of pERK was inhibited by K252a, an inhibitor of tyrosine protein kinase activity, which suggests that neurotrophic factors are able to activate pathways that may induce differentiation in hES cell-derived neuronal precursors.

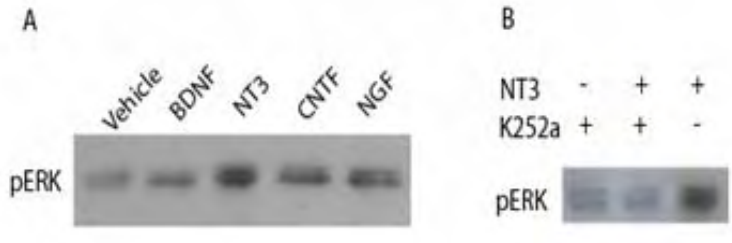

Fig. 11. Activation of the MAPK pathway in hES cell-derived cells. **(A)** Induction of phosphorylated ERK following exposure to neurotrophic factors. **(B)** Inhibition of neurotrophin-induced phosphorylation of ERK by K252a.

4.8 EXPRESSION OF TELENCEPHALIC IDENTITY IN HUMAN ES-CELL DERIVED NEURONS

In order to identify the positional character of neurons that were generated in the presence of neurotrophic factors, the expression of various transcription factors which are known to have important roles in brain development was examined. In hES cell-derived neuronal progenitors, the proneural transcription factor Mash1, which is involved in the regulation of telencephalon dorsal/ventral fates in the developing forebrain, was expressed **(Fig. 12).** Moreover, other transcription factors including Dlx1, Dlx2, Gsh2 and Gbx2 were found, which serve as markers of neurons of developing telencephalon[264, 265]. In order to characterize telencephalic identities further, the expression of two LIM-homeobox genes Lhx6 and Lhx8 was analyzed, which serve as markers of the medial ganglionic eminence (MGE) area. Exposure to CNTF induced Lhx6, a marker of neurons in the subventricular and submantle zones of MGE[266], whereas Lhx8, which is involved in the specification of many cholinergic neurons[267] and is a marker of the submantle and mantle zone of the MGE, was induced in hES cell-derived neuronal cells by BDNF and NGF.

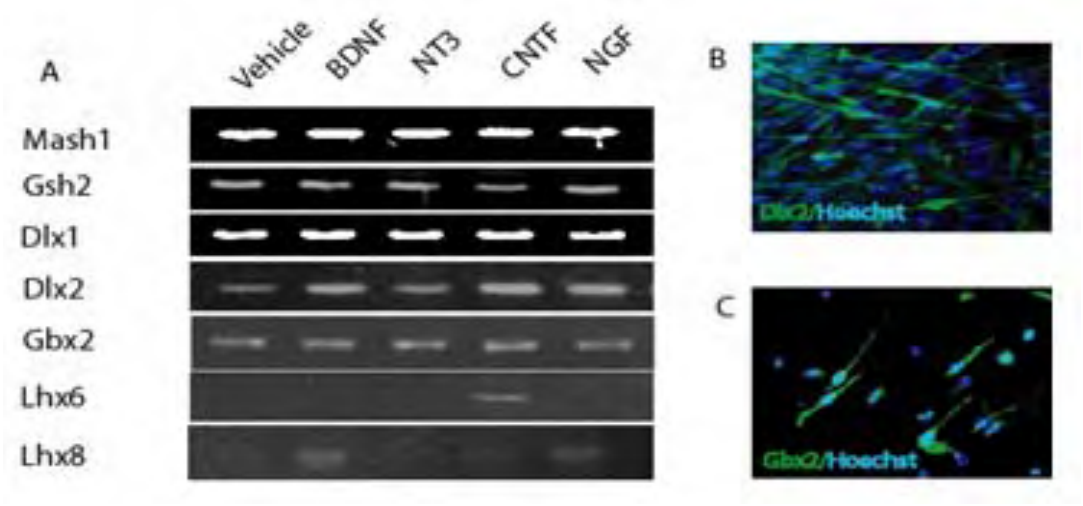

Fig. 12. (A) Expression of subregional markers in differentiated hES cells. After 18 days of differentiation with BDNF, NT3, CNTF and NGF, cells expressed transcripts for several telencephalic transcription factors including Mash1, Dlx1, Dlx2, Gbx2 and Gsh2. **(B and C)** Immunofluorescence of Dlx2- and Gbx2-positive cells, respectively, 18 days after plating, (at 40 ×).

Expression of transcription factors (Mash1, Dlx1, Dlx2, Gbx2, Gsh2, Lhx6 and Lhx8) was observed in our differentiated hES cell cultures, that specify the identity of progenitors and neuronal cells in the developing telencephalon, indicating that this culture system offers a way to generate forebrain neuronal populations. Since basal

forebrain cholinergic neurons originate from developing telencephalic neuronal precursor cells, these findings suggest that our hES cell-derived neuronal progenitors may have the potential to differentiate into a population of neurons which exhibit a similar positional identity to neurons that are markedly reduced in AD.

4.9 CHOLINERGIC DIFFERENTIATION OF HUMAN ES CELL-DERIVED NEURONS BY NEUROTROPHIC FACTORS

The prototype of neurotrophins, NGF, is well-known for its ability to prevent and reverse degeneration in adult and aging brain. Brain-derived neurotrophic factor also has important functions in maturation of the basal forebrain *in vivo*[268], and it increases survival and the levels of ChAT and AChE in cultured embryonic and early postnatal cholinergic neurons[269-271]. In addition, the cytokine CNTF upregulates ChAT *in vitro*[272], which suggests that several neurotrophic factors can mediate cholinergic differentiation in NPCs and neurons.

Accordingly, we found that the levels of transcripts encoding ChAT and the proportion of ChAT-immunopositive cells were increased following exposure to neurotrophic factors **(Fig. 13)**.

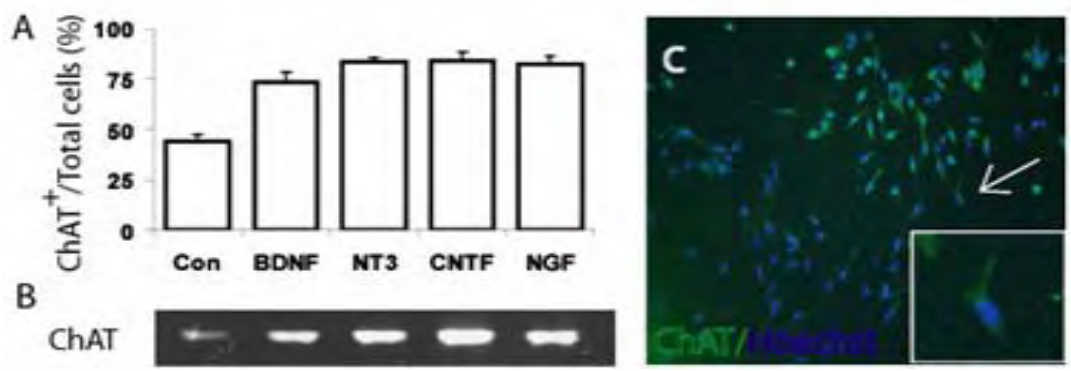

Fig. 13. Cholinergic differentiation for 18 days of hES cell-derived neurons. **(A)** Proportion of ChAT-positive cells following exposure to neurotrophic factors. **(B)** RT-PCR analysis of ChAT in hES cell-derived neurons. **(C)** Immunostaining of hES cell-derived cells following CNTF exposure (at 20 ×). Inset, higher magnification of the cell indicated with an arrow.

The increased expression of pERK following stimulation with neurotrophic factors suggests that the MAPK pathway may be involved in cholinergic differentiation of hES cell-derived neuronal precursors. Increasing the number of cholinergic neurons

generated from hES cells is of great interest for understanding developmental biology programs and for future cell-based replacement therapeutic strategies.

Neuronal nAChRs and mAChRs have important roles for many cognitive functions, and in primary cultures of stem and progenitor cells from embryonic mouse cortex, functional nAChRs have been reported[20].

In differentiated hES, transcripts encoding the α3, α4 and α7 nAChR subunits were found in cell-derived neuronal cells **(Fig. 14A)**. Expression of the subunits was differentially regulated by various neurotrophic factors. Exposure to BDNF, NT3, CNTF and NGF increased the levels of transcript for the α3 nAChR subunit, whereas small reductions were observed as regards the α4 nAChR subunit following BDNF and CNTF treatment.

The expression of these nAChR subunits is of particular interest since the majority of high affinity nAChRs in the brain are composed of the α4(β2) and α3 nAChR subtypes, and principally the α7 nAChR is found early in the developing human brain[12, 29].

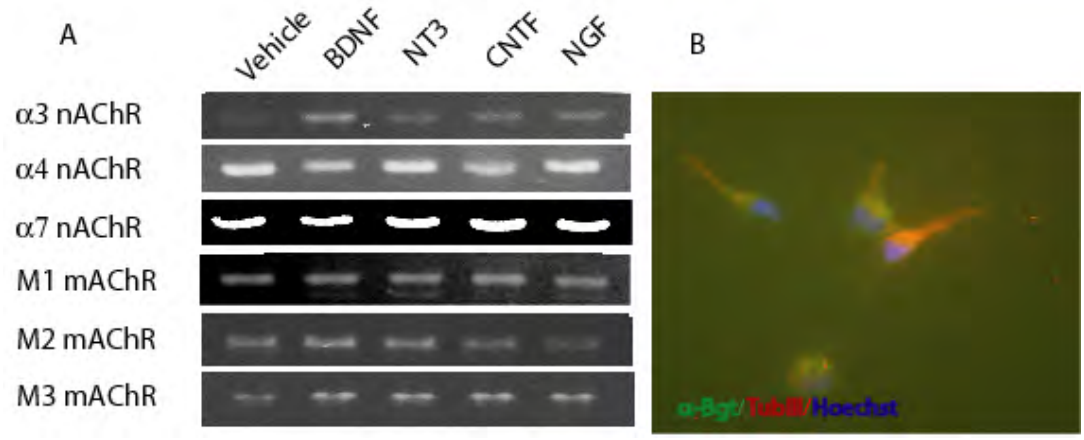

Fig. 14. Expression of markers of cholinergic receptors in hES-cell derived neuronal cells. **(A)** Differential regulation of transcripts encoding nAChRs and mAChRs in the presence of neurotrophic factors after 18 days. **(B)** Immunostaining of cells differentiated for 48 hr on polylysine/laminin-coated coverslip with α-BTX and βIII-tubulin. Nuclei were stained with Hoechst.

Immunocytochemical analysis showed that the α7 nAChR subtype was expressed in 5-10% of the cells. Since α7 nAChRs in particular have a high permeability to calcium[21], and calcium signaling is known to affect a variety of developmental processes including cell proliferation, neurite outgrowth and retraction[22-25], the

findings suggest that α7 nAChRs have important functions in early neuronal development and plasticity.

In addition, transcripts encoding the M1, M2 and M3 mAChR subtypes were found in hES cell-derived neuronal cells **(Fig. 14A)**. Activation of mAChRs may be associated with proliferation and differentiation of neural precursor[34, 273]. In the neuroepithelium of embryonic rat forebrain, the M1 mAChR subtype is expressed in neuronal progenitors and newly generated neurons[33], suggesting that these receptors are involved in the development of the nervous system prior to the onset of synaptogenesis.

4.10 CHOLINERGIC RECEPTORS MEDIATE CYTOSOLIC CALCIUM

In order to examine the functionality of the cholinergic receptors expressed in hES cell-derived neuronal cells, cytosolic calcium was imaged.

Upon ACh administration to living cells, an increase in cytosolic calcium was found. The proportion of responding cells was dose-dependent up to a certain concentration of ACh (1 μM), which evoked a calcium increase in a maximum of 50 to 60% of imaged cells **(Fig. 15)**. Since the proportion of responding cells varied according to the ACh concentration, there is probably a heterogeneous population of cholinergic receptor subtypes expressed in hES cell-derived neurons.

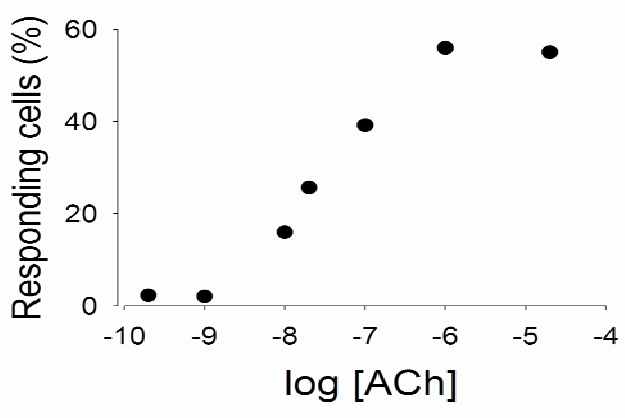

Fig. 15. Cholinergic receptors mediate the calcium increase evoked by ACh in hES-cell derived neurons. The fraction of responding cells is plotted against the logarithm of the concentration of ACh. Each point corresponds to the counting of more than 50 cells (2-6 experiments).

In order to investigate the source of the ACh-evoked cytosolic calcium, we applied ACh in calcium-free conditions (**Fig. 16A**). The absence of extracellular calcium was ineffective in abolishing the increase in cytosolic calcium evoked by ACh, indicating that calcium permeability was not a major contributor to the cellular response. Moreover, the calcium response was abolished by the AChR antagonist atropine (**Fig. 16B**), which suggests the presence and involvement of G-protein coupled receptors in mobilization of calcium from intracellular stores. Together, these data suggest that the calcium response was mainly mediated by mAChRs. A nicotinic contribution in augmentation of cellular calcium was not observed in our experimental conditions, which may reflect the different kinetics of the ACh-evoked calcium responses mediated by mAChRs and nAChRs. Another explanation may be that some receptor subtypes remain in intracellular compartments, with a negligible presence on the plasma membrane.

The expression of functional mAChRs in hES cell-derived neurons is important because of the crucial role of the cholinergic system in normal cognitive functioning and in age-related dementia disorders. Furthermore, an age-related reduction in the density of M1 mAChRs has been observed in rat hippocampus[274]. Since postsynaptic activation of M1 mAChRs in the hippocampus enhanced LTP of excitatory synaptic transmission[275], these receptors may have the ability to modulate synaptic plasticity.

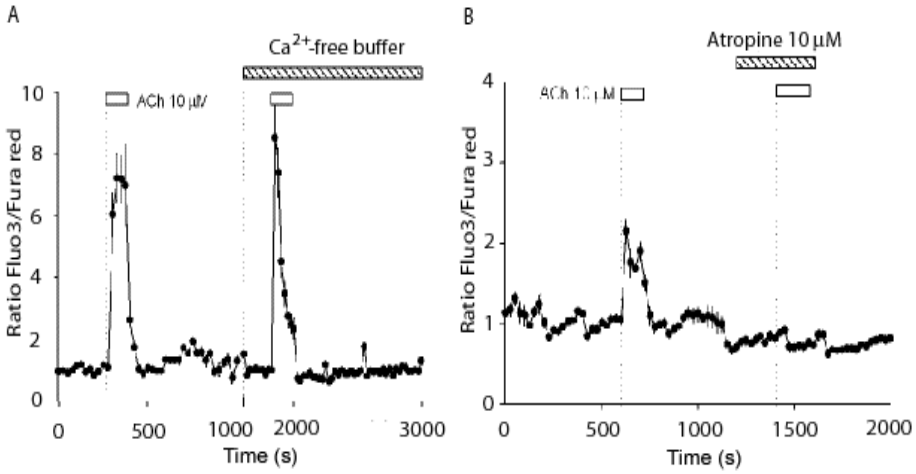

Fig. 16. (A) Human ES cell-derived neuronal cells were exposed twice to ACh (10 μM). The first application was in a physiological buffer supplemented with 20 mM glucose (KRH-glc). The second time, ACh was applied in the absence of external calcium. The trace corresponds to an average of 11 cells and is a representative of three experiments. **(B)** Cells were exposed twice to ACh (10 μM). The first application shows the calcium increase elicited by ACh. The second application of ACh was performed in the presence of atropine (10 μM).

4.11 PHARMACOLOGICAL REDUCTION OF APP LEVELS *IN VIVO*

As the amyloid plaques of AD are primarily composed of the Aβ peptide, which is a proteolytic product derived from APP[58, 276, 277], the majority of studies on Aβ have been focused on its neurotoxicity. Although the underlying mechanisms of Aβ neurotoxicity are of major interest in AD research, thorough understanding of the biological role of APP has remained elusive. Earlier studies have shown that the expression of APP is elevated after brain injury[278] and in rats with lesions of the forebrain cholinergic system[279], suggesting an important role of APP in the brain under both normal and diseased conditions.

Interaction between APP and cholinergic neurotransmission have been suggested, as the release of soluble APP from human cell lines and brain tissue has been reported to be increased through activation of M1 and M3 receptors[125, 126]. Interestingly, earlier *in vitro* studies have demonstrated that some of the AChEIs regulate the expression and processing of APP and reduce the level of Aβ. These effects on APP were found to be mediated independently of the ChEI activities of the drugs[280–282].

(–)-Phenserine is a novel AChEI which currently is in clinical trials for treatment of AD. Experimental studies have also demonstrated that treatment with (–)-phenserine reduced the APP protein expression in neuronal cells and improves cognitive performance in rodents[140, 141, 283]. This action is not because of the AChEI activity of phenserine, since the (+)-enantiomeric form of phenserine, which lacks AChEI activity, has a similar effect on the suppression of APP[141]. Recently, PET measurements have shown that treatment with (–)-Phenserine affects Aβ and glucose metabolism in the brains of AD patients[143].

We found that treatment of wild-type and APP23 transgenic mice with (+)-phenserine reduced the protein expression of APP in the hippocampus of the APP23 transgenic mice **(Fig. 17A)**. The treatment was carried out in 3- to 4- month old mice before the onset of Aβ plaque pathology at about six months of age[284]. Our findings are consistent with earlier reports demonstrating that alteration of APP expression is not dependent on the catalytic activity of phenserine. The results suggest that non-catalytic functions of AChEIs could be utilized to modulate the level of APP. Since phenserine is dose-limited by its AChEI activity, the cholinergically inactive enantiomer allows administration of higher doses. Based on the fact that over-expression of APP in APP23 transgenic mice may be associated with inflammatory reactions[284], the protein level of GFAP was detected. Following treatment with (+)-

phenserine the expression of GFAP was also reduced **(Fig. 17B)**, suggesting that this treatment may interfere with gliosis in these animals.

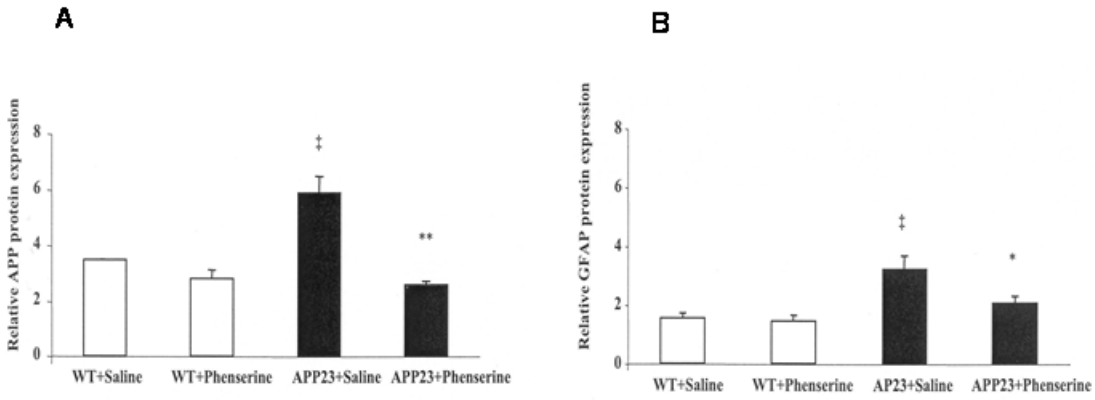

Fig. 17. Relative protein levels of total sAPP **(A)** and GFAP **(B)** in hippocampal tissue of APP23 and non-transgenic mice, following (+)-phenserine or saline treatment for 14 days. * P <0.05 and ** P <0.01 indicate significant differences versus saline-treated mice. ‡ P <0.05 indicates significant differences within the saline-treated groups (ANOVA). Values are expressed as mean ± s.e.m.

4.12 *IN VIVO* DIFFERENTIATION OF HUMAN NEURAL PRECURSORS

Stem cell transplantation studies in animal models of neurodegeneration have indicated that stem cells are able to migrate to sites of injury and differentiate into neurons and glial cells in the brain. Disturbances in the microenvironment due to brain injury or pathology could affect the microenvironment of the brain and expose the cells to cues that are different from those in normal conditions, leading us to examine the differentiation patterns of human NPCs under pathological conditions.

The increase in APP expression during the development of the CNS coincides with a peak in neuronal differentiation. In addition, APP plays a regulatory role in neural survival, proliferation and neurite outgrowth, which suggests that it may have important physiological functions in stem cell biology and neurogenesis. It has also been demonstrated that human NPCs *in vitro* differentiate into astrocytes rather than neurons following exposure to high doses of secreted APP[285], suggesting that regulation of APP expression in the brain is crucial in the development of future neuroreplacement strategies.

Following transplantation of human NPCs into the lateral ventricle of (+)-phenserine- or saline-treated APP23 and wild-type mice, reduced glial cell differentiation was observed in hippocampal regions of APP23 transgenic mice treated with (+)-phenserine compared with controls (**Fig. 18)**. We also found a negative correlation between APP protein expression and the number of implanted NPCs that differentiated into astrocytes in the hippocampus of APP23 transgenic mice **(Fig. 18D)**.

Since there was no difference in caspase-3 immunoreactivity of transplanted cells derived from human NPCs in brain sections from APP23 transgenic mice treated with (+)-phenserine or saline, we concluded that (+)-phenserine did not have any significant toxic effects on the implanted cells.

In contrast to the reduced glial differentiation of transplanted human NPCs in APP23 transgenic mice that received (+)-phenserine, we observed significant increases in the number of βIII-tubulin/BrdU-positive cells in the hippocampal CA1 and CA2 regions, and also in the motor and the somatosensory cortices of APP23 mice after (+)-phenserine treatment compared with the number of such cells found following saline treatment (**Fig. 19)**. These findings indicate that neuronal differentiation of implanted NPCs was stimulated after (+)-phenserine-induced APP protein reduction in the brains of APP23 mice.

In an earlier study it was demonstrated that human NPCs which were transplanted into aged rat brain not only differentiated into neuronal cells but were also able to reverse age-associated cognitive impairment[210], suggesting that the aged brain may provide necessary conditions for implanted human NPCs. Other studies also indicated that human NPCs may hold great promise in the replacement of degenerative cholinergic neurons, which may improve the deficits associated with AD[207-209].

Interestingly, a dramatic increase of APP in cholinergic projection areas in rats with forebrain cholinergic lesions has been found[279], indicating that the regulatory control of APP may be important.

Based on the findings in our study involving drug treatment and transplantation of human NPCs into APP23 mice, we suggest that modulation of altered levels of APP protein, which have a regulatory role in the migration and differentiation of human NPCs, may have implications for the development of future cell-therapy programs.

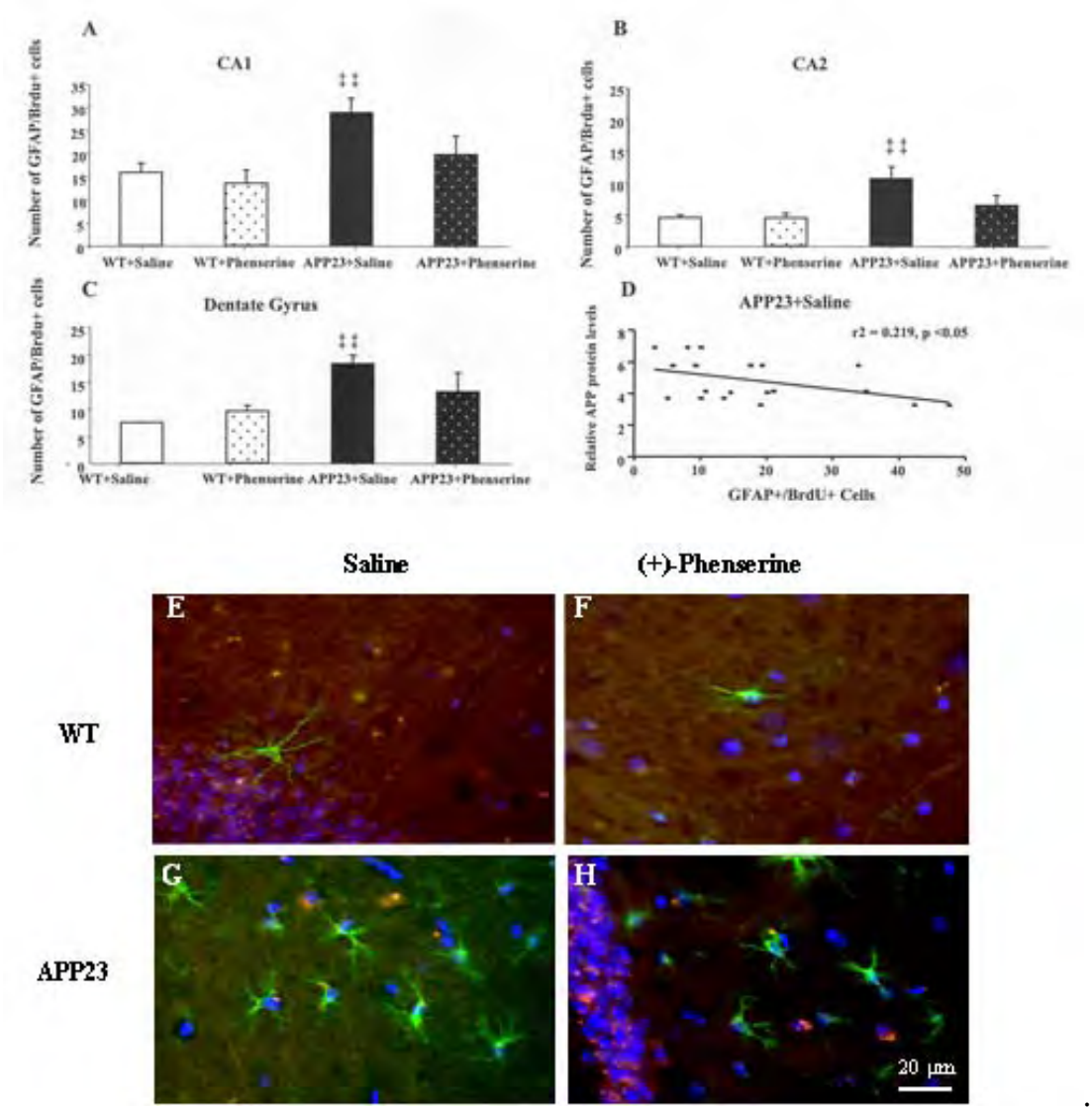

Fig. 18. Differentiation of transplanted human NPCs into astroglial cells *in vivo* following treatment with saline or (+)-phenserine. After six weeks of differentiation in hippocampal regions of 6- to 7-month old APP23 and non-transgenic mice, the ratios of transplanted NPCs that were immunopositive for GFAP were estimated in the CA1 region **(A)**, the CA2 region **(B)**, and the dentate gyrus **(C).** Values are expressed as mean ± s.e.m. (n = 6 or 7 within each group), measured in four to six sections for each mouse. ‡‡ $P < 0.01$ indicates a significant difference between the saline-treated groups. **(D)** Correlation between APP protein level and the number of GFAP/BrdU-positive cells in the hippocampus of 6- to 7-month old APP23 transgenic mice that received saline only**. (E-H)** Immunofluorescent images in the CA1 region of the hippocampus of 6- to 7-month old APP23 transgenic and non-transgenic mice stained for GFAP and BrdU. Nuclei were stained with DAPI.

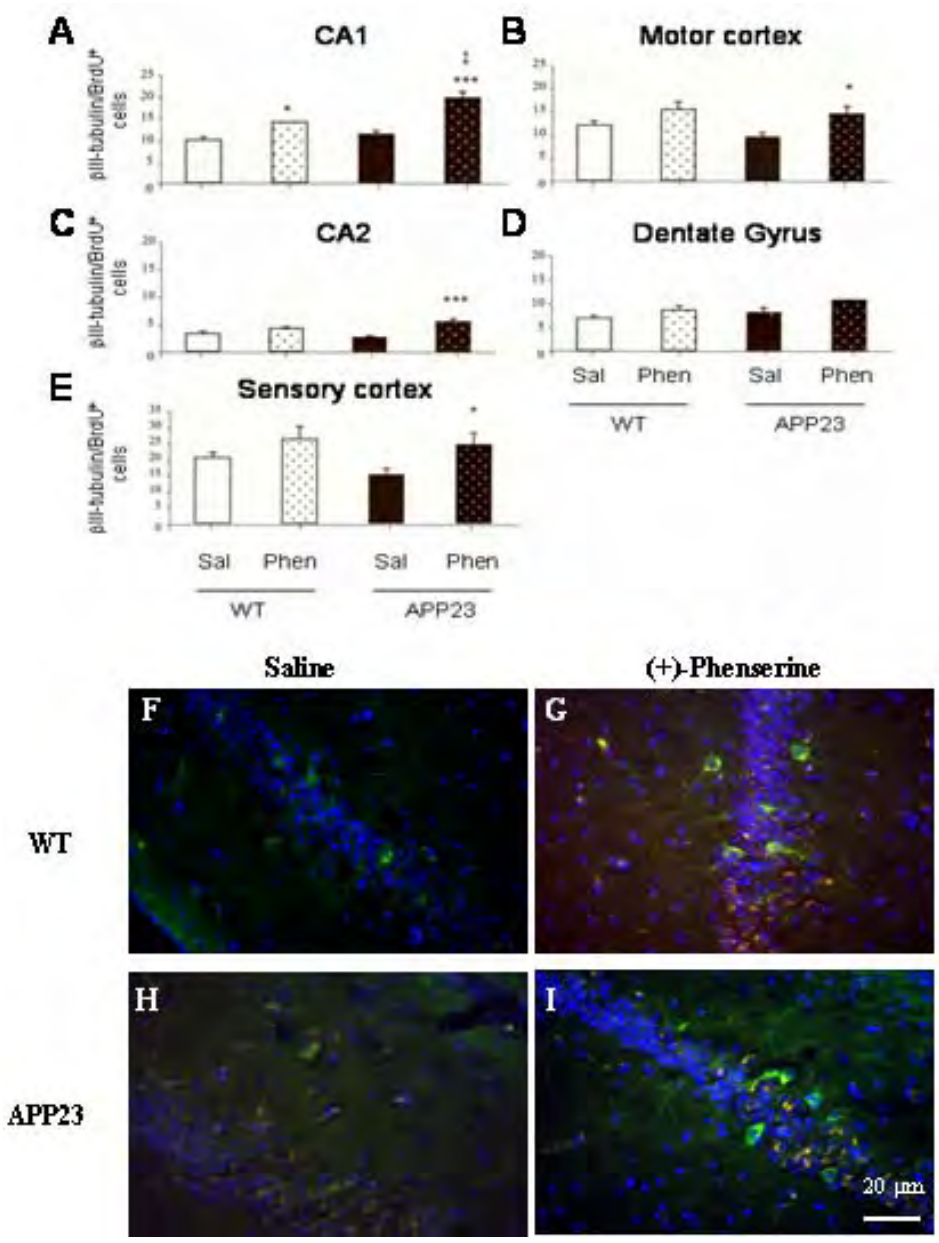

Fig. 19. Differentiation for 6 weeks of transplanted human NPCs into neuronal cells in hippocampal and cortical regions of APP23 transgenic and non-transgenic mice treated with saline or (+)-phenserine. Neuronal cells in the CA1 region, the CA2 region and the dentate gyrus (**A, C** and **D**), and the motor and sensory cortices (**B** and **E**). **P* < 0.05, and ****P* < 0.0001, indicate significant differences versus the saline-treated group. ‡ *P* < 0.05, indicates a significant difference between (+)-phenserine-treated groups. Values are expressed as mean ± s.e.m. (n = 6 or 7 within each group), measured in four to six sections for each mouse. **(F-I)** Immunofluorescent images in the CA1 region of the hippocampus of 6- to 7-month old APP23 transgenic and non-transgenic mice stained for βIII-tubulin and BrdU. Nuclei were stained with DAPI.

5 CONCLUDING REMARKS

The cholinergic system has essential roles in a variety of cognitive functions. Owing to the close association between loss of cholinergic activity and cognitive decline, it is crucial to understand the regulatory mechanisms for the stimulation and maintenance of cholinergic neurotransmission under both normal and pathological conditions.

In the present work, cells from six different hES cell lines all demonstrated the capacity to differentiate into neuroepithelial and radial glial cells in adherent and suspension cultures. As both neuroepithelial and radial glial cells serve as neural progenitors in the developing forebrain[162], these cells, generated *in vitro*, and cultured in controlled feeder-free and serum-free conditions may serve as a source of neurogenic cells for further neuronal differentiation. It is also tempting to speculate that radial glial cells, besides their classical role in neuronal migration, may also have a regulatory function in regional neuronal differentiation.

Although considerable efforts have been made to understand the cellular contribution of the ganglionic eminences to the neuronal subtypes in the telencephalon, little is known about the signals and mechanisms that control their generation. Since forebrain cholinergic neurons arise from the ventral telencephalon, including the MGE, the expression of transcription factors in hES cell-derived neurons that specify neuronal identity of this region was of particular interest. Differentiated hES cells expressed the ACh-synthesizing enzyme ChAT and its expression was increased following stimulation with BDNF, NT3, CNTF and NGF, probably through the activation of the MAPK pathway. We report here, for the first time, that differentiated hES cell-derived neurons express α3, α4 and α7 nAChR subunits and M1, M2 and M3 mAChR subtypes, and their expression was differentially regulated following treatment with neurotrophic factors. In addition, the septal SN56 cell line was demonstrated to be a useful model to study the regulation of ChAT and the different nAChR subunits. Consistent with the hES cell-derived neurons, stimulation with RA and NGF regulated the various nAChR subunits differentially in SN56 cells. Because of the importance of ACh for neuronal survival, proliferation and neurite outgrowth, increased knowledge of the cellular elements which influence the developmental regulation and modulation of the expression of

nAChRs and mAChRs is essential in order to understand their roles in neuronal development and plasticity.

The functionality of cholinergic receptors in hES cell-derived neurons was demonstrated by way of an ACh-evoked increase in cytosolic calcium. Upon exposure to various concentrations of ACh, the proportion of responding cells varied, indicating that multiple subtypes of cholinergic receptors were functional in these cells. Additionally, the ACh-evoked calcium increase was abolished by the mAChR antagonist atropine.

Amyloid precursor protein may not only have a key role in AD pathology, but it may also have regulatory functions for stem cells and neurogenesis. We found that treatment with (+)-phenserine suppressed the protein expression of both APP and GFAP in the hippocampus of APP23 transgenic mice, suggesting that this drug not only has the capacity to lower the level of the precursor of Aβ independently of its AChEI activity, but it may also have the potential to interfere with gliosis. Treatment with (+)-phenserine also increased the neuronal differentiation of transplanted human NPCs in hippocampal and cortical regions of APP23 mice, which indicates that a combined approach of drug treatment and stem cell transplantation may be crucial in order to further explore the mechanisms of stem cell differentiation and migration under the pathophysiological conditions of AD.

Altogether, the findings in the present work demonstrate that hES cells may serve as an important model to study cellular mechanisms in early human neural development. Exploring the mechanisms of neurotrophic factors that are present in the cellular microenvironment, acting as inductive signals and regulators of neuronal differentiation processes and plasticity of human ES cells, NPCs and neurons, may also lead to future development of novel strategies for therapeutic intervention in cases of AD.

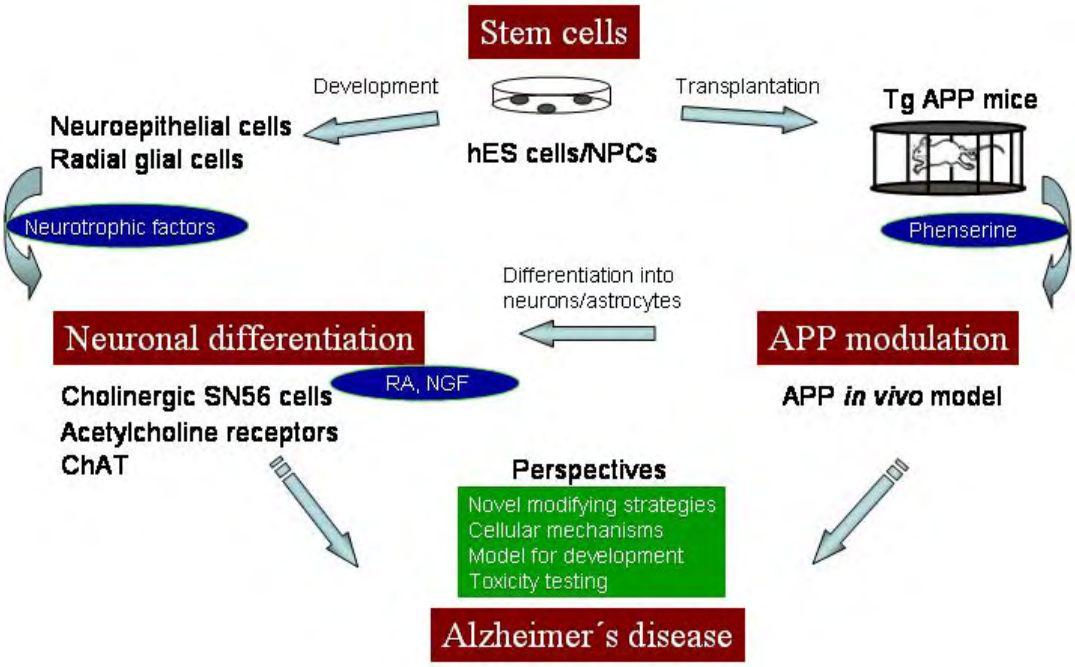

Fig. 20. Summary and perspectives of the present work.

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