Molecular mechanisms for lineage-restricted differentiation of adult neural progenitors

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ABSTRACT

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Aims: In the central nervous system of several species, including humans, neurogenesis persists even in the adult life in discrete neurogenic regions of the brain. Adult neural stem cells derived from these neurogenic areas are proliferating cells, which can differentiate to neurons, astrocytes, and oligodendrocytes. The molecular mechanisms and signaling pathways regulating the lineage commitment and differentiation of neural stem cells is now unfolding. Understanding the mechanism underlying these events is essential for the potential future use of neural stem cells for cell therapy in neurodegenerative diseases. In the present thesis, we investigated the role of bone morphogenetic proteins (BMP), apoptosis signaling-regulating kinase 1 (ASK1) and the nuclear receptors, all-trans retinoic acid (ATRA) and TLX, in neural differentiation of adult hippocampus-derived progenitor cells (AHPs).

Results: Overexpression of dominant negative BMP type I (Alk2, 3, and 6) receptors in adult neural progenitors revealed that Alk6 signaling is necessary for differentiation and survival of astrocyte and suppression of oligodendrocyte fate. Blockage of Alk3, on the other hand, increased Alk6 expression, resulting in an increased survival and differentiation towards astrocyte lineage. Blockage of any of the receptors did not alter the neuronal differentiation.

In order to investigate the role of ASK1, we overexpressed either a constitutively active or a kinase mutant form of ASK1. In this study we provide evidence for ASK1 via p38 MAPK activation induces neuronal lineage commitment while inhibiting glial differentiation. We determined that the ASK1-induced glial inhibition was due to a direct repression of the GFAP promoter in a STAT3-independent way.

In search for further downstream mechanisms of ASK1-induced neuronal differentiation, we found that ASK1 in a p38-dependent manner phosphorylated and thereby activated MEF2C. This transcription factor was recruited to the MASH1 promoter along with CaMKII and the coactivator CBP, while the corepressors HDAC1 and 4 were dismissed. Moreover, we combined ASK1 expression with ATRA treatment. Consequently, we observed a synergistic increase in neuronal differentiation. ATRA also activated the MASH1 promoter however, via the transcription factor Sp1.

Finally, we investigated the role of the orphan nuclear receptor, TLX. By means of overexpressing TLX, we found that TLX induced a transient increase in neural progenitor proliferation and an increase in the number of differentiating and mature neurons, while suppressing glial differentiation. Similar to ATRA signaling, Sp1 was necessary for TLX-induced MASH1 activation.

Conclusions: The results presented in this thesis suggest a new role for both ASK1 and TLX in the regulation of neuronal and astroglial differentiation of adult hippocampus-derived neural progenitors. In addition, we have demonstrated that ASK1 in combination with ATRA yield synergistic effect on the generation of mature neurons. Our results indicate that the Alk6 signaling has an important role for astrocyte survival and differentiation. We have determined the mechanisms involved in these signaling pathways, which might potentially be of benefit for future therapies of neurodegenerative diseases.

List of Publications

This thesis is based on the following articles, which are referred to by their Roman numerals in the text:

I. Brederlau A, Faigle R*, **Elmi M***, Zarebski A, Sjöberg S, Fujii M, Miyazono K, Funa K. The bone morphogenetic protein type Ib receptor is a major mediator of glial differentiation and cell survival in adult hippocampal progenitor cell culture.
Mol Biol Cell. 2004 Aug;15(8):3863-75 ***joint second authors**

II. Faigle R, Brederlau A, **Elmi M**, Arvidsson Y, Hamazaki TS, Uramoto H, Funa K. ASK1 inhibits astroglial development via p38 mitogen-activated protein kinase and promotes neuronal differentiation in adult hippocampus-derived progenitor cells.
Mol Cell Biol. 2004 Jan;24(1):280-93

III. **Elmi M**, Faigle R, Yang W, Matsumoto Y, Rosenqvist E, Funa K. Mechanism of MASH1 induction by ASK1 and ATRA in adult neural progenitors.
Mol Cell Neurosci. 2007 Oct;36(2):248-59

IV. **Elmi M**, Matsumoto Y, Yang W,Uemura A, Nishikawa S, Funa K. Nuclear receptor TLX promotes neuronal differentiation in adult hippocampus-derived progenitor cells.
Manuscript

Table of Contents

List of Abbreviations

AHPs	adult rat hippocampus-derived progenitors
Alk	activin receptor-like kinase
ASK1	apoptosis signal-regulating kinase 1
ATRA	*all trans* retinoic acid
FGF	fibroblast growth factor
bHLH	basic helix-loop-helix
BMP	bone morphogenetic protein
CaMKII	Ca^{2+}/calmodulin-dependent protein kinase
CBP	CREB- binding protein
C/EBP	CCAAT enhancer binding protein
ChIP	chromatin immunoprecipitation
Co-Smad	common-mediator Smad
CNS	central nervous system
CNTF	ciliary neurotrophic factor
CRABP	cellular retinoic-acid-binding protein
DCX	doublecortin
DIV	days *in vitro*
DMSO	dimethylsulphoxide
E12	embryonic day 12
ERK	extracellular signal-regulating kinase
FACS	fluorescence-activated cell sorting
FITC	fluorescein isothiocyanate
GalC	galactocerebroside
GFAP	glial fibrillary acidic protein
GCL	granular cell layer
HA	haemagglutinin
HAT	histone acetyl transferase
HDAC	histone deacetylase
Hes1	*hairy/enhancer of split homologue1*
Id	inhibitor of differentiation
JNK	c-Jun N-terminal kinase
LDH	lactate dehydrogenase
LIF	leukemia inhibitory factor
MAP2	microtubule-associated protein 2
MAPK	mitogen-activated protein kinase
Mash1	mammalian *achaete-scute homologue1*
m.o.i	multiplicity of infection
mTOR/FRAP	mammalian target of rapamycin/FKBP12-rapamycin-associated protein
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide
Ngn	neurogenin
NMDA	N-methyl-D-aspartate

NPC	neural progenitor cell
NSC	neural stem cell
PBS	phosphate buffered saline
PCAF	p300/CBP-associated factor
PCR	polymerase chain reaction
PDGF	platelet-derived growth factor
RA	retinoic acid
RAR/RXR	retinoic acid receptor
R-Smad	receptor-regulated Smad
SDS	sodium dodecylsulphate
Sp1	stimulatory protein1
SGZ	subgranular zone
STAT	signal transducer and activator of transcription
SVZ	subventricular zone
TBS	tris-buffered saline
TGF-β	transforming growth factor-β
TNF-α	tumor necrosis factor-α
TRAF2	TNF-receptor-associated factor 2
Trx	thioredoxin
XIAP	X-chromosome-linked inhibitor of apoptosis protein

Introduction

Neurogenesis

Neurogenesis, which means birth of neurons, is a process by which new neurons are created. During mammalian development, neurogenesis is required for the formation of the central nervous system (CNS). The main cell types in the CNS, namely astrocytes, neurons, and oligodendrocytes, are all generated in a stereotyped sequential manner (Temple, 2001). Neural stem cells (NSCs) are defined as cells that have the ability to self-renew, and give rise to the three major cell types of the mammalian CNS (Gage, 2000). The term "neural progenitors" on the other hand, has been used less stringently, describing all neural cells that have the ability to divide and differentiate (Zhao et al., 2008).

Embryonic Neurogenesis

Before the formation of the nervous system the neural plate and neural tube are composed of a layer of cells, called neuroepithelial cells, which form the neuroepithelium and are considered to be NSC (Merkle and Alvarez-Buylla, 2006). The neuroepithelial cells undergo symmetric proliferative divisions generating daughter stem cells to later divide asymmetrically, giving rise to more differentiated cells that are able to develop into terminally differentiated postmitotic cells (Merkle and Alvarez-Buylla, 2006). In mouse and rat CNS development, the first neurons are being formed around embryonic day (E) 12 when neural progenitor cells (NPCs) proliferate in the ventricular zone.

After neurogenesis has been initiated, the neuroepithelial cells give rise to a distinct but related cell type — the radial glial cells. These are the only glial cells that can be detected prior to E16 (Rakic, 1972). Radial glial cells, having both neuroepithelial as well as astroglial properties, represent more fate-restricted progenitors compared to neuroepithelial cells (Campbell and Gotz, 2002; Merkle and Alvarez-Buylla, 2006). Immature neurons while differentiating use radial glial cells as guides and migrate along the glial extensions to the cortex (Rakic, 1972). The very first cortical astrocytes are formed around E16 and the very first oligodendrocytes are generated around birth. However, the majority of these cell types is produced and differentiate at later stages after most neurons are born (Kandel et al., 2000).

Previously, the process of neurogenesis was thought to be restricted only to the developing brain and not to occur in the adult brain. Today, it is recognized that neurogenesis continues in specific areas of the adult brain (Gage, 2000).

Adult Neurogenesis

In the early 20th century, investigations examining dividing cells in the adult rodent brain revealed cells in the hippocampus to be mitotically active (Altman and Das, 1965). However, at this time neurogenesis was not believed to occur in the adult mammalian brain. Although the

existence of dividing cells in the postnatal CNS was suggested, it was at this time impossible to trace the fate of those rare dividing cells and to prove that the newborn cells were in fact neurons rather than glia (reviewed in Gross, 2000). The main repair mechanisms in the CNS were thought to be postmitotic, such as sprouting of axon terminals, changes in neurotransmitter receptor expression, and synaptic reorganization (Lie et al., 2004). No replacement of degenerating neurons was believed to occur, and this became a central dogma in neuroscience for almost a century (Gross, 2000).

Four decades ago, Altman and his colleagues used autoradiography, a method to detect DNA synthesis by incorporation of tritiated thymidine into the DNA of dividing cells, to show that generation of new neurons was indeed occurring in the dentate gyrus of the adult rodent hippocampus (Altman and Das, 1965) and the olfactory bulb (OB) (Altman, 1969). However, little notice was given to these studies, mainly due to the fact that the fate of these proliferating cells was not clear and also perhaps because they were considered to lack functional relevance. It was not until in the early 1980s that Kaplan and Bell (1983) could demonstrate the fate of the new neurons in the adult hippocampus, that these cells could survive for a long period of time and receive synaptic inputs. Around the same time, studies of adult neurogenesis in songbirds showed evidence for functional roles of postnatal neurogenesis in seasonal song learning (reviewed in Nottebohm, 2004).

The idea of local adult mammalian neurogenesis was fully accepted in the early 1990s, and since then a large body of work has demonstrated that new neurons are indeed born in restricted regions of the adult mammalian CNS (Lois and Alvarez-Buylla, 1993; Luskin, 1993; Gage, 2000; Alvarez-Buylla and Garcia-Verdugo, 2002) including human CNS (Eriksson et al., 1998). Using retroviral-based lineage tracing and electrophysiological studies, evidence showed that newborn neurons in the adult mammalian CNS are functional and synaptically integrated (Ming and Song, 2005).

Neurogenesis in the Adult Hippocampus

In the Subgranular zone (SGZ) of the hippocampus, progenitors are closely opposed to a dense layer of granule cells that includes both immature and mature neurons. The progenitor cells that reside here divide continuously, giving rise to both neurons and glial cells. The newly generated neuronal progenitors migrate into the granular cell layer (GCL) and become mature functional neurons. The proliferating cells in the SGZ are classified into different types of neural progenitors, distinguishable by their morphological and phenotypical appearances. The type 1 cells are referred to as the radial glia-like cells that have a triangular cell body and a long process reaching into the molecular layer (Kempermann et al., 2004). Moreover, type 1 cells are rarely dividing cells that express nestin and the marker glial fibrillary acidic protein (GFAP). Although, GFAP is an astrocytic marker, type 1 cells are morphologically and functionally different from mature astrocytes. In the SGZ, as well as in the Subventricular zone (SVZ), it has been suggested that it is this distinct population of cells, which possess these astrocytic features, that are the true stem cells (Doetsch et al., 1999; Seri et al., 2001).

The type 2 cells are believed to be the progeny of type 1 cells, having only short processes and lack GFAP expression. These cells are highly proliferative and capable of migration. Other features have been described along the neuronal maturation process, referring them to type 3-6, where type 6 cells being the mature stage (Kempermann et al., 2004). In addition to these cells there are also astrocytes, oligodendrocytes, and other types of cells interacting with the progenitor cells, creating a so called neurogenic niche. This microenvironment will be described in more detail later in this thesis.

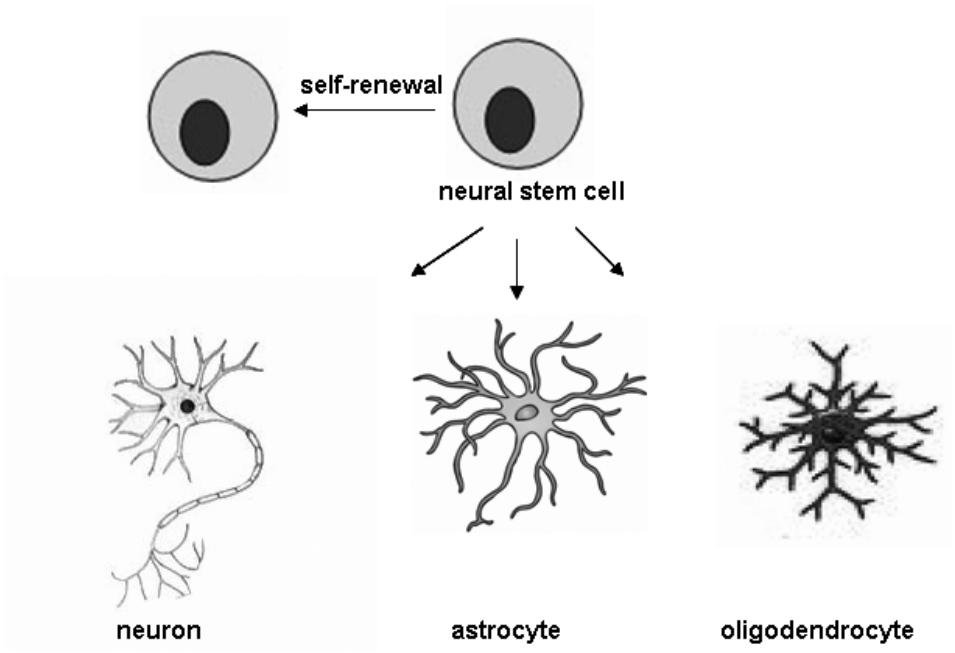

Figure 1. Neural stem cells can renew themselves and give rise to neurons, astrocytes and oligodendrocytes.

Adult Neural Stem Cells *In Vitro*

Neural stem cells from the adult CNS can be cultured *in vitro*. They were first isolated from the adult CNS of rodents, and later from humans (Reynolds and Weiss, 1992; Kukekov et al., 1999; Palmer et al., 1999). The standard method of isolating neural stem and progenitor cells *in vitro* is to dissect out a region of the adult brain, for example the SVZ or the hippocampus. Usually the tissue is enzymatically and mechanically disaggregated, and the dissociated cells are plated either directly (Reynolds and Weiss, 1992) or after partial purification to remove major contaminants (Palmer et al., 1999). Afterwards they are exposed to high concentrations of mitogens, such as fibroblast growth factors (FGFs) and/or epidermal growth factors (EGFs), which are the two most commonly used growth factors to maintain self-renewal, in either a defined or supplemented medium (Reynolds and Weiss, 1992; Palmer et al., 1999). In this condition, the NSCs will have a preferential growth compared to other cell types. The continuous culturing in the presence of

growth factors and serial passaging will result in expansion of the proliferating cells that have properties of neural stem and progenitor cells, constituting the major cell population of the culture. Two types of progenitor culture paradigms are commonly used. One condition allows progenitors to expand in culture in the form of cellular aggregates called neurospheres, where individual neural progenitors proliferate on a non-adhesive substrate and generate suspended clusters of cells (Reynolds and Weiss, 1992). Another way to culture neural progenitors is to grow them as an adhesive monolayer on surfaces coated with substrates such as polyornithine and laminin, and produce large clones containing mainly stem cells, but also neurons and glia (Palmer et al., 1999).

Other regions of the adult CNS, such as the adult eye and spinal cord, are not considered to be proliferative in general. They have been demonstrated to contain progenitors, and continuously dividing stem cells have been cultured from these regions using a method similar to that for neurospheres (Shihabuddin et al., 1997; Tropepe et al., 2000). These and other studies have suggested the presence of adult NSCs throughout the entire neuraxis (Weiss et al., 1996; Arsenijevic et al., 2001; Lie et al., 2002). However, a limitation to this culture method is the fact that proliferating cells derived from the adult brain have been primarily analyzed following long-term exposure to high concentrations of growth factors that can lead to changes of their epigenetic program (Kondo and Raff, 2000).

Markers defining NSCs are now being developed (Uchida et al., 2000; Rietze et al., 2001; Coskun et al., 2008), however, due to the lack of markers have made it difficult to identify and acutely isolate adult NSCs. Consequently, it has been difficult to establish the relationship between the *in vivo* proliferating cells and the *in vitro* cultured cells. Protocols have been developed that allow the enrichment of NSCs in culture, thereby allowing *in vitro* characterization soon after isolation (Palmer et al., 1999; Rietze et al., 2001). *In vitro* studies using these methods have confirmed that *in vivo* proliferating cells from gliogenic regions have the ability to give rise to neurons in culture without long-term exposure to mitogens, thereby providing additional support for the idea of a broad presence of NSCs in the adult mammalian brain (Lie et al., 2002).

Adult Rat Hippocampus-Derived Progenitor (AHP) Cells

In this thesis adult NPCs termed AHPs were used. We refer to these cells as “progenitor cells” instead of “stem cells”, since these terms should be used with strict scientific definitions. Only single cells, genetically or otherwise marked, can fulfill the criteria to be called a NSC. However, within the cell population that is termed progenitor cells there is probably a pool of true stem cells.

AHP cells are derived from the adult rat hippocampus using the method described earlier (Palmer et al., 1997). They have been cultured and maintained as an adhesive monolayer and shown to have normal diploid karyotype for up to 35 population doublings. Multipotency of NPCs is the ability of a single cell to give rise to both neurons and glial cells. To investigate the multipotency of AHPs, single cells were infected with a replication-incompetent retroviral vector and clonal

analysis of single cells was conducted after the proliferation and differentiation of AHPs (Palmer et al., 1997). The phenotype of the cells that originated from the initial genetically-marked cell was investigated with immunocytochemical staining using lineage specific markers. It was found that cells from all three lineages present in the CNS, namely neurons, astrocytes, and oligodendrocytes could be derived from one initial AHP cell, and thereby retrospectively characterizing it as a NSC.

Cell-grafting experiments showed that when AHPs were transplanted back into the adult rat hippocampus, the AHPs differentiated into neurons of the GCL (Gage et al., 1995). Furthermore, when AHPs were grafted into the developing eye they had the ability to develop into even non-hippocampal neuronal phenotypes, integrating well into the retinal microstructure and differentiating into Muller, amacrine, bipolar, horizontal, and photoreceptor cells (Takahashi et al., 1998). Previously, most studies described how NPCs differentiated into neurons and glial cells spontaneously as they were plated onto an adhesive substrate (Palmer et al., 1997; Galli et al., 2003). Today easy access and defined culture conditions allow manipulation of adult neural progenitors in adherent or neurosphere cultures. This allows precise analyses of the intrinsic and extrinsic mechanisms that control the various steps of neurogenesis, including proliferation, survival, fate specification, neuronal migration, maturation, and synapse formation (Song et al., 2002; Deisseroth et al., 2004; Raineteau et al., 2004).

Regulation of Adult Neurogenesis

The process of neurogenesis is regulated at different levels, *i.e.* stem cells in the adult brain are controlled by intracellular and extracellular factors. It is clear today that the environment is important for the process of neurogenesis. The role of environmental factors in NSC fate choice has been demonstrated by transplantation studies. Adult hippocampus-derived NSCs, when transplanted into the rostral migrotary stream, generated tyrosine-hydroxylase-positive interneurons in the olfactory bulb, a phenotype never seen in the hippocampal GCL (Suhonen et al., 1996). Furthermore, spinal progenitors, which are typically gliogenic in their native environment, can differentiate into neurons when transplanted to the pro-neurogenic environment of the hippocampus (Shihabuddin et al., 2000). On the other hand, neurogenic hippocampal and olfactory progenitors cease neurogenesis once transplanted to non-neurogenic regions of brain (Suhonen et al., 1996).

These findings show that adult NSCs from different regions are not fate-restricted by intrinsic programs, but that extrinsic cues in the local environment control the fate of adult NSCs. Adult stem cells in the neurogenic niches interact with their environment. The cellular elements of this niche are composed of astrocytes, endothelial cells, and ependymal cells. Through their interaction, the niche regulates neurogenesis, *i.e.* proliferation and fate choice of adult NSCs, as well as survival of newly generated neurons (Lim and Alvarez-Buylla, 1999; Song et al., 2002; Shen et al., 2004). In addition, the neurogenic areas have been found to be in close proximity of blood vessels, suggesting that vasculature- or blood-derived factors regulate neurogenesis (Palmer et al., 2000; Louissaint et al., 2002). Since the cell types that contribute to neurogenesis

have been identified, it is of enormous interest to identify signaling molecules in the cellular microenvironment that permits neurogenesis.

Growth Factors and other Extrinsic Signals

A large number of growth factors have been implicated in the control of neurogenesis. The growth factors FGF and EGF are mitogens used for propagating adult neural progenitors *in vitro* (Reynolds and Weiss, 1992; Palmer et al., 1995). Analysis of EGF and FGF2 responsiveness in the developing forebrain shows that early growth factor choice is regulated over time. FGF2 response is present in mice at E8.5, while at this time point EGF receptors are not expressed in NSCs. By E14.5 NSCs expressing EGF receptors emerge (Tropepe et al., 1999). In the adult, a subpopulation of proliferating cells in the SVZ expresses the EGF receptor. A null mutation of the EGF receptor-ligand, transforming growth factor α (TGF-α), leads to decreased stem cell proliferation in the SVZ (Tropepe et al., 1997). Moreover, infusion of EGF or FGF2 to the adult rodent brain increases cell proliferation in the SVZ. When recombinant EGF were infused in the lateral ventricles of adult rodents, EGF increased proliferation of cells in the SVZ, however this was not the case in the SGZ. In the SGZ, EGF influenced the fate of the cells, and resulted in more glial cells and fewer neurons (Craig et al., 1996; Kuhn et al., 1997).

Leukemia inhibitory factor (LIF) and ciliary neurotrophic factor (CNTF) growth factors, which both belong to the gp130 cytokine family, have been shown to exert numerous effects on CNS precursors and their progeny (Turnley and Bartlett, 2000). LIF and CNTF both signal through a receptor heterodimer consisting of gp130 and LIF receptor-β subunits, although CNTF requires an additional soluble CNTF receptor subunit to bring about a signal (reviewed in Heinrich et al., 2003). It has been reported that CNTF maintain adult NSCs in an undifferentiated state, inhibiting differentiation by activating the CNTF/LIF/gp130 receptor-heterodimer complex (Shimazaki et al., 2001). On the other hand, signaling through the LIF receptor has been demonstrated to promote the differentiation of NSCs into astrocytes (Johe et al., 1996; Bonni et al., 1997). In agreement with this, neural precursors cells from LIF receptor-β null embryos show a delayed generation of GFAP-positive astrocytes (Koblar et al., 1998).

Adult NSCs express members of the bone morphogenic protein (BMP) family and their receptors. The BMP family instructs adult NSCs to adopt a glial fate, which is therefore the “default path” as astrocytes. In the neurogenic niche, however, the local astrocytes and ependymal cells express and secrete signals antagonizing BMP and inhibit astroglial differentiation. Ependymal cells secrete a BMP-antagonist termed noggin (Lim et al., 2000), which binds BMPs, thereby preventing their activation (Gross et al., 1996). Antisense oligonucleotides against noggin decrease cell proliferation in the dentate gyrus of adult rats, indicating that endogenous noggin activity is important for cell proliferation to occur naturally (Fan et al., 2004). However, although noggin blocks gliogenic signals, it alone is insufficient to induce the neuronal differentiation of progenitors.

Another molecule antagonizing BMP and glial differentiation is the secreted factor Neurogenesin 1, expressed by astrocytes (Ueki et al., 2003). Hippocampal astrocytes have also been found to express Wnt, instructing NSCs in the dentate gyrus to adopt a neuronal fate (Lie et al., 2005). Conversely, blocking astrocyte-derived Wnt signaling decreases neuronal differentiation of adult NSCs. Interestingly, the NSCs themselves secrete Wnt-inhibitors such as secreted frizzled related protein 2 and 3 (Lie et al., 2005). Although astrocytes produce factors that promote neurogenesis, there are also astrocyte-derived BMPs that suppress neurogenesis (Gross et al., 1996). It appears that astrocytes have both positive and negative effects on neurogenesis. Ultimately, in these neurogenic environments, it is the balance between competing signaling pathways that control adult NSC proliferation and differentiation.

Hormones and Neurotransmitters

Certain hormones and neurotransmitters have been identified to promote neurogenesis. For instance, it has been found that in songbirds testosterone induces the expression of vascular endothelial growth factor, an angiogenic protein which increases angiogenesis (Louissaint et al., 2002). Subsequently, the newly generated endothelial cells stimulate neurogenesis by increasing the levels of brain-derived neurotrophic factors in the neurogenic area, which enhances the proliferation of progenitors (Louissaint et al., 2002). However, other hormones such as glucocorticoids have an inhibitory role on neurogenesis. Studies have shown that in aged rats with high levels of circulating glucocorticoids, cell proliferation in the hippocampus decreases (Kuhn et al., 1996; Cameron and McKay, 1999).

In terms of neurotransmitters, glutamate, which is the major excitatory neurotransmitter, is important for migration and differentiation of neurons (Rakic and Komuro, 1995). Glutamate appears to have complex effects on proliferation when acting through N-methyl-D-aspartate (NMDA) receptors. Pharmacological blockade of NMDA receptors by antagonists increases proliferation in the hippocampus (Nacher and McEwen, 2006). By contrast, NMDA blockers inhibit seizure- and stroke-induced proliferation of NPCs in the hippocampus (Arvidsson et al., 2001). However, new evidence suggests that, depending on the level of NMDA receptor activation, the outcome can be either proliferation or neuronal differentiation (Joo et al., 2007).

Transcriptional Regulation

Extracellular and intracellular signals target a variety of downstream transcriptional factors in order to regulate gene expression. Transcription factors usually contain DNA-binding domains and other domains such as those responsible for activation or protein interactions. The regulatory sequences of a gene, referred to as the promoter region which is located in close vicinity upstream of the transcription initiation site, contain elements binding specific transcription factors. Several transcription factors have been shown to play critical roles in adult neurogenesis.

A number of transcription factors of the basic helix-loop-helix (bHLH) family regulate neuronal differentiation both positively and negatively. A subfamily of the bHLH transcription factors acts in a proneural fashion including mammalian *achaete-scute homolog* (Mash1), Neurogenin (Ngn), and NeuroD. They usually heterodimerize with ubiquitously expressed E proteins, such as

E12/E47, and bind to a specific DNA sequence named E-box on the target genes, and subsequently act as transcriptional activators (Ross et al., 2003). Studies have shown that these transcription factors are required for the induction of neuronal lineages (Guillemot et al., 1993; Cau et al., 2002). The induction of neuronal differentiation involves a coordinate expression of proneural bHLH activity. Mash1 and Ngn are expressed in neural progenitors and in early differentiating neurons, while NeuroD is expressed in later stages of neuronal differentiation (Lo et al., 1991; Cau et al., 1997).

The inhibitory family of bHLH includes *hairy/enhancer of split homologue* (Hes) and inhibitor of differentiation (Id) transcription factors. These factors antagonize the ability of proneural bHLH factors to prevent neuronal differentiation, maintaining cells in a proliferative, undifferentiated state. Hes1 binds to a target DNA sequence on the Mash1 promoter, named the N-box, exerting an inhibitory effect on the Mash1 transcription (Davis and Turner, 2001). However, recent findings provide evidence that it is the phosphorylated status of Hes1, and not only the binding, that is decisive for the inhibitory role of Hes1 on the Mash1 promoter (Ju et al., 2004).

Id transcription factors act through a different mechanism for inhibiting differentiation of neural progenitors. Ids inhibit gene transcription by forming dimers with the E-proteins, thereby preventing them from interacting with the proneural bHLHs (Ross et al., 2003). Other bHLH transcription factors regulating neural lineage fates are the Olig-1/2 factors. Their expression is associated with early specification of the oligodendrocyte lineage. Olig-1/2 are the first genes involved in the oligodendrocyte lineage determination, expressed either concomitantly or several days before the other established oligodendrocyte progenitor markers appear (Lu et al., 2000b; Zhou et al., 2000).

Non-bHLH transcription factors, such as Pax6 and Myocyte enhance factor (MEF), have also been implicated in regulating neuronal differentiation. Pax6 transcription factors contain two types of DNA binding domains, paired box and homeobox. The Pax6 gene plays a crucial role in the development of the vertebrate CNS. Mutations in the Pax6 gene, producing nonfunctional proteins, results in multiple CNS defects in the eye, forebrain, cerebellum, and spinal cord (Stoykova et al., 1996; Burrill et al., 1997; Guillemot, 2005).

MEF2C, a member of the MADS transcription factor family, is expressed in neurons of the CNS and the level of MEF2C expression increases in differentiating neurons in the developing brain. MEF2C regulates expression of genes that are critical for survival of newly differentiated neurons. One of the MEF2C target gene, is the Mash1 promoter, and studies have shown that MEF2C upregulates Mash1 expression (Skerjanc and Wilton, 2000). Moreover, physical and functional interaction between MEF2C and Mash1 proteins has been reported (Black et al., 1996).

Epigenetic Regulation

Epigenetics describes mechanisms controlling gene expression and interaction during development independently of changes in DNA sequence. Epigenetic changes can be caused by modification of the DNA, such as phosphorylation, acetylation, and methylation (reviewed in

Jaenisch and Bird, 2003). The above described identified extracellular and intracellular signaling mechanisms partly act through epigenetic mechanisms. Epigenetic and chromatin modifications of target genes responding to environmental stimuli might serve as a major source of alteration in gene expression and function.

Histone acetylation mediated by histone acetyl transferases (HATs) cause relaxation of the chromatin, resulting in access of transcription factors for their target genes. Transcriptional coactivators, such as p300, CREB- binding protein (CBP), and p300/CBP-associated factor (PCAF), display intrinsic HAT activity, which further relaxes the chromatin. Histone deacetylases (HDACs) on the other hand, deacetylates the histones, making the chromatin densely packed, and in association with corepressors, such as N-CoR, causes transcriptional repression (Hsieh and Gage, 2004). Inhibition of HDAC has been shown to mediate neuronal protection through the activation of signal transduction pathways, such as the extracellular signal-regulated kinase (ERK) pathway (Hsieh et al., 2004). It induces neuronal differentiation mediated in part by the proneuronal bHLH transcription factors.

Methylation of DNA, such as genomic imprinting, is stable and may be involved in the long-term maintenance of certain regions of the genome (Peters and Schubeler, 2005). Methylation of the DNA results in further recruitment of HDAC repressors and leads to transcriptional repression (Hsieh and Gage, 2004, 2005). Several recent studies suggest that DNA methylation plays an extensive role in the CNS (Hsieh and Gage, 2004, 2005).

In conclusion, in the adult CNS the neurogenic niches provide a milieu in which the fate-choice of the NSCs is influenced by a cohort of proliferating, gliogenic, and neurogenic signals. The study of neurogenesis in the adult brain has become one of the most exciting and most rapidly developing areas of neuroscience today, mainly due to the fact that these findings have fueled our hopes to treat and cure neurodegenerative diseases in the CNS. However, a prerequisite for the potential use of NSCs in cell-based therapeutic strategies is that NSCs must demonstrate the ability to differentiate into appropriate lineages. Of great interest is finding optimal methods that will direct and control the differentiation of adult NSCs by addition of relevant signaling. Hence, the focus of the present thesis is to investigate the signaling pathways regulating NSC differentiation, and to elucidate possible *in vitro* manipulations in order to induce neuronal differentiation. The pathways investigated in this thesis are those of the BMP, apoptosis signal-regulating kinase (ASK1), *all-trans* retinoic acid (ATRA), and the orphan nuclear receptor TLX.

Bone Morphogenetic Protein

BMPs are molecules that belong to the diverse TGF-β superfamily that includes numerous secreted growth factors sharing highly conserved common sequence elements and structural features. They regulate a wide range of biological functions in all animals. So far the TGF-β superfamily ligands include more than 20 members such as the TGF-βs themselves, the activins and inhibins, Müllerian inhibiting substance, growth differentiation factors, and the BMPs, which constitutes the largest family (reviewed in Massague, 1998).

BMPs are conserved broadly across the animal kingdom, including vertebrates, arthropods, and nematodes. In vertebrates, BMPs play role in dorsal-ventral patterning of the early embryonic mesoderm and specification of epidermis. Moreover, BMPs are involved in generation of primordial germ cells, tooth development, and regulation of apoptosis. In addition, they have been found to regulate cell division, apoptosis, cell migration, and differentiation (Balemans and Van Hul, 2002). The activity of BMPs can be regulated by various secreted proteins such as chordin, noggin, and Gremlin (Balemans and Van Hul, 2002).

The BMP ligands exert their effects by activating a tetramer complex of transmembrane type I and type II serine/threonine kinase receptors. The type II receptor is primarily a ligand-bidning component; both the BMP receptor II (BMPRII) and the Activin receptor II (ActRII) are functional type II receptors for BMPs. Type I receptors also have BMP-binding properties and are responsible for transducing the signal into the cell. BMPRIA (activin receptor-like kinase 3 – Alk3), BMPRIB (Alk6), and ActRI (Alk2) are all known to transduce BMP signals (Massague, 1998).

The basic BMP signaling process is started by homo- or heterodimeric BMP ligands. Upon cooperatively binding to both type I and type II receptors, the formation of a heteromeric complex is induced. The constitutively active type II receptors then transphosphorylate and subsequently activate the type I receptors. In turn, the activated type I receptors phosphorylate specific intracellular receptor substrates, known as Smads, which act as transcription factors by forming multisubunit complexes. The Smads that transmit the signal are referred to as receptor-regulated Smads (R-Smads). There are two groups of R-Smads, one group consisting of Smad2 and -3 that transduce activin/TGF-β signals, and the other group of Smad1, -5, and -8 that are mainly transducers of BMP signals. Following the activation of R-Smads, they form heteromeric complexes with the common-mediator (Co-) Smad4. These tetrameric complexes then move into the nucleus, either alone or in combination with other non-Smad transcription factors, where the Smads bind directly to GC-rich Smad binding elements within target gene promoters in order to control gene expression (Heldin et al., 1997).

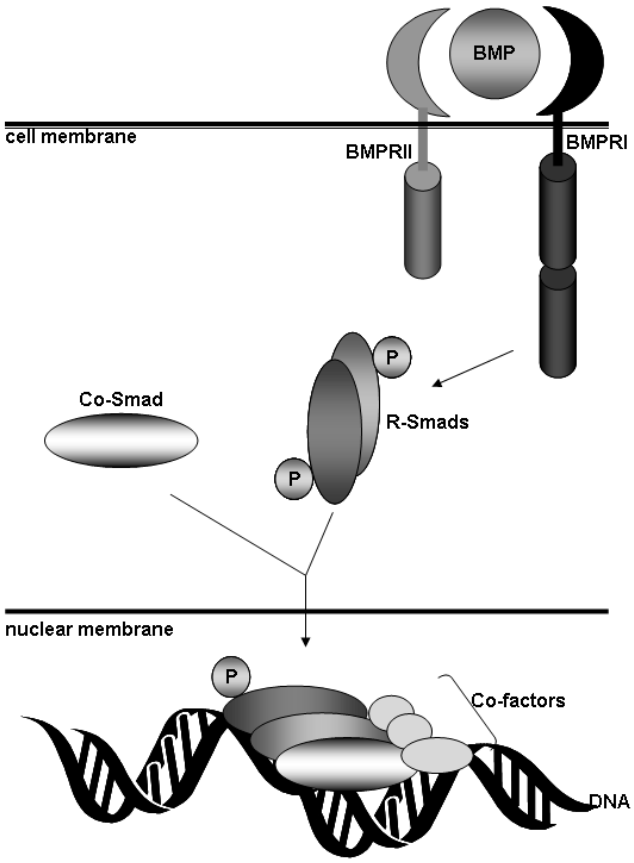

Figure 2. Schematic illustration of BMP signaling pathway.

The Role of BMP Signaling in Neurogenesis

The BMP signaling pathway plays multiple roles in CNS and perifer nervous system development. During early development BMP signaling suppresses neural cell fate and promotes epidermal fate (Wilson and Hemmati-Brivanlou, 1995). The formation of neural ectoderm requires the active repression of BMP signals, by noggin or chordin (Liu and Niswander, 2005). Later on, however, BMPs help to define the regions of the dorsal neural tube and also distinct NPC domains along the dorsoventral axis. Non-neural ectodermal cells flanking the neural tube secrete BMPs that will induce dorsal cell types, such as neural crest stem cells, sensory neurons and roof plate cells (Liu and Niswander, 2005). In cortical development, the effects of BMPs on the differentiation of vertebrate neuroepithelial or stem cells appear to be rather complex and BMPs have been shown to induce alternative fates depending on the stage of embryonic development (Mehler et al., 2000).

Mechanism of BMP Signaling in Neurogenesis

The decision of neuronal or astroglial differentiation is based on a network of positive and negative regulation. One study provides a mechanism by which LIF and BMPs can synergistically cooperate to induce gliogenesis. In this mechanism glial differentiation in response to BMPs requires the p300/CBP-coactivators in complex with Smad1 and the LIF/CNTF responsive transcription factor signal transducer and activator of transcription 3 (STAT3). The binding and activation of this complex to the GFAP promoter enhances the differentiation of NPCs towards astroglial lineage (Nakashima et al., 1999). In another study it was demonstrated that BMP treatment of NSCs, having been cultured in high-density, leads to the activation of Smads and a protein known as mammalian target of rapamycin/FKBP12-rapamycin-associated protein (mTOR/FRAP). The mTOR/FRAP then binds and phosphorylates STAT3, which selectively promotes the generation of glia (Rajan et al., 2003). However, although BMP activates Smad proteins, the activation of STAT3 and consequent glial differentiation may occur even when Smad signaling is inhibited (Rajan et al., 2003).

BMP exposure results in down-regulation of Olig-2 expression (Mekki-Dauriac et al., 2002), however, when cells are exposed to low amounts of BMPs, Olig-2 is able to inhibit the formation of the GFAP activator complex STAT3-p300/CBP-Smad1 (Fukuda et al., 2004). By this repression, Olig-2 inhibits astrocytic differentiation. Furthermore, although the GFAP promoter is activated when bound by the STAT3-p300/CBP-Smad1complex in glial differentiation, the NeuroD promoter involved in neurogenesis is also strongly driven by binding of Smad1-p300/CBP but in complex with Ngn1 (Sun et al., 2001). Ngn1 was found to block gliogenesis and promote neurogenesis by sequestering the complex of p300/CBP coactivators and Smad1 from association with STAT proteins (Sun et al., 2001). The decision of NSCs to generate either neurons or glia may be influenced by competition between Ngn1 and STAT proteins for coactivator complexes, thereby giving BMP signals another role in neurogenesis as well. This mechanism may also explain why glial differentiation cannot be induced in early progenitors by LIF/CNTF, which later in development is strongly gliogenic (Molne et al., 2000).

BMP-responsive transcription factors such as Id1 and Zic1 are known to repress neuronal differentiation through the repression of neurogenic bHLH genes. BMP signals lead to enhanced expression of Id proteins, which in turn bind and sequester the neurogenic bHLH transcription factors Ngn1 and Mash1 (Nakashima et al., 2001). Similarly, BMP-induction of the Id proteins results in inhibition of oligodendrocyte differentiation, in which Id2 and -4 act as dominant-negative binding partners for Olig-1/2 (Samanta and Kessler, 2004). These studies have shown that self-renewal and gliogenesis use molecular mechanisms that generally antagonize the actions of neurongenic molecules and bias the response of progenitor cells to BMPs.

Apoptosis Signal-Regulating Kinase 1

Mitogen-Activated Protein Kinase

The mitogen-activated protein kinase (MAPK) cascade is one of the most ancient and evolutionarily conserved signaling pathways. These pathways can be found in almost all eukaryotic organisms, including fungi, plants and animals. MAPK cascades are multifunctional and respond to various extracellular and intracellular stimuli in a variety of cellular responses, including cell cycle arrest, DNA repair, cytokine production and apoptosis (Widmann et al., 1999).

The transmission of signals is achieved by sequential phosphorylation and activation of the components specific for respective cascade. The MAPK cascade is typically organized in a set of three sequentially acting proteins. Briefly, MAPKs are activated by MAPKK-catalyzed phosphorylation of threonine and tyrosine residues in the activation loop of the kinase domain. MAPKKs are activated in turn by MAPKKKs-catalyzed phosphorylation of serine/threonine residues in the kinase domain. The activated MAPKs targets transcription factors, other kinases, and other enzymes. Activated MAPKs are then dephosphorylated and thereby deactivated by a battery of protein phosphatases, some of which have been named MAPK phosphatases (Johnson and Lapadat, 2002).

There are four major groups of MAP kinases in mammalian cells. These include the ERK1/2, ERK5, the c-Jun N-terminal kinase (JNK) and p38 MAPK. In general, the ERK pathway preferentially regulates cell proliferation, differentiation, and cell survival in response to various hormones, growth factors, and morphogens. JNK and p38 are preferentially activated by cytotoxic stresses, such as X-ray/UV irradiation, heat/osmotic shock, and oxidative stress as well as by proinflammatory cytokines such as tumor necrosis factor-α (TNF-α) and interleukin-1 (IL-1). One of the crucial biological responses mediated by the stress activated MAP kinase pathways appears to be the decision of cell fate by regulation of apoptosis (Chang and Karin, 2001). It should be mentioned that this is just a tendency and not a rule.

ASK1/MAPKKK5 is ubiquitously expressed activator of the JNK and p38 pathways by phosphorylating and thereby activating their respective MAPKKs, MKK4 (SEK1)/MKK7 and MKK3/MKK6 (Ichijo et al., 1997). Various ASK1-interacting proteins have been shown to regulate ASK1 activity. Thioredoxin (Trx), a ubiquitously expressed reduction/oxidation (redox)-regulatory protein, was the first identified ASK1-interacting protein playing a critical role in the regulation of ASK1 activity. Trx inhibits ASK1 kinase activity by binding to the N-terminal region of ASK1 (Saitoh et al., 1998; Liu and Min, 2002). The mechanism of ASK1 activation involves homo-oligomerization. In the resting state, ASK1 constitutively forms a homo-oligomer through its C-terminal coiled-coil domain, and carries Trx bound to it. Upon exposure to reactive oxygen species, such as the formation of hydrogen peroxide, intramolecular disulfide bonding is formed in Trx and, subsequently, Trx dissociates from ASK1. Furthermore, the release of Trx leads to autophosphorylation of a threonine residue located in the so-called activation loop of the kinase domain (Saitoh et al., 1998; Liu and Min, 2002).

TNF-α is a pleiotropic cytokine that plays important roles in inflammation, immune responses, and apoptosis. It has been reported that upon TNF-α treatment, ASK1 is first activated and then JNK and p38 cascades are set forth (Ichijo et al., 1997; Liu and Min, 2002). This activation is mediated by the recruitment of TNF-receptor-associated factor 2 (TRAF2), an adaptor protein that couples TNF-α receptors, directly interacting with the C-terminal domain of ASK1 (Liu and Min, 2002). Moreover, ASK1 was also reported to be involved in Fas signaling, through an adaptor protein named Daxx (Chang et al., 1998). Activated Fas receptor recruits the Daxx protein which then through interaction with ASK1, activates its kinase activity. In Fas-signaling, ASK1 serves as a critical regulator of the JNK and p38 pathways.

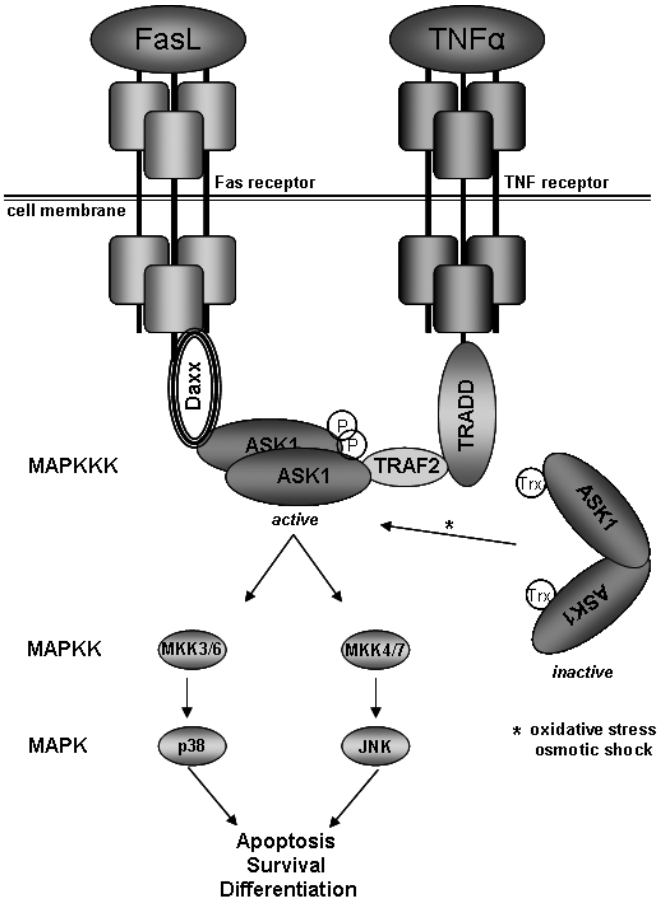

Figure 3. Schematic illustration of ASK1 signaling pathway.

The Role of MAPK Signaling in Neurogenesis

Cells are exposed to many extracellular signals and have to integrate these signals in order to choose an appropriate response. Therefore, the manner how MAPK activation is interpreted depends on the cell type or biological context in which the signal has been mediated. However, MAPK activation might also lead to opposing responses in the same cell type, suggesting that signal specificity is also determined by regulatory mechanisms other than the selective activation of a MAPK module (Schaeffer and Weber, 1999).

The activation of ERK pathway has been shown to play roles during nerve growth factor (NGF)-induced neuronal differentiation. In studies where the ERK pathway was blocked, the NGF-induced neurite outgrowth was inhibited, while neurite outgrowth was induced by constitutively active MEK (Cowley et al., 1994; Fukuda et al., 1995; Pang et al., 1995). Furthermore, it has been suggested that sustained ERK activation is required in PC12 cells for neuronal differentiation, while transient activation of ERK is not sufficient to induce differentiation but rather leads to proliferation (Qui and Green, 1992; Traverse et al., 1992; Marshall, 1995). However, there are findings showing opposite results – that blockade of sustained ERK activation do not result in inhibition of NGF-induced neurite outgrowth. This would suggest that the sustained activation of ERK is not necessarily required for NGF-induced neuronal differentiation (York et al., 1998). Furthermore, neuronal differentiation of PC12 cells by treatment with BMP2 was induced in the absence of ERK activation (Iwasaki et al., 1996). These studies suggest that signaling pathways other than the ERK cascade also contribute to neuronal differentiation of PC12 cells (Takeda et al., 2000).

Activation of the JNK and/or p38 pathway has been observed in response to deprivation of trophic factors in differentiated PC12 cells and other neuronal cells, suggesting the possible involvement of JNK and p38 in neuronal death (Xia et al., 1995; Kummer et al., 1997; Watson et al., 1998; Le-Niculescu et al., 1999; Takeda et al., 2000). On the other hand, evidence has suggested that the JNK and p38 pathways are involved in neuronal differentiation (Heasley et al., 1996; Leppa et al., 1998). The MAPK p38 has recently been shown to be activated in response to NGF and to be required for NGF-induced differentiation of PC12 cells (Morooka and Nishida, 1998; Xing et al., 1998). Furthermore, it was reported that p38 is activated by treatment with BMP2 in PC12 cells and that activation of p38 might be sufficient to induce neuronal differentiation of PC12 cells (Iwasaki et al., 1999). These observations suggest that the JNK and p38 pathways mediate important biological signals not only for neuronal cell death but also for neuronal differentiation.

Mechanims of MAPK Signaling in Neurogenesis

Studies on neuronal differentiation of cultured cortical progenitor cells have shown that MEK-CCAAT enhancer binding proteins (C/EBPs)-signaling is required (Menard et al., 2002). Progenitors cultured in the presence of FGF2 revealed a phosphorylated and active form of ERK, which increased upon addition of platelet-derived growth factor (PDGF). Furthermore, blockage of MEK inhibited the induction of neuronal genes, even in the presence of both FGF2 and PDGF. This study shows that upon MEK activation the downstream kinase Rsk is phosphorylated, which

in turn phosphorylates and activates the transcription factor C/EBP. The C/EBP then promotes neurogenesis by direct transcriptional activation of neuronal genes. The MEK–C/EBP pathway was demonstrated to promote the generation of neurons over glia, providing a mechanism by which growth factor signaling could regulate neuronal determination (Ferguson and Slack, 2003).

It has been shown that neurite outgrowth induced by constitutively active ASK1 in PC12 cells is mediated by p38 and not ERK. Furthermore, these cells survived in serum-starved condition, suggesting that ASK1 may mediate signals leading to both differentiation and survival of PC12 cells (Takeda et al., 2000). Calcium signaling plays important roles in regulating neuronal functions, and calcium-dependent activation of MAPK has been shown to be involved in processes such as synaptic plasticity (Thomas and Huganir, 2004). For example, calcium influx results in binding of calcium to calmodulin (CaM), and activation of the ERK pathway. In this cascade, CaM-binding proteins, such as the Ca^{2+}/calmodulin-dependent protein kinase IV (CaMKIV), positively modulate ERK1/2 activation induced by NGF or membrane depolarization (Thomas and Huganir, 2004). In *C. elegans,* it is shown that, NSY-1, an orthologue of the mammalian ASK1, is required to induce asymmetric expression of a certain type of odorant receptor to act downstream of CaMKII UNC-43 (Sagasti et al., 2001). Furthermore, it was reported that SEK-1 MAPKK (an orthologue of mammalian MKK3 and MKK6, the downstream kinases of ASK1) is also required for asymmetric expression in neurons, and that SEK-1 acts in a pathway downstream of UNC-43 and NSY-1 (Tanaka-Hino et al., 2002). Thus, the CaMKII–NSY-1–SEK-1 pathway is essential for asymmetric expression of the odorant receptor gene, *str-2* and, as a result, neuronal differentiation.

Nuclear Receptors

The nuclear receptor superfamily is a related but diverse group of transcription factors that are responsible for sensing the presence of hormonal ligands and certain other molecules. These nuclear receptors work in concert with other proteins to regulate the expression of specific genes, regulating several biological processes, including cell proliferation, differentiation, and cellular homeostasis. Today, there are more than 150 different known members of the nuclear receptor superfamily, encompassing all of the known nuclear hormone receptors, and spanning a large diversity of animal species from worm to human (Mangelsdorf et al., 1995; Evans, 2005). Unlike the water-soluble peptide hormones and growth factors, which bind to cell surface receptors, the fat-soluble hormones are able to both diffuse from a source and to traverse the plasma membrane in order to interact with their associated receptors, thereby transducing signals by binding DNA for the purpose of modulating transcription (Mangelsdorf et al., 1995).

The superfamily is divided into the steroid receptor family and the thyroid/retinoid/vitamin D (or nonsteroid) receptor family. Each type of receptor constitutes a subfamily, *e.g.*, the retinoic acid (RA) receptor subfamily, and the receptor subtypes are the products of individual genes (Mangelsdorf et al., 1995). Some nuclear receptors have no known endogenous ligands, and are therefore referred to as orphan receptors. Some may be activated by interacting with ligands,

while others may be constitutive activators, repressors, or factors whose activity is modulated by posttranslational modification (Mangelsdorf and Evans, 1995; Mangelsdorf et al., 1995).

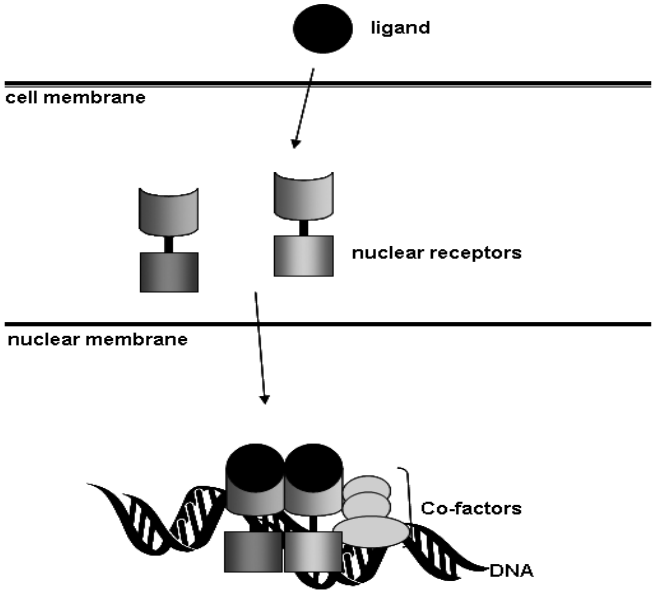

Figure 4. Schematic illustration of nuclear receptor signaling.

Retinoic Acid

There are two isomers of RA, ATRA and 9-cis-RA. It is not known whether they are produced by two separate enzymatic pathways, from all-trans-retinol and 9-cis-retinol, respectively, or if they can be interconverted by isomerization (Maden, 2002). The newly synthesized RA is bound to cytoplasmic proteins called cellular retinoic-acid-binding proteins 1 and 2 (CRABP1 and 2). Assisted by CRABP2, RA enters the nucleus and regulates gene activity by binding to ligand-activated nuclear transcription factors. There are two classes of these nuclear transcription factors — the RA receptors (RARs) and the retinoid X receptors (RXRs). In human, there are three subtypes of RARs (RARα, RARβ, RARγ) and three subtypes of RXRs (RXRα, RXRβ, RXRγ), with additional isoforms resulting from alternate promoter usage and splicing. Each of these nuclear receptors are encoded by different genes (Maden, 2002).

The functional receptor unit is a heterodimer consisting of one RAR paired with one RXR, *e.g.*, RARα–RXRβ, and they recognize consensus DNA sequences known as RA-response elements (RAREs) or retinoid X response elements (RXREs) located within the promoter of target genes. However, RXRs can also function as promiscuous heterodimerization partners for numerous nonsteroidal nuclear receptors other than RARs. *In vitro* binding studies have demonstrated that ATRA and 9-*cis*-RA are high-affinity ligands for RARs, whereas only 9-*cis*-RA has been shown to bind RXRs (Chambon, 1996; Mark et al., 2006).

In addition to ligand binding, phosphorylation of these receptors and recruitment of coactivators or corepressors is necessary for inducing or repressing gene transcription (Mangelsdorf and Evans, 1995). Furthermore, there are several other levels at which a cell's response to ATRA could be regulated, such as the uptake of retinol from the blood, regulation of ATRA metabolism, and the presence of the receptors RARs and RXRs (Blomhoff et al., 1992; Chambon, 1996).

The Role of ATRA Signaling in Neurogenesis

ATRA-signaling has been shown to have critical roles in the regulation of body axis formation, and development of heart and lungs. Both gain- and loss-of-function studies have shown that ATRA is required for appropriate specification and patterning of several tissues, including the hindbrain, spinal cord, and eye (reviewed in Drager et al., 2001; Maden, 2002; Wilson and Maden, 2005). In the early developing nervous system, ATRA contributes to the patterning of both the neural plate and the neural tube. Moreover, in the developing nervous system ATRA has a role in neuronal differentiation and axon outgrowth (Maden, 2002). The role of ATRA in neuronal differentiation has been studied extensively in *in vitro* models, and it has been shown that stem cells from different tissues, such as embryos or blood, can be directed towards neural differentiation using combinations of ATRA and growth factors or neurotrophins (Maden, 2007). ATRA induces the differentiation of various types of neurons and glia by activating the transcription of many genes, including ones that encode transcription factors, cell signaling molecules, structural proteins, enzymes, and cell-surface receptors.

Exposure of ATRA to adult CNS stem cells increased the proliferation of progenitor cells and increased neurogenesis (Wang et al., 2005). In the olfactory system ATRA has been shown to be involved in the maintenance of neuronal plasticity. In accordance to these results under conditions of vitamin A deficiency or blockage of RARα, resulted in a decrease in the number of mature olfactory sensory neurons (Yee and Rawson, 2000; Asson-Batres et al., 2003; Hagglund et al., 2006). In the hippocampus, ATRA is also involved in the regulation of neuronal plasticity. Deficits in spatial and recognition working memory have been demonstrated in rats and mice that have been deprived of vitamin A, and in mice mutated in subtypes of RAR and/or RXR genes. In all, cognitive impairment was restored when vitamin A was supplemented to the diet (Chiang et al., 1998; Misner et al., 2001; Cocco et al., 2002; Etchamendy et al., 2003). These results suggest that ATRA is necessary for the maintenance of memory mechanisms. Although *in vitro* studies have suggested ATRA to have important roles in neuronal differentiation, ATRA is also involved in the differentiation of astroglial cells (Wohl and Weiss, 1998). It is therefore believed that ATRA acts as a general differentiation factor rather than specifically a mechanism favoring the differentiation towards a certain lineage.

Mechanism of ATRA Signaling in Neurogenesis

Nuclear receptors exert transcriptional functions through the recruitment of coactivators and corepressors. In the absence of a ligand, RAR/RXR heterodimers bind to their consensus DNA-binding sequences repressing the transcription of target genes through regulatory domains responsible for the interaction with coregulators. In the repressive mode RAR/RXR recruit corepressors such as Sin3, N-CoR, and SMRT, which form complexes with the HDACs and

thereby modifying the chromatin structure of the target genes (Mangelsdorf and Evans, 1995; Nagy et al., 1997; Wei, 2003). However, ligand binding to the RAR/RXR-heterodimers results in conformational changes within the receptor complex. This leads to the release of the corepressors and instead to the recruitment of coactivators with intrinsic HAT activity such as p300/CBP and PCAF. Consequently, histone acetylation results in chromatin decompaction, allowing the initiation of transcription (Wei, 2003).

TLX

The orphan nuclear receptor, TLX (also called NR2E1), was first identified less than two decades ago (Yu et al., 1994). TLX is the vertebrate homologue of the Drosophila *tailless* gene. It is a member of the nuclear receptor gene superfamily, encoding ligand activated transcription factors.

The Role of TLX Signaling in Neurogenesis

In Drosophila, *tailless* is expressed in the embryonic brain and is required for brain development. In mice, expression of TLX is restricted to progenitor cells in the developing telencephalon, eye, and nasal placode from E8.5. Transcription peaks at E13.5 in ventricular zone and SVZ and then decreases to almost undetectable levels in the perinatal brain, again to be expressed in the adult brain (Monaghan et al., 1995). In adult animals, transcripts become localized to certain areas, including the SVZ, the hippocampus, and the retina (Yu et al., 2000; Shi et al., 2004).

Tlx-knockout mice are viable and appear normal at birth and are indistinguishable from their littermates. However, the adult mutant animals show severe behavioral abnormalities – aggressiveness, altered maternal instincts, late onset epilepsy and reduced learning abilities. Consistent with these behavioral observations, mutant mice have significantly reduced volumes of cerebral hemispheres (Monaghan et al., 1997) as well as severe visual defects (Yu et al., 2000).

The *Tlx*-knockout mice studies have shown that TLX is required for the formation of superficial cortical layers in embryonic brains (Land and Monaghan, 2003), that serves to regulate the timing of neurogenesis in the cortex (Roy et al., 2004) and to control patterning of lateral telencephalic progenitor domains during development (Stenman et al., 2003). TLX is a key component of retinal development and is essential for vision (Yu et al., 2000).

On the cellular level, TLX has been shown to be an essential regulator of NSC maintenance and self-renewal in the adult brain (Shi et al., 2004), and it maintains adult NSCs in the undifferentiated and self-renewable state. The TLX-expressing cells isolated from adult *Tlx*-heterozygote brains can proliferate, self-renew, and differentiate into all neural cell types *in vitro*. By contrast, *Tlx*-null cells isolated from the brains of adult *Tlx*-knockout mice fail to proliferate. Furthermore, TLX represses the expression of astrocyte markers, such as GFAP, in NSCs, suggesting that TLX by transcriptional repression maintains the undifferentiated state of NSCs (Shi et al., 2004; Sun et al., 2007). In contrast, in the postnatal mouse retina, TLX is strongly expressed in the proangiogenic astrocytes and acts as a proangiogenic switch in response to hypoxic condition (Uemura et al., 2006).

Mechanism of TLX Signaling in Neurogenesis

How TLX is modulated by transcriptional coregulators remains largely unknown. A recent study, however, demonstrated that TLX repression of target genes in NSCs is associated with HDACs, targeting the genes *p21* and *pten* (Sun et al., 2007). The TLX-mediated repression of pten and p21 expression serves as a regulator for maintaining NSCs in a proliferative condition (Sun et al., 2007). Furthermore, the transcription factor Pax2, known to be involved in retinal development, has been shown to be a direct target of TLX. In chick embryos, ectopic expression of TLX repressed Pax2 expression (Yu et al., 2000).

In other nuclear receptors, such as RAR/RXR, the interaction with HDACs often occurs by corepressors, such as SMRT and N-CoR. So far, TLX has not been shown to recruit these corepressors, but another corepressor, atrophin, has been reported to interact with TLX, and could potentially mediate the TLX-HDAC association (Zhang et al., 2006).

Aims of the Studies

General Aim

To identify molecules involved in determining lineage commitment of adult neural progenitor cells (AHPs), and elucidate the signaling pathways implicated in this process in order to find a target of therapy or for manipulation. To direct the adult neural progenitors towards a neuronal specification in order to obtain neurons suitable for cell-replacement strategies in neurodegenerative diseases.

Specific Aims

- To investigate the differences in signaling and specific roles of BMP type I receptors on survival and lineage commitment of AHP cells.
- To study the effect of ASK1-signaling on survival, differentiation, and cell specification of AHPs.
- To optimize the differentiation of AHPs towards neurons by combination of ASK1 and ATRA, and to determine their downstream signaling mechanisms.
- To study the role and mechanism of TLX in the proliferation and neuronal differentiation of AHPs.

Methods

Cell Culture – Adult Hippocampal Progenitors

The procedure of isolating neural progenitors from the adult rat hippocampus (AHPs) has previously been described (Palmer et al., 1995). Clonally derived AHPs were kindly provided by F.H. Gage (Salk Institute, La Jolla Ca.) and maintained as described previously (Palmer et al., 1995). For proliferation conditions, progenitor cells were cultured in DMEM:F12 (Invitrogen) with 20 ng/ml recombinant human bFGF (PeproTech EC), 1% N2-supplement (Life Tecnologies), and 1% Ultra-Glutamine in plastic flasks coated with polyornithine. bFGF is used to keep the cells in a proliferating and undifferentiated state, so for differentiation experiments, AHPs were cultured in the absence of bFGF and plated onto dishes coated with polyornithine and laminin.

AHPs have been shown to differentiatie into the three major components of the CNS, *i.e.* neurons, astrocytes and oligodendrocytes. In addition, clonally derived cells have a stable phenotype in long-term culture, retaining identical immunocytological characteristics for more than 30 passages (Gage et al., 1995). For our experiments we have used AHPs with passage number 10-20 postcloning.

Transient Transfection

AHPs were transfected on DIV 2 with reporter plasmids and/or expression vectors using FuGENE-6^{TM} transfection reagent (Roche) according to the manufacturer's protocol. The total amount of DNA ranged from 0.25 µg to 2.0 µg. Transfection was used to introduce exogenous plasmid-DNA into the cells.

Silencing-RNA Transfection

In paper IV in order to study the effect of sequence-specific knockdown of gene expression, small interfering RNA (siRNA) was introduced into the cells by transfection. AHPs were transfected on DIV 2 with siRNA using HiPerfect (Qiagen) according to manufacturer's protocol. The final concentration of siRNA was kept at 10 nM per well.

Adenoviral Vectors

Recombinant adenoviruses were used to transiently express genes encoding β-galactosidase (LacZ), different constructs of the Alk receptors, different constructs of the ASK1 gene, and the nuclear receptor TLX. Gene transfer via adenovirus vectors gives high transduction efficiency and expression that last longer compared with plasmid-DNA transfection.

The adenovirus vectors used in this thesis have a deletion of the E1 and E3 genes and because of the lack of the E1 gene the virus is considered to be incapable of replication and propagation in ordinary cells. However, in order to propagate the recombinant virus, it was infected into the

human embryonic foetal kidney cell line 293, which continuously expresses the E1 gene. To propagate the virus, the cell line 293 was infected for 3-4 days, harvested and lysed by five cycles of freeze-thawing. The debris was removed by centrifugation and the virus stock was stored at -80°C. The titer was assayed by judging cytopathic effect on 293 cells that were seeded in microtiter plates and infected by serially diluted virus.

[^{3}H]-Thymidine Incorporation Assay

To monitor proliferation of AHP cells, DNA synthesis was assayed by detection of [^{3}H]-thymidine. [^{3}H]-thymidine substitutes the endogenous DNA base, thymidine, which ensures specific labeling of only the dividing cells.

AHPs were cultured on 24-well plates as described previously. At the time of analysis, the cells were pulsed with 0.5 or 0.6 µCi /ml of [*methyl*-[^{3}H]thymidine (Amersham) of medium for another 4 or 24 h. The cells were then washed once with ice-cold phosphate buffered saline (PBS) and precipitated with 5% trichloracetate on ice for 30 min. The precipitate was then dissolved in 1 M NaOH, neutralized with 1 M HCl, and transferred to vials containing 5-10 ml of scintillation fluid. Radioactivity was counted by using a β-counter.

Cell Survival Assays

MTT Assay

In order to assess cell survival a method based on the breakdown of the yellow tetrazolium salt, dimethylthiazol-diphenyl-tetrazolium bormide (MTT), into a blue formazan salt was used. The product, formazan, can be quantified spectrophotometrically. The color change reflects the enzyme activity of the mitochondrial dehydrogenases in living cells. AHPs were cultured on 24-well plates as described. A volume of 60 µl of 5 mg/ml MTT solution (Sigma Aldrich) was added to the medium, and the mixture was cultured for another 4 h at 37°C. Solubilization buffer was added, and the mixture was cultured for another 30 min to release the formazan salt. The absorbance was measured at 570nm.

LDH-Cytotoxicity Assay

As a measure of cell death a photometric method that measure the release of lactate dehydogenase (LDH) from dying cells was used. Cellular release of LDH was determined in 96-well plates with the CytoTox 96 NonRadioactive Cytotoxicity Assay kit (Promega), according to the manufacturer's protocol.

Propidium Iodide Staining

Propidium iodide (PI) intercalates into double-stranded nucleic acids. PI is membrane impermeable and excluded from viable cells but can penetrate cell membranes of dying or dead cells. PI staining was performed using the Cycle TEST PLUS DNA kit (Becton Dickinson) according to the manufacturer's protocol. Fluorescence was analyzed using a fluorescence-activated cell sorting (FACS) Calibur flow cytometer (Becton Dickinson).

Immunocytochemistry

Immunocytochemisty with antibodies is a powerful method of demonstrating the presence and distribution of a particular antigen of interest. The two different methods 3,3-Diaminobenzidine staining (DAB) and immunofluorescence (IF) have been used in this thesis.

3,3-Diaminobenzidine Staining

Cells were fixed in 4% paraformaldehyde for 20 min at room temperature. After several washes with PBS, the cells were quenched with 3% hydrogen peroxide for 10 min. After additional washing, cells were blocked with Tris-buffered saline (TBS)-based blocking buffer containing 0.1% Triton X-100, 3% Bovine Serum Albumin (BSA), and 3% horse serum for 1 h at room temperature. The cells were then incubated with the respective primary antibodies in the same blocking buffer (see Table1). After three washes with TBS, the cultures were incubated for 1 h at room temperature with biotinylated secondary antibody (Vector Laboratories) diluted 1:200. The cells were washed, and labeling was visualized by incubation with an ABC Elite kit (Vector Laboratories) for 1 h at room temperature and finally with 0.05% 3,3-diaminobenzidine-0.03% H_2O_2 for 5 min.

Immunofluorescence

Different protocols for IF were used in this thesis. AHPs were seeded and treated as described above. For IF analysis the AHP cells were fixed in 4% paraformaldehyde for 20 min at room temperature or in ice-cold methanol for 10 min. Cells were incubated in TBS-based blocking buffer containing 0.1% Triton X-100, 3% BSA, and 3% normal horse or goat serum, for 1 h at room temperature. For detection of surface proteins, Triton X-100 was omitted in the blocking buffer. The cells were incubated with primary antibodies diluted in blocking buffer overnight at 4°C. After three washes with TBS, cells were incubated with Alexa Fluor-488, Alexa Fluor-594 (Molecular Probes), fluorescein isothiocyanate (FITC)-, or Texas red-labeled secondary antibodies. For nuclear counterstain, cells were incubated with 1 µg/ml Hoechst 33258 for 20-30 min.

Western Blot

Western blotting, also referred to as immunoblotting, is an analytical method that involves the determination of relative amounts of a protein of interest using a specific primary antibody.

Cultured cells were lysed and the protein content of the cell lysate was determined using DC protein assay (Bio-Rad). AHP cells were washed with PBS and lysed on ice in freshly prepared Triton X-100-based lysis buffer containing phosphatase and proteas inhibitors. Cellular debris was removed by centrifugation and equal amounts of protein were separated by SDS 8% - 12% polyacrylamide gel electrophoresis and then electroblotted onto a polyvinylidine difluoride membrane by semi-dry transfer. After blocking with 5% BSA or 5% skim milk in TBS with 0.05% Tween 20 (TBS-T), the membranes were probed with primary antibodies (see Table1). The secondary antibodies were directed against either mouse-, rabbit-, or goat-IgG and

conjugated with horseradish peroxidase (Amersham Bioscence). Signals were detected by enhanced chemiluminescence (LAS-1000 Plus, Fuji).

Immunoprecipitation

In order to increase the levels of certain proteins before running the samples on SDS gel for western blot analysis, the protein of interest was pulled down with a specific primary antibody and a secondary protein G coupled antibody.

For detection of Smad2- and MEF2C-phosphorylation cells were harvested in either a buffer containing Tris pH 8, 1 mM EDTA, 129 mM NaCl, 0.5% NP-40, 10% glycerol, 1mM PMSF, and 1mM aprotinin or another containing 25 mM Tris pH 8.0, 137 mM NaCl, 2.7 mM KCl, 10% TritonX-100, 1 mM PMSF, 1 mM Aprotinin, and 1 mM DTT respectively. The lysates were cleared from insoluble material by centrifugation for 15 min at 15,000 rpm. Sample protein was incubated with an antibody raised against Smad2/3 or MEF2C, followed by precipitation with protein-G-plus agarose. The immunoprecipitates were collected via centrifugation for 2-3 min at 1000 x g, washed four times with lysis buffer, and boiled in sample buffer. The immunoprecipitates were separated using 12% SDS-PAGE, and Western blotting was performed as described above. Membranes were probed with rabbit-polyclonal PS2 raised against phosphorylated Smad2 or rabbit-polyclonal SED raised against Smad2 or rabbit-polyclonal anti-p-serine.

In Vitro Kinase Assay

In vitro kinase assay is a method to measure kinase activity. In paper I, in order to show the inactivity of the dnAlk3 receptor kinase, in vitro kinase assay was performed.

For in vitro kinase analysis of Alk-autophosphorylation, the cells were harvested in buffer containing 20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% Triton X-100, 2.5 mM EDTA, 1% glycerol, 100 U/ml Trasylol, 1mM PMSF, 1 mM dithiothreitol, and 0.1 mM sodium orthovanadate. The samples were mixed with protein A-Sepharose-coupled antibodies against the HA-tag bound to protein A-Sepharose for 3 h at 4°C. Immune complexes were precipitated and in vitro kinase assays performed as described previously (Mori et al., 1991). Signals were detected by Image Analyzer (FLA 2000, Fuji).

Primary Antibodies	Source	Application	Dilution	Company	Paper
Alk6	Rabbit polyclonal	WB	1µg/ml	Gift from Dr CH Heldin	I
β_{III}-tubulin	Mouse monoclonal	WB	1:500	Sigma Aldrich	I, II
β-actin	Mouse monoclonal	WB	1:2000	Sigma Aldrich	I-IV
CaMKII	Mouse monoclonal	IP	4µg/ml	Santa Cruz Biotechnology	III
CBP	Rabbit polyclonal	IP	4µg/ml	Santa Cruz Biotechnology	III
DCX	Mouse monoclonal	IF	1:1000	BD Biosciences Pharmingen	IV
GalC	Mouse monoclonal	DAB/IF	1:1000/ 1:100	Chemicon	I
GFAP	Rabbit polycloonal	DAB/IF/WB	1:1000/ 1:400/ 1:1000	DakoCytomation	I, II, IV
GAPDH	Mouse monoclonal	WB	1:5000	Chemicon	III
HA	Mouse monoclonal	WB	1:500	Roche	I, II
HDAC1	Rabbit polyclonal	IP	4µg/ml	Sigma Aldrich	III, IV
HDAC4	Rabbit polyclonal	IP	4µg/ml	Santa Cruz Biotechnology	III
Hes1	Rabbit polyclonal	IP	4µg/ml	Santa Cruz Biotechnology	III
Mash1	Mouse monoclonal	IF/ WB	1:500/1:1000	BD Pharmingen	II-IV
MAP2ab	Mouse monoclonal	IF	1:1000	Sigma Aldrich	I-IV
MEF2C	Goat polyclonal	IP/WB	4µg/ml/1:500	Santa Cruz Biotechnology	III
p38	Rabbit polyclonal	WB	1:1000	Cell Signaling	II
PCAF	Goat polyclonal	IP	4µg/ml	Sigma Aldrich	III, IV
Phoshpo-p38	Rabbit polyclonal	WB	1:1000	Cell Signaling	II
Phospho-Smad2 (PS2)	Rabbit polyclonal	WB	1:1000	Gift from Dr CH Heldin	I
Phospho Smad1/5/8	Rabbit polyclonal	IF/ WB	1:500/1:2000	Gift from Dr CH Heldin	I, II
Phospho-serine	Rabbit polyclonal	WB	1:500	Invitrogen	III
Phospho Stat3	Rabbit polyclonal	WB	1:1000	Cell Signaling	II
Prominin1	Mouse monoclonal	IF	1:1000	MACS	IV
Smad2 (SED)	Rabbit polyclonal	WB	1:500	Gift from CH Heldin	II
Sp1	Rabbit polyclonal	IP	4µg/ml	Santa Cruz Biotechnology	III, IV
STAT3	Rabbit polyclonal	WB	1:1000	Cell Signaling	II
Tlx	Rabbit plolyclonal	IF	1:500	Sigma Aldrich	IV
Tlx	Mouse monoclonal	IF	1:500	MBL	IV

Table 1. Comprehensive list of antibodies used in this thesis.

Promoter-Reporter (Luciferase) Assay

This method allows quantification of in vitro gene expression. Cells are transfected with an expression vector carrying a *luciferase* gene under control of the promoter of interest. In this thesis we used plasmid-DNA constructs in which the promoter-sequence for GFAP and Mash1 was fused to the *luciferase* gene. Depending on the promoter-activity the enzyme luciferase will be expressed and by quantifying the amount of light produced by the luciferase-based enzymatic reaction, it is possible to monitor transcriptional activation of this promoter. Quantification is performed by using a luminometer (Victor Wallac).

AHP cells were transfected with reporter plasmids and expression vectors by using Fugene6^{TM}. At the time of analysis cells were harvested and processed for luciferase assay with Luciferase Reporter Assay System (Promega), according to the manufacturer's protocol. Absolute luciferase-activity was normalized to β-galactosidase activity or total amount of protein.

Chromatin Immunoprecipitation Assay

Chromatin immunoprecipitation (ChIP) is a method for identifying proteins associated with specific regions of the genome by using specific antibodies that recognize the proteins of interest.

For ChIP assay AHPs were cultured as described above. At the time of analysis the initial step was to cross-link protein-protein and protein-DNA in live cells with formaldehyde at a final concentration of 1% for 10 min at room temperature. Following cross-linking, the cells were lysed in Buffer X (50 mM Tris–HCl at pH 8.0, 1 mM EDTA, 120 mM NaCl, 0.5% NP-40, 10% glycerol, and 1 mM PMSF) for 15 min on ice. The lysate was sonicated to shear the DNA. Proteins together with cross-linked DNA were subsequently immunoprecipitated overnight at 4°C with 2 μg of antibodies against proteins of interest and also mouse or rabbit IgG as negative control. Immune complexes were collected by incubation with 15 μl Protein G–agarose (Santa Cruz) for 1 h at 4°C. The pellets were washed once with 1 ml Buffer X, once with high salt Buffer X (500 mM NaCl), once with LiCl buffer (10 mM Tris, 1 mM EDTA, 0.25 M LiCl, 1% NP-40, 1% sodium deoxycholate, pH 8.1), and twice with TE (10 mM Tris, 1 mM EDTA, pH 8.0). Immune complexes were eluted twice with 250 μl of elution buffer (0.1 M $NaHCO_3$, 1% SDS). Protein-DNA cross-links in the eluted samples were then reversed when incubated with 0.2 M NaCl for 4–5 h at 65°C. Samples were digested with Proteinase K (0.04 mg/ml) for 2 h at 45°C and then with RNase A (0.02 mg/ml) for 30 min at 37°C. DNA was purified with phenol and chloroform followed by ethanol precipitation. Purified DNA was resuspended in 10 μl H_2O and serially diluted. Aliquots of 2 μl were analyzed by PCR with the appropriate primer pairs (se Table2). Amplification was performed for a pre-determined optimal number of cycles. PCR products were separated by electrophoresis on 2% agarose gels.

Reverse Transcriptase-PCR

Reverse Transcriptase PCR (RT-PCR) is a sensitive method for detecting and semi-qualitatively determining the amount of a specific RNA-transcript in cells at very low levels.

Total RNA was extracted using a protocol based the guanidinium thiocyanate method of Chomczynski and Sacchi (Chomczynski and Sacchi 1987) Cells were homogenized using a denaturing buffer containing 4 M guanidiumthiocyanate, 0.1 M β-mercaptoethanol, 25 mM sodium citrate, and 0.5% sarcosyl. Followed by the addition H_2O-saturated phenol and chloroform:isoamyl alcohol (24:1). After centrifugation, the upper phase (containing total RNA) was transferred to a new tube, precipitated using isopropanol and redissolved in RNase free H_2O. Total RNA was quantified spectrophotometrically

An aliquot of total RNA from sample was transcribed into c-DNA with random primers and M-MLV reverse transcriptase (Invitrogen). The PCR was carried out using standard protocol with Taq polymerase (Fermentas). For nucleotide primers see Table2. The samples were run 1.5-2.0% agarose gel containing ethidium bromide and analyzed using a FLA 2000 plate reader (Fujifilm). β-actin was used as a control housekeeping gene, allowing semi-quantitative evaluation of the mRNA levels.

Statistics

All experiments were replicated at least three times. Overall significance was determined by submitting data to one-way analysis of variance (ANOVA). Significance of between-group differences was determined by the Scheffé post-hoc test.

Gene	Primer sequence	Paper
β-actin	5'-AAG ATG ACC CAG ATC ATG TTT GAG-3' 5'- AGG AGG AGC AAT GAT CTT GAT CTT-3'	I, II, IV
ActRII	5'-CTG GAT ATC TAG CGA GAA C-3' 5'-ACG ACA CTC CCG TTA TAC AC-3'	I
ActRIIB	5'-GAG ACG ATG TCA CGA GGC C-3' 5'-CTC GGT AGT TGA AGG TCT CT-3'	I
Alk2	5'-TCT GTG CTA ATG ATG GCT CTC C-3' 5'-TTC TGC GAT CCA GGG AAG GAT TTC-3'	I
Alk3	5'-GGA GGA ATC GTG GAG GAA TAT-3' 5'-CAT ACG CAA AGA ACA GCA TGT C-3'	I
Alk6	5'-CGG CTG AAT CAC AAC CAT TTG G-3' 5'-CTA GAC ATC CAG AGG TGA CAA CAG-3'	I
BMP2/4	5'-TAT GTG GAC TTC AGT GAT GTG G-3' 5'-CAG AGT CTG CAC TAT GGC ATG GTT-3'	I
BMP5/6/7	5'-TGG CAG GAC TGG ATC ATT GCA C-3' 5'-ATG GCG TGG TTG GTG GCA TTC AT-3'	I
BMPRII	5'-GGA GAA ATC AAA AGG GGA C-3' 5'-CCT ATG TCT TAC AAC TGT CC-3'	I
Mash1	5'-GGA ACT GAT GCG CTG CAA AC-3' 5'-CCT GCT TCC AAA GTC CAT TCC-3'	II, IV
Mash1 promoter	5'-TTA TTC AGC CGG GAG TCC GG-3' 5'-TGC GGC CTT TTC AAT GG-3'	III, IV
TLX	5'-TGA ATG GGA CCC CAA TGT AT-3' 5'-GGC CCA TTG TGC TAT TCC TA-3'	IV

Table 2. Comprehensive list of primers.

Results

Paper I

The BMP molecules signal via a tetrameric receptor complex consisting of two BMP type II receptors and two type I receptors. The type I receptors Alk2, 3, and 6 act downstream of the type II receptors and can phosphorylate Smad 1, 5, and 8 once activated. In order to study functional differences for the BMP type I receptor signaling in NPCs, we used adenovirus constructs encoding two mutant variants of the receptors. Constitutively active (ca) Alk receptors constantly phosphorylate Smad 1/5/8, and dominant negative (dn) Alk receptors possess a non-functional intracellular kinase domain and are incapable of activating downstream molecules. Overexpression of dnAlk receptors deplete the medium of BMPs, thereby blocking BMP receptor signaling.

Overexpression of caAlk 3 and 6 receptors had almost identical effects on AHP cells, resulting in an increased number of GFAP-positive cells while the number of MAP2ab-positive cells was unaffected. Although there was a discrepancy in the morphology between the caAlk3 and caAlk6-induced GFAP positive cells, we were not able to distinguish them with additional markers for glial lineage. It is possible that Alk3 also induced proliferation of GFAP-positive progenitors, since those cells exhibited relatively round and flat cytoplasms with shorter processes. In contrast, caAlk6-induced cells had a differentiated appearance with longer fibers. Staining with several markers such as nestin, S100β, and Prominin-1 might have helped to clarify their identity.

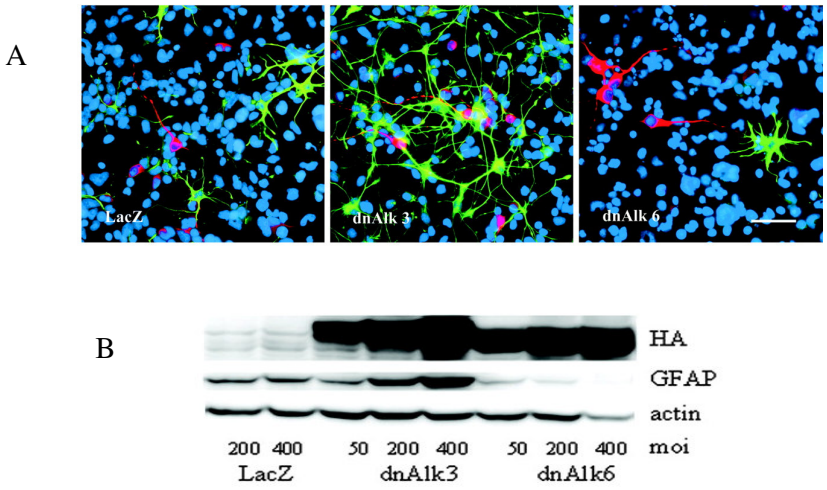

Figure 5. Effect of dnAlk3 and dnAlk6 in AHPs. (A) Immunocytochemical staining following overexpression of dnAlk receptors in AHPs after 5 days in culture. GFAP- (green), MAP2ab-positive cells (red) and nuclei staining (blue). (B) Western blot analysis of GFAP expression after dnAlk-infection with different moi after 4 days in culture.

Blockage of Alk6 signaling in the presence of BMP6 resulted in a diminished phosphorylation of Smad 1/5 and an moi-dependent decrease of GFAP expression. This suggests that BMP6 was

bound to the overexpressed nonfunctional receptor and not available for signaling via the endogenous Alk3 or Alk6 receptors. While astroglial differentiation was suppressed in dnAlk6-infected cultures, the number of oligodendrocytes increased. These results suggest that BMP signaling in AHPs suppresses oligodendroglial differentiation while promoting astroglial fate, which is in accordance with previous results (Gross et al., 1996; Mabie et al., 1997). Moreover, our finding shows that Alk6 signaling is important for cell survival. It is possible that the dnAlk6-induced decrease in GFAP expression that we observe might be partly due to an increased death of GFAP-expressing cells.

Blockage of Alk3 signaling was followed by an increase of Smad 1/5 phosphorylation, GFAP expression, and cell survival. Furthermore, when dnAlk3 was overexpressed, AHP cells responded by an increase of endogenous Alk6 expression. These findings in conjunction with the results from dnAlk6 overexpression, show that BMP6 has a higher affinity for Alk6 than Alk3 since overexpression of dnAlk3 could not deplete BMP6 from endogenous Alk2 or Alk6. The mechanism by which Alk6 is upregulated is not known and will be the focus of future studies. In contrast to these findings, a previous study using embryonic stem cells described an upregulation of Alk6 upon increased Alk3 signaling (Panchision et al., 2001). We ascribe the differences in the results to the different cell types used which differ in their intrinsic properties.

In studies with dnAlk2, we found an almost identical but weaker effect in comparison with dnAlk6. None of the dnAlk or caAlk receptors had any effect on neuronal differentiation of AHPs, as observed with the neuronal markers MAP2ab and β_{III}-tubulin. In the adult SVZ BMPs inhibit neurogenesis (Lim et al., 2000), however, in our AHP culture system BMPs exert their gliogenic effect without being anti-neurogenic.

In conclusion, our findings suggest that it is Alk6 rather than Alk3 that has the capacity to recruit molecules important for progenitor survival and astroglial differentiation.

Paper II

ASK1 is an intracellular MAPKKK, which can activate the downstream MAP kinases p38 and JNK pathways. Activated p38 and JNK translocate to the nucleus and activate transcription by phosphorylating transcription factors. ASK1 was first identified as an inducer of apoptosis in response to TNFα-signaling and oxidative stress. However, overexpression of activated ASK1 has been shown to promote differentiation and survival and not apoptosis (Takeda et al., 2000). None of these reports used neural progenitors within the CNS. For this reason we set out to investigate the effect of ASK1 signaling on survival, proliferation, and differentiation in adult neural progenitor cells.

Since ASK1 induces apoptosis in certain conditions, we first examined the effect of ASK1 overexpression in AHPs. We used different variants of ASK1, the constitutively active and kinase mutants ASK1, ASK1-ΔN and ASK1-KM, respectively. Moderate expression of ASK1-ΔN, *i.e.* moi 10 and 25, did not affect cell viability as measured by MTT and LDH assays. In order to

analyze the effect of ASK1 on AHP cells, we performed immunocytochemistry and western blot using markers characteristic for either neuronal or glial lineage. AHP cultures infected with ASK1-ΔN displayed a significantly higher number of cells expressing MAP2ab, and an increased expression of β_{III}-tubulin. These results support previous findings of ASK1 promoting differentiation rather than apoptosis in certain conditions (Takeda et al., 2000; Sayama et al., 2001).

Moreover, we found a novel role for ASK1 as a potent repressor of glial lineage commitment. Protein analysis from cultures overexpressing ASK1-ΔN, showed a clear moi-dependent decrease in GFAP expression 4 days after infection. However, this effect disappeared 10 days after infection. We concluded that the most likely explanation is that as long as the progenitors are expressing ASK1, glial lineage commitment remains to be suppressed. However, since adenoviral constructs will not be integrated into the host genome, for each cell division the level of ASK1-ΔN protein expression decreases. As a consequence, the inhibitory effect of ASK1 on glial differentiation eventually decreases and cells are able to continue an astroglial fate.

We sought to investigate the downstream molecules of ASK1-induced differentiation of AHPs. A likely candidate of ASK1 was p38, given that many studies account p38 to be a mediator of ASK1 signaling (Ichijo et al., 1997; Takeda et al., 2000). Our finding that ASK1-ΔN overexpression resulted in increased *Mash1* transcript had shown that ASK1 could target the early pro-neuronal gene MASH1. In order to study the role of p38 in ASK1-induced neuronal differentiation AHPs transfected with the MASH1-promoter reporter were pre-treated with p38 inhibitors, prior to infection. The luciferase measurement showed that the ASK1-induced increase in MASH1 promoter activity was abolished when p38 was blocked. Consistent with this result, the increase of MAP2ab-expressing cells due to ASK1-ΔN overexpression was gone when p38 was inhibited.

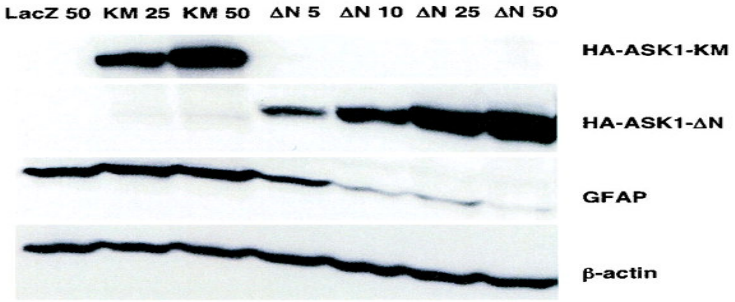

Figure 6. ASK1 represses GFAP expression. AHPs were infected with different moi of ASK1-ΔN and ASK1-KM at DIV 2 and processed for western blot analysis of GFAP expression after additional 4 days in culture.

The reduction of glial cells observed in ASK1-ΔN-infected cultures could have been due to a selective apoptosis of glial cells. However, since we did not observe any increase in apoptosis, we

hypothesized that ASK1 directly suppressed the GFAP promoter. Gene-reporter analysis supported this concept. ASK1-ΔN could significantly suppress GFAP promoter activity, even in the presence of the glial inducing factors, LIF and BMP. Moreover, blockage of the p38 pathway disinhibited the ASK1 suppression of GFAP promoter activity and protein expression. The cytoplasmic proteins STAT1 and STAT3 are well-known activators of the GFAP promoter. We elucidated whether ASK1 targeted these proteins in order to suppress GFAP expression. However, we found that ASK1-induced repression of GFAP expression is independent of the STAT3 pathway.

In conclusion, our results suggest a p38 MAPK-dependent mechanism by which ASK1 induces neuronal differentiation and suppresses glial lineage commitment in neural progenitors.

Paper III

All-trans RA (ATRA) is an important regulator of developmental neurogenesis. It has also been shown to induce differentiaton of embryonic stem cells towards the neuronal lineage *in vitro* (Maden, 2007). Having determined the role of ASK1 in AHP cells, we investigated the combined effect of ASK1 and ATRA on neural differentiation of adult neural progenitors to yield an increased number of neurons. Furthermore, we attempted to understand the signaling mechanisms elicited by ASK1 and ATRA, separately and together, that induce neuronal lineage commitment.

Infection of the AHP cells with ASK1-ΔN construct, followed by ATRA treatment, resulted in a synergistic increase in MASH1 promoter activity and number of MAP2ab positive cells. The potential candidate effector molecules of ASK1 and ATRA were MEF2C, Sp1 and HES1, since these transcription factors have putative binding sites on the MASH1 promoter. Although both ASK1 and ATRA, separately and in combination, may significantly upregulate MASH1 promoter activity, our findings suggest that the molecules use two different pathways, converging on the transcriptional level.

Overexpression of MEF2C activated the MASH1 promoter, and by using a GAL4 reporter assay, we show here for the first time that ASK1 stimulates the MEF2C transactivation domain in a p38-dependent manner. Furthermore, phosphorylation as well as an increased protein level of MEF2C was observed in ASK1-ΔN infected cultures. Whether this increase in MEF2C protein is due to phosphorylation-induced protein stabilization or ASK1-induced MEF2C protein expression remains to be determined. *In vivo* analysis of the MASH1 promoter using ChIP assay revealed that in cultures overexpressing ASK1-ΔN, MEF2C was recruited to the promoter DNA. To our knowledge there is no evidence for p38 facilitating MEF2C DNA binding. However, since ASK1 is a kinase far upstream, it cannot be excluded that kinases other than p38 induce MEF2C recruitment to the MASH1 promoter. A possible kinase would be CaMK, since evidence proposed that CaMKII facilitates MEF2 DNA-binding domain activation (Lu et al., 2000a). In support of this notion we found CaMKII recruited to the MASH1 promoter upon ASK1 overexpression, suggesting responsiveness of CaMKII to ASK1 signaling.

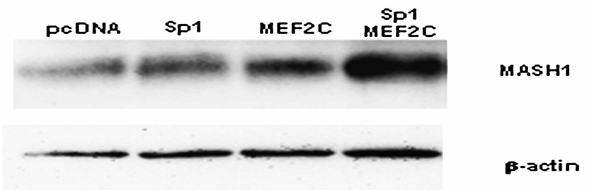

Figure 7. MEF2C and Sp1 synergistically induce MASH1 expression. AHP cells were transfected to overexpress Sp1 and/or MEF2C at DIV 2. Cells were harvested for MASH1 immunoblot after additional 2 days in culture.

ATRA did not activate MEF2C or its recruitment to the MASH1 promoter. However, we found that ATRA targeted the transactivating domain of Sp1 in a p38-independent manner. ChIP analysis showed that Sp1 was constitutively bound to the MASH1 promoter. ATRA activity on the MASH1 promoter was significantly blocked by mithramycin, an Sp1 DNA binding inhibitor, suggesting the necessity of Sp1 DNA binding for ATRA-induced MASH1 activation. ATRA induced a rapid transient increase of MASH1 protein, and it is therefore conceivable that ATRA by Sp1 modulation, rather than induction of Sp1, upregulates MASH1 expression. The precise ATRA-modulation of Sp1 was not investigated in the present study.

Overexpressing MEF2C and Sp1 synergistically increased MASH1 promoter activity, thus reproducing a similar effect to that of ASK1-ΔN and ATRA on MASH1 promoter. Furthermore, ChIP analysis of the MASH1 promoter showed that its activation by ASK1 and ATRA required the dismissal of the transcriptional repressors HDAC1 and 4, and recruitment of coactivators, such as CBP, PCAF, and CaMKII. Interestingly, ASK1 and ATRA activation of MASH1 promoter did not require dismissal of HES1, a suppressor of MASH1 transcription. Constitutive binding of HES1 is in concert with recent findings (Ju et al., 2004), suggesting that it is the phosphorylation status of HES1 that is decisive for its effect on MASH1 transcription.

Paper IV

TLX is a member of the *tailless* class of orphan nuclear receptors, a highly conserved family in both vertebrates and invertebrates, and has been shown to be important for CNS development. In the CNS of adult mice, TLX is expressed in the subventricular zone and subgranular zone, both neurogenic regions. Recent studies provide evidence that TLX is an inhibitor of glial differentiation and suggests TLX to be a maintainer of undifferentiated adult NSCs (Shi et al., 2004). Although the role of TLX in adult neural stem cells is now unfolding, little is known about its role in neuronal differentiation. Therefore, in the present study, the role and mechanism of TLX in neuronal differentiation of AHP cells were investigated by means of TLX overexpression.

In proliferating or differentiating cultures, we found no difference neither in the number of adult neural progenitors expressing TLX nor in TLX transcripts by RT-PCR analysis. Moreover, when

TLX was overexpressed in AHPs in the presence of FGF, the number of cell expressing the neural stem cells marker Prominin1 was significantly reduced. In contrast to previous findings, our results suggest that overexpressed TLX does not maintain AHP cells in an undifferentiated state. Therefore, we sought to investigate the fate of cells induced to overexpress TLX and its mechanism.

AHPs cultured in differentiation condition and infected with TLX, transiently increased cell proliferation. In this study, for the first time, we provide evidence that exogenous TLX promotes neuronal differentiation. We found that TLX overexpression in AHPs increased the number of cells expressing the neuronal markers MASH1, DCX, and MAP2ab. In addition, TLX significantly suppressed GFAP expression even in the presence of the glial inducing factors, LIF and BMP6, confirming previous findings (Shi et al., 2004).

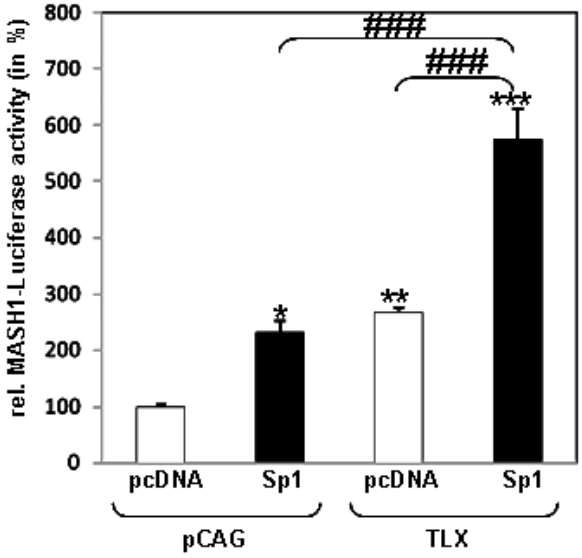

Figure 8. TLX and Sp1 synergistically induce MASH1 promoter activity. AHPs in differentiation condition were transfected with MASH1-Luc together with Sp1 and/or TLX at DIV 2 and processed for luciferase assay after additional 2 days in culture.

In order to see whether the neural-inducing effect of TLX is an indirect effect of glial inhibition, we performed luciferase assay using the MASH1-Luc promoter, and found that TLX could significantly increase promoter activity. In addition to this, *in vivo* chromatin analysis using ChiP assay, revealed that TLX could associate with the MASH1 promoter. Hence, TLX in a direct fashion inhibits glial differentiation, while activating MASH1 expression and inducing commitment towards a neuronal lineage.

Elucidating the mechanism by which TLX activates the MASH1 promoter, we investigate the possible implication of p38 in TLX signaling. Our results showed that blocking p38 did not

change the TLX-dependent activity on the MASH1 promoter. However, when AHPs were treated with mithramycin, which inhibits the DNA binding of Sp1, the TLX activity on the MASH1 promoter was significantly blocked. These results suggest that TLX works in a similar fashion as the nuclear-receptor ligand ATRA to induce neuronal differentiation.

Discussion

Neural Lineage Determinants

MAP Kinases and Nuclear Receptors

The findings of adult neural progenitors have raised hope to find treatments for neurodegenerative diseases. A simple solution would be to replace the neurons that are lost. However, in order to do so one has to be able to direct and control the differentiation of NPCs toward the desired lineage before grafting the cells into the diseased brain. Hence, one of the most important issues in NSCs biology today is to understand the molecular mechanisms underlying cell specification and cell fate.

The aim of this thesis was to investigate candidate molecules and the signal pathways involved in directing NPCs to differentiate into neuronal cells. In paper II – IV, we found that by means of overexpressing ASK1 or TLX we were able to obtain significantly increased numbers of neurons in cultures of AHPs *in vitro*, compared with control cultures. Furthermore, ASK1 in combination with ATRA treatment further yielded a significant increase in the number of mature neurons, compared with ASK1 or ATRA alone. This suggests that these molecules are good candidates for manipulating NPCs into becoming neurons. However, with these outcomes it is still important to keep in mind that ASK1 is a kinase high up in the MAPK signaling cascade and under certain circumstances also an inducer of apoptosis. In addition, ATRA is a potent morphogen involved in the regulation of many developmental processes *in vivo*, and is believed to act as a general differentiator rather than a lineage instructor. With such molecules it is difficult to harness the signaling cascades toward a specific lineage commitment and differentiation. A possible setting to avoid unwanted side effects would thus be to find the downstream effector molecules involved in neuronal differentiation for therapeutic use.

This concept of activating the downstream targets rather than molecules high up in the chain of command appears to be more convincing. However, there is a possibility that one might neglect pathways activated by TLX, ASK1, or ATRA, other than those we studied and which works in favor of neuronal differentiation. Our findings show that when p38 is inhibited, the ASK1-induced neuronal differentiation as well as the ASK1 anti-glial effect is aborted. This suggests that p38 is essential for the observed ASK1 effect. However, this does not rule out that additional pathways, yielding the same outcome, are also activated. There will have to be a balance between minimizing the unwanted side effects, while at the same time keeping the positive aspects of TLX, ASK1, and/or ATRA signaling on neuronal differentiation.

More evidence is now emerging showing that ASK1 signaling is implicated in neuronal regulation. The CaMKII/UNC-43-NSY-1 pathway in *C. elegans*, described in the introduction section, is also conserved in mammalian cells. It is shown that calcium signaling molecules regulate the ASK1-p38 cascade in mammalian neurons (Takeda et al., 2004). In PC12 cells and primary neurons, ASK1 was activated in response to influx of Ca^{2+} ions evoked by membrane depolarization. Consistent with the results from *C. elegans*, CaMKII has an important role in the

$Ca2^+$ activated ASK1-p38 signaling. These results indicate that the CaMKII-ASK1-p38 MAP kinase cascade transmit calcium signal in neurons (Takeda et al., 2004).

Our findings, however, suggest that CaMKII is activated by ASK1, since ASK1 could facilitate the recruitment of CaMKII to the MASH1-promoter. It is therefore possible that ASK1 and CaMKII function in a reciprocal fashion, mutually activating one another. Furthermore, in the same study we identified MEF2C as the downstream transcription factor of ASK1-p38 signaling. These observations, that ASK1 activity is regulated by calcium, and is able to activate MEF2C and CaMKII, raise the possibility that ASK1 mediates neuronal differentiation and survival by way of these two molecules.

TLX, like ASK1, has a dual role in neural progenitors, where it promotes neuronal commitment while suppressing glial differentiation. We and others have shown that TLX works as a negative regulator of glial differentiation suppressing the GFAP promoter (Shi et al., 2004). In addition to this, in Paper IV we show that TLX promotes neuronal differentiation by inducing early transcription machinery by way of activating the MASH1 promoter. Considering the important role of TLX described here in regulating neural progenitors, it is of great interest to screen for a potential ligand and/or activator of TLX. The identification of such a molecule can have possible implications for drug discovery in treatment of patients suffering from neurodegeneration.

BMP Receptors

With regard to the fact that BMP receptors and molecules of the BMP signaling cascade are broadly expressed in a large variety of tissues and organs, the simple linear pathway of BMP signaling described in the introduction is not to be expected in reality. The pleiotropic functions of BMPs suggest a strong need for regulation of BMP signaling. Many factors have been recognized to influence BMP signals, such as combinations of different type I and type II BMP receptors, formation of a variety of different Smad complexes, and interaction of receptors and Smads with accessory proteins (Nohe et al., 2004). Cross-talk with other signaling pathways may also provide a high degree of signaling specificity and flexibility (Herpin and Cunningham, 2007).

Alternative pathways for BMP receptor signaling that are Smad-independent, have been described. Studies have shown that the mode of oligomerization of receptors prior to ligand-binding results in different downstream targets. Prior to ligand binding, BMP receptors can be found as preformed homo- and hetero-oligomers (Gilboa et al., 2000). Evidence suggest that the Smad pathway is initiated at pre-existing receptor complexes, which exist in an inactive state in the plasma membrane and become activated upon binding of BMP2 (Nohe et al., 2002; Massague, 2003). On the other hand, BMP receptors not present in pre-existing receptor complexes do not appear to assemble into oligomers until BMP2 binds to the type I receptor. This causes dimerization of BMPRI and recruitment of BMPRII into the signaling complex, followed by the activation of downstream targets involving p38 (Gilboa et al., 2000). Similarly, another alternative signaling pathway has been described in which the X-linked inhibitor of apoptosis protein (XIAP) functions as an adaptor protein bridging Alk3 receptors and the MAPK signaling

of TGFβ activated kinase (TAK1) which in turn can activate p38 and JNK (Shirakabe et al., 1997; Yamaguchi et al., 1999; Kimura et al., 2000)

It is possible that overexpression of dnAlk receptors recruit type II receptors into non-functional complexes. Thus, overexpression of one dnAlk can in turn indirectly impair the signaling of the other Alk receptors by depleting BMPRII from the cell membrane. Furthermore, in the light of the observation of pre-existing receptor complexes, there might be additional explanations for our results on the BMP receptor study. It was reported that in the absence of ligand there is a discrepancy between Alk3 and Alk6 associating into preformed complexes with type II receptors (Gilboa et al., 2000). Our results show that dnAlk6, but not dnAlk3, significantly block Smad phosphorylation, suggesting that dnAlk6 associates with preformed complexes. However, the implication of dnAlks on ligand-induced heteromeric receptor complex formation, which induces p38 signaling, has not been elucidated. It would therefore be of interest to investigate the activation of p38 upon dnAlk3 and dnAlk6 overexpression.

Taken as a whole, our use of dnAlk receptors has provided a tool to further understand the complex role of BMP signaling in adult neural progenitors. Furthermore, our results indicate that blocking type I receptors, using dnAlk, as therapeutic genes to induce neuronal differentiation is not a suitable option. Overexpression of dnAlk6 did not have any effect on neuronal differentiation, however we found that the number of oligodendrocytes increased. In some diseases, such as multiple sclerosis and spinal cord injuries, an increased oligodendroglial differentiation is of potential interest. However, the increased cell death caused by dnAlk6 renders it inappropriate for use in therapy.

Neural Progenitor Cultures

The process of neurogenesis involves a sequential and combination of molecular signals. It is likely that in order to direct NPCs down the neuronal lineage and yield a high number of neurons *in vitro* the expression of more than one molecule, which involves commitment to the neuronal lineage, facilitation of survival and maturation, is required.

Clonal analysis of AHP cells has shown that these cells have the characteristics of multipotent NSCs that can differentiate and mature into the three major CNS lineages (Palmer et al., 1997). In a cell culture of AHPs grown with FGF, the cell population can be expected to be heterogeneous due to spontaneous differentiation, consisting of stem cells, immature progenitors, as well as more committed cells. In Paper IV we showed that in our AHP cells, although passaged between 11-20 times, 60% of the cells expressed the NSC marker Prominin1 when cultured in FGF. This would indicate that the progenitor cultures contain a majority of cells that have the characteristics of NSCs. AHPs cultured for two days without FGF had a reduced number (29%) of Prominin1-positive cells. Considering that we infected/transfected AHPs at this time point, overexpression of ASK1, TLX, Alk receptors, and treatment with ATRA occurred in cultures containing a majority of cells more committed than NSCs. It is possible that a substantial number of AHPs at the time of infection contain cells that are already lineage committed. Both ASK1 and TLX suppress glial differentiation, however whether they are able to reverse glial lineage fate is not known.

Therefore, the possibility remains that the glial committed cells could reduce the effect of ASK1 and TLX in neurons. In order to avoid this, cells could be infected while still kept in FGF containing medium, and remove FGF after 8 h — the earliest time point in which appreciable amounts of exogenous protein expression can be detected. By doing so, the probability of infecting NSCs could be enhanced.

In this study, we have examined only the early neural commitment of AHPs. It would be of interest to monitor the long-term differentiated phenotypes of cells expressing ASK1, ATRA, and TLX. This is feasible since various phenotypical markers on differentiating hippocampal progenitors have been described (Kempermann et al., 2004).

In Vivo Application

Pre-differentiation

Integration of functional neurons in neural networks is believed to occur through a highly regulated sequential process. The first step is proliferation of the NSC, followed by generation of rapidly amplifying progenitor cells, and differentiation into immature neurons. The process continues with migration to the final location, growth of axons and dendrites and formation of synapses with other neurons in the circuits. The final step is the maturation into fully functional neurons (Lie et al., 2004). Grafting undifferentiated AHPs into the neurogenic regions of the normal healthy brain has shown the ability of AHPs to integrate and differentiate according to environmental cues (Gage et al., 1995; Suhonen et al., 1996). However, in neurodegenerative diseases, the process of neurogenesis is disrupted and the CNS environment altered. Therefore, in this thesis we elucidated the possibility of pre-differentiating the AHPs *in vitro*. Overexpression of TLX, ASK1, and treatment with ATRA each was sufficient to induce maturation of AHPs into MAP2ab-expressing neurons. However, *in vivo* studies are necessary to decide whether these cells once transplanted will be able to migrate and project dendrites and axons in such a fashion that they can form synapses and mature into functional neurons, fully integrated into the neural circuitry of a diseased brain. Furthermore, it would be interesting to elucidate which types of neurons are produced after overexpression of ASK1, TLX, or ATRA treatment. This is important when treating neurodegenerative diseases, since different types of neurons are affected depending on the disorder.

In Paper II we found that ASK1 via p38 suppressed glial differentiation of AHPs, but after long-term culture this effect was gone. We hypothesized that the most likely explanation to the increase of GFAP-positive cells was a reduction of exogenous ASK1 expression. We used adenoviral vectors as a mean of transduction. Adenovirus does not integrate into the host genome, and consequently every cell division will reduce the number of ASK1-DNA in each cell, resulting in a diminishing ASK1 expression. The persistent expression of ASK1 and TLX is necessary to suppress glial differentiation in AHP cultures. Lentivirus could be a potential vector since they can integrate with the host genome even in non-dividing cells, thereby inducing a stable expression. Although gene transduction of stem cells using lentiviral vectors are commonly utilized in *in vitro* systems, there are some serious concerns in using this type of vectors — *e.g.*

frequent transgene silencing *in situ*, and integration of the transgene activating a nearby oncogene, leading to selection of abnormally growing cells.

Cell Therapy Strategies

Stem cells are presently believed to be a promising cell source for cell replacement strategies in neurodegenerative diseases. However, this poses multiple specific challenges and problems to be solved. There are two possible scenarios on how our results may be used as tools for future therapies — targeting NPCs either *in situ* or *ex vivo*.

Adult NPCs have been proposed as an endogenous cellular source for the treatment of CNS diseases. One way to mobilize these cells would be to genetically engineer endogenous stem cells, to overexpress for instance ASK1 or TLX or any of their potential downstream target molecules, inducing their differentiation toward neuronal lineages. The advantage with ASK1 and TLX is that they have dual roles, where they can both promote neuronal differentiation while repressing glial differentiation. This is of interest in gliogenic regions where there are stem cells but normally restricted to a glial fate due to the extrinsic milieu.

In some CNS diseases, the anatomical and functional relationships between cell components might be disturbed, thereby impairing the repair capacity of the endogenous stem cells. The consequence is that trying to mobilize endogenous precursors *in vivo* might prove to be difficult. Depending on the number of NPCs remaining in the diseased or damaged brain and the efficiency of transducing NPCs *in vivo*, the question arises as how likely it is that ASK1 or TLX are capable of mobilizing a substantial number of cells to reconstitute a damaged neural circuitry. Overexpression of TLX induces transient proliferation of neural progenitors and a subsequent differentiation. Even though TLX transiently induces proliferation it seems unlikely that the population/group of transduced cells would be sufficient to regenerate damaged brain tissue. Therefore, transplantation of cells might represent an alternative, and possibly a more successful approach.

Due to the limitations in recruiting endogenous NPCs, it might be necessary to genetically engineer these cells *ex vivo*, to expand and differentiate towards the desired lineage in a monitored and controlled environment, optimized to support cell survival, neuronal differentiation, and maturation. NPCs can be expanded in growth factor containing cultures, transduced to overexpress TLX, ASK1 and treated with ATRA directing the cells towards a neuronal maturation.

Considering the fact that some diseases are caused by a recurrent ongoing process, transplanted genetically altered cells might still be affected by a hostile environment, which after a while would also cause them to degenerate. Studies have shown that when undifferentiated NPCs were transplanted into animal models of CNS diseases, the transplanted NPCs could in many cases ameliorate the disease symptoms (Martino and Pluchino, 2006). However, this was not due to a production of new neurons, since very few transplanted cells gave rise to terminally differentiated neural cells. It is speculated that the transplanted NPCs release molecules such as neurotrophins,

cytokines, and chemokines which exerts a neuroprotective effect on the remaining cells modulating the hostile environment. It is to be noted that in transplantation studies using NPCs, inappropriate or undesired cells have never been observed, such as tumor formation, despite their extensive multipotency and their possession of all the stem cell attributes. These studies suggest that the ideal solution would not only be to replace neurons that have died. In order to also reconstitute a damaged tissue, undifferentiated NPCs that detoxify the environment will be needed.

In addition to this, studies have shown that the degree of differentiation of transplanted cells could have an effect on their ability to integrate into the CNS. While undifferentiated NPCs failed to differentiate into neurons upon transplantation into adult mouse neocortex after targeted neuronal degeneration, embryonic neuroblasts were able to differentiate and integrate to replace lost callosal projection neurons. Later-stage neurons were even more efficient in this process. However, the survival of the transplanted neurons was lower with an increased differentiation state (Komitova et al., 2006).

In conclusion, complex disorders require complex solutions, and the answer for one disease does not necessarily extrapolate to another, even a presumably similar disease. Ultimately, transplantation of genetically transduced neural progenitors would constitute one part, probably an indispensable component of a therapy, combined with transplantation of undifferentiated neural progenitors, as well as stimulation of endogenous cells.

Conclusions

I. The three BMP type I receptors, Alk2, 3, and 6, play different roles in AHP cells. Alk6 signaling is indispensable for survival of and differentiation towards astrocyte lineage while inhibiting oligodendrocyte. In agreement with this, blockage of Alk3- or Alk2-signaling induces Alk6 expression. Blockage of any of these receptors did not alter the neuronal differentiation.

II. Overexpression of ASK1 in AHPs induces neuronal lineage commitment towards neurons by targeting MASH1 induction while in parallel, ASK1 repressing the GFAP promoter, consequently inhibiting glial differentiation. These effects depend on p38 MAPK phosphorylation by ASK1.

III. ASK1 and ATRA yielded a synergistic increase in neuronal differentiation of AHPs. We showed that ASK1 and ATRA induce early neuronal commitment by activation of two different transcription factors, MEF2C and Sp1, separately. In combination, these factors interact with each other, bind and activate the MASH1 promoter by recruiting co-activators.

IV. Expression of TLX activated a transient cell division and suppression of glial differentiation. As monitored by neural stem cell and postmitotic neural markers we could demonstrate that neuronal differentiation was not a default pathway but actively promoted by TLX. The mechanism is somewhat similar to ATRA, that TLX requires an interaction with Sp1 to bind and activate the MASH1 promoter.

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