Leukemia inhibitory factor enhances neurogenin´s pro-neural effect during mouse cortical development

Dissertation

zur Erlangung des Doktorgrades

der Naturwissenschaften

vorgelegt beim Fachbereich Biologie und Informatik

der Johann Wolfgang Goethe-Universitaet

in Frankfurt am Main, Deutschland

von

Sascha Hasan

aus Heidelberg, Deutschland

Frankfurt am Main 2007

Vom Fachbereich Biologie und Informatik der

Johann Wolfgang Goethe-Universitaet als Dissertation angenommen

Dekan: Prof. Dr. R. Wittig

Gutachter: Prof. Dr. W. Volknandt

Prof. Dr. Y. Sun

Datum der Disputation:

Table of contents

1 Introduction.....6

1.1 Neural stem cells and cortical development 7

1.2 Intrinsic factors that promote cortical neurogenesis: proneural basic helix-loop-helix transcription factors.....10

1.3 Extrinsic factors that regulate cortical development.....15

1.4 Mechanisms by which neurogenin inhibits gliogenesis and promotes neurogenesis.....16

1.5 The role of SWI/SNF chromatin remodeling protein Brg1 in promoting neurogenesis.....18

1.6 Therapeutic potential of NSCs in neurodegenerative diseases.....19

1.7 Overview of project experiments and findings.....21

2 Matherial and methods.....23

2.1 Cell culture and reagents.....24

2.2 Mice.....25

2.3 Expression vectors and adenovirus constructions.....26

2.4 PLCγ and PKCδ siRNA.....27

2.5 Immunocytochemistry.....28

2.6 Immunohistochenistry.....29

2.7 Western-blot analysis and immunoprecipitation.....30

2.8 Dual luciferase reporter assay.....31

2.9 Chromatin Immunoprecipitation assays.....32

3 Results.....36

3.1 LIF enhances neurogenin's transcriptional activation in cortical NPCs...37

3.2 LIF promotes neurogenesis and inhibits gliogenesis in cortical NPCs in the presence of *Ngn1* and *Ngn2 in vitro*.....40

3.3 Potential signaling pathways mediating the neurogenic effect of LIF.....42

3.4 PLCγ/PKC mediate the effect of LIF on neurogenesis.....45

3.5 PLCγ/PKC promote association between *Ngn1*, CBP/p300 and *Brg1*.....47

3.6 PKCδ might be the mediator of LIF in cortical NPCs during neurogenesis.....50

3.7 PLCγ and PKCδ siRNA abolishes the pro-neural effect of Ngn and LIF.53

3.8 LIF promotes neurogenesis *in vivo*.....56

3.9 LIF heterozyogte and knock out mice display less binding of the Ngn1-CBP cotranscriptional complex to the *NeuroD* promoter.....59

4 Discussion.....60

4.1 Novel pro-neurogenic role of LIF during cortical neurogenesis is solely dependent on the expression of bHLH factors Ngn1 and Ngn*2*.....62

4.2 LIF promotes CBP-Ngn1 association, leading to enhanced *NeuroD* transcription and therefore to increased neurogenesis.....63

4.3 LIF-induced PKC activity is necessary for CBP-Ngn association.....65

4.4 The role of Brg1 during mouse cortical neurogenesis.....68

4.5 *In vivo* evidence for LIF's pro-neural role during mouse cortical neurogenesis.....69

4.6 Implication of this work for regenerative medicine.....71

5 Summary (German).....73

6 Reference.....78

7 Acknowledgement.....93

8 Lebenslauf.....94

9 Declaration.....95

Chapter 1

Introduction

1.1 Neural stem cells and cortical development

During mammalian development the major cell types comprising the cerebral cortex of the central nervous system (CNS) arise from a single layer of proliferating neuroepithelial cells that line the ventricles and form the ventricular zone (VZ). Neurons, astrocytes and oligodentrocytes differentiate sequentially from these neural progenitor cells (NPSs, also referred to as neural stem cells, NSCs), with most neurons being generated before glial cells (Figure 1a; Bayer and Altman, 1991; Sauvageot and Stiles, 2002; Sun et al., 2003).

When NPCs are isolated from the embryonic cortex this pattern of differentiation is recapitulated *in vitro*. NPCs can be expanded in culture either as monolayers on a coated surface, or as neurospheres, which are clusters of floating cells (Johe et al., 1996; Reynolds and Weiss, 1996). When derived from early embryos (e.g., mouse embryonic day (E) 10-11) NPCs give rise exclusively to neurons after short-term culture, while cortical progenitors isolated after E13-14 become predominantly astrocytes under the same culture conditions (Qian et al., 2000). However, to maintain these progenitor cells in a proliferating state, mitogenic growth factors, such as basic fibroblast growth factor (bFGF), or epidermal growth factor (EGF), must be added to a well-defined culture medium (Gage et al., 1995). Further, NPCs derived from early embryos switch from being predominantly neurogenic to predominantly gliogenic over time *in vitro*, which implies that intrinsic changes can regulate the neural versus glial cell fate (Figure 1b; Reynolds and Weiss, 1992; Sauvageot and Stiles, 2002; Sun et al., 2003).

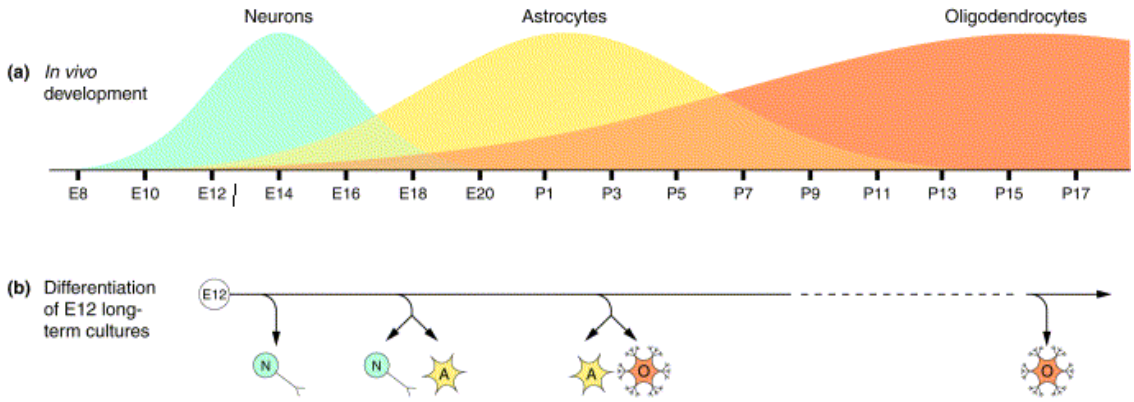


Figure 1: Cortical progenitor cells follow an intrinsic developmental sequence *in vivo* and *in vitro*.

(a) The generation of the three cell types within the brain occurs in a temporally distinct yet overlapping pattern. **(b)** *In vitro* cultures mimic the differentiation pattern seen *in vivo*, suggesting that these cells are intrinsically primed for a given fate at a given developmental period. Long-term cultures of cells isolated at E12 will sequentially give rise to neurons, then astrocytes, and finally oligodendrocytes.

Notably, during development, neuroepithelial cells first undergo symmetric, proliferative divisions, each of which generates two daughter stem cells (Rakic, 1995; McConnell, 1995). As cortical development proceeds, the length of the cell cycle increases primarily through the extension of the G1 phase. Concurrently, cells begin to undergo asymmetric cell division, and the fraction of cells that begin to differentiate into neurons increases, whereas the proportion of cells remaining as progenitors decreases (Caviness and Takahashi, 1995).

Upon cell cycle exit, a cell must migrate out of the ventricular zone (VZ) towards the developing neocortex. About 80-90% of cortical neurons arise from the VZ of the dorsal telencephalon and migrate radially to their place in the cortex. Although radial migration accounts for the bulk of cortical neurons, studies have shown that a subpopulation of neurons moves tangentially across the plane of the glial fiber system (Figure 2). These cells originate in the subpallium and include the majority of γ-

aminobutric acid-expressing (GABAergic) interneurons and some cortical oligodentrocytes (Kriegstein and Noctor, 2004; Marin and Rubinstein, 2001). Radial glia, which are columnar cells that have their cell body in the VZ but extend their processes to the pial surface, function as scaffolding for brain development by guiding developing neurons from the VZ to their final destination. In addition, radial glia not only provide the primary pathways for directed migration, but also themselves are the neural progenitors (Kriegstein and Noctor, 2004). Furthermore, they could give rise to adult subventricular zone (SVZ) stem cells that maintain the neurogenic potential in the adult brain (Doetsch, 2003). However, at about E11, the first group of neurons that migrate out of the ventricular zone constitutes the preplate. A subsequent wave of neuronal migration at about E13 splits the preplate into two layers: the more superficial marginal zone, which consists of the Cajal-Retzius cells born in the first migration wave, and the deeper subplate, which consists of the rest of the primordial cells. The development of the cerebral cortex progresses with successive waves of migration that position neurons within the different layers in the cortical plate (Hatten, 1999)

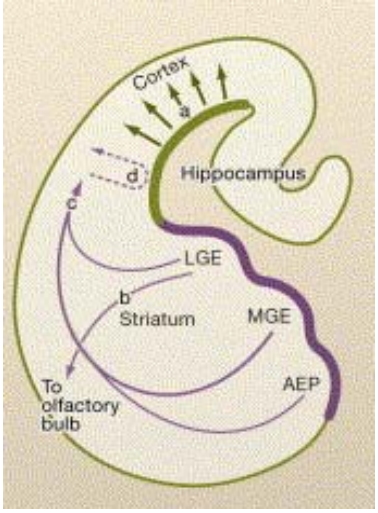


Figure 2: Primary migration routes.

Projection neurons migrate radially from the dorsal ventricular zone (a). Interneurons expressing GABA originate from the subpallium structure - the lateral ganglionic eminence (LGE), medial ganglionic eminence (MGE), and anterior entopeduncular area (AEP) - and migrate tangentially into the olfactory bulb (b) or the cortex (c and d). When they arrive at the cortex, some of these neurons are directed towards the VZ before radially migrating into the cortex (d).

(Ayala et al., 2007)

Interestingly, studies that examine the birth date of neurons have shown that each time a new wave of neurons migrates towards the cortical plate, the cells migrate past the neurons previously located in the cortical plate and come to rest right below the marginal zone. Thus the layers in the cortical plate (future adult layers II-VI) are established according to an inside-out pattern, where the earliest waves of neuronal migration will go on to form the deeper layer (V-VI), while the last wave of neurons will be localized to the more peripheral layer II and III (Marin and Rubinstein, 2003).

The potential for controlled differentiation into all neuronal tissues makes NSCs very attractive for regeneration therapy in the nervous system. There are yet many obstacles to overcome and procedures to improve. Direct transplantation of undifferentiated NPCs into an injured nervous system may result in uncontrolled differentiation that could be counterproductive. To ensure progress in the medical use of stem cells increased basic knowledge of the cellular mechanisms that govern the cell fate choice made by NSCs will significantly facilitate the development of more effective and directed production of the cell types required for specific neural repair purposes.

1.2 Intrinsic factors that promote cortical neurogenesis: proneural basic helix-loop-helix transcription factors

At the time of neurogenesis a subset of cortical progenitors becomes restricted to a neuronal lineage. It is likely that this restriction involves asymmetric division in which one cell is maintained as a NSC, while the other differentiates into a neuron within a few rounds of cell division (Caviness and Takahashi, 1995). Recent insights into the

molecular mechanisms of cortical development have shown that proliferation, specification, and differentiation of cortical NSCs are controlled, to a large degree, by transcription factors with basic helix-loop-helix (bHLH) motifs (Figure 3). These are the structural motives that mediate DNA binding and form heterodimeric complexes with ubiquitously expressed bHLH proteins, or E protein, such as E12 or E47. Through their positively charged basic domain, these heterodimers then bind to a core hexanucleotide E box element, CANNTG (Gradwohl et al., 1996). To promote neuronal differentiation and activate tissue-specific gene expression E-box binding is critical for bHLH proteins (Cau et al., 1997).

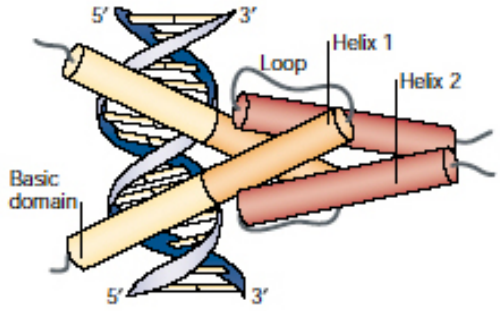


Figure 3: Structure of pro-neural bHLH proteins. Schematic representation of the structure of a bHLH dimer that is bound to DNA via its basic region. (Bertrand et al., 2002)

Further, coactivators, such as p300/CBP and PCAF, mediate transcriptional activation through their interaction with bHLH heterodimers. These coactivators recruit a large complex that includes the basal transcriptional machinery. In addition, coactivators facilitate transcription by acetylating histones, leading to the unrevealing of DNA from a tightly packed structure to one that is accessible to the transcriptional machinery (Roth at al., 2001).

It was shown in Drosophila and vertebrates that only a small number of proneural bHLH transcription factors are both necessary and sufficient to initiate the development of neuronal lineages (Ma et al, 1996; Ross et al, 2003). Three proneural genes *Neurogenin 1* (*Ngn1*), *Neurogenin 2* (*Ngn2*), and *Mash1*, are expressed exclusively in the cortical ventricular zone (Guillemont and Joyner, 1993; Lo et al., 1991; Sommer et al., 1996). While neural progenitors are being specified these bHLH factors are expressed at low levels, followed by a transient increase in their expression that results in the initiation of neurogenesis. Consistent with this idea, proneural bHLH factors are expressed in the ventricular zone, where progenitors begin differentiation, but not in the cortical plate, where fully differentiated neurons are situated. While *Ngn1* and *Ngn2* are expressed in the dorsal telencephalon, which gives rise to glutamatergic neurons, *Mash1* is predominantly expressed in the ventral telencephalon, which gives rise to GABAergic and cholinergic neurons, suggesting that different neuronal subtypes are specified by different proneural genes (Wilson and Rubinstein, 2000).

Overexpression of *Ngn1*, like *Mash1*, can induce the expression of neuronal markers, such as β-tubulin, indicating that either factor can initiate a program of gene expression that results in a neuronal phenotype (Sun et al., 2001; Farah et al, 2000). However, different bHLH factors seem to activate different signaling cascades. *Ngn1* and *Ngn2*, but not *Mash1* control expression of neuronal differentiation bHLH factors, such as *NeuroD*, which is necessary for mediating terminal differentiation after neurogenesis is initiated (Cau et al., 1997). Like proneural bHLH factors, bHLH differentiation proteins act as E-box binding transcriptional activaters, and, when overexpressed, are sufficient to induce cell cycle arrest and neuronal differentiation in culture (Farah et al., 2000). The

programs of gene expression that are mediated by Mash1 activity in the ventral telencephalon are still unknown. By defining the subset of genes that are common to *Mash1* and *Ngn1/Ngn2* versus those that are different to each proneural gene one would gain great value in understanding how pan-neuronal and subtype-specific programs of gene expression work together to mediate neurogenesis.

The important role of proneural bHLH factors in the CNS has been supported by several knockout studies. *Mash1* knockout mice display a loss of GABAergic interneurons in the telencephalon (Casarosa et al., 1999, Yun et al, 2002), whereas *Ngn1* or *Ngn2* single mutant mice show reduced expression of several transcription factors specifically expressed by cortical neurons (*Math2*, *Nscl1*, *NeuroD*, *NeuroD2*, *Tbr1*, *Tbr2*). These transcription factors were even more severely downregulated in *Ngn1/Ngn2* double-knockout mice (Schuurmans et al, 2004). Further, *vesicular glutamate transporter1* and *2* (*VGLU1* and *2*), which load glutamate into synaptic vesicles (Fremeau et al., 2001), are also downregulated in *Ngn* mutant mice. Thus *Ngns* are required to activate cortical- and glutamatergic-specific differentiation programs in early-born cortical plate neurons, likely acting downstream of cortical patterning genes, such as *Gli3*, *Pax6* and *Emx2,* that are normally expressed in *Ngn* mutants. In addition, *Ngn* mutant mice show an increase in ventral telencephalic transcription factors in cortical progenitors, such as *Mash1*, *Dlx1* and *Dlx2*, and an increase in GABAergic neuron markers (Schuurmans et al., 2004). When both *Ngn2* and *Mash1* are disrupted neural progenitors switch from producing neurons to becoming astrocytes, further indicating that a decrease in proneural bHLH activity leads to an astrocyte fate (Nieto et al., 2001).

As shown by our own and other laboratories, *Ngn2* knockout mice display strong neuronal migration defects, suggesting that bHLH transcription factors have dual functions to couple neuronal migration with neurogenesis, enabling newborn neurons to migrate (Ge et al., 2006; Hand et al., 2005). Moreover, bHLH genes regulate the neurogenic and migratory machineries via partially distinct mechanisms because mutations in bHLH genes were found to specifically affect either migration or neurogenesis.

The way the expression of *Ngn1/2* and *Mash1* is regulated is still not clear. Members of the *Hes* family (for example *Hes1* and *Hes5*) are known bind to N boxes (CACNAG) and repress the expression of target genes, such as *Mash1* (Chen at al., 1997; Davis and Turner, 2001). Recent finding suggest that HMG-box proteins, such as *Sox1* and *Sox2*, might function upstream of proneural genes inhibiting these genes' expression (Graham et al., 2003). The paired homeodomain transcription factor *Pax6* binds to a conserved regulatory element on the promoters of *Ngn1* and *Ngn2* (Scardigli et al., 2003). Nevertheless, the signaling pathways and mechanisms that positively regulate the activity of proneural factors are largely unknown. Furthermore, we have no knowledge about mechanisms that downregulate the expression of proneural genes at a later developmental stage.

Additionally, it was shown that *Ngn1, Ngn2* and *Mash1* all have consensus sequences in the C-terminus that correspond to consensus glycogen synthase kinase 3 (GSK3) phosphorylation sites. It has been suggested that this may represent a common mechanism to regulate the activity of these bHLH factors (Moore et al., 2002). Because *Wnt* and FGF signaling is well known to regulate the activity of GSK3, it is tempting to

speculate that extrinsic signals and intrinsic pathways regulate bHLH factors through GSK3-mediated phosphorylation. Whether phosphorylation of bHLH factors leads then to ubiquination followed by degradation by proteasomes, similar as *β-catenin* regulation by GSK3 phosphorylation, still needs to be explored.

1.3 Extrinsic factors that regulate cortical development

Cortical precursors exhibit distinct biological responses to various extracellular stimuli. Proliferation of cortical NSCs is primarily controlled by the action of basic fibroblast growth factor (bFGF), which leads to an expansion of the precursor cell population (Gosh et al., 1995).

Differentiation of cortical NSCs into neurons, on the other hand, is controlled by neurotrophic factors, such as neurotrophin-3 (NT-3) and brain-derived neurotrophic factor (BDNF), and growth factors, such as platelet-derived growth factor (PGDF) (Gosh et al., 1995; Menard et al., 2002; Barnabe-Heider et al., 2003). As shown by Menard et al, PDGF promotes the generation of neurons *in vitro* via activation of an MEK-RSK-C/EBP (MAP kinase kinase - ribosomal S6 kinase - CAAT enhancer-binding protein) pathway. In addition, PDGF inhibits CNTF-mediated gliogenesis, while inhibition of C/EBP-dependent transcription enhances CNTF-mediated gliogenesis, leading to precocious glia cell differentiation. Further, recent findings demonstrate that cortical NSCs express BDNF and NT-3, as well as their preferred tyrosine kinsase B (TrkB) and TrkC receptors. Moreover, these endogenously produced neurotrophins signal via Trk receptors to activate the phosphatidyl inositol-3-kinase (PI3-kinase) and MEK pathways, which have

distinct functions, with PI-3 kinase being essential for progenitor survival and MEK for the differentiation of neurons but not glia (Barnabe-Heider et al, 2003).

Additionally, bone morphogenic proteins (BMPs) induce neuronal differentiation in primary cultured, early (E11-12), neurogenic cortical progenitors, but induce astroglial differentiation in relatively late (E16-17) progenitors (Li et al., 1998; Mabie et al., 1999), depending on the presence or absence of Ngn1 (Sun et al., 2001).

Other astroglial-inducing factors, such as leukemia inhibitory factor (LIF), Notch-Delta, and bFGF can initiate the astrocyte differentiation program only in E15 or older cortical progenitors, but not in early neurogenic progenitors (Gaiano et al., 2000; Ge et al., 2002; Kamakura et al., 2004; Molne et al., 2000; Song and Gosh, 2004; Takizawa et al., 2001; Tanigaki et al., 2001; Viti et al., 2003). In E11 CNS neural progenitors, both Notch-delta and bFGF function instead as pro-proliferation factors (Bartlett et al., 1998; Chambers et al., 2001; Gaiano et al., 2000; Ge et al., 2002; Kuhn et al., 1997).

1.4 Mechanisms by which Neurogenin inhibits gliogenesis and promotes neurogenesis

The treatment of cortical progenitors with cytokines, such as LIF or CNTF, promotes astrocyte differentiation (Bonni et al., 1997; Johe et al., 1996; Park et al., 1999). The effect of these cytokines is depended on the Janus kinase (JAK) - Signal Transducers and Activators of Transcription (STAT) signaling pathway. Phosphorylation by JAKs results in the dimerization and translocation of STATs into the nucleus, where they

initiate transcription of astrocyte-specific genes (Bonni et al., 1997; Nakashima et al., 1999a; Stahl and Yancopoulos, 1994). Additional treatment with BMPs potentiates astrocyte differentiation by activating Smad transcription factors, which form a complex with STAT1/3 that is bridged by the p300/CBP coactivators (Nakashima et al., 1999b). However, our laboratory demonstrated that *Ngn1* blocks cytokine-induced astrocyte formation by disrupting this STAT1/3-Smad-CBP transcriptional coactivator complex (Figure 4; Sun et al., 2001). The interaction of CBP with STAT1/3 is blocked by the association of CBP with Ngn1, inhibiting STAT1/3-mediated transcription. In addition Ngn1 can also block the phoshorylation of STAT1/3 after cytokine treatment.

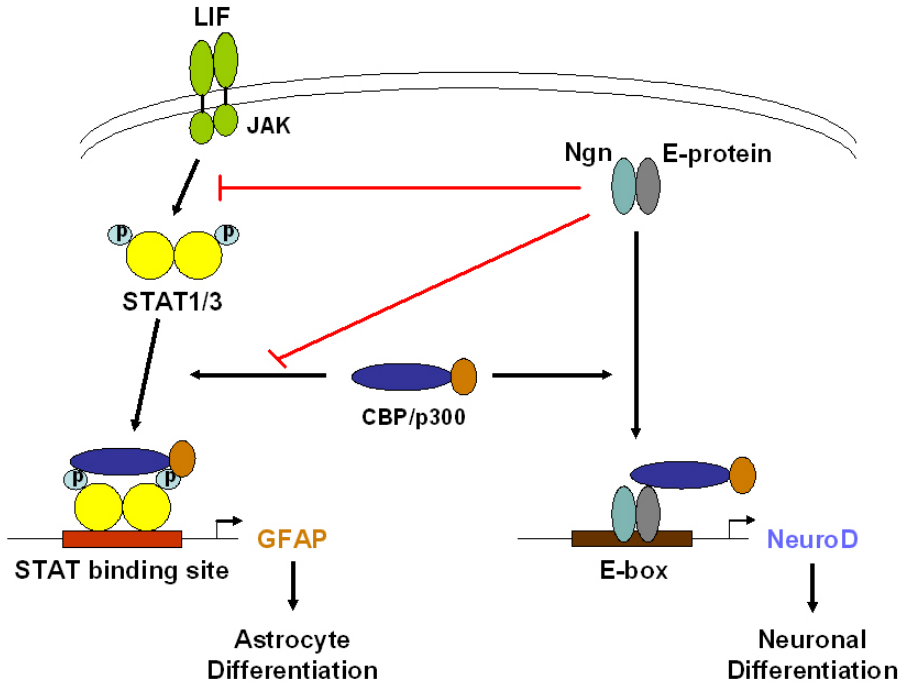


Figure 4: Ngn1 inhibits gliogenesis via two different mechanisms.

By inhibiting STAT1/3 phosphorylation, and by sequestering CBP-Smad1 away from glia specific genes *Ngn1* inhibits glia cell differentiation. In the absence of *Ngn1*, CBP/p300 binds to STAT1/3, initiating transcription of glia-specific genes, such as GFAP. If Ngn1 is present, CBP/p300 and Smad1 (separately or together) bind to Ngn1 and promote transcription of neuron-specific genes, such as NeuroD.

1.5 The role of SWI/SNF chromatin remodeling protein Brg1 in promoting neurogenesis

Factors that modulate the chromatin structure, such as chromatin-remodeling complexes, histone acetyltransferases (HATs) and deacetylases (HDACs) play important roles in transcriptional regulation. The SWI/SNF complex was the first chromatin-remodeling complex identified and characterized (Kadonaga, 1998; Martens and Winston, 2003). It consists of 7-13 subunits and uses energy provided by ATP hydrolysis to locally disrupt histone-DNA associations and relocate nucleosomes to alternate positions (Kingston and Narlikar, 1999; Whitehouse et al., 1999). In mammalian cells, SWI/SWF complexes have either one or two catalytic subunits, Brahma (*Brm*) or Brahma-related gene 1 (*Brg1*), but not both (Martens and Winston, 2003). It was recently demonstrated that *Brg1* plays a critical role in primary neurogenesis in *Xenopus* (Seo et al, 2005). Inhibition of *Brg1* resulted in an expansion of proliferating NSC and inhibition of neuronal differentiation. The studies showed that *Brg1* interacts with *xenopus Ngn* and *NeuroD*, and that the ability of these pro-neural bHLH proteins to promote neurogenesis and transactivate their target genes required *Brg1* activity. Also, Gerald Crabtree's group (Lessard et al., 2007) showed in a recently published paper that *Brg1,* depending on the subunit it is bound to, is necessary for NPC proliferation as well as neuronal differentiation. When *Brg1* was bound to Brg/Brm-associated factor 45a (BAF45a) or BAF53a, NPCs remained in a proliferative state by inducing the expression of several components of the Notch signaling pathway. On the other hand, as soon BAF45b/c or BAF 53b bound to *Brg1* neuronal differentiation was induced.

1.6 Therapeutic potential of NSCs in neurodegenerative diseases

Neural stem cells (NSCs) of the central nervous system (CNS) have recently received much attention and interest because of their therapeutic potential for neurological disorders. An important issue is the potential of NSC to differentiate into different neuronal subtypes, which makes these cells applicable for replacement therapies of diseases, such as Parkinson's disease, Huntington's disease, Alzheimer's disease and stroke.

Parkinson disease is characterized by the progressive loss of dopominergic neurons in the substantia nigra of the midbrain. Transplantation of dopamine (DA) neuron precursors into the brain of patients suggests that neuronal replacement therapy may be a possible treatment (Lindvall et al., 2004). However, transplantation trials with Parkinson disease patients raised concerns about both the therapeutic benefit and the consequences of fetal cell grafting (Freed, et al., 2001; Olanov et al., 2003; Hagell et al., 2002). For further development of a transplantation therapy a constant source of DA neurons is necessary, and clear evidence of DA function in preclinical models must be shown. Additional knowledge of neuron developmental might be helpful in effectively differentiating NSC into neurons, and ultimately into a certain neuronal subtype.

Huntington's disease (HD) is a fatal disorder that is characterized by chorea (excessive spontaneous movements) and progressive dementia. It is caused by the death of projection neurons in the striatum. Stem cell therapy aims to restore or preserve brain function by replacing and protecting straital neurons, a strategy that might be insufficient because patients also suffer progressive neocortical degeneration. In animal HD models,

cell replacement, using fetal striatal neurons, promotes functional recovery, and some evidence from clinical trials indicate that this can also occur in patients (Lindvall et al., 2004).

Alzheimer's disease (AD) is the most common form of degenerative dementia that affects many elderly people with progressive symptoms of memory loss (Marin et al., 2002; Rossner et al., 1994; Selkoe, 1999). AD is characterized by neuronal and synaptic loss throughout the brain, involving the basal forebrain cholinergic system, amygdala, hippocampus and several cortical areas (Auld et al., 2002). The pathology of AD includes neuritic plaques composed of beta-amyloid peptide (Aβ), and neurofibrillary tangles composed of hyperphosphorylated tau. Current therapies, such as treatment with acetylcholinesterase inhibitors to enhance cholinergic function, provide only partial and temporary easing of symptoms. In theory, transplanting cholinergic neurons generated from NSC *in vitro* could prevent cognitive decline caused by the degeneration of basal forebrain cholinergic neurons. But to provide a long-lasting benefit, this approach would require the existence of functional target cells that are like to be damaged over time. However, to counteract cholinergic neuronal death, basal forebrain grafts of fibroblasts that produce nerve growth factor (NGF) have been of some benefit in patients with AD (Tuszynski et al., 2005).

Stroke is caused by the blockage of a cerebral artery, leading to the loss of neurons and glia cells, which causes motor, sensory or cognitive impairments. However, it was demonstrated that transplantation of human fetal NSC into the brains of stroke-damaged rates resulted in migration of new neurons towards the damaged area (Kelly et al., 2004). Furthermore, studies showed that monkey ES-cell-derived NSC transplanted

into the brains of mice after stroke can differentiate into neurons and glia cells and re-establish connections with target areas (Hayashi et al., 2006).

1.7 Overview of project experiments and findings

During mammalian CNS development, neural progenitor cells switch from early neurogenic to late gliogenic state (Sauvageot and Stiles, 2002). Various extracellular factors are required to regulate various processes during development. Interestingly, my studies revealed that one common factor, LIF, can serve different developmental functions by activating different down-stream signaling pathways in a cell-context dependent manner. Our laboratory has previously reported that, during cortical development, LIF-induced JAK-STAT signaling is essential for glia cell differentiation, but that Ngn1 can counteract this signaling pathway and induce transcription of neuronal markers. However, applying luciferase assay, western blot and immunocytochemistry our results demonstrated that LIF not only promotes gliogenesis, but also neurogenesis in early as well as in late mouse cortical NPCs, when *Ngn1/2*, but not *Mash1*, is expressed/overexpressed. Further molecular and biochemical analyses indicated that LIF enhances the association between Ngn1 and its transcriptional co-activator CBP/p300. In addition, LIF enhances association between *Ngn1* and the SWI/SNF chromatin-remodeling complex subunit *Brg1* in order to activate gene transcription leading to neuronal differentiation. To identify the mechanism by which LIF promotes neurogenesis three signaling pathways were examined: the Ras-MAP kinase, the PI3 kinase-Akt, and the PLCγ-PKC pathway in *Ngn1*-overexpressing and control NPCs. Administration of

small molecule drugs and siRNA to inhibit individual pathways showed that LIF specifically promotes neurogenesis via PLCγ-PKC. More specifically, PKCδ seems to be a likely candidate as mediator for the pro-neural LIF effect in NPCs. Furthermore, chromatin-immunoprecipitation (ChIP) data demonstrated that LIF promotes the association of the *Ngn1*/CBP transcriptional complex to the *NeuroD* promoter.

To observe the in vivo relevance of LIF with regards to neurogenesis, brains/cortices of E13, LIF knockout, heterocygote, and wildtype mice were isolated and compared. While immunohistochemistry of coronal brain section showed neuron expression in all three genotypes, western blot analysis, a more quantitative method, showed that LIF knockout and heterocygote mice express less neurons as suggested by a decreased protein level of neuronal markers, such as TuJ1 and MAP2a/b. In addition, ChIP analysis demonstrated reduced binding of the *Ngn1*-CBP transcriptional complex to the *NeuroD* promoter.

These experiments elucidate an elegant regulation mechanism by which a single extracellular factor may promote opposite cell fates solely depending on the presence and absence of an intracellular factor, an observation, which might have important implications in directing the differentiation of neural stem cells to neurons that can be used for neural repair.

Chapter 2

Materials and Methods

2.1 Cell culture and reagents

To prepare E11/E12 cortical progenitor cell cultures we used timed pregnant Balb/c mice. Mouse embryo cortices were dissected out in hank's balanced salt solution (HBSS, Invitrogen) and dissociated mechanically by triturating using a flame-polished pasture pipette. Dissociated cells were plated onto poly-ornithine (PO, Sigma, 15 μg/ml in H_2O) and fibronectin (FN, 2 μg/ml in phosphate-buffered saline or PBS) coated 10 cm dishes at a density of 1 million cells/dish in serum-free medium (DMEM/F12 supplemented with B27 supplement [Invitrogen], penicillin-streptomycin [50 μg/ml and 50 U/ml, respectively]). Basic fibroblast growth factor (bFGF, PeproTech) at a final concentration of 10 ng/ml was added to the culture every day to expand the progenitor cell population. After 5-7 days in culture, the cells were scraped off the plate and plated onto a new PO/FN-coated plate at a density of 1 million cells per dish, and cultured under the same condition as the primary cells. After two passages (1-2 weeks), the cultures were relatively homogenous, composed predominantly of Nestin-positive multipotent progenitor/stem cells.

For immunocytochemistry and immunoblotting analyses of differentiated NSCs, cells at various passages were plated on PO/FN-coated glass coverslips at a density of 25,000-50,000 cells per well in a Costar 24-well plate, and on PO/FN coated 6-well plates at a density of 1 million cells per well. bFGF was added at the time of plating. Usually, 1-2 days after culturing, cells were treated with Ngn-Adenovirus and LIF (100ng/ml, R&D Systems) over night (about 24 hours), then fixed and subjected to immunostaining, or lysed and proteins extracted.

2.2 Mice

The strain of LIF knockout mice on a C57BL/6 background was obtained from Dr. David Anderson's laboratory (California Institute of Technology). A polymerase chain reaction (PCR)-based method was used to determine the genotype of the mice. Genomic DNA was isolated from ear biopsies. These biopsies were digested over night at 37°C using DNA lysis buffer (100 mM Tris-HCl [pH 8.1], containing 0.2% SDS, 200mM NaCl, 5mM EDTA, 10mg/ml proteinase K). DNA was precipitated with 100% ethanol, and dissolved in 200 µl TE buffer. 100-150 ng was subjected to PCR amplification (5 min at 95°C, hold at 4°C; 38 cycles of 30 s at 95°C, 45 s at 63°C, 45 s at 72°C; and 10 min at 72°C, then hold at 4°C). The reaction mixture contained 1× PCR buffer (10 mM Tris–HCl [pH 8.3], 50 mM KCl), 3.0 mM $MgCl_2$, 0.1 mM dNTPs, 0.1 U/µl Taq-polymerase and 0.4 pmol/µl primers (each) in a total volume of 50 µl. Two specific DNA fragments were co-amplified: a 192-bp LIF gene fragment (using a LIF sense primer, 5′-cgcctaacatgacagacttcccat-3′, and a LIF antisense primer, 5′-aggcccctcatgacgtctatagta-3′) and a 541-bp neomycin gene fragment (using a Neo-sense aggcccctcatgacgtctatagta-3′) and a 541-bp neomycin gene fragment (using a Neo-sense primer, 5′-ccagctcttcagcaatatcacggg-3′, and a Neo-antisense primer, 5′-cctgtccggtgccctgaatgaact-3′). LIF WT mice contained only the LIF product (192 bp); the heterozygotes had both bands (192 and 541 bp); and the LIF-deficient mice had only the larger fragment (541 bp).

2.3 Expression vectors and adenovirus constructions

Mouse *neurogenin2* (*Ngn2*) was amplified via PCR (5 min at 95°C, hold at 4°C; 35 cycles of 40 s at 94°C, 60 s at 53°C, 60 s at 72°C; and 10 min at 72°C, then hold at 4°C). The reaction mixture contained 1× PCR buffer (10 mM Tris–HCl [pH 8.3], 50 mM KCl), 3.0 mM $MgCl_2$, 0.1 mM dNTPs, 0.1 U/µl Taq-polymerase and 0.4 pmol/µl primers (each) in a total volume of 50 µl using the following primers: Ngn2 sense primer, 5'-cgggatccgccgccatgttcgtcaaatctg-3'; Ngn2 antisense primer, 5'-ggaattcctagatacagtccctggcg-3'. The PCR fragment was then cloned into a TOPO-TA-vector (TOPO TA Cloning System; Invitrogen) following the manufacturer's protocol, then cut and ligated (Quick Ligation Kit, NEB) into the *Bam*H1/*Eco*R1 sites of an adenoviral shuttle vector, pMZL6, containing a GFP expression cassette. Transformation into DH5α competent cells was performed using standard protocols, while mini-prep (Promega), and maxi-prep (Invitrogen) were performed following the instructions of the manufacturer's protocol.

The mouse *Mash1* construct was obtained from Dr. David Anderson's laboratory (California Institute of Technology) and cloned into the *Eco*R1 site of pMZL6.

Myc-tagged Neurogenin1 was also cloned into *Eco*R1 sites of pMZL6 and has been described previously (Sun et al., 2001).

Recombinant adenoviruses of *Ngn*1, *Ngn*2 and *Mash*1 were made by co-transfection (Lipofectamin 2000; Invitrogen) of the shuttle plasmids with the plasmid pBHG10 into HEK293 cells. Viruses were amplified by infecting HEK293 cells, followed by the harvesting of the virus-containing supernatant after about 2-3 days. Supernatant was then

tittered and frozen at -80°C. 4 hours after infection with adenovirus, cell medium was usually changed to DMEM /N2 minimal medium, and cells were treated with LIF.

2.4 PLCγ and PKCδ siRNA

PLCγ and PKCδ siRNA duplex were obtained from IDT DNA Technologies. The following duplex sequences were used: PLCγ sense: 5'-uaaagcuacaggacaaugutt-3', PLCγ antisense: 5-acauuguccuguagcuuuatt-3'.

The duplex-siRNA was then transfected via electroporation using The Nucleofector Device (Amaxa Biosystems), the siRNA Test Kit (Amaxa Biosystems), following the manufacturer's protocol. Cells were co-transfected with a pmaxGFP vector as a transfection control.

2.5 Immunocytochemistry

Cell cultures were routinely fixed for 3 minutes with methanol/acetone (vol/vol 1:1) or 20 minutes with 4% paraformaldehyde at room temperature (20-25°C). After 3 times washing with 1×PBS, cells were permeablized in 1×PBS containing 0.04% Triton X-100 at room temperature for 30 minutes to 1 hour. For staining, the fixed and permeablized cells were incubated in a blocking buffer, Tris-buffered saline (TBS) with 0.02% Tween-20 plus 10% milk and 1% normal goat serum, at room temperature for 1 hour. Primary

antibodies were diluted with a dilution buffer (TBS with 0.02% Tween-20 plus 3% bovine serum albumin [BSA]) and added to the cells after incubation with blocking buffer. Cells were usually incubated with primary antibodies at 4°C overnight and were washed 3 times with 1×PBS before incubating with Cy2- or Cy3-conjugated secondary antibodies (Jackson Immunoresearch). This incubation was performed at room temperature for 1-2 hours in dark. After the final 3 times 1×PBS washes, coverslips were mounted onto glass slides with a mounting solution containing an antifluorescent bleaching reagent, n-propyl-gallate (5% in PBS:glycerol, 1:1).

Neurons were labaled with mouse monoclonal anti-neuronal specific βIII tubulin (TuJ1) (Babco/Covance, 1: 500 dilution), while astrocytes were labeld with mouse monoclonal anti-GFAP antibody (Sigma, 1:400 dilution).

Images were taken with an Olympus fluorescent microscope.

2.6 Immunohistochemistry

The embryonic (E13) brains from LIF knockout, heterocygote and wildtype mice were removed from the skull, fixed in 4% paraformaldehyde (PFA) in PBS overnight at 4°C. All brains were cryoprotected with 20% sucrose for 24 hours, embedded in O.C.T., frozen liquid nitrogen, cut in the coronal plane on a cryostat at 10 μm, and then mounted onto Superfrost (plus) slides. Slides were then stored at -80°C. For tissue staining, the brain slides were removed from the –80°C freezer followed by a 30 min equilibration to room temperature. Afterwards, the slides were rinsed three times (10 min each time) on a

shaker in 0.1M PBS. For post-fixation, the slides were incubated in 4% PFA followed by three 10 min washes in 0.1 PBS. Next, the brain slides were removed from the staining dishes, the PBS drained with vacuum, and then placed in a staining tray that contained wet paper towels that kept the chamber humidified. As a blocking step, slides were covered with about 200 µl blocking solution (3% normal donkey serum in 0.1M PBS) for 1 hour at room temperature. After blocking the tissue slides, the blocking solution was removed and the primary antibody cocktail (mouse monoclonal anti-neuronal specific βIII tubulin (TuJ1), Babco/Covance,1:500; PSA-NCAM, Chemicon, 1:500 in blocking solution) was applied, and slides incubated over night at 4°C.

The following day, the slides were washed three times for 10 min in 0.1M PBS on a shaker, then removed from the staining dish, PBS carefully removed with a vacuum, slides placed in black staining tray, and covered with the secondary antibody cocktail (Cy3-conjugated secondary antibodies (Jackson Immunoresearch) at a 1:200 dilution in blocking buffer) for 1 hour at room temperature. After the secondary antibody exposure, the slides were washed three times for 10 min with 0.1M PBS on a shaker. Next, slides were removed from PBS, the excess PBS drained with a paper towel, two drops of Vectashield (Vector Labs) applied, and then coverslips placed on top of them. Slides were then dried for about 1 hour at room temperature, and then stored at 4°C. Images were captured with an Olympus fluorescent microscope.

2.7 Western-blot analysis and immunoprecipitation

When cells were confluent adenoviruses were added for about 4 hours. Afterwards the medium was changed from DMEM/B27 to DMEM/N2 minimal medium and cells were treated with LIF and certain cell signaling inhibitors (Calphostin C, Calbiochem, 300 nM; Ro-32-0432, Calbiochem, 1 μM; Rottlerin, Calbiochem, 3 μM; PD 98059, Calbiochem, 10 μM; LY 294002, Calbiochem, 50 μM). Cells were rinsed in 1×PBS, lysed in 0.7% NP40 lysis buffer (50 mM Tris-HCl [pH 8.0], 0.1 mM EDTA [pH 8.0], 250mM NaCl, 10% glycerol, 0.2 mM Na_3Vo_4, 50 mM NaF, 1 mM PMSF, 10 mM DTT, and a cocktail of protease inhibitors (Roche Applied Science)) and centrifuged at 13,000 rpm for 15 min at 4°C. After the determination of protein concentration (Bio-Rad), the resulting supernatants were size-separated by 10% SDS-PAGE and transferred to nitrocellulose membranes (Bio-Rad). Western blotting was performed using standard protocols.

For immunoprecipitation cells grown on a 6-well plate were lysed in 150 μl 0.5% NP40 lysis buffer, centrifuged at 13,000 rpm for 15 min at 4°C, and pre-cleared with 30 μl protein A agarose beads (Upstate) (in IP lysis buffer (1:1 ratio)) for 1 hour at 4°C. After adjustment of protein concentration and saving of about 25 μl protein sample as a control, about 1 μg antibody was added to each sample, which a was then rocked over night at 4°C. The next day, 30 μl protein A agarose beads were added and the samples rocked for one further hour at 4°C. The beads were then washed three times with 500 μl IP lysis buffer, and the samples boiled for 5 min after 80 μl IP buffer and 20 μl 5x protein loading buffer was added. Proteins were separated on a 10% SDS-PAGE.

The antibodies used for western-blot analyses were the following: mouse monoclonal anti-GFAP (Sigma), mouse monoclonal anti-neuronal specific βIII tubulin (TuJ1) (Babco/Covance), rabbit anti-neurogenin-1 (gift from Dr. Jane E. Johnson, University of Texas Southwestern), rabbit anti-neurogenin-2 (gift from Dr. David J. Anderson, California Institute of Technology), rabbit anti-CBP (Santa Cruz Biotechnology), rabbit anti-Akt (Cell Signaling Technology), rabbit anti-phospho-Akt (Cell Signaling Technology), rabbit anti-p42 MAPK (Cell Signaling Technology), rabbit anti-phospho-p44/p42 MAPK (Cell Signaling Technology), rabbit anti-PKC (Cell Signaling Technology), rabbit anti-phospho-PKC (pan) (Cell Signaling Technology), mouse monoclonal anti-MAP2 (Sigma), rabbit anti-Brg1 (gift from Dr. Weidong Wong, National Institutes of Health), mouse anti-Myc (Santa Cruz Biotechnology), rabbit anti-Phospholipase C gamma (Upstate), mouse monoclonal anti-GAPDH (Abcam).

Secondary goat anti-mouse or anti-rabbit IgG-horseradish peroxidase conjugated anibodies (CalBiochem) were used, and detection was performed using the ECL plus chemiluminescense (PerkinElmer) on X-Omat Blue films (Kodak).

2.8 Dual luciferase reporter assay

The NeuroD, NeuroD E-box mutant and GFAP promoter-luciferase reporter constructs (NeuroD-pGL3; NeuroD E-box-mut-pGL3, GFAP-pGL3) have been previously described, as has the Ngn1-pcDNA3 (Invitrogene) construct (Sun et al., 2001; Bonni et al., 1997*). Mash1* and *Ngn2* plasmid-constructs were described above.

To study the promoter activity, neural progenitor cells were plated onto PO/FN-coated 96 well plates at a density of 5 million cells per plate. Transient transfection into neural porgenitor cells was performed using FuGENE-6 reagent (Roche) in accordance with the manufacturer's instructions. The TK-pRL Renilla luciferase constructs (Promega) were used as transfection controls in the dual luciferase assays. 4 hours after Ngn1-pcDNA3/ control-pcDNA3, or Ngn2-pMZL6/control-pMZL6, or Mash1-pMZL6/conrol-pMZL6 plasmids were co-transfected with TK-pRL Renilla constructs into neural progenitor cells, cell medium was changed to DMEM/N2 minimal medium, cells were treated with LIF (100ng/ml) and then lysed after further 24 hours incubation. Luciferase assays were performed using the dual-luciferase reporter system (Promega). The results shown indicate luciferase activities normalized against an internal luciferase reporter of Renilla luciferase (Promega).

Statistical analyses were carried out using Microsoft Excel.

2.9 Chromatin Immunoprecipitation assays

Day 1:

Cultured neural progenitor cells were harvested using a modified protocol from Upstate Biotechnologies. Cells were grown on 10cm dishes, and, when dense, infected with Ngn1- or control-Adenovirus. About 4 hours after, medium was changed and cells were treated with LIF (100ng/ml). Next, medium was removed, and after two washes with iced-cold PBS cells were cross-linked with 1% formaldehyde in 1×PBS for 15 min at

room temperature. After two washes with iced-cold PBS, cells were scraped into a 15ml tube on ice, harvested by centrifugation (2000 rpm, 5 min, 4°C), and lysed with 0.3 ml SDS lysis buffer (1g/ml) containing protease inhibitor cocktails (Roche) and 1 mM DTT for about 10 min on ice. Lysates were were sonicated at 4°C using a microtip (Branson sonifier 450) to generate DNA fragments 200-1000 base pairs in length. Then the cell lysate was transferred to a new eppendorf tube and centrifuged for 10 min at 13,200 rpm at 4°C. Equal amounts (35 μg DNA by OD 260 nm reading) of chromatin were diluted in 100μl SDS lysis buffer containing 10% glycerol. 5% of lysate per IP was saved for an input control. As a next step, chromatin was diluted to 1 ml with ChIP dilution buffer, and then pre-cleared with 30 μl protein A agarose beads (Upstate) for 1 hour at 4°C. Immunoprecipitation was performed over night (for about 15 hours) at 4°C with 2-3 μg antibodies. Immunoprecipitation with normal mouse or rabbit IgG (Santa Cruz) was used as a negative control.

Day2:

Immunoprecipitated DNA/protein complexes were collected by adding 40 μl protein A agarose beads (rotation for 1 hour, at 4°C), and centrifuged at 1000 rpm for 1 min at 4°C. After the supernatant was carefully removed, beads were washed with 1ml under rotation for 5 min at 4°C with low salt, high salt, LiCl, and TE buffer. Next, 120 μl freshly prepared elution buffer (ddH_2O containing 1% SDS, 0.1M $NaHCO_3$) was added to the beads, briefly vortexed, rotated for 15 min at room temperature, and then centrifuged at 1000 rpm for 1 min. 100 μl were transferred to a new 1.5 ml tube, and the previous step

repeated (200 µl total). 8 µl of 5M NaCl were then added to the 200 µl total eluate. To reverse the cross-link the DNA/protein complex was incubated at 65°C for at least 6 hours to over night. Included were also the saved input samples, which were diluted to 200 µl total volume with elution buffer.

Day 3:

To each sample (200 µl) 4 µl EDTA (0.5M), 8 µl Tris-HCl (1M, pH 6.5) and 1 µl proteinase K was added, followed by incubation at 37°C for 1-2 hours. To purify the DNA, 220 µl phenol was added, the samples vortexed for about 10 sec, and then centrifuged at 13,200 rpm for 10 min at room temperature. The upper aqueous phase was then transferred to a new tube, and 200 µl chloroform was added. The samples were vortexed again for about 10 sec, and then centrifuged at 13,200 rpm for 10 min at room temperature. The upper phase was transferred to a new tube. To precipitate the purified DNA (200 µl), 20 µl 3M NaOAc (0.1 Vol.), then 1 µl glycogen (20 µg/µl), and then 500 µl 100% EtOH (2.5 Vol.) was given to each sample, followed by incubation for 1 hour at -80°C. Samples were then centrifuged for at 13,200 rpm for 10 min at 4°C. The DNA pellet was washed with 1 ml 70% cold EtOH, air-dried, and then dissolved in 50 µl TE buffer. 2.5 to 5 µl of the IP DNA was then used for each semi-quantitative hot-start PCR reaction (5 min at 95°C, hold at 4°C; 35 cycles of 45 s at 95°C, 45 s at 54°C, 45 s at 72°C; and 10 min at 72°C, then hold at 4°C) using primers designed to amplify DNA within the NeuroD promoter region.

The reaction mixture contained 1× PCR buffer (10 mM Tris–HCl [pH 8.3], 50 mM KCl), 3.0 mM $MgCl_2$, 0.1 mM dNTPs, 0.1 U/µl Taq-polymerase and 0.4 pmol/µl primers (each) in a total volume of 50 µl using the following primers: NeuroD sense primer: 5'-ccccagatgtcctctgtctt-3'; NeuroD antisense primer: 5'-gaaccacgtgacctgcctat-3'.

Antibodies used for the chromatin immunoprecipitation were rabbit anti-neurogenin-1 (gift from Dr. Jane E. Johnson, University of Texas Southwestern), rabbit anti-CBP (Santa Cruz Biotechnology), and mouse anti-Brg1 (gift from Dr. Weidong Wong, National Institutes of Health).

Chapter 3

Results

3.1 LIF enhances Neurogenin's transcriptional activation in cortical NPCs

In the cerebral cortex *Ngn1* and *Ngn2* are detected exclusively in the ventricular zone, where precursor cells reside, and only during the period of cortical neurogenesis (Guillemont and Joyner, 1993; Lo et al., 1991; Sommer et al., 1996). While *Ngn1* and *Ngn2* are expressed in the dorsal telencephalon, which gives rise to glutamatergic neurons, *Mash1* is predominantly expressed in the ventral telencephalon, which gives rise to GABAergic and cholinergic neurons (Wilson and Rubinstein, 2000). As previously shown, *Ngn1* promotes neurogenesis by binding as a transcriptional complex together with CBP/p300 to the *NeuroD* promoter (Sun et al., 2001). However, the programs of gene expression that are mediated by *Mash1* activity in the ventral telencephalon are still unknown.

To examine whether LIF can enhance the neurogenic effect of *Ngn1*, *Ngn2* and *Mash1* on a transcriptional level, neural progenitor cells (NPCs) from mouse E11 cortices were co-transfected with a *NeuroD* promoter-luciferase reporter and *Ngn1*, *Ngn2*, or *Mash1* expression constructs. Coexpression of *Ngn1* and *Ngn2* significantly enhanced *NeuroD* promoter activity, while LIF even further enhanced this effect (Figure 1A). On the other hand, *Mash1* expression did not lead to any *NeuroD* promoter activity in cortical NPCs whether cells were treated with LIF or not (Figure 1A).

Since the induction of *NeuroD* transcription could have been due to a positive feedback of *NeuroD* binding to its own promoter and therefore further enhancing its own expression, NPCs were also co-transfected with a *NeuroD*-expressing plasmid and the

NeuroD promoter-luciferase reporter, followed by the treatment with LIF. Compared to the effect of LIF and *Ngn1* or *Ngn2*, ectopic expression of *NeuoD* did not show any significant increase in this gene's own promoter activity, although *NeuroD* is capable of binding to E box elements. The treatment of *NeuroD*-transfected NPCs with LIF did not induce promoter activity either (Figure 1B).

To examine whether E box binding of Ngn1 to the *NeuroD* promoter is required for the proneural Ngn/LIF effect, NPCs were transfected with *Ngn1* and a *NeuroD* promoter-luciferase construct that contained mutations at its E box elements. As shown in Figure 1C, E box binding of *Ngn1* is very critical for *NeuroD* transcription, as well as for enhanced, LIF induced transcriptional activity of Ngn1.

The possibility that *Ngn1* and *Ngn2* instructively promote neural cell fate determination implies that both genes induce neurogenesis at the expense of gliogenesis. We found that ectopically expressed *Ngn1* or *Ngn2*, but not control, were able to inhibit cytokine LIF-induced GFAP promoter activity in cortical precursor cell cultures (Figure 1D).

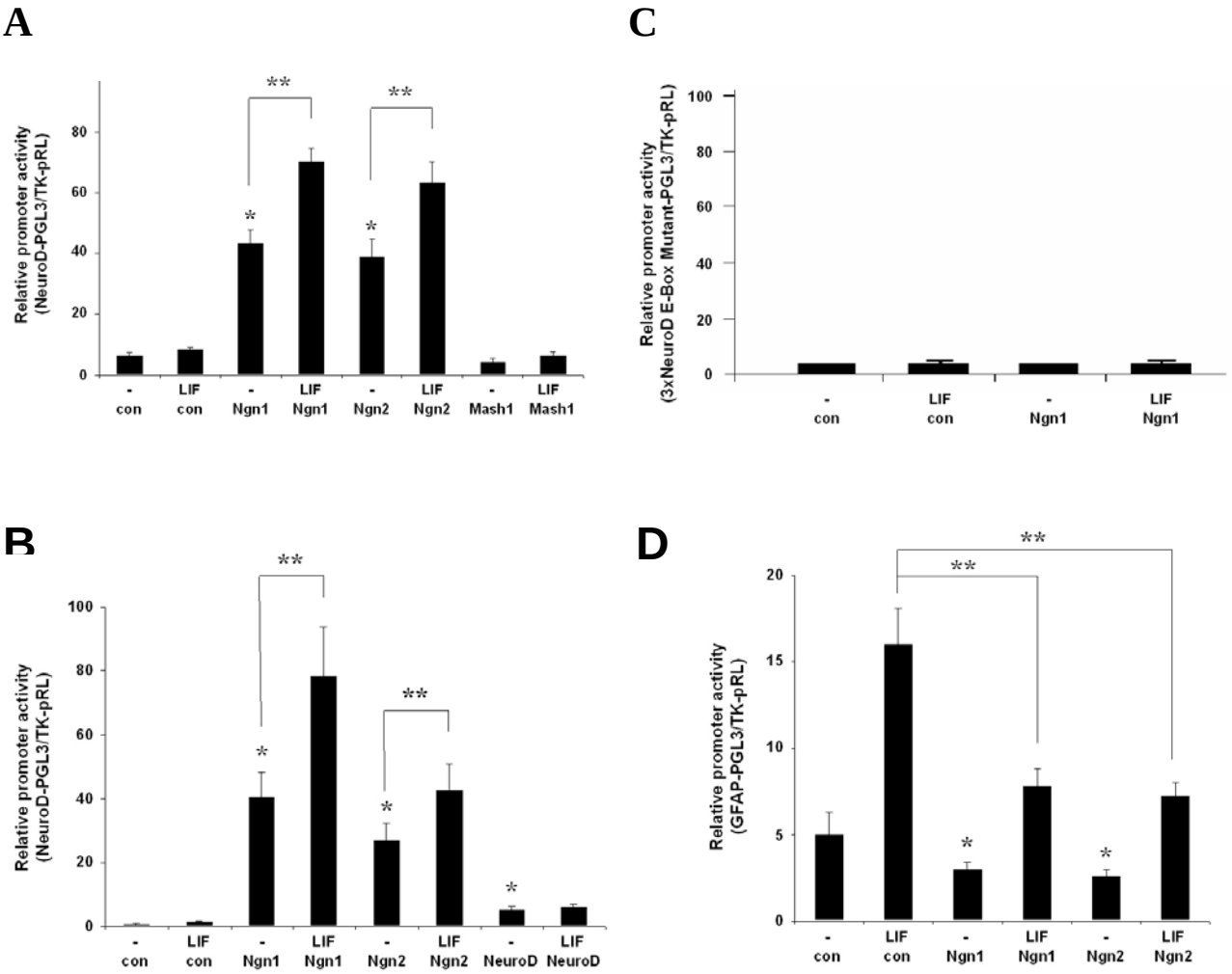


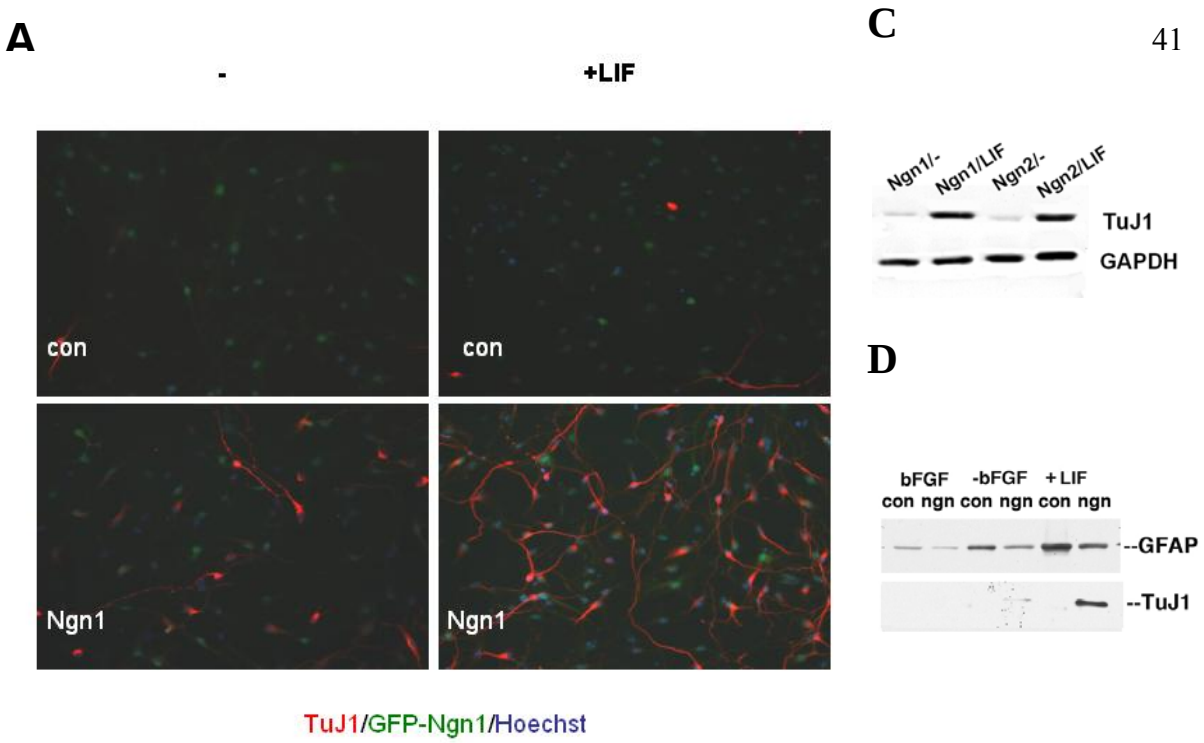
Figure 1: LIF promotes neurogenesis on the transcriptional level when proneural bHLH factors *Ngn1* or *Ngn2* are expressed.

(A) *NeuroD* luciferase constructs were co-transfected with *control*, *Ngn1*, *Ngn2*, or *Mash1* expression constructs in cortical NPCs, either untreated or treated with LIF for 1 day. (*, P<0.05 compared to control group; **, P<0.05 compared to LIF untreated group). **(B)** Luciferase assay of *NeuroD* promoter in NPCs transfected with *Ngn1*, *Ngn2*, or *NeuroD*. (*, P<0.05 compared to control group; **, P<0.05 compared to LIF untreated group). **(C)** Luciferase activity of *NeuroD*-E-box-mutant promoter with or without *Ngn1* and/or LIF **(D)** The 1.9 kb GFAP promoter-luciferase-reporter construct was co-transfected with *control*, *Ngn1* or *Ngn2* expression constructs into cortical NPCs, either treated or untreated with LIF for 1 day. (*, P<0.05compared to LIF treated group).

3.2 LIF promotes neurogenesis and inhibits gliogenesis in cortical NPCs in the presence of *Ngn1* and *Ngn2 in vitro*

To investigate the effect of *Ngn1*/*Ngn2* and LIF on both the expression of neuronal markers such as neuron-specific type III β-tubulin (recognized by the antibody TuJ1), and on the expression of astrocyte-specific marker GFAP, an adenoviral vector was generated to introduce exogenous *Ngn1* or *Ngn2* into neural precursor cells cultured from mouse E11 cortices. The neural stem cell cultures, because they are homogenous and display high (>90%) infection efficiency to adenoviruses, are amenable to gene expression studies. When cortical neural precursor cells (NPC) were infected with the *Ngn1*, *Ngn2* or control viruses, we found that exogenous *Ngn1/2* expression led to a significant increase in the number of precursors that became neurons, while treatment with LIF even further increased the number of *Ngn1/2* infected cells that differentiated into neurons (Figure 2A, B, C). To further explore the possibility that *Ngn1/2* and LIF decrease GFAP expression not only on a transcriptional level, but also on the protein level, western analyses were performed. The results revealed that expression of both *Ngn1* and *Ngn2* greatly reduced GFAP expression. As determined by cell counting and western blot, LIF had only a minimal positive/promoting effect on GFAP expression when proneural *Ngn1* and *Ngn2* were expressed in NPCs (Figure 2B, D).

In addition, *Ngn1/2*-expressing cells displayed a neuronal-like morphology (small, round cell bodies with one or two simple processes) that is quite distinct from the stellate GFAP-positive astrocytes, suggesting that Ngn1/2 inhibit various aspects of the glial differentiation program.



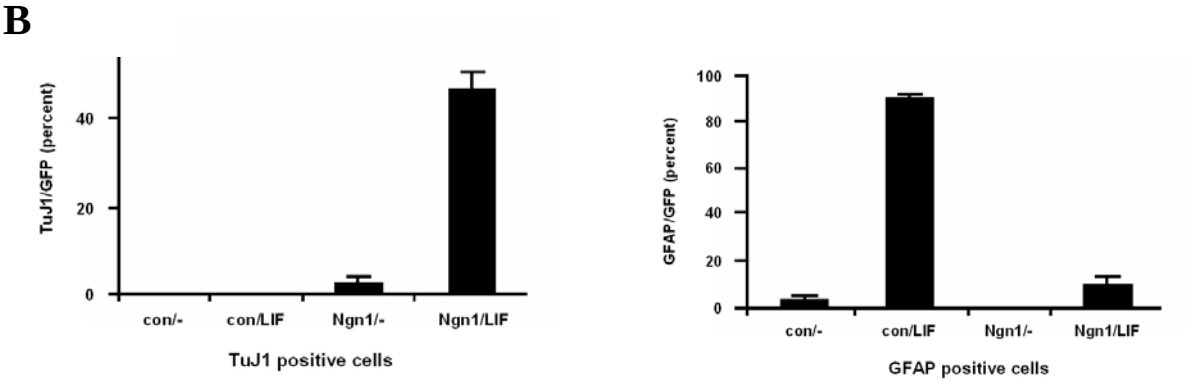


Figure 2: LIF enhances the proneural effect of *Ngn1* and *Ngn2*

(A) Expression of TuJ1 (red) in mouse cortical NPCs infected with adenoviruses containing *control*/*Ngn1*-expression cassettes, and treated with LIF for 24 hours. **(B)** Quantification of *Ngn1*/Tuj1 positive cells (neuronal) and GFAP (astrocytic) positive cells after infection with *control/Ngn1*-adenovirus, with or without LIF treatment. **(C)** Western blot analysis showing TuJ1 expression after NPCs were infected with *Ngn1* or *Ngn2* adenoviruses, and treated with LIF. **(D)** Western blot of E11 mouse cortical NPCs, infected with *control* or *Ngn1* gene expression viruses for 24 hours, and treated with bFGF (mitogenic) or LIF. The levels of cell type specific markers were determined by using anti-TuJ1 and anti-GFAP antibodies.

3.3 Potential signaling pathways mediating the neurogenic effect of LIF

LIF is a member of the family of interleukin-6 type cytokines, which have overlapping, pleiotropic effects on a variety of different cell types and activate target genes involved in survival, apoptosis, proliferation and differentiation, as well as suppression of differentiation (Heinrich P. C., 2003). LIF binds directly to the low-affinity LIF receptor β (LIFRβ) that subsequently heterodimerizes with the signal transducer glycoprotein 130 (gp130). The formation of this high-affinity trimeric complex composed of two class I cytokine receptors, LIFRβ and gp130, and LIF leads to the activation of several downstream pathways essential for regulating biological responses: the JAK-STAT, the MAPK, the Akt/PI3K, and the PLCγ/PKC pathways (Figure 3; Kristensen et al., 2005). However, since it has already been shown that a LIF activated JAK-STAT pathway promotes glial cell differentiation in late NPC, we investigated the effects of the latter three signaling pathways with regards to pathway activation and correlation to neurogenesis.

As shown by David Turner's lab (Vojtek et al., 2003), endogenous Akt kinase plays an essential role in neurogenesis in mouse P19 teratocarcinoma cells not only by promoting neuronal survival, but also by enhancing the complex formation of CBP/p300 with ectopically expressed *Ngn3*. In addition, inhibition of the Akt/PI3K pathway abolished this complex formation.

The MAPK pathway is important to keep fetal NSCs and ES cell derived NSCs in a proliferative state, with growth factors, such as bFGF, EGF, IGF I, II, and III being important activators of this signaling pathway (Colombo et al., 2006). On the other hand,

factors such as BDNF and NT-3 can activate MEK and induce neurogenesis by mechanisms that are still not well understood (Gosh et al., 1995; Menard et al., 2002). Though it was shown in NPCs that activated MEK could activate C/EBP, which directly promoted the transcription of at least one early pan-neural gene, Tα1 α-tubulin, a LIF-induced/MEK-dependent activation of *Ngn* could not be shown yet (Miller, F; 2005).

Protein kinase C (PKC) is a family of kinases composed of about 10 different isozymes that have been categorized into three groups - the conventional PKCs, the novel PKCs and the atypical PKCs - depending on their ligand sensitivities and structural properties (Newton et al., 2001; Nakashima et al, 2002; Ohno and Nishizuka, 2002). It has been shown that PKCs are involved in diverse cellular processes such as long term potentiation in the hippocampal CA1 region, synaptic plasticity, development and apoptosis (Malinow et al., 1989; Abbeliovich et al., 1993; Hu et al., 1987; MacDonald et al., 1989; Perez et al, 2001; Gutcher et al., 2003). The expression of different PKC isoforms varies greatly between tissues, but certain isoforms, for instance PKCα, βII, γ, δ, ε, θ and ζ, are more widely expressed in the CNS (Battaini, 2001).

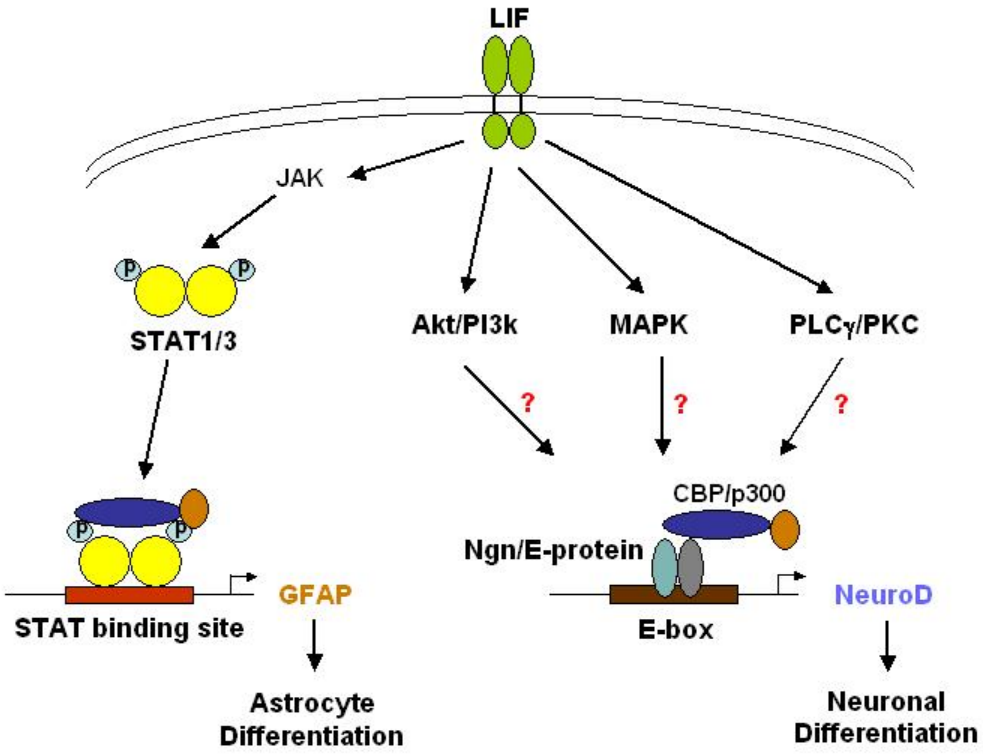


Figure 3: LIF-induced signaling pathways that might promote neurogenesis

Ngn1 inhibits glia cell differentiation by sequestering CBP/p300 away from glia specific genes. If *Ngn1* is present, CBP/p300 binds to it and promotes transcription of neuron-specific genes, such as NeuroD, a process that is enhanced by LIF through activation of either the Akt/PI3, the MAPK, or the PLCγ/PKC signaling pathway.

3.4 PLCγ/PKC mediate the effect of LIF on neurogenesis

To address the relationship between LIF and its neurogenic effects, E11, (passage 3) NPCs were cotransfected with a *NeuroD* promoter-luciferase reporter and an *Ngn1* expression plasmid. About 4 hours after transfection, progenitor cells were treated with LIF (100ng/ml) and certain drugs that inhibited the signaling pathways mentioned above. To inhibit the Akt/PI3K pathway we applied LY 294002 (50 μM – Calbiochem), for MAPK signaling pathway inhibition PD 98059 (10 μM – Calbiochem), and for PKC pathway inhibition a combination of Calphostin C (300 nM – Calbiochem) and Ro-32-0432 (1 μM – Calbiochem). Using luciferase assays, we observed that the effect of *Ngn1*/LIF on NeuroD promoter activity was diminished by inhibition of all three signaling pathways. However, inhibition of the PKC led not only to the strongest promoter activity inhibition, but also abolished the pro-neural effect of LIF (Figure 4A).

Consistent with the luciferase assay results, western blot analyses demonstrated a similar outcome on the protein level. The expression of the neuronal-specific marker class III β-tubulin/ TuJ1 was diminished by application of all drugs, with PKC inhibitors Calphostin C/ Ro-32-0432 leading to the strongest TuJ1 expression inhibition (Figure 4B, C). Also, when progenitors where treated with PKC inhibitors, LIF did not cause any pro-neural effect as it did when cells where treated with Akt/PI3K or MAPK inhibitors.

Taken together, these results suggest that the pro-neural effect of *Ngn* and LIF in cortical neural progenitors is mediated by the PLCγ/PKC signaling pathway, rather than by the Akt/PI3K or MAPK pathways.

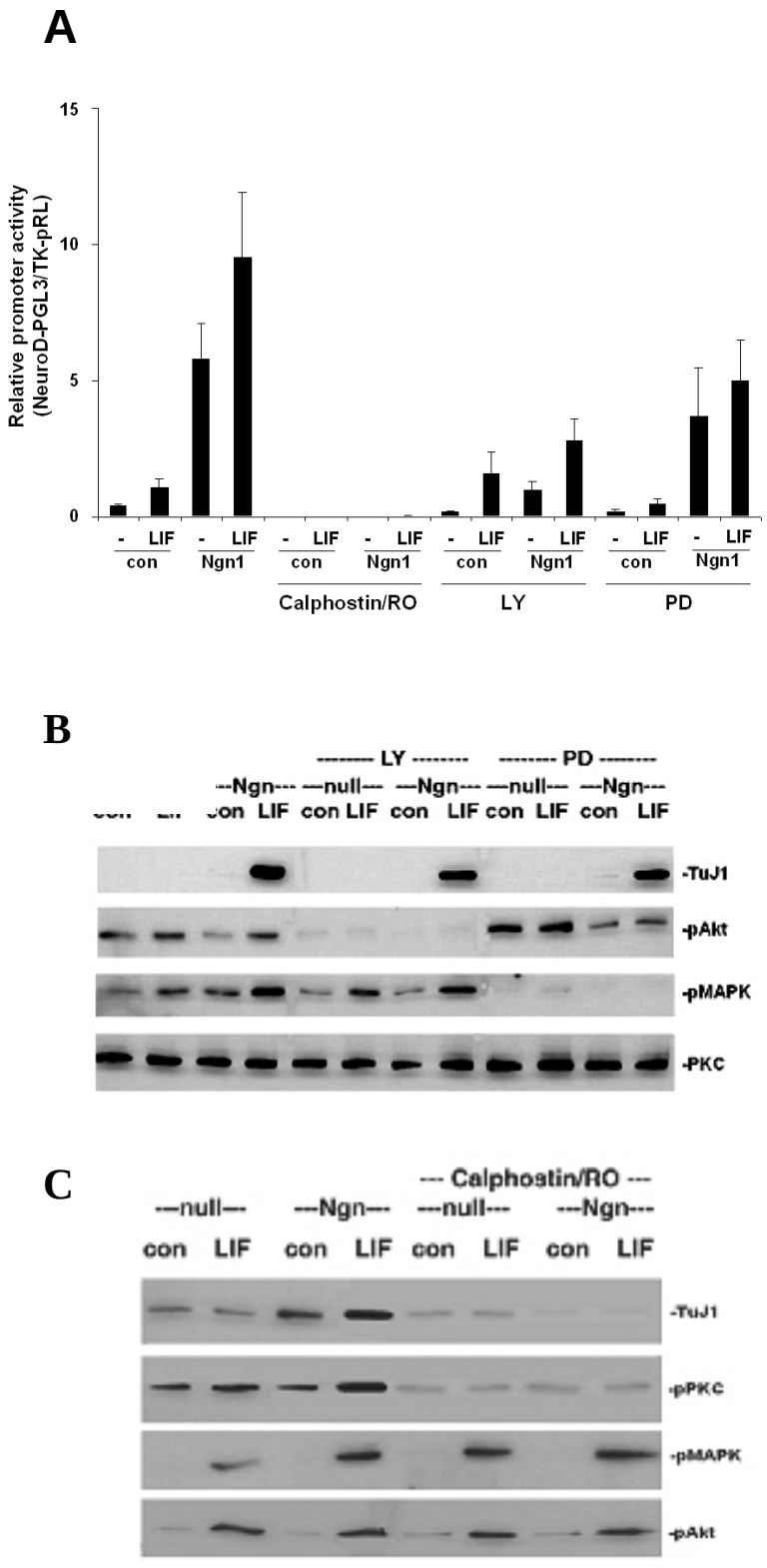


Figure 4: PKC mediates LIF's pro-neural effect

(A) *NeuroD* luciferase constructs were co-transfected with *control* or *Ngn1* expression constructs in cortical NPCs, either untreated or treated with LIF for 1 day, and PKC (Calphostin/RO), Akt (LY), or MAPK (PD) pathways inhibited using specific drugs. **(B) and (C)** Western blot analyses of NPCs infected with *control* or *Ngn1* adenoviruses viruses, untreated or treated with LIF. Signaling pathways were inhibited using specific drugs (LY: Akt/PI3K; PD: MAPK; Calphostin/RO: PKC). The western blot shows protein expression of a neural specific marker, TuJ1, with regards to activation/ phosphorylation and inhibition of certain signaling pathway members. pAkt: phospho-Akt; pMAPK: phospho-MAPK; pPKC: phospho-PKC.

3.5 PLCγ/PKC promote association between *Ngn1*, CBP/p300 and *Brg1*

As previously shown by our lab, *Ngn1* competes with STAT3 for CBP/p300 binding (Sun et al, 2001), which is necessary for transcriptional activity. At later stages of cortical development, when progenitor/stem cells give rise to astrocytes, CBP/p300 binds to STAT3 to induce glial differentiation, while during earlier development, when neurogenesis is at its peak, *Ngn* sequesters CBP/p300 away from STAT3 and induces transcription of proneural genes, such as *NeuroD*.

To further explore the mechanism by which LIF enhances *NeuroD* promoter activity, we hypothesized that LIF might enhance *Ngn1*-CBP/p300 association, leading to an increased binding of this complex to the *NeuroD* promoter. We performed coimmunoprecipitation to investigate *Ngn1*-CBP association in NPCs upon ectopic expression of *Ngn1* and treatment of cells with LIF. The result shows that LIF significantly increases the binding of *Ngn1* to CBP (Figure 5A). However, this interaction is inhibited by the application of the PKC inhibitors Calphostin C/Ro-32-0432, suggesting that LIF promotes association of *Ngn1* to CBP via the PLCγ/PKC signaling pathway (Figure 5B).

In addition, we examined *Ngn1* association to Brahma-related gene 1 (*Brg1*). Seo et al (2005) previously showed that in *Xenopus Brg1*, a catalytic subunit of the SWI/SWF complex, binds to *Ngn*, and that this cooperation is necessary to induce target gene activation during neurogenesis. Therefore, we further hypothesized that *Brg1* might also associate with the *Ngn*/CBP cotranscriptional complex in mouse cortical progenitors. Coimmunoprecipitation analysis indicated that *Brg1* forms indeed a complex with *Ngn1*

and CBP (Figure 5C). To further explore the possibility that LIF promotes *Ngn1-Brg1* association, we treated cells with LIF in the same experiment. The result shows that LIF treatment also increased that association, suggesting that *Brg1* might also play an important role during mouse cortical neuron development (Figure 5C). However, in following experiments the focus remained on *Ngn* and CBP.

After analyzing the association between *Ngn1* and CBP upon LIF treatment, we started to analyze the association of this complex to the *NeuroD* promoter. Cortical NPCs were treated with *Ngn1* adenovirus and LIF. Chromatin immunoprecipitation (ChIP) assays with antibodies against *Ngn1* and CBP clearly showed that LIF treatment caused an increased binding of the *Ngn1*-CBP complex to the *NeuroD* promoter in these cells, presumably responsible for the increased transcriptional activation of this gene, and therefore also very likely responsible for the increase in neuronal differentiation (Figure 5D).

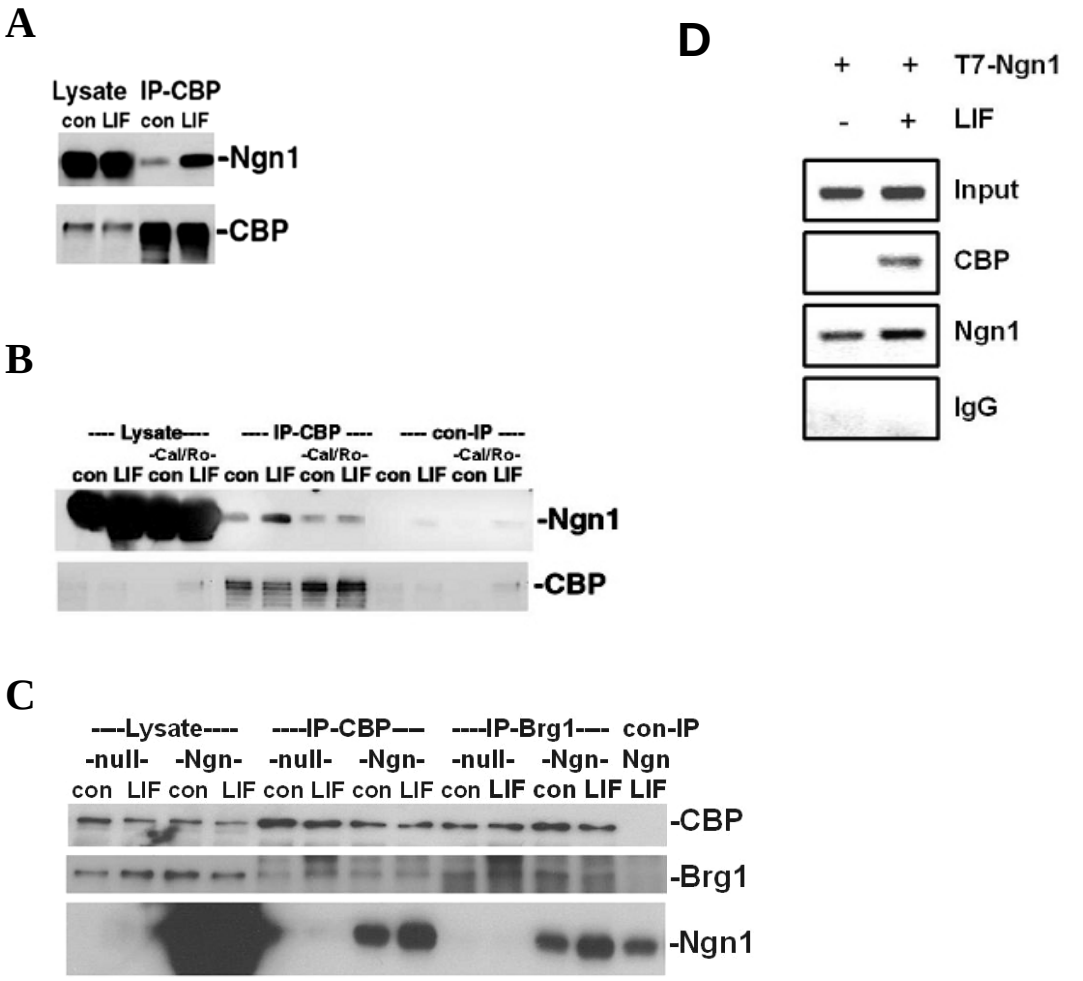


Figure 5: Association of *Ngn1* to CBP and *Brg1* is enhanced through LIF induced PKC activation

(A) Western blot analysis of *Ngn1* and CBP in mouse NPCs in the presence of Ngn1 (+/- LIF treatment) after immunoprecipitation of CBP. **(B)** Western blot analysis of Ngn1 and CBP in *Ngn1*-overexpressing progenitors, untreated or treated with LIF, and PKC signaling pathway inhibited using Calphostin C/Ro-32-0432. **(C)** Western blot analysis of CBP, *Ngn1*, and *Brg1* after cells were immunoprecipitated with CBP or *Brg1*. NPCs were infected with *control* or *Ngn1* adenoviruses, untreated or treated with LIF for 24 hours. **(D)** ChIP assay showing that the association of the *Ngn1*/CBP complex to the *NeuroD* promoter is enhanced by LIF.

3.6 PKCδ might be the mediator of LIF in cortical NPCs during neurogenesis

Several PKC isoforms are expressed in the developing brain during neurogenesis. However, it was shown that in multipotent PCC7-Mz1 embryonic carcinoma stem cells retinoic acid (RA) induces neurogenesis while upregulating the expression of PKC betaII, delta, epsilon, and eta. The expression of other isoforms (alpha, betaI, gamma, theta, mu, lamda and zeta) remained unchanged. In addition, after neurogenesis, the expression of PKC betaII, delta and eta decreased again, implying that these isoforms might play an essential role during neurogenesis. Of those isoforms, PKC delta appeared to be mainly membrane-bound, implying that it was also in an active state (Oehrlein et al, 1998). Furthermore, it was shown that RA induces PKC delta (PKCδ) expression in NT2 human teratocarcinoma cells, and that this expression is necessary for neuronal differentiation (Patel et al, 2006).

To test our hypothesis that PKCδ might mediate LIF signaling in cortical NPCs, we investigated first the expression of this enzyme during mouse cortical development. At the peak of mouse neurogenesis, at E14, NPCs expressed the highest level of PKCδ (Figure 6A). It increased from E12 to E14, and then decreased again at the end of neurogenesis.

With regards to TuJ1 expression and neuronal differentiation, we next tested a PKCδ specific inhibitor, Rottlerin, on NPCs, after cells were infected with *Ngn1*-adenovirus, and treated with LIF. The Figure 6B shows a similar result as that of NPCs treated with the general PKC inhibitors Calphostin C/Ro-32-0432. Similar to the general

PKC inhibitors, Rottlerin did not allow neuronal differentiation upon *Ngn1* infection/LIF treatment.

To investigate the mechanism by which Rottlerin inhibited TuJ1 expression/neurogenesis, we performed luciferase assays on the *NeuroD* promoter. NPCs were cotransfected with a *NeuroD* promoter-luciferase reporter and an *Ngn1* expression plasmid. 4 hours after transfection, progenitor cells were treated with LIF and Rottlerin. The result shows that the effect of *Ngn1*/LIF on *NeuroD* promoter activity was diminished by inhibition of PKCδ. Further, Rottlerin, like Calphostin C/Ro-32-0432, abolished the pro-neural effect of LIF (Figure 6C).

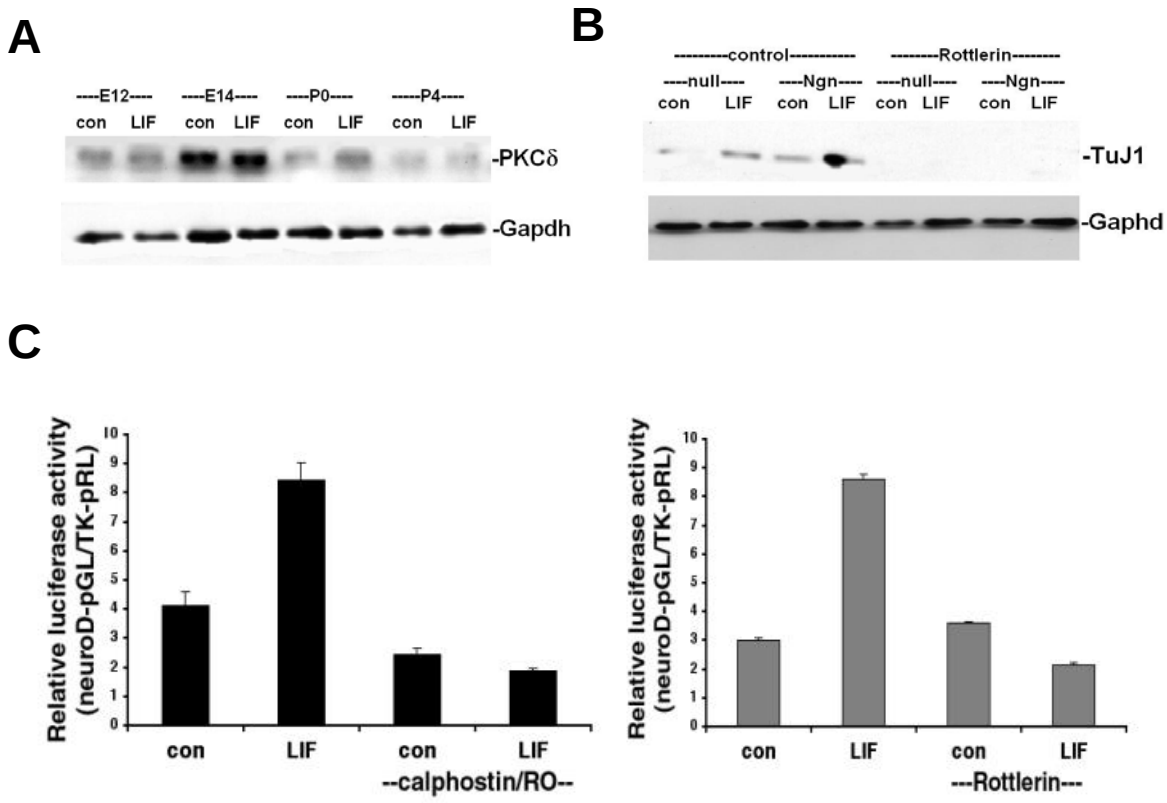


Figure 6: Inhibition of PKCδ inhibits the pro-neural effect of LIF.

(A) Western blot analysis of PKCδ in mouse cortices during development - from embryonic day 12 (E12) to postnatal day 4 (P4) - showing highest level of PKCδ at the peak of neurogenesis (at E14). **(B)** Western blot analysis of TuJ1 in NPCs infected with control/*Ngn1* adenoviruses, untreated or treated with LIF, +/- PKCδ-inhibitor Rottlerin. **(C)** *NeuroD* luciferase constructs were co-transfected with *Ngn1* expression constructs in cortical NPCs, either untreated or treated with LIF for 1 day, and either PKC (Calphostin/RO), or PKCδ (Rottlerin) pathways inhibited using specific drugs.

3.7 PLCγ and PKCδ siRNA abolishes the pro-neural effect of Ngn and LIF

There are 10 PKC genes, which code for isozymes classified into three groups: Conventional/classical PKCs, novel PKCs and atypical PKCs. Conventional PKCs can be activated by calcium and phorbol esters or diacylglycerol (DAG), whereas novel PKCs, a group to which PKCδ belongs, can only be activated by phorbol esters or DAG. Atypical PKCs are unresponsive to either calcium or DAG. Increases in plasma-membrane DAG levels functions for the intracellular relocalization and activation of conventional and novel PKCs, occurring in response to receptor tyrosine-kinase stimulation through their coupling to PLCγ that then cleaves the lipid phosphoinositide 4,5-biphosphate into the soluble second messenger inositol triphosphate and the membrane lipid DAG (Griner et al., 2007).

In order to inhibit conventional/novel PKC as well as PKC delta activity without potentially inhibiting the function of other enzymes, we knocked down PLCγ and PKCδ expression using duplex RNA interference (RNAi). RNAi is the process of sequence-specific, post-transcriptional gene silencing in animals and plants, initiated by double-stranded RNA (dsRNA) that is homologous in sequence to the silenced gene (Hammond et al., 2001; Sharp et al, 2001). The mediators of sequence-specific messenger RNA degradation are 21- and 22-nucleotide small interfering RNAs (siRNAs) generated by ribonuclease III cleavage from longer dsRNAs (Elbashir et al, 2001). However, it was also shown that 21-nucleotide siRNA duplexes can specifically suppress

expression of endogenous and heterologous genes in different mammalian cell lines, including HEK293 cells and HeLa cells (Elbashir et al, 2001).

Neural progenitors (E11, passage 2 [P2]) were first transfected with PLCγ and PKCδ siRNA and then, after two days, infected with Ngn adenovirus followed by treatment with LIF. Western blot analysis shows downregulation of PLCγ and PKCδ expression and, consistent with previous results, also downregulation of the neuronal marker TuJ1 (Figure 7A). However, treatment with LIF lead also to an increase in TuJ1 protein expression in PLCγ or PKCδ-siRNA transfected cells, suggesting that only a certain percentage of progenitor cells were transfected with RNAi. To further investigate the impact PLCγ and PKCδ siRNA on NPCs, we performed immunocytochemistry on cells that were cotransfected with siRNA and GFP, then infected with Ngn1 adenovirus and treated with LIF. As shown in Figure 7B, only some progenitors were GFP/RNAi-positive. Cell counting revealed that only about 30% of NSCs were transfected with PLCγ/PKCδ-siRNA. Nevertheless, none of the cells that were GFP/PLCγ-siRNA or GFP/PKCδ-siRNA-positive expressed TuJ1 (Figure 7B, C). Even treatment with LIF did not lead to any GFP-TuJ1 double-labeled cells, implying again that LIF's pro-neural effect might be mediated by the PLCγ/PKC, or, more specific, by the PLCγ/PKCδ signaling pathway.

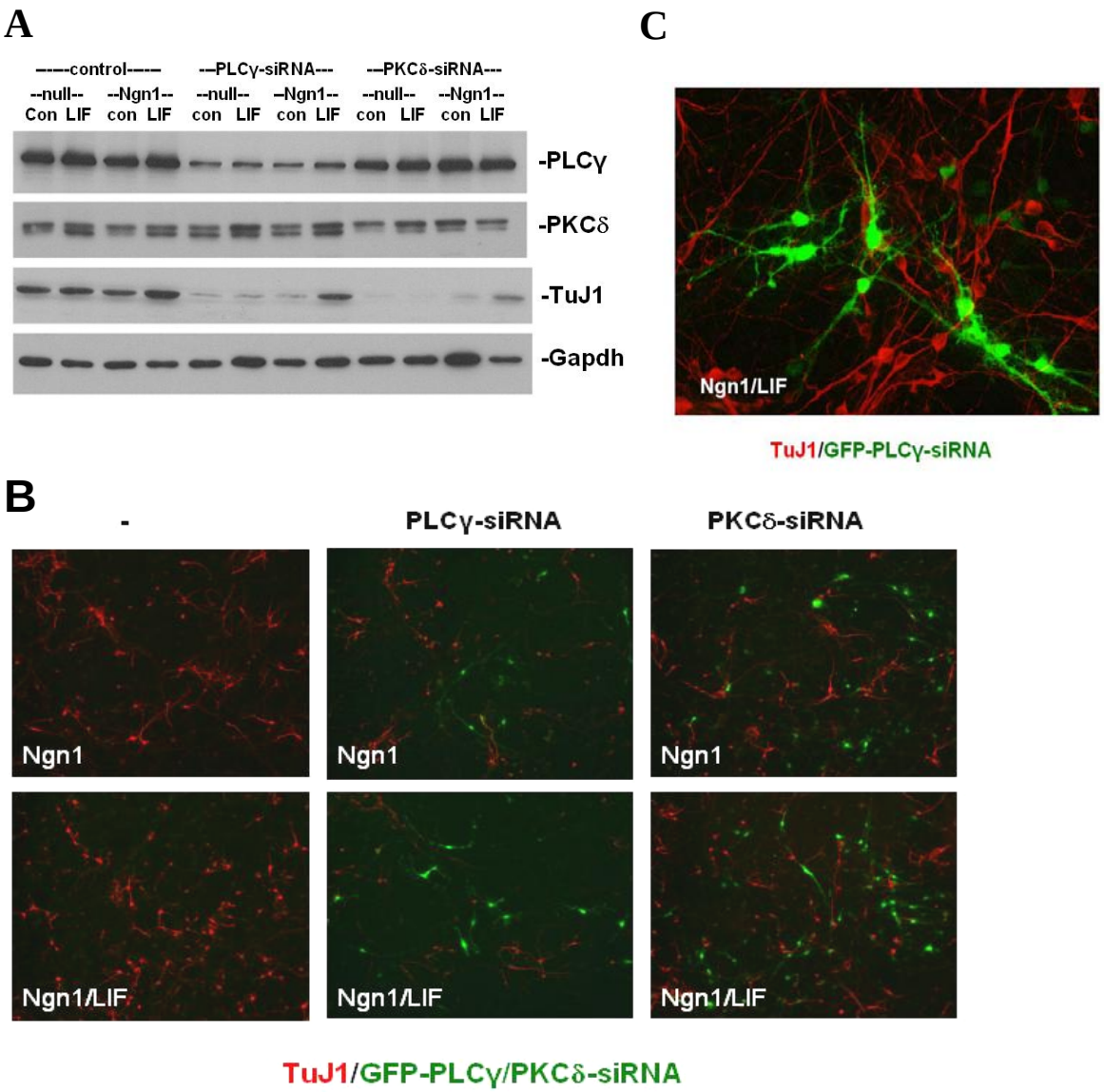


Figure 7: Downregulation of PLCγ or PKCδ using siRNA abolishes LIF's pro-neural effect

(A) Western blot analysis of NPCs that were infected with *control*/*Ngn1* adenoviruses, untreated or treated with LIF, and transfected with either PLCγ or PKCδ-siRNA shows protein levels of PLCγ and PKCδ with regards to TuJ1 expression. **(B)** Expression studies of TuJ1 (red) in mouse cortical NPCs infected with adenoviruses containing *Ngn1*-expression cassettes, untreated or treated with LIF for 24 hours, in absence or presence of PLCγ-siRNA **(C)** or PKCδ-siRNA, does not show TuJ1 protein expression in siRNA transfected progenitors.

3.8 LIF promotes neurogenesis *in vivo*

In vivo, extrinsic and intrinsic mechanisms also have a role in the sequential generation of neurons and glia cells during development. It has been suggested that LIF, synthesized prenatally by neural progenitors, might act in an autocrine/paracrine manner promoting astrocyte differentiation. (Chang et al, 2004). Regarding gliogenesis, it has been shown that in LIF KO mice cortical gliogenesis seems to occur normally, even though adult astrocyte numbers are reduced in some discrete areas such as the hippocampus (Barnabe-Heider et al, 2005; Koblar, et al, 1998). In contrast, mice lacking gp130 or its co-receptor LIFR exhibited a marked deficit in astrocytes (Koblar et al, 1998; Ware et al, 1995). It seems, although knocking out individual cytokines does not produce a phenotype that is notably different from wild type, blocking the effect of the entire cytokine family reveals its important role in gliogenesis *in vivo.* However, with regards to neurogenesis, it has been shown in mice lacking both LIF and CNTF that these two factors seem to be primarily responsible for promoting the survival and maintenance of developing motor neurons, although deletion of the CNTF gene had more pronounced effects than LIF gene deletion on this cell type (Sedtner et al, 1990 and 1996).

To test the hypothesis that LIF is required for cortical neuron differentiation *in vivo*, we compared the expression of neuronal markers, such as TuJ1, MAP2 and PSA-NCAM in embryonic day 13 (E13) LIF knock out (KO), heterozygote (Het) and wild type (WT) mice. First, we studied the expression pattern of neuron-specific class III β-tubulin (recognized by antibody TuJ1) on mouse E13 coronal brain sections. This antibody has been shown to label postmitotic neurons of the CNS very early, during or

immediately after the last mitotic cycle (Lee at al, 1990). However, as shown in Figure 8A, no major differences between WT, Het and KO mice could be observed, although it seemed that TuJ1 was slightly less expressed in the pre-plate of Het and KO mice compared to that of WT mice. To further investigate neuronal differentiation we labeled E13 cortical neurons with an additional neuron-specific marker, PSA-NCAM. The anti-PSA-NCAM antibody recognizes polysialic acid (PSA), which, in vertebrates, is linked to the extracellular domain of the neural cell adhesion molecule (NCAM). PSA-NCAM, the highly polysialated form of NCAM, is predominantly expressed in embryonic and neonatal neural tissue and is a marker for immature neuronal-committed progenitors. Staining neurons with anti-PSA-NCAM did not reveal major phenotype differences either. However, similar to TuJ1 staining, PSA-NCAM expression seemed to be slightly diminished in Het and KO mice when compared to WT mice (Figure 8B).

In order to gain more quantitative results, we applied western blot analysis investigating expression of TuJ1 and microtubule-associated protein 2 (MAP2) in mouse E13 cortices. Our data show that cortical TuJ1 expression was significantly decreased in LIF Het and KO mice compared to LIF WT mice, indicating that LIF might promote neuronal differentiation not only *in vitro*, but also *in vivo* (Figure 8C). Further labeling with an anti-MAP2 antibody supported this idea. The antibody recognizes MAP2-isoforms MAP2a, MAP2b and MAP2c. While MAP2c is predominantly found in growing dendrites of immature neurons, the isoforms MAP2a and MAP2b are expressed in dendrites of mature neurons (Belanger et al, 2001). As shown in figure 8C, E13 LIF Het and KO mice display less MAP2a/b than WT mice, suggesting that, during cortical development, LIF might be an important factor promoting neuronal maturity.

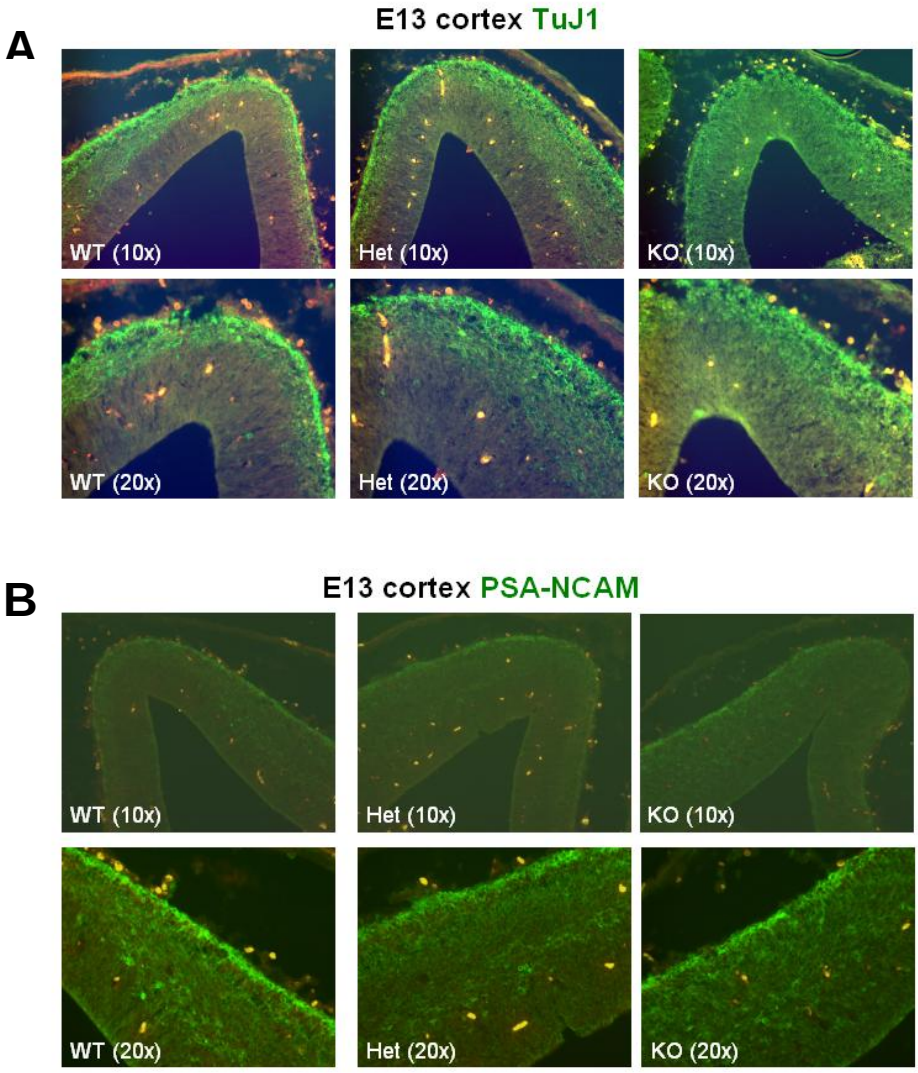


Figure 8: LIF promotes expression of pro-neural markers *in vivo*

(A) and **(B)** Immunohistochemistry of mouse E13 LIF WT, Het and KO coronal brain sections. Tissues were stained with pro-neural markers for TuJ1 and PSA-NCAM. **(C)** Western blot analysis of mouse E13 LIF WT, Het and KO cortices showing a decrease of the neuronal markers TuJ1 and MAP2 a/b in Het and KO mice compared to WT mice, while *Ngn1* and *Ngn2* levels remained the same.

3.9 LIF heterozyogte and knock out mice display less binding of the *Ngn1*-CBP cotranscriptional complex to the *NeuroD* promoter

After analyzing the association of the *Ngn1*-CBP cotranscriptional complex to the *NeuroD* promoter *in vitro* in previous experiments, we started to analyze the association of this complex to the *NeuroD* promoter *in vivo* (cortical tissues), comparing E13 LIF Het and KO mice to WT mice. Chromatin immunoprecipitation (ChIP) assays with antibodies against *Ngn1* and CBP revealed that LIF Het and KO mice display less binding of the *Ngn1*-CBP complex to the *NeuroD* promoter, presumably responsible for a decreased transcriptional activation of this gene, and therefore also very likely responsible for the decrease of neuron-specific markers such as MAP2a/b and TuJ1 (Figure 9).

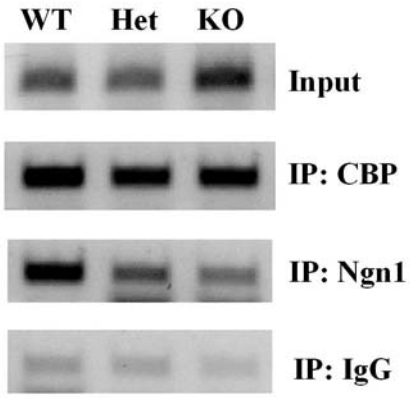


Figure 9: Association of the *Ngn1*-CBP cotranscriptional complex to the *NeuroD* promoter is enhanced by LIF *in vivo*

ChIP assay showing the association of the *Ngn1*/CBP complex to the *NeuroD* promoter *in vivo*. Cortices of E13 LIF Het and KO mice were isolated and compared to WT mice.

Chapter 4

Discussion

The development of the mammalian CNS, including the cerebral cortex, is a temporally and spatially well-organized process. Previous studies have shown that during cortical development, the neurogenic machinery is largely composed of a cascade of transcriptional activation events mediated by neurogenic bHLH factors. Subsequent steps in the differentiation process are mediated by successive waves of neuronal bHLH differentiation genes. Neurogenesis is therefore mediated by two broad categories of bHLH factors, pro-neural bHLH factors (e.g, *Ngn1* and *Ngn2*), which are involved in initiating neurogenesis, and neuronal differentiation bHLH factors (e.g., *NeuroD*), which are involved in mediating terminal differentiation.

Our studies suggest a novel function of cytokine LIF during cortical development. We demonstrated that LIF, a factor that is well-known for promoting gliogenesis, also promotes neurogenesis *in vitro* and *in vivo*. Our *in vitro* data showed that LIF, through activation of the PLCγ/PKC signaling pathway, and probably through activation of PKCδ, enhanced the association of ectopically expressed *Ngn* with CBP, leading to an increased association of this transcriptional complex to the *NeuroD* promoter. Also, LIF seemed to increase the binding of *Ngn* to *Brg1*, a factor, which might also be important for enhanced *NeuroD* transcription. However, increased *NeuroD* transcriptional activation, in turn, led to increased expression of neuron-specific markers such as neuron-specific class III β-tubulin. In addition, our *in vivo* data demonstrate that, compared to wildtype (WT) mice, LIF knock-out (KO) and LIF heterozygote (Het) mice display less mature neurons, as suggested by a decrease of neuron-specific markers MAP2a/b as well as class III β-tubulin, presumably caused by a decreased association of *Ngn1*-CBP to the *NeuroD* promoter.

4.1 Novel pro-neurogenic role of LIF during cortical neurogenesis is solely dependent on the expression of bHLH factors *Ngn1* and *Ngn2*

LIF has been known for some time to have the role in determining cell phenotype, and was originally characterized in the nervous system for its ability to induce a switch from noradrenergic to cholinergic phenotype in cultured sympathetic neurons (Yamamori et al., 1989). LIF and CNTF induce premature generation of astrocytes in vitro through activation of the JAK-STAT and MAPK pathways (Bonni et al., 1997; Chang et al., 2004). Interestingly, LIF mediates astrogliogenesis in late (>E15), but not in early (E12-E14), cortical progenitors in mice (Chang et al., 2004; Barnabe-Heider et al., 2005). We showed that this might be due to a developmental increase of *Ngn1/2* during cortical neurogenesis. As previously shown, *Ngn* not only promotes neurogenesis, but also inhibits gliogenesis (Sun et al., 2001). Also, it was shown that cortical expression of *Ngn1/2* is positively regulated by factors such as *Pax6*, while, on the other hand, the action of *Ngns* is negatively regulated by Id bHLH factors, which bind to E-proteins and thereby inhibit the activity of pro-neural bHLH factors, including *Ngn1* and *Ngn2*, that require E-proteins for their activity (Toresson et al., 2000; Norton, 2000). However, the signaling pathways and mechanisms that positively regulate the activity of pro-neural factors *Ngn1/2* are still unknown. Our results suggest a novel function of LIF by promoting neuronal differentiation in cortical NPCs, but only during neurogenesis, when *Ngn* is expressed.

It has previously been shown by our lab that *Ngn* inhibits glial differentiation via several mechanisms. First, it inhibits STAT1/3 phosphorylation via a DNA-binding

independent, still unknown mechanism, which is not enhanced by LIF (Sun et al, 2001). Second, *Ngn* sequesters CBP/p300 away from STAT1/3, a process that is, as our work shows, enhanced by LIF. However, since through this action LIF diminishes the CBP pool available for STAT-binding, which is necessary to induce glial gene activation, LIF can even be considered as an inhibitor of glial differentiation during cortical neurogenesis and *Ngn* expression. Third, as recently shown by our lab, *Ngns* might suppress alternative cell fates through directly turning on the expression of microRNAs (such as miR-9) by binding to E-box elements in the promoter region of such microRNAs (He et al., unpublished data). In turn, these microRNAs negatively regulate the signaling molecules (like gp130) of the JAK-STAT pathway by binding to the 3'UTR of its target mRNAs. Since binding of the Ngn/CBP-transcriptional complex to the *NeuroD*-promoter is enhanced by LIF, it is be intriguing to speculate that binding of this complex to promoter regions of certain microRNAs is enhanced by LIF as well. Through posttranscriptional downregulation of, for example, gp130, LIF might thereby negatively trigger its own pro-neural action during neurogenesis.

4.2 LIF promotes CBP-*Ngn1* association, leading to enhanced *NeuroD* transcription and therefore to increased neurogenesis

The pro-neural bHLH factor *Ngn* interacts with coactivators CBP as well as p300, which facilitate the assembly of active transcription factor complexes in part by bringing transcription factors and basal components of the transcriptional machinery into

proximity, as well as by acetylation of histones and transcription factors through an intrinsic acetyltransferase activity (Sun et al., 2001; Eckner et al., 1996; Koyano-Nakagawa et al., 1999; Goodman et al., 2000). Our data indicate that LIF-induction increases transcriptional activation of ectopically expressed *Ngn1/2*. Furthermore, LIF could increase the amount of *Ngn1* (and possibly *Ngn2*) that is associated with CBP, providing a likely mechanism for the increased transcriptional activity. The possibility that increased *Ngn1*-CBP association is caused by increased LIF-induced *Ngn1* expression can be ruled out, since our data demonstrate that *Ngn1* levels remained unchanged. However, increased *NeuroD* expression, which is necessary for terminal neuronal differentiation, led in turn to an increase in generation neurons, as shown by the expression of class III β-tubulin, which is recognized by the antibody TuJ1. This antibody has been show to label postmitotic, immature/mature neurons of the CNS very early, during or immediately after the last mitotic cycle (Lee et al., 1990). Thus it seems that LIF plays an important role in initiating terminal neuronal differentiation, rather than in initiating neurogenesis.

The fact that ectopically expressed *Ngn* reflects the function of endogenous *Ngn* is supported by previous findings that show that, first, endogenous Ngn sequesters CBP away from STATs in extracts from E14 cortices, where *Ngn* is expressed at physiological levels, second, during cortical development, gliogenesis does not begin before *Ngn* levels fall and CBP becomes available for STAT-binding, and third, knock-out of *Ngn* results in inhibition of neurogenesis and increased glial differentiation (Sun et al., 2001; Nieto et al., 2001; Tomita et al., 2000).

4.3 LIF-induced PKC activity is necessary for CBP-Ngn association

LIF is a member of the family of interleukin-6 type cytokines, which have overlapping, pleiotropic effects on a variety of different cell types and activate target genes involved in survival, apoptosis, proliferation and differentiation, as well as suppression of differentiation (Heinrich P. C., 2003). LIF binds directly to the low-affinity LIF receptor β (LIFRβ) that subsequently heterodimerizes with the signal transducer glycoprotein 130 (gp130). The formation of this high-affinity trimeric complex composed of two class I cytokine receptors, LIFRβ and gp130, and LIF leads to the activation of several downstream pathways essential for regulating biological responses: the JAK-STAT, the MAPK, the Akt/PI3K, and the PLCγ/PKC pathways (Kristensen et al., 2005).

Here we showed that, during neurogenesis, PKC is required for the pro-neural LIF effect in mouse cortical NPCs, in which *Ngn* was ectopically expressed. First, we demonstrated that pharmacological inhibitors of MAPK, Akt/PI3K, and PKC displayed the strongest inhibition of neuronal differentiation when PKC inhibitors were applied. Not only was *NeuroD* transcription inhibited by PKC inhibition, but also the pro-neural LIF effect abolished. This was not the case when applying drugs against MAPK and Akt/PI3K, suggesting that the pro-neural effect of LIF might be mediated by PKC. In addition, protein expression of the neuronal marker class III β-tubulin was most diminished upon administration of PKC inhibitors, where LIF treatment did not cause, as expected, a further increase of this marker either. It might therefore be of interest to further investigate possible *Ngn* and/or CBP consensus sites that can get potentially

phosphorylated by PKC during neurogenesis, and maybe even show that mutation of these sites abolishes LIF's pro-neural function.

As previously shown, MAPK and Akt/PI3K signaling pathways are important for mouse cortical neurogenesis and survival, respectively. Although it was shown by David Turner's lab (Vojtek et al., 2003) that beside being involved in neuronal survival, Akt1 and Akt2 promote neuronal differentiation in mouse P19 teratocarcinoma cells by activating ectopically expressed *Ngn3*, the targeted disruption of either Akt1 or Akt2 in mice had no apparent effect on neuron formation or differentiation, reflecting differences between P19 cells and neural progenitors on the level of Akt signaling (Chen et al., 2001; Cho et al., 2001a; Cho et al., 2001b). Regarding MAPK signaling, Paquin et al. (2005) showed that MEK-induced C/EBP phosphorylation/activation is essential for cortical neurogenesis in *vivo*, proposing that activated C/EBP might collaborate with pro-neuronal bHLH factors to ultimately promote neurogenesis by activating transcription of neuron-specific genes such as Tα1 α-tubulin. However, this group did not provide any evidence showing how MEK could activate, or partially activate any bHLH factors, or, more specifically, activate *Ngn*.

Our work indicates that PKC might be the mediator of LIF's pro-neural effect in cortical NPCs. We also showed that PKCδ, a PKC isoform, is upregulated during neurogenesis, and downregulated again during gliogenesis, implying a potentially important role during neuronal differentiation. Oehrlein et al. (1998) previously showed that PKCδ is upregulated during neurogenesis in mouse PZZ-7 Mz1 embryonic carcinoma cells, where neuronal differentiation was induced by retinoic acid. In addition, the same group showed that growth-associated protein 43 (GAP-43), which has an

important function in regulating the growth state of axon terminals, and was described as a postmitotic, neuron-specific PKC substrate, is actually already expressed in rat forebrain NPCs during neurogenesis, supporting the idea for the role of PKC during cortical neuronal development (Esdar et al., 1999, Burry, 1998). In transgenic mice, overexpression of GAP-43 led to spontaneous formation of new synapses and enhanced sprouting after injury. GAP-43 appears to be involved in transducing intra- and extra-cellular signals to regulate cytoskeletal organization in nerve endings. Phosphorylation by protein kinase C is particularly significant in this regard, and is linked with both nerve-terminal sprouting and long-term potentiation.

When we infected NPCs with *Ngn* adenovirus and stimulated them with LIF, inhibition of PLCγ expression using siRNA led to inhibition of class III β-tubulin expression in the same way as inhibition of PKCδ expression did. This suggests that PKCδ might be a very likely mediator of the pro-neuronal LIF effect. Among the PKC isoforms, PKCδ is unique in that its overexpression results in inhibition of cell growth and initiation cell differentiation (Miyamoto et al., 2002). Because of the reasons mentioned above, one could imagine that LIF-induced PKC/PKCδ activation promotes cortical neuronal differentiation not only by enhancing *NeuroD* transcription, but also by enhancing GAP-43 phosphorylation. Furthermore, it would be therefore also of interest to demonstrate how dependent (or independent) both events are on (of) each other.

4.4 The role of *Brg1* during mouse cortical neurogenesis

Our *in vitro* data showed that *Brg1*, a subunit of SWI/SNF chromatin remodeling protein complex, associates with the *Ngn1/*CBP transcriptional complex during cortical neurogenesis. In addition, we showed that LIF could enhance even this association. Seo et al (2005) previously demonstrated that in *Xenopus Brg1* binds to *Ngn*, which, in turn, induces activation of target genes during neurogenesis. In contrast, Matsumoto et al. (2006) showed that in mice *Brg1* is not expressed during early neurogenesis, until E13, but then gets upregulated during late neurogenesis and gliogenesis. This group concluded that *Brg1* is necessary for inducing the neurogenesis to gliogenesis switch, and also shows that *Brg1* downregulation leads to precocious maturation of NPCs. In addition, the same group also observed a dramatic loss of neurons, and abnormalities in cortical layering and pyramidal neuron morphology *Brg1*-deficient E18 cortices. The authors conclude that this loss was likely due to, first, a loss of trophic support as a result of the loss of glia, which were also less expressed in *Brg1* mutants, and second, because many NPCs were already exhausted before E14. However, our results indicate that *Brg1* is probably necessary during neurogenesis in the mouse cortex. Further support for this hypothesis comes from Gerald Crabtree's group (Lessard et al., 2007). In their recently published paper, the authors showed that *Brg1,* depending on the subunit that it is bound to, is necessary for NPC proliferation as well as neuronal differentiation. When *Brg1* was bound to Brg/Brm-associated factor 45a (BAF45a) or BAF53a, NPCs remained in a proliferative state by inducing the expression of several components of the Notch signaling pathway. On the other hand, as soon as BAF45b/c or

BAF 53b bound to *Brg1* neuronal differentiation was induced. It would therefore be very interesting to investigate first, whether Brg/Ngn association is required for Ngn's transcriptional activity, second, whether binding of *Brg1* to *Ngn* requires BAF 45b/c, and BAF53b, and third, whether BAF 45a/53a expression abolishes *Ngn1-Brg1* association. In addition, it would be interesting to see whether BAF 43b/c and BAF53b are part of the *Ngn*/CBP/*Brg* cotranscriptional complex.

4.5 *In vivo* evidence for LIF's pro-neural role during mouse cortical neurogenesis

Upon demonstrating the pro-neurogenic effects of LIF *in vitro*, we next examined the effect of LIF on cortical neurogenesis by taking an *in vivo* "loss of function" approach. Specifically, we compared the cortical tissue from E13, LIF knockout (KO), LIF heterocygote (Het), and wildtype (WT) mice with regards to neuron-specific markers. While immunohistochemistry of coronal brain sections showed seemingly unchanged neuronal differentiation pattern in all three genotypes, western blot analysis, a more quantitative method, showed that cortical tissue from LIF KO and Het mice contained less neurons as suggested by the decreased protein levels of neuronal markers, such as class III β-tubulin (TuJ1) and MAP2a/b, but not MAP2c. While MAP2c is predominantly found in growing dendrites of immature neurons, the isoforms MAP2a and MAP2b are expressed in dendrites of mature neurons (Belanger et al, 2001; Don et al., 2004)). Class III β-tubulin, recognized by the antibody TuJ1, has been shown

to be a marker for postmitotic, immature/mature neurons of the CNS very early, during or immediately after the last mitotic cycle (Lee et al., 1990). However, the obtained results are supporting the idea that LIF promotes neuronal maturity during mouse cortical development. Though genes such as *Ngn1* and *Ngn2* are well known for being initiators of neuronal differentiation, genes like *NeuroD* are involved in mediating terminal differentiation. Taken together, our data suggest that once *Ngn* is expressed in cortical NPCs, it mediates the transcription of terminal differentiation bHLH factor *NeuroD* under positive regulation of LIF, resulting in neuronal differentiation/maturation to appropriately build the functional CNS.

As previously shown, knocking out individual cytokines did not produce a phenotype that is notably different from wildtype with regards to gliogenesis, but blocking the action of the entire cytokine family (e.g., by knocking out gp130 or its coreceptor LIFR) led to a deficit in astrocyte formation (Koblar et al., 1998; Nakashima et al., 1999). These data indicate that, in single knockout mice, compensatory mechanisms might occur, as has been described for motor neuron survival (Sendtner et al., 1996; Holtmann et al., 2005). It might be therefore possible that these compensatory mechanisms also occur in LIF KO/Het mice during cortical neurogenesis, and factors such as CNTF may partially compensate for the lack of LIF. Based on this possibility, it might be conceivable that LIF/CNTF KO/Het mice may display even less class III β-tubulin and/or MAP2a/b expression in the E13 cortex.

Furthermore, it might be also of interest to elucidate the effect of LIF and *Ngns* on other inducible genes. For example, one of the downstream effectors regulated by *Ngn2* in developing cortical neurones is neuroligin 1, a postsynaptic cell adhesion

molecule that has been shown to contribute to synapse maturation and function (Mattar et al., 2004; Varoqueaux et al., 2006). Berninger et al. (2007) made recently the observation that *Ngn2*-expressing neurones undergo synaptogenesis more readily compared with controls or *Mash1*-transduced cells, which may thus be accounted for by the capacity of *Ngn2* to upregulate neuroligins. It might be possible that LIF plays a positive-regulator role in this process as well.

4.6 Implication of this work for regenerative medicine

The results of this work might have an impact on studies that focus on the generation of neurons suitable for stem cell therapy. For example, Parkinson's disease is characterized by the progressive loss of dopominergic (DA) neurons in the substantia nigra of the midbrain. Transplantation of DA neuron precursors into the brain of patients suggests that neuronal replacement therapy may be a possible treatment for Parkinson's disease (Lindvall et al., 2004). Recently, Andersson et al. (2007) showed that overexpression of both Ngn2 and Nurr1 in combination was required to promote the generation of DA neurons from expanded multi-passaged neurosphere cultures, which originated from the ventral midbrain, with Nurr1 being a transcription factor that is involved in establishing the DA neurotransmitter identity in midbrain DA neurons (McKay, 2004; Zetterstrom et al., 1997). However, the number of DA neurons was still limited, and one could imagine that treatment of these cells with LIF might even further promote the generation of DA neurons *in vitro*.

The case above was just one example for the potential impact that LIF might have in generating large amounts of specific neurons. Nevertheless, understanding the molecular control of neuronal differentiation will allow us to direct the differentiation of neural stem cells more efficiently, so that these cells will be suitable in cell replacement therapies for patients suffering from neurodegenerative disorders such as Parkinson's and Alzheimer's disease

5. Zusammenfassung

Die Entwicklung von unterschiedlichen Zelltypen waehrend der embryonalen ZNS-Entwicklung ist abhaengig von zellintrinsischen und positionsabhaengigen, aeusseren Einfluessen. Dabei bilden sich die verschiedenen Zellen in nacheinander ablaufenden bzw. sich teilweise ueberlappenden Zeitraeumen. Zuerst entstehen Radiaglia und Neuronen, nachfolgend Astrozyten und zuletzt Oligodendrozyten. Werden neurale Stammzellen/Vorlaeuferzellen (NPCs – neural precursor cells) zu unterschiedlichen Zeitpunkten entnommen und ohne den Einfluss von Wachstumsfaktoren kultiviert, so entwickeln sich diese Zellarten in der gleichen Reihenfolge.

Die Neurogenese, die bei Mausembryos am Tag E11-12, nach dem Etablieren der Radialglia, beginnt, findet an E14 ihren Hoehepunkt. Zu diesem Zeitpunt werden die Gene *Neurogenin1* (*Ngn1*) und *Ngn2* in den neuralen Vorlaeuferzellen der Ventrikularzone des dorsalen Cortexes in hohem Masse exprimiert. Wie aus Untersuchungen von unserm Labor gezeigt wurde, beguenstigt es die Entstehung von Neuronen und blockiert gleichzeitig Pro-Astrozyten-Einfluesse. Zum einen inhibiert *Ngn* den JAK/STAT Signalweg, dessen Aktivierung fuer die Gliogenese noetig ist, indem es die Phosphoylierung von STAT1/3 auf bisher noch unbekannte Weise blockiert. Ausserdem bindet der Transkriptions-Coaktivator cAMP-response element binding protein (CBP), welches auch von den STATs fuer die Transkription benoetigt wird, bevorzugt an *Ngn* sobald dieses von den Vorlaeuferzellen exprimiert wird.

Mit dem Tag E16 nimmt die Neurogenese *in vivo* wieder stark ab und es setzt die Gliogenese ein, bei der zunaechst ueberwiegend Astrozyten gebildet werden.

Faktoren wie leukemia inhibitory factor (LIF) sowie ciliary neurotrophic factor (CNTF) beguenstigen dabei die Astrozytogenese indem sie den JAK/STAT Signalweg aktivieren. Die Bindung von LIF/CNTF fuehrt zur Phosphorylierung von STAT-Transkriptionsfaktoren, die ihrerseits dann an den CBP/p300 Komplex binden und schliesslich die Expression von Astrozyten-spezifischen Genen aktivieren. Die STAT-Faktoren koennen aber erst nach Abfall des *Ngn*-Spiegels an den Transkriptions-Coaktivator binden, da sich die Bindungsstellen dieser beiden ueberlappen.

Um die Hypothese zu ueberpruefen, dass LIF auch die Neurogenese, oder spezifischer, die Wirkung von *Ngn* positiv beeinflusst, wurden cortikale NPCs von murinen Embryos entnommen und der Wirkung von LIF via Luciferase Assay untersucht. Dabei wurden die Vorlaeuferzellen mit *Ngn* und einem Reporter transfiziert, welcher den *NeuroD*-Promoter beinhaltete. *NeuroD*-Expression findet in der Regel gegen Mitte/Ende der Neurogenese statt und ist wichtig fuer die Reifung von Neuronen. Der Promoter von *NeuroD* beinhaltet ein E-box Element, an welches *Ngn* bindet und die Transkription einleitet. Wie unsere ersten Versuche zeigten, verstaerkt LIF die Transkriptionsaktivitaet von *Ngn* und somit die Transkription von *NeuroD*. Wenn aber im selben Versuch ein *NeuroD*-Reporter transfiziert wurde, dessen E-box mutiert war, wurde keine Transkriptionsaktivitaet gemessen, was wiederum bestaetigte, dass der pro-neurale LIF-Effekt ueber *Ngn* lief und E-box-Bindung noetig war.

Um den Einfluss des pro-neuralen Effekts von LIF auf Proteinebene zu testen, wurden NPCs mit *Ngn*-Adenovirus infiziert und mit LIF stimuliert. Dabei wurden die Zellen auf die Expression von Neuron-spezifischem class III β-tubulin (TuJ1) untersucht. Die Ergebnisse zeigten, dass LIF bei Zellen, die *Ngn* exprimierten, die Rate der Neuronen

von etwa 5% auf etwa 50% anstiegen liess, waehrend LIF bezueglich der Gliogenese (gezeigt durch die Expression von GFAP) in *Ngn*-exprimierenden Vorlaeuferzellen kaum Wirkung zeigte.

Als naechstes sollte untersucht werden ueber welchen Signalweg LIF Ngn aktivierte. LIF bindet zunaechst an LIF receptor β (LIFRβ), der dann an glycoprotein 130 (gp130) bindet. Diese Bindung fuehrt dann zur Aktivierung mehrerer Signalkaskaden: dem JAK/STAT, dem MAPK, dem Akt/PI3K und dem PLCγ/PKC Signalweg. Da der JAK/STAT Signalweg fuer die Gliogenese wichtig ist, lag unser Fokus auf den anderen Signalwegen. Deren Aktivierung wurde dann mit spezifischen Inhibitoren blockiert und, wie auch in den Vorversuchen, die Wirkung von LIF auf Transkriptionsebene (*NeuroD*) in neuralen Vorlaeuferzellen bestimmt. Dabei zeigte sich, dass die Blockierung des PLCγ/PKC Signalweges die *NeuroD*-Promoteraktivitaet am starksten inhibierte, waehrend auch LIF´s pro-neurale Wirkung verloren ging. Dementsprechend zeigte die Western Blot Analyse, dass die Expression von class III β-tubulin (TuJ1) durch die Anwendung der PKC Inhibitoren am staerksten inhibiert wurde, wobei auch hier die Stimulation durch LIF keine erhoehte Neurogenese mit sich zog.

In weiteren Versuchen konnten wir dann mit Hilfe von Immunoprezipitation demonstrieren, dass LIF die Bindung von *Ngn* an CBP verstaerkte (eine Bindung, welche durch PKC Inhibitoren aufgehoben wurde), was wiederum zu einer erhoehten Bindung dieses Transkriptionskomplexes an den *NeuroD* Promoter fuehrte, wie unsere Chromatin Immunoprezipitation (ChIP) Daten beweisen. Dies wiederum laesst darauf schliessen, dass womoeglich diese erhoehte *Ngn*-CBP/*NeuroD*-Promoter Bindung der Grund fuer die erhoehte *NeuroD*-Transkriptionsaktivitaet ist daher auch fuer die erhoehte neuronale

Differenzierung. Interessanterweise konnten wir auch zeigen, dass Brahma-related gene 1 (*Brg1*), eine katalytische Untereinheit des SWI/SWF Komplexes, an den *Ngn*/CBP cotranscriptionalen Komplex bindet und dass diese Bindung durch LIF-Stimulation verstaerkt wurde. Dies suggeriert wiederum, dass auch *Brg1* eine wichtige Rolle waehrend der murinen, cortikalen Neurogenese spielt. Dennoch, in folgenden Experimenten verblieb der Fokus auf *Ngn* und CBP.

Um unsere Hypothese zu bestaetigen, dass PKCδ ein moeglicher Mediator des LIF-Effekts sein koennte, zeigten wir zunaechst, dass die PKCδ-Expression in cortikalen NPCs waehrend der Neurogenese erhoeht ist. Desweiteren demonstrierten wir, dass die Inhibition von PKCδ einen aehnliche Wirkung zeigte wie die Inhibition von PKC mit einem generellen PKC Inhibitor: weder war nach PKCδ-Inhibition eine LIF-induzierte *NeuroD*-Transkription erzielbar, noch wurde nach LIF-Stimulation der pro-neurale Marker class III β-tubulin/TuJ1 in *Ngn1*-infizierten NPCs exprimiert. Um aber mehr spezifisch die PKC- und PKCδ-Aktivitaet/Expression zu blockieren transfizierten wir NPCs mit PLCγ oder PKCδ siRNA. Unsere Daten zeigten hierbei, dass siRNA-transfizierte Zellen kein class III β-tubulin mehr aufweisen, was darauf hindeuted, dass PKCδ der potentielle Mediator des pro-neuralen LIF-Effekts ist.

Durch unsere *in vivo* Daten demonstrierten wir schliesslich, dass LIF auch hierbei fuer die Neurogenese von Bedeutung ist. Verglichen wurden die Cortices von E13 LIF Het (heterozygote) und KO (knock out) Maeusen mit denen von WT (wild type) Maeusen. Durch Immunohistologie von Hirnschnitten konnten dabei keine groesseren Unterschiede bezueglich der Expression neuraler Marker beobachtet werden, waehrend aber mit Hilfe der Western Blot Analyse, eine quantitativere Methode, gezeigt wurde,

dass LIF Het und KO Maeuse weniger pro-neurale Marker im Cortex exprimieren wie WT Mause. Um auch zu beweisen, dass dies auf eine verringerte Transkription von *NeuroD* zurueckzufuehren ist, demonstrierten wir mit Hilfe des ChIP Assay, dass LIF Het und KO Maeuse weniger *Ngn*1-CBP Bindung an den *NeuroD*-Promoter aufweisen wie WT Maeuse.

Diese Experimente veranschaulichen einen eleganten Regulationsmechanismus, durch welchen ein einzelner, extrazellulaerer Faktor die unterschiedliche Differenzierung einer Zelle verstaerkt, abhaengig von der Anwesenheit oder Abwesenheit eines einzelnenn intrazellulaeren Faktors. Auch koennen durch die erlangten Resultate Strategien entworfen werden, durch die in Zukunft die Produktion bestimmter Neurone zur Heilung von verschiedenen, neurodegenerativen Krankheiten erhoeht wird.

6. Reference

Abeliovich, A., Chen, C., Goda, Y., Silva, A. J., Stevens, C. F., Tonegawa, S., (1993). Modified hippocampal long-term potentiation in PKC gamma-mutant mice, Cell *75*, 1253–1262

Andersson, E. K., Irvin, D. K., Ahlsioe, J., Parmar, M. (2007). Ngn2 and Nurr1 act in synergy to induce midbrain dopaminergic neurons from expanded neural stem and progenitor cells. Exp Cell Res *313*, 1172-1180

Auld D.S., Kornecook T.J., Bastianetto S., Quirion R. (2002). Alzheimer's disease and the basal forebrain cholinergic system: relations to β-amyloid peptides, cognition, and treatment strategies. Prog Neurobiology *68*, 209-245

Ayala, R., Shu, T., Tsai, L. H. (2007). Trekking across the brain: The journey of Neuronal migration. Cell *128*, 29-43

Barnabe-Heider, F., Wasylnka, J. A., Fernandez, K. J., Porsche, C., Sendtner, M., Kaplan, D. R., Miller, F. D. (2005). Evidence that embryonic neurons regulate the onset of cortical gliogenesis via cardiotrophin-1. Neuron *48*, 253-65

Bartlett, P. F., Brooker, G. J., Faux, C. H., Dutton, R., Murphy, M., Turnley, A., and Kilpatrick, T. J. (1998). Regulation of neural stem cell differentiation in the forebrain. Immunol Cell Biol *7*6, 414-418

Bayer, S.A., and Altman, J. (1991). Neocortical Development (new York, Raven Press).

Casarosa, S., Fode, C., and Guillemot, F. (1999). Mash1 regulates neurogenesis in the ventral telencephalon. Development *126*, 525-534

Belanger, D., Abi-Farah, C., Nguyen, M. D., Lauzon, M., Cornibert, S., Leclerc, N. (2001). The projection domain of MAP2b regulates microtubule protrusion and process formation in Sf9 cells. J Cell Sci *115*, 1523

Berninger, B., Guillemot, F., Goetz, M. (2007). Directing neurotransmitter identity of neurons derived from expanded adult neural stem cells. Europ J Neurosci 25, 2581-2590

Bertrand, N., Castro, D. S., and Guillemot, F. (2002). Proneural genes and the specification of neural cell types. Nat Rev Neurosci *3*, 517-530

Bonni, A., Sun, Y., Nadal-Vicens, M., Bhatt, A., Frank, D. A., Rozovsky, I., Stahl, N., Yancopoulos, G. D., Greenberg, M. E. (1997). Regulation of gliogenesis in the central nervous systemby the JAK-STAT signaling pathway. Science *278*, 477-483

Burry, A. W. (1998). PKC activators (phorbol ester or bryostatin) stimulate outgrowth of NGF-dependent neurites in a subline of PC12 cells. J Neurosci Res *53*, 214-222

Cau, E., Gradwohl, G., Fode, C., Guillemont, F. (1997). Mash1 activates a cascade of bHLH regulators in olfactory neuron progenitors. Development *124*, 1611-1621

Caviness, V. S., Jr., and Takahashi, T. (1995). Proliferative events in the cerebral ventricular zone. Brain Dev. *17*, 159163

Chambers, C. B., Peng, Y., Nguyen, H., Gaiano, N., Fishell, G., and Nye, J. S. (2001). Spatiotemporal selectivity of response to Notch1 signals in mammalian forebrain precursors. Development *128*, 689-702

Chang, M. Y., Park, C. H., Son, H., Lee Y. S., Lee, S. H. (2004). Developmental stage-dependant self-regulation of embryonic cortical precursor cell survival and differentiation by leukemia inhibitory factor. Cell Death Differ *11*, 985-996

Chen, W. S., P. Z. Xu, K. Gottlob, M. L. Chen, K. Sokol, T. Shiyanova, I. Roninson, W. Weng, R. Suzuki, K. Tobe, T. Kadowaki, N. Hay (2001). Growth retardation and increased apoptosis in mice with homozygous disruption of the Akt1 gene. Genes Dev. *15*, 2203-2208

Chen, H., Thiagalingam, A., Chopra, H., Borges, M. W., Feder, J. N., Nelkin, B. D., Baylin, S. B., and Ball, D. W. (1997). Conservation of Drosophila lateral inhibition pathway in human lung cancer: a hairy-related protein (Hes-1) directly represses achaete-scute homolog-1 expression. Proc Natl Acad Sci USA *94*, 5355-5360

Cho, H., J. Mu, J. K. Kim, J. L. Thorvaldsen, Q. Chu, E. B. Crenshaw III, K. H. Kaestner, M. S. Bartolomei, G. I. Shulman, M. J. Birnbaum (2001a). Insulin resistance and a diabetes mellitus-like syndrome in mice lacking the protein kinase Akt2 (PKB beta). Science *292*, 1728-1731

Cho, H., J. L. Thorvaldsen, Q. Chu, F. Feng, M. J. Birnbaum. (2001b). Akt1/PKBα is required for normal growth but dispensable for maintenance of glucose homeostasis in mice. J. Biol. Chem. *276*, 38349-38352

Colombo, E., Giannelli, S. G., Galli, R., Tagliafico, E., Foroni, C., Tenedini, E., Ferrari, S., Ferrari, S., Corte, G., Vescovi, A., Cossu, G., Broccoli V (2006). Embryonic stem-derived versus somatic neural stem cells: a comparative analysis of their developmental potential and molecular phenotype. Stem Cells *24*, 825-835

Davis, A. L., and Turner, D. L. (2001). Vertebrate hairy and Enhancer of split related proteins: transcriptional repressors regulating cellular differentiation and embryonic patterning. Oncogene *20*, 8342-8357

Doetsch, F. (2003). The glia identity of neural stem cells. Nat Neurosci *6*, 1127-1134

Farah, , M. H., Olson, J. M., Sucic, H. B., Hume, R. I., Tapscott, S. J., and Turner, D. L. (2000). Generation of neurons by transient expression of neural bHLH proteins in mammalian cells. Development *127*, 693-702

Don, S., Verrillis, N. M., Liaw, T. Y. E., Liu, M. L. M., Norris, M. D., Haber, M., Kavallaris, M (2004). Neurona-associated microtubule proteins class III β-tubulin and MAPc in neuroblastoma: Role in resistance to microtubule-targeted drugs. Mol Cancer Therap *3*, 1137-1146

Eckner, R., T. P. Yao, E. Oldread, and D. M. Livingston (1996). Interaction and functional collaboration of p300/CBP and bHLH proteins in muscle and B-cell differentiation. Genes Dev. *10***,** 2478-2490

Elbashir, S. M., Lendeckel, W. & Tuschl, T. (2001). RNA interference is mediated by 21 and 22 nt RNAs. Genes Dev. *15,* 188–200

Elbashir, S. M., Harborth, J., Lendeckel, W., Yalcin, A., Weber, K., Tuschl, T. (2001). Duplexes of 21-nucleotide RNAs mediate RNA interference in cultures mammalian cells. Nature *411,* 494-498

Esdar, C., Oehrlein, S., Reinhardt, S., Maelicke, A., Herget, T. (1999). The protein kinase C (PKC) substrate GAP-43 is already expressed in neural precursor cells, colocalizes with PKCη and binds calmodulin. Eur J Neurosci *11*, 503-516

Freed CR, Greene PE, Breeze RE et al (2001). Transplantation of embryonic dopamine neurons for severe Parkinson's disease. N Engl J Med *344*, 710–719

Fremeau Jr R. T., Troyer, M. D., Pahner, I., Nygaard, G. O., Tran, C. H., Reimer, R. J., Bellocchio, E. E., Fortin, D., Storm-Mathisen, J., Edwards, R. H. (2001). The expression

of vesicular glutamate transporters defines two classes of excitatory synapse. Neuron *31*, 247-260

Gage, F. H., Ray, J., and Fisher, L. J. (1995). Isolation, charactarization, and use of stem cells from the CNS. Annu Rev Neurosci. *19*, 159-192

Gaiano, N., Nye, J. S., and Fishell, G. (2000). Radial glia identity is promoted by Noth1 signaling in the murine forebrain. Neuron *26*, 395-404

Ge, W., Martinovich, K., Wu, X., He, F., Miyamoto, A., Fan, G., Weinmaster, G., and Sun, Y. E. (2002). Notch signaling promotes astrogliogenesis via direct CSL-mediated glial gene activation. J Neurosci Res *69*, 848-860

Ge, W., He, F., Kim, K. J., Blanchi, B., Coskun, V., Nguyen, L., Wu, X., Zhao, J., Heng, J. I., Martinowich, K., *et al.* (2006). Coupling of cell migration with neurogenesis by proneural bHLH factors. Proc Natl Acad Sci USA *103*, 1319-1324

Goodman, R. H., S. Smolik (2000). CBP/p300 in cell growth, transformation, and development. Genes Dev. *14*, 1553-1577

Gosh, A., Greenberg, M. E. (1995). Distinct Roles for bFGF and NT-3 in the Regulation of Cortical Neurogenesis. Neuron 15, 89-103

Gradwohl, G., Fode, C., Guillemont, F. (1996). Restricted expression of a novel murine atonal-related bHLH protein in undifferentiated neural precursors. Dev Biol *180*, 227-241

Graham, V., Khudyakov, J., Ellis, P., and Pevny, L. (2003). SOX2 functions to maintain neural progenitor identity. Neuron *39*, 749-765

Griner, E. M., Kazanietz, M. (2007). Protein kinase C and other diacylclycerol effectors in cancer. Nature Rev Cancer *7*, 281-294

Guillemot, F., and Joyner, A. L. (1993). Dynamic expression of the murine Achaete-Scute homologue Mash-1 in the developing nervous system. Mech Dev *42*, 171-185

Gutcher, I., Webb, P. R., Anderson, N. G. (2003). The isoform-specific regulation of apoptosis by protein kinase C. Cell Mol Life Sci. *60,* 1061–1070

Hagell P, Piccini P, Bjorklund A et al. (2002). Dyskinesias following neural transplantation in Parkinson's disease. Nat Neurosci *5*, 627–628

Hammond, S. M., Caudy, A. A. & Hannon, G. J. (2001). Post-transcriptional gene silencing by double-stranded RNA. Nature Rev. Genet. *2*, 110–1119

Hand, R., Bortone, D., Mattar, P., Nguyen, L., Heng, J. I., Guerrier, S., Bout, E., Peters, E., Barnes, A. P., Parras, C. et al (2005). Phosphorylation of Neurogenin2 specifies the migration properties and the dentritic morphology of pyramidal neurons in the neocortex. Neuron *48*, 45-62

Hatten, M. E. (1999). Central nervous system neuronal migration. Annu Rev Neurosci. *22*, 511-539

Hayashi, J., et al. (2006). Primate embryonic stem cells-derived neuronal progenitors transplanted into the ischemic brain. J Cereb Blood Flow Metab

Heinrich, P. C., Behrmann, I., Haan, S., Hermanns, H. M, Muller-Newen, G., Schaper, F. (2003). Principles of interleukin (IL)-6-type cyokine signaling and its regulation. Biochem J *374*, 1-20

Holtmann, B. et al. (2005). Triple knockout of CNTF, LIF, and CT-1 defines cooperative and distinct roles of these neurotrophic factors for motoneuron maintenance and function. J Neurosci *25*, 1778-1787

Hu, G. Y., Hvalby, O., Walaas, S. I., Albert, K. A., Skjeflo, P., Andersen, P., Greengard, P. (1987). Protein kinase C injection into hippocampal pyramidal cells elicits features of long term potentiation, Nature *328*, 426–429

Johe, K. K., Hazel, T. G., Muller, T., Dugich-Djordjevic, M. M., and McKay, R. D. (1996). Single factors direct the differentiation of stem cells from the fetal and adult central nervous system. Genes Dev *10*, 3129-3140.

Kadonaga, J. T. (1998). Eukaryotic transcription: an interlaced network of transcription factors and chromatin-modifying machines. Cell *92*, 307 -313

Kamakura, S., A., Oishi, K., Yoshimatsu, T., Nakafuku, M., Masuyama, N., and Gotoh, Y. (2004). Hes binding to STAT3 mediates crosstalk between Notch and JAK-STAT signaling. Nat Cell Biol *6*, 547-554

Kelly, S. et al. (2004). Transplanted human fetal neural stem cells survive, migrate, and differentiate in ischemic rat cerebral cortex. Proc Natl Acad Sci USA *101*, 11839-11844

Kingston, R. E. and Narlikar, G. J. (1999). ATP-dependent remodeling and acetylation as regulators of chromatin fluidity. Genes Dev *13*, 2339 –2352

Kobler, S. A. (1998). Neural precursor differentiation into astrocytes requires signaling through the leukemia inhibitory factor receptor. Proc Natl Acad Sci USA *95*, 3178-3181

Koyano-Nakagawa, N., D. Wettstein, C. Kintner (1999). Activation of Xenopus genes required for lateral inhibition and neuronal differentiation during primary neurogenesis. Mol. Cell. Neurosci. *14*, 327-339.

Kriegstein, A. R., and Nector, S. C. (2004). Patterns of neuronal migration in the embryonic cortex. Trends Neurosci. *27*, 392-399

Kristenson, D. M., Kalisz, M., Nielsen, J. (2005). Cyrokine signaling in embryonic stem cells. APMIS *113*, 756-772

Kuhn, H. G., Winkler, J., Kempermann, G., Thal, L. J., and Gage, F. H. (1997). Epidermal growth factor and fibroblast growth factor-2 have different effects on neural progenitors in the adult rat brain. J Neurosci *17*, 5820-5829

Lee, M. K., Tuttle, J. B., Rebhun, L. L., Cleveland, D. W., Frankfurter, A (1990). The expression and posttranslational modification of a neuron-specific β-tubulin isotype during chick embryogenesis. Cell Motil Cytoskel *17*, 118-132

Lessard, J., Wu, J. I., Ranish, J., Wan, M., Winslow, M. M., Staahl, B. T., Wu, H., Aebersold, R., Graef, I. A., Crabtree, G. R. (2007). An essential switch in subunit composition of a chromatin remodeling complex during neural development. Neuron *55*, 201-215

Li, W., Cogsswell, C. A., and LoTurco, J. J. (1998). Neuronal differentiation of precursors in the neocortical ventricular zone is triggered by BMP. J Neurosci *18*, 8853-88623

Lindvall O, Bjorklund (2004) A. Cell therapy in Parkinson's disease. NeuroRx *1*, 382–393

Lo, L., Dormand, E., Greenwood, A., and Anderson , D. J. (2002). Comparison of generic neuronal differentiation and neuron subtype specification functions of mammalian achaete-scute and atonal homologs in cultured neural progenitor cells. Development *129*, 1553-1567

Ma, Q., Kinter, C., and Anderson, D. J. (1996). Identification of neurogenin, a vertebrate neuronal determination gene. Cell *87*, 43-52

Mabie, P. C., Mehler, M. F., and Kessler, J. A., (1999). Multiple roles of bone morphogenic protein signaling in the regulation of cortical cell number and phenotype. J Neurosci 19, 7077-7088.

Malinow, R., Schulman, H., Tsien, R. W. (1989). Inhibition of postsynaptic PKC or CaMKII blocks induction but not expression of LTP, Science *285*, 862–866

Marin, O., and Rubenstein, J. L. (2001). A long remarkable journey: tangential migration in the telencephalon. Nat Rev Neurosci *2*, 780-790

Marin D. B., Sewell M. C., Schlechter A. (2002): Alzheimer's disease. Accurate and early diagnosis in the primary care setting. Geriatrics *57*, 36-40

Marin, O., and Rubenstein, J. L. (2003). Cell migration the forebrain. Annu Rev Neurosci *26*, 441-483

Martens, J. A. and Winston, F. (2003). Recent advances in understanding chromatin remodeling by Swi/Snf complexes. Curr Opin Genet Dev *13*,136 –142

Matsumoto, S., Banine, F., Struve, J., Xing, R., Adams, C., Liu, Y., Metzger, D., Chambon, P., Rao, M. S., Sherman, L. S. (2006). Brg1 is required for murine neural stem cell maintenance and gliogenesis. Dev Biol *289*, 372-383

Matter, P., Britz, O., Johannes, C., Nieto, M., Ma, L., Rebeyka, A., Klenin, N., Polleux, F., Guillemot, F., and Schuurmans, C. (2004). A screen for downstream effectors of Neurogenin2 in the embryonic neocortex. Dev Biol *273,* 373-389

McConnell, S. K. (1995). Constructing the cerebral cortex: neurogenesis and fate determination. Neuron *15*, 761-768

MacDonald, J. F., Mody, I., Salter, M. W. (1989). Regulation of N-methyl-D-aspartate receptors revealed by intracellular dialysis of murine neurons in culture, J. Physiol *414*, 17–34.

McKay, R. D. (2004). Stem cell biology and neurodegenerative disease. Philos Trans R Soc Lond B Biol Sci 29, 851-856

Menard, C., Hein, P., Raquin, A., Savelson, A., Y, X. M., Lederfein, D., Barnabe-Heider, F., Mir, A., Sterneck, E., Peterson, A. C., Johnson, P. F., Vinson, C., Miller, F. D. (2002). An essential Role for a MEK-C/EBP pathway during Growth Factor-Regulated Cortical Neurogenesis. Neuron *36*, 597-610

Miyamoto, A., Nakayam, K., Imaki, H., Hirose, S., Jiang, Y., Abe, M., Tsukiyama, T., Nagahama, H., Ohno, S., Hatakeyama, S., Nakayama, K. I. (2002). Increased proliferation of B cells and auto-immunity in mice lacking protein kinase Cδ

Molne, M., Studer, L., Tabar, V., Ting, Y. T., Eiden, M. V., and McKay, R. D. (2000). Early cortical precursors do not undergo LIF-mediated astrocytic differentiation. J Neurosci Res *59*, 301-311

Moore, K. B., Schneider, M. L., and Vetter, M. L. (2002). Posttranslational mechanisms control the timing of bHLH function and regulate retinal cell fate. Neuron *34*, 183-195

Nakashima, K., Yanagisawa, M., Arakawa, H. and Taga, T. (1999). Astrocyte differentiation is mediated by LIF in cooperation with BMP2. FEBS Lett *457*, 43–46 a

Nakashima, K., Yanagisawa, M., Arakawa, H., Kimura, N., Hisatsune, T., Kawabata, M., Miyazono, K. and Taga, T. (1999). Synergistic signaling in fetal brain by STAT3-Smad1 complex bridged by p300. Science *284*, 479–482 b

Nakashima, K. et al (1999). Developmental requirement of gp130 signalingin neuronal survival and astrocyte differentiation. J Neurosci *19*, 5429-5434

Nakashima, S. (2002). Protein kinase Cα (PKCα): regulation and biological function, *J.* Biochem. *132*, 669–675

Newton, A. C. (2001). Protein kinase C: structural and spatial regulation by phosphorylation, cofactors, and macromolecular interactions, Chem. Rev. *101*, 2353–2364

Nieto, M., Schuurmans, C., Britz, O., and Guillemot, F. (2001). Neural bHLH genes control the neuronal versus glial fate decision in cortical progenitors. Neuron *29*, 401-413.

Norton, J. D. (2000). ID helix-loop-helix proteins in cell growth, differentiation and tumorigenesis. J Cell Sci *113*, 3897-3905

Oehrlein, S. A., Maelicke, A., Herget, T. (1998). Expression of protein kinase C gene family members is temporally and spatially regulated during neural development in vitro. Eur J Cell Biol *77*, 323-337

Ohno, S., Nishizuka, Y. (2002). Protein kinase C isotypes and their specific functions: prologue, J. Biochem. *132*, 509–511

Olanow CW, Goetz CG, Kordower JH et al. (2003) A double-blind controlled trial of bilateral fetal nigral transplantation in Parkinson's disease. Ann Neurol *54*, 403–414

Paquin, A., Barnabe-Heider, F., Ryoichiro, K., Miller, F. D. (2005). CCAAT/enhancer-binding protein phosphorylation biases cortical precursors to generate neurons rather astrocytes in vivo. J Neurosci *25*, 10747-10758

Park, J. K., Williams, B. P., Alberta, J. A., and Stiles, C. D. (1999). Bipotent cortical progenitor cells process conflicting cues for neurons and glia in a hierarchical manner. J Neurosci *19*, 10383-10389

Patel, N. A., Song, S. S., Cooper, D. A. (2006). PKC delta alternatively spliced isoforms modulate cellular apoptosis in retinoic acid-induced differentiation of human NT2 cells and mouse embryonic stem cells. Gene Expression *13*, 73-84

Perez, J. L., Khatri, L., Chang, C., Srivastava, S., Osten, P., Ziff, E. B. (2001). PICK1 targets activatd protein kinase Cα to AMPA receptor clusters in spines of hippocampal neurons and reduces surface levels of the AMPA-type glutamate receptor subunit 2, J. Neurosci. *21*, 5417–5428

Qian, X., Shen, Q., Goderie, S. K., He, W., Capela, A., Davis, A. A., and Temple, S. (2000). Timing of CNS cell generation: a programmed sequence of neuron and glial cell production from isolated murine cortical stem cells. Neuron *28*, 69-80

Rakic, P. (1995). A small step for the cell, a giant leap for mankind: a hypothesis of neocortical expansion during evolution. Trends Neurosci *18*, 383-388

Reynolds, B. A., and Weiss, S. (1992). Generation of neurons and astrocytes from isolated cells of the adult mammalian nervous system. Science *255*, 1707-1710

Ross, S. E., Greenberg, M. E., Stiles, C. D. (2003). Basic helix-loop-helix factors in cortical development. Neuron 39, 13-25

Rossner S., Schliebs R., Bigl V.(1994). Ibotenic acid lesion of nucleus basalis magnocellularis differentially affects cholinergic, glutamatergic and GABAergic markers in cortical rat brain regions. Brain Res *668*, 85-99

Roth, S. Y., Denu, J. M., and Allis, C. D. (2001). Histone acetyltransferases. Annu Rev Biochem *70*, 81-120

Sauvageot, C. M., and Stiles, C. D. (2002). Molecular mechanisms controlling cortical gliogenesis. Curr Opin Neurobiol *12*, 244-249

Scardigli, R., Baumer, N., Gruss, P., Guillemot, F., and Le Roux, I. (2003). Direct and concentration-dependent regulation of the proneural gene Neurogenin2 by Pax6. Development *130*, 3269-3281

Schuurmans, C., Armant, O., Nieto, M., Stenman, J. M., Britz, O., Klenin, N., Langevin, L. M., Seibt, J., Brown, C., Tang, H., Cunningham, J. M., Cyck, R., Walsh, C., Campbell, K., Polleux, F., Guillemot, F. (2004). Sequential phases of cortical specification involve neurogenin-dependent and -independent pathways. EMBO J *23*, 2892-2902

Selkoe D.J. (1999). Translating cell biology into therapeutic advances in Alzheimer's disease. Nature *399*, A23-31,

Seo, S., Richardson, G. A., and Kroll, K. L. (2005). The SWI/SNF chromatin remodeling protein Brg1 is required for vertebrate neurogenesis and mediates transactivation of Ngn and NeuroD. Development *132*, 105-115

Sendtner, M. et al. (1996). Cryptic physiological trophic support of motoneurons by LIF revealed by double gene targeting of CNTF and LIF. Curr Biol *6*, 686-694

Sharp, P. A. (2001). RNA interference. Genes Dev. *15*, 485–490

Sommer, L., Ma, Q., and Anderson, D. J. (1996). Neurogenins, a novel family of atonal-related bHLH transcription factors, are putative mammalian neuronal determination genes that reveal progenitor cell heterogeneity in the developing CNS and PNS. Mol Cell neurosci *8*, 221-241

Song, M. R., and Gosh, A. (2004). FGF2-induced chromatin remodeling regulates CNTF-mediated gene expression and astrocyte differentiation. Nat Neurosci *7*, 229-235

Stahl, N. and Yancopoulos, G.D. (1994). The tripartite CNTF receptor complex: activation and signaling involves components shared with other cytokines. J. Neurobiol. *25*, 1454–1466

Sun, Y., Nadal-Vicens, M., Misono, S., Lin, M. Z., Zubiaga, A., Hua, X., Fan, G., and Greenberg, M. E. (2001). Neurogenin promotes neurogenesis and inhibits glial differentiation by independent mechanisms. Cell *104*, 365-376

Sun, Y. E., Martinovich, K., and Ge, W. (2003). Making and repairing the mammalian brain-signaling toward neurogenesis and gliogenesis. Semin Cell Dev Biol *14*, 161-168

Takizawa, T., Nakashima, K., Namihira, M., Ochiai, W., Uemura, A., Yanagisawa, M., Fujita, N., Nakao, M., and Taga, T. (2001). DNA methylation is a critical cell-intrinsic determinant of astrocyte differentiation in the fetal brain. Dev Cell 1, 749-758

Tomita, K., Moriyoshi, K., Nakanishi, S., Guillemot, F., and Kageyama, R. (2000). Mammalian achaete-scute and atonal homologs regulate neuronal versus glial fate determination in the central nervous system. EMBO J. *19*, 5460-5472

Tanigaki, K., Nogaki, F., Takahashi, J., Tashiro, K., Kurooka, H., and Honjo, T. (2001). Notch1 and Notch3 instructively restrict bFGF-responsive multipotent neural progenitor cells to an astroglial fate. Neuron *29*, 45-55

Toresson, H., Potter, S. S., Campbell, K. (2000). Genetic control of dorsal-ventral identity in the telencephalon: opposing roles for Pax6 and Gsh2. Development *127*, 4361-4371

Tuszynski, M. H. et al. (2005). A phase 1 clinical trial of nerve growth factor gene therapy for Alzheimer disease. Nature Med *11*, 551-555

Varoqueaux, F., Aramuni, G., Rawson, R.L., Mohrmann, R., Missler, M., Gottmann, K., Zhang, W., Südhof, T.C. & Brose, N. (2006) Neuroligins determine synapse maturation and function. Neuron *51*, 741–754

Viti, J., Feathers, A., Phillips, J., and Lillien, L. (2003). Epidermal growth factor receptors control competence to interpret leukemia inhibitory factor as an astrocyte inducer in developing cortex. J Neurosci *23*, 3385-3393

Vojtek, A. B., Taylor, J., DeRuiter, S., Yu, J. Y., Figueroa, C., Kwok, R. P., Turner, D. L. (2003). Akt regulates basic Helix-Loop-Helix transcription fator-coactivator complex formation and activity during neuronal differentiation. Mol Cell Biol *23*, 4417-4427

Whitehouse, I., Flaus, A., Cairns, B. R., White, M. F., Workman, J. L. and Owen-Hughes, T. (1999). Nucleosome mobilization catalysed by the yeast SWI/SNF complex. Nature *400*, 784 -787

Wilson, S. W., and Rubinstein, J, L. (2000). Induction of dorsoventral patterning of the telencephalon. Neuron *18*, 553-562

Ware, C. B., et al (1995). Targeted disruption of the low-affinity leukemia inhibitory factor receptor gene causes placental, skeletal, neural and metabolic defects and results in perinatal death. Development *121*, 1283-1299

Yamamorei, T. et al (1989). The cholinergic neuronal differentiation factor from heart cells is identical to leukemia inhibitory factor. Science *246*, 1412-1416

Zetterstrom, R. H., Solomin, L. Jansson, L., Hoffer, B.J., Olson, L., Perlmann, T. (1997). Dopamine neuron agenesis in Nurr1-deficient mice. Science *276*, 248–250

Acknowledgement

I would like to thank Prof. Dr. Yi Sun for her support and advice, and for giving me the opportunity to carry out my Ph.D. in her laboratory.

I am also very grateful to Prof. Dr Walter Volknandt for stepping in as a reviewer of my thesis.

Especially I would like to thank Jing Zhao for her help, support and encouragement during the last couple of years. Without her help this work would have never come to an end. Thanks Jing!

Special thanks to Dr. Volkan Coskun for teaching me various laboratory techniques, and for giving me suggestions for writing this thesis. I think I still owe you three coffees for that.

Best regards to all the past and present members of our lab for providing a great atmosphere.

Last but not least a big “Thank you” to my parents, who supported me the entire time of my studies.

Lebenslauf

Sascha Hasan

Geboren am 7. Januar 1976 in Heidelberg

1982-1986	Grundschule Ludwigshafen/Oggersheim
1986-1995	Theodor-Heuss Gymnasium, Ludwigshafen
1995-1996	Zivildienst BG-Unfallklinik, Ludwigshafen
1996-2001	Biologiestudium an der Johann Wolfgang Goethe-Universitaet in Frankfurt am Main. Hauptfach: Genetik, Nebenfaecher: Neurobiologie und Physiologie
2001-2002	Praktikum an der University of California, Los Angeles, Department of Psychiatry
Seit Juni 2002	Externe Dokorarbeit in der Arbeitsgruppe von Prof. Dr. Yi Sun, University of California, Los Angeles, Department of Molecular and Medical Pharmacology
Seit Sept. 2004	Staff Research Associate II, University of California, Los Angeles, Department of General Surgery

Eidesstattliche Versicherung

Ich erklaere hiermit an Eides Statt, dass ich die vorgelegte Dissertation

“Leukemia inhibitory factor enhances neurogenin´s pro-neural effect during mouse cortical development”

selbststaendig angefertigt und mich anderer Hilfsmittel als der in ihr angegebenen nicht bedient habe.

Frankfurt am Main, den Sascha Hasan