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Academic dissertation

**Generation of cells for cell replacement therapy:
Specification of neural precursors *in vivo* and
in neural stem cell cultures**

by

Josephine Hebsgaard

*With the approval of the Faculty of Medicine at Lund University this thesis will
be defended on March 14, 2008 at 9.15 in Segerfalksalen,
Wallenberg Neuroscience Center, Lund, Sweden*

Faculty opponent

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Smilow Neuroscience Program and
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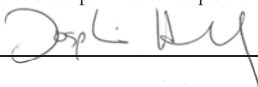


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Abstract <p>Cell replacement therapy of neurodegenerative disorders aims to substitute the degenerating cells with new functional neurons. Clinical trials with patients suffering from Parkinson's or Huntington's disease have provided proof-of-principle that neural precursors taken from the developing human brain can survive upon grafting to the diseased brain and provide long-lasting symptomatic relief. However, further development of this type of therapy critically depends on the generation of an unlimited and standardized source of neural precursors that after transplantation differentiate into the proper neuronal subtypes. This requires knowledge on the molecular mechanisms responsible for the specification of neurons during development, and how cells with the potential for regional specific neuronal differentiation can be expanded in culture. The work presented in this thesis is focused on the role of the proneural gene Neurogenin2 in specification of the midbrain dopaminergic (mesDA) neurons, the cell population that degenerate in Parkinson's disease. Additionally, we have studied to what extent neural stem cells isolated from the developing brain and expanded under growth-factor stimulation in culture maintain their regional specification. We show that Neurogenin2 is required <i>in vivo</i> for proper development of the mesDA neuron system, more specifically for the immature mesDA neuron precursors to adopt a neuronal fate. Furthermore, we successfully applied a new culture system for expansion of neural stem cells, the neural stem cell (NS cell) cultures, to neural precursors from different regions of the developing brain. We showed that even after extensive expansion cells in the NS cell cultures retain their capacity to form neurons. Furthermore, the expanded cells harbor regional differences in their growth properties and to some extent in their gene expression profile. This show that the NS cell culture system is an attractive alternative to the traditionally and more commonly used neurosphere culture system for expansion of fetal neural stem cells. Unfortunately, our investigations also showed that neither in the neurosphere nor in the NS cell culture system cells with the characteristic of mesDA neuron precursors are expandable. These results are valuable for further progression in neural stem cell research and particular for improvement of the existing protocols for generating mesDA neurons from expanded neural stem cells.</p>		
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Academic dissertation

**Generation of cells for cell replacement therapy:
Specification of neural precursors *in vivo* and
in neural stem cell cultures**

Josephine Hebsgaard

Neurobiology
Wallenberg Neuroscience Center
Department of Experimental Medical Science
Lund University, Lund, Sweden

Lund 2008



Neurobiology



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Cover

Neural stem cells from the developing ventral midbrain co-expressing the neural progenitor marker nestin (green) and the radial glial markers GLAST (red) and BLBP (blue) in a NS cell culture.

Cover artwork by Bengt Mattsson and Josephine Hebsgaard

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To Morten

*To dare is to lose one's footing momentarily.
To not dare is to lose oneself.*

Søren Kierkegaard

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Original papers and manuscripts

This thesis is based on the following papers that will be referred to by their roman numbers:

- I** Neurogenin2 identifies a transplantable dopamine neuron precursor in the developing ventral mesencephalon
Lachlan Thompson*, Elin Andersson*, Josephine B Jensen, François Guillemot, Malin Parmar and Anders Björklund. *Exp Neurol.* 2006, 198(1):183-98
- II** Development of the mesencephalic dopaminergic neuron system is compromised in the absence of Neurogenin2
Elin Anderson*, Josephine B Jensen*, Malin Parmar, François Guillemot and Anders Björklund. *Development* 2006, 133(3):507-16
- III** Striatal neuron differentiation from neurosphere-expanded progenitors depends on Gsh2 Expression
Josephine B Jensen, Anders Björklund and Malin Parmar.
J. Neurosci. 2004, 24(31):6958-6967
- IV** Regional differences in radial glia-like neural stem (NS) cell cultures obtained from subregions of the developing brain
Josephine B Hebsgaard*, Audrey Dufour*, Krisatian K Hansen, Anders Björklund and Malin Parmar. *Manuscript in preparation*

*Equal contribution

Summary

Cell replacement therapy of neurodegenerative disorders aims to substitute the degenerating cells with new functional neurons. Clinical trials with patients suffering from Parkinson's or Huntington's disease have provided proof-of-principle that neural precursors taken from the developing human brain can survive upon grafting to the diseased brain and provide long-lasting symptomatic relief. However, further development of this type of therapy critically depends on the generation of an unlimited and standardized source of neural precursors that after transplantation differentiate into the proper neuronal subtypes. This requires knowledge on the molecular mechanisms responsible for the specification of neurons during development, and how cells with the potential for regional specific neuronal differentiation can be expanded in culture. The work presented in this thesis is focused on the role of the proneural gene *Neurogenin2* in specification of the midbrain dopaminergic (mesDA) neurons, the cell population that degenerate in Parkinson's disease. Additionally, we have studied to what extent neural stem cells isolated from the developing brain and expanded under growth-factor stimulation in culture maintain their regional specification. We show that *Neurogenin2* is required *in vivo* for proper development of the mesDA neuron system, more specifically for the immature mesDA neuron precursors to adopt a neuronal fate. Furthermore, we successfully applied a new culture system for expansion of neural stem cells, the neural stem cell (NS cell) cultures, to neural precursors from different regions of the developing brain. We showed that even after extensive expansion cells in the NS cell cultures retain their capacity to form neurons. Furthermore, the expanded cells harbor regional differences in their growth properties and to some extent in their gene expression profile. This shows that the NS cell culture system is an attractive alternative to the traditionally and more commonly used neurosphere culture system for expansion of fetal neural stem cells. Unfortunately, our investigations also showed that neither in the neurosphere nor in the NS cell culture system cells with the characteristic of mesDA neuron precursors are expandable. These results are valuable for further progression in neural stem cell research and particular for improvement of the existing protocols for generating mesDA neurons from expanded neural stem cells.

Dansk populærvideenskabelig sammenfatning

Neurodegenerative sygdomme kendetegnes af tab af en eller flere typer nerveceller. I Parkinsons sygdom, der påvirker mere en 1% af befolkningen over 60 år, er det specifikt de dopaminproducerende nerveceller i mellemhjernen som dør. Nervecellerne anvender dopamin som signalstof og deres funktion er vigtig for at en person har et normalt bevægelsesmønster. Der findes ingen helbredelse for Parkinsons sygdom og de eksisterende behandlingsformer giver ofte alvorlige bivirkninger efter en 5-10-årig behandlingsperiode. Det specifikke tab af en type nerveceller i et lokaliseret hjerneområde åbner dog muligheden for at udvikle cellebaserede behandlingstyper for netop Parkinsons sygdom. Ideen bag denne form for behandling er at de celler som dør erstattes af nye nerveceller via transplantation af umodne stam celler til hjernen. Kliniske forsøg udført i Lund og andre steder rundt om i verden har vist at princippet fungerer, da patienter, som har modtaget umodne celler isoleret fra mellemhjernen af aborterede fostre, har opnået vedvarende symptomatiske forbedringer. Anvendelsen af væv fra aborterede fostre er dog yderst problematisk, både etisk men også ret praktisk, da mængden er meget begrænset og ikke mulig at standardisere. Etableringen af en cellebaseret behandling for Parkinsons sygdom eller for andre neurodegenerative sygdomme afhænger derfor i høj grad af udviklingen af kultursystemer til vækst af celler med potentiale for dannelsen af specifikke typer nerveceller efter transplantation til hjernen. I denne afhandling har vi undersøgt forskellige systemer for vækst af celler isoleret fra den embryonale hjerne og undersøgt i hvilken grad cellerne bevarer deres evne til at danne netop den type nerveceller som er karakteristisk for det område i hjernen de kommer fra, med fokus på de dopaminproducerende nerveceller i mellemhjernen. Vi har også undersøgt mekanismerne bag dannelsen af de dopaminproducerende nerveceller under fosterudviklingen. Denne type forskning er vigtig netop for udviklingen af alternative kilder af celler til at muliggøre en cellebaseret terapi for Parkinsons sygdom.

Abbreviations

bFGF	basic fibroblast growth factor
bHLH	basic helix-loop-helix
BLBP	brain lipid-binding protein
BrdU	bromo-deoxyuridine
CNS	central nervous system
CTX	cortex
DA	dopaminergic
DARPP-32	dopamine and cAMP-regulated phosphoprotein
E	embryonic day
EGF	epidermal growth factor
En	engrailed
ES	embryonic stem
FACS	fluorescence activated cell sorting
FGF8	fibroblast growth factor 8
GE	ganglionic eminences
GFP	green fluorescence protein
L-dopa	L-3,4-dihydroxyphenylalanine
LGE	lateral ganglionic eminences
mesDA	midbrain dopaminergic
MGE	medial ganglionic eminences
Ngn2	neurogenin2
NS cell(s)	neural stem cell(s)
P	postnatal day
SHH	sonic hedgehog
SN	substantia nigra
TH	tyrosine hydroxylase
VM	ventral midbrain
VTA	ventral tegmental area

Introduction

Cell replacement therapy for neurodegenerative disorders

Ideas and principles

Cell replacement therapy aims to substitute diseased or degenerating cells with new functional cells. The prospect of replacing dying neurons is particularly attractive for neurodegenerative and acute neurological diseases as the brain does not, for the most part, possess any regenerative capacity. Currently, only symptomatic treatments exist for neurodegenerative diseases such as Alzheimer's, Parkinson's and Huntington's disease. As age is the main risk factors for both Alzheimer's and Parkinson's disease, the two most-common neurodegenerative disorders, and the age of the world's population will increase over the coming decades these age-related diseases will become an increasing burden on our societies.

Parkinson's and Huntington's disease are ideal diseases for cell replacement therapy. Both diseases are slowly progressive and characterized by the loss of preferentially one type of neurons: the dopaminergic (DA) neurons of the midbrain in Parkinson's disease and GABAergic medium sized spiny neurons of the striatum in Huntington's disease. In both disorders open-label clinical trials have provided proof-of-principle that neural precursors taken from the developing brain can survive upon grafting to the diseased brain and provide long-lasting symptomatic relief (Lindvall et al., 1994; Freeman et al., 1995; Defer et al., 1996; Bachoud-Levi et al., 2000; Bachoud-Levi et al., 2006). However, further development of cell a replacement therapy for these diseases critically depends on generation of an unlimited and standardized source of transplantable neural precursors.

Parkinson's disease

James Parkinson was in 1817 the first to describe and document the symptoms of Parkinson's disease. The disease affects more than 1% of the population over 65 years, and classical symptoms of the disease are muscle rigidity, slowness of movements (hypokinesia) and resting tremor (Lang and Lozano, 1998b). In 5-10% of the cases Parkinson's disease is believed to have a genetic component, but most often the cause of the disease is unknown, termed idiopathic Parkinson's disease (Olanow and Tatton, 1999). Parkinson's disease results from a progressive loss of DA neurons in the midbrain. Small numbers of DA neurons also exist in other sites of the central nervous system (CNS), such as the olfactory bulb and the

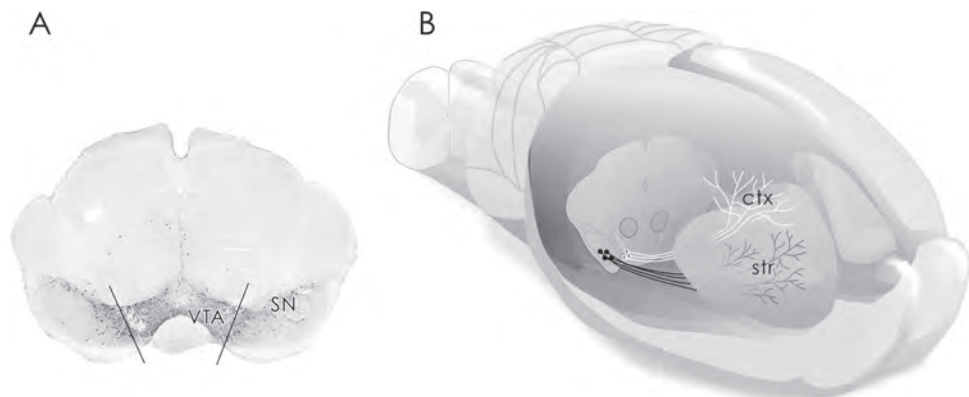


Figure 1. The DA neurons in the adult rat ventral midbrain as detected by TH expression (A). SN neurons are located laterally, VTA neurons medially. Schematic illustration of the projection of the SN and VTA neurons to the forebrain (B).

diencephalon, however, these neurons are not affected to the same extent as the midbrain dopaminergic (mesDA) neurons. The mesDA neurons are located in three nuclei: the substantia nigra (SN, cell group A9), the ventral tegmental area (VTA, cell group A10) and the retro-rubral area (cell group A8) (Dahlstrom and Fuxe, 1964; Hökfelt, 1984) (figure 1A). DA neurons in the SN project to the dorso-lateral striatum, and it is the loss of this innervation that is the leading cause of the debilitating motor symptoms in Parkinson's disease (Lang and Lozano, 1998b). The more medially located VTA neurons project to the limbic areas and parts of the cerebral cortex, and are implicated in motivational and cognitive behavior (figure 1B).

The standard treatment for Parkinson's disease is administration of the dopamine precursor L-3,4-dihydroxyphenylalanine (L-dopa), which is taken up by the remaining mesDA neurons and converted to dopamine, thus increasing dopamine signaling. In early- and mid-stages of the disease, symptoms are effectively alleviated by L-dopa treatment, however, as the disease progresses L-dopa loses its efficacy, and many patients develop severe side effects, such as involuntary movements (dyskinesias) (Lang and Lozano, 1998a; Ahlskog and Muenter, 2001). Cell replacement therapy aims to restore dopamine signaling by replacing the degenerating SN mesDA neurons with DA neurons grafted into the striatum –the normal target of the mesDA neurons. Studies done in rodents have shown that transplanted DA neuron precursors

can re-establish functional innervation and restore dopamine neurotransmission in the deinnervated striatum. The grafted neurons are spontaneously active, release dopamine in an impulse-dependent manner, and reverse motor impairments in animal models (Rioux et al., 1991; Nikkhah et al., 1994; Winkler et al., 1999; Winkler et al., 2000). Recent work from our laboratory, show that it is the mesDA neurons with characteristics of SN neurons that are responsible for the reinnervation of the striatum, which is in agreement with the knowledge that the striatum is the natural target of these neurons (Thompson et al., 2005). Together with the results from the clinical trials these results strengthen the belief that with the right type of cell, development of a cell-based therapy for Parkinson's disease is plausible.

Developmental neurobiology and cell replacement therapy

To develop unlimited and standardized sources of transplantable neural precursors that have the capacity to differentiate into mesDA neurons after grafting, it is crucial to be able to control the proliferation, specification and differentiation of these cells in cultures prior to transplantation. This requires knowledge on the molecular mechanisms responsible for the generation of the mesDA neurons during development, and also insight into how cells with the potential for mesDA neuron differentiation can be expanded in culture. The work presented in this thesis is focused on the role of the proneural gene *Neurogenin2* (*Ngn2*) in specification of the mesDA neurons, and on the possibility that neural stem cells from the developing CNS can maintain their regional specification after expansion *in vitro* under growth-factor stimulation. We have performed our studies in the mouse, as murine tissue allows for faster analysis due to more rapid growth and better availability of the tissue. Additionally, genetically modified mice enable mechanistic studies of genes of interest. Results obtained on mouse tissue need of course to be confirmed on a proper human tissue source, but the history has shown that results from basic developmental studies on rodents to a large extent can be applied also to human tissue.

Development of mesDA neurons

When the work of this thesis was initiated two groups of genes were known to be important for mesDA neuron development. The first group is important for the initial formation of the midbrain, and the second group for the terminal differentiation and survival of young postmitotic DA neurons present in this region. However, little was known about the mechanism of specification of the DA neurons within the ventral midbrain. Our research on the role of the proneural gene *Ngn2* in mesDA neuron development, combined with findings

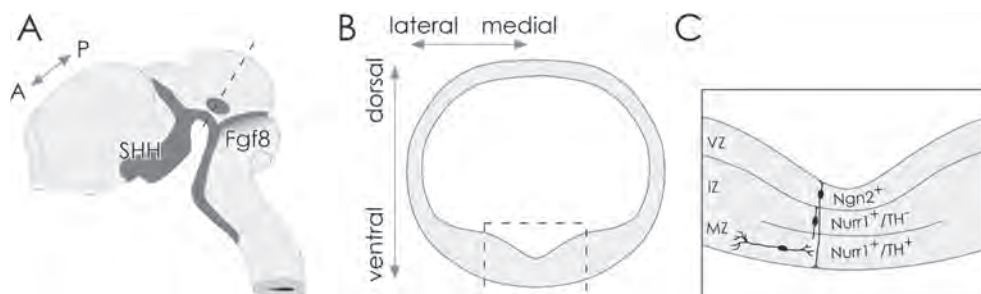


Figure 2. Schematic presentation of the DA neurons within the developing ventral midbrain (A-C). Sagittal view of the CNS, showing the location of the mesDA neurons in the direct vicinity of SHH and FGF8 secreting regions (A). Line in A indicates the section through the midbrain represented in B. Distribution of immature and differentiating DA neuron precursors and TH-expressing DA neurons in the ventral midbrain (C). A: anterior; P: posterior.

from other research groups, have within the past years dramatically increased the knowledge of how the DA neurons of the midbrain are specified.

Early specification of the ventral midbrain

The mesDA neurons develop in the ventral part of the midbrain in the immediate vicinity of the mid-hindbrain boundary and within the floor plate at the ventral midline (figure 2A). Early in development the mid-hindbrain boundary is positioned by the expression of two homeodomain transcription factors: *Otx2* (expressed in the fore- and midbrain) and *Gbx2* (expressed in the hindbrain) (Broccoli et al., 1999; Millet et al., 1999). The transcription factors *Engrailed* (*En*), *Pax2/5* and the diffusible factors *Wnt1* and fibroblast growth factor 8 (FGF8) are induced at the interface of the *Otx2* and *Gbx2* expression and their expression is critical for the further development of the mid-hindbrain region (Wurst et al., 1994; Bally-Cuif et al., 1995; Lee et al., 1997; Schwarz et al., 1997; Meyers et al., 1998). Ventrally, the floor plate secretes the ventralizing factor sonic hedgehog (SHH) that together with FGF8 position the DA neuron domain within the mid-hindbrain region (Hynes et al., 1995b; Hynes et al., 1995a; Ye et al., 1998).

Postmitotic development of mesDA neurons

In mice, the bulk of the mesDA neurons are born between embryonic day (E) 10.5 and 12.5, and tyrosine hydroxylase (TH), the rate-limiting enzyme in dopamine synthesis, is

detectable from around E10.5-E11 (Bayer et al., 1995; Kawano et al., 1995). The mesDA neurons are born at the ventral midline in the proliferative layer lining the ventricle, the ventricular zone. Unlike the situation at more posterior levels of the CNS the floor plate cells in the midbrain appear to be neurogenic and give rise to the DA neurons (Ono et al., 2007). When the precursors become postmitotic they migrate ventrally into an intermediate zone consisting of immature neuron precursors (figure 2C). At this stage the cells express *Nurr1*, an orphan receptor expressed by immature and mature DA neurons (Zetterstrom et al., 1996). As the neurons mature they start to express TH and continue their migration ventrally into the mantle zone and finally laterally to populate both the VTA and SN (Kawano et al., 1995). Loss-of-function studies have shown that genes such as *Nurr1*, *Pitx3*, *Lmx1b*, and *En1/2*, all expressed in immature and mature DA neurons, are important for the terminal differentiation and survival of the mesDA neurons (Zetterstrom et al., 1997; Saucedo-Cardenas et al., 1998; Smidt et al., 2000; Simon et al., 2001; Nunes et al., 2003; van den Munckhof et al., 2003; Alberi et al., 2004). *Pitx3* stands out by being the only known gene exclusively expressed by mesDA neurons in the brain. Furthermore, despite being expressed both by DA neurons of the VTA and SN, it is mainly the SN neurons that are lost in the absence of *Pitx3* (Nunes et al., 2003; Smidt et al., 2004; Maxwell et al., 2005).

MesDA neuron specification

In 2006 the groups of Perlmann and Ericson published a paper in which they identify two homeodomain transcription factors responsible for the specification of mesDA neurons, *Lmx1a* and *Msx1* (Andersson et al., 2006). Homeodomain transcription factors are known for determining the specification of neurons in various regions of the CNS through combinatorial expression patterns (Briscoe et al., 2000; Jessell, 2000). Within the ventral midbrain the expression of *Lmx1a* and *Msx1* is restricted to the DA neuron domain. Both genes are expressed in the mitotic cells of the ventricular zone and *Lmx1a* expression is induced by SHH one day before the first mesDA neurons are born. *Lmx1a* was identified as a mesDA neuron determinant, whereas *Msx1* appeared to be involved in the timing of DA neuron formation and repression of other neuronal fates by repression of *Nkx6.1*. Later studies have placed *Lmx1a* downstream of *Otx2* and shown that in contrast to *Otx2*, ectopic expression of *Lmx1a* in the floor plate of the mouse hindbrain is insufficient in generating neurons with a complete mesDA neuron phenotype (Prakash et al., 2006; Ono et al., 2007). *Otx2* has also been shown to repress the expression of the homeodomain transcription factor *Nkx2.2*, this repression is necessary for the formation of mesDA neurons (Puelles et al., 2004; Prakash et al., 2006). However, other factors are likely to be added to this signaling

cascade before the specification of the mesDA neurons are fully understood.

Proneural genes and neuronal commitment

In this thesis we studied the role of the proneural gene *Ngn2* in ventral midbrain development. The proneural genes, which include *Ngn1*, *Ngn2* and *Mash1*, are part of a family termed basic helix-loop-helix (bHLH) transcription factors (Lo et al., 1991; Guillemot and Joyner, 1993; Ma et al., 1996; Sommer et al., 1996). They promote neuronal differentiation (Fode et al., 2000; Nieto et al., 2001; Sun et al., 2001) and are in some cases also involved in neuronal subtype specification (Ma et al., 1999; Fode et al., 2000; Scardigli et al., 2001; Parras et al., 2002). The proneural genes are initially expressed at low levels in the dividing cells of the proliferative zones. As the level of expression increases, the cells become postmitotic and committed to a neuronal fate, after which the expression of the proneural genes is down-regulated (Bylund et al., 2003; Nguyen et al., 2006). The expression and function of the proneural genes have been studied in detail at the level of the forebrain and spinal cord, but at the time this work was started much less was known about their expression and function at the level of the midbrain.

Neural stem cell cultures

In the clinical trials discussed above, the cells used for transplantation were obtained from the developing brain of aborted fetuses. Each patient received tissue from 1-7 fetuses (Winkler et al., 2005), which is not only problematic ethically but also practically. Thus, for future development of cell replacement based therapies for any neurodegenerative disorder, there is a need for an unlimited on-demand source of transplantable cells that can be standardized and quality tested prior to transplantation. Cultured neural stem cells might hold this promise. Neural stem cells can be isolated and cultured from the adult and fetal CNS (Reynolds et al., 1992; Reynolds and Weiss, 1996); from differentiation of embryonic stem (ES) cells, that harbors the potential to form a new embryo (Lee et al., 2000; Tropepe et al., 2001; Ying et al., 2003); and from adult non-neural tissue stem cells (e.g. bone marrow stem cells) that “trans-differentiate” into neural stem cells (Brazelton et al., 2000; Mezey et al., 2000). The latter method, however, is still being questioned (Terada et al., 2002; Ying et al., 2002). ES cells are responsive to developmental factors and can be differentiated to various neuronal subtypes (Kawasaki et al., 2000; Lee et al., 2000; Wichterle et al., 2002; Bibel et al., 2004). However, undifferentiated ES cells, which often persist in the cultures upon differentiation, represent a risk for tumor formation upon transplantation to the brain (Bjorklund et al., 2002;

Morizane et al., 2006). Additionally, due to the broad differentiation potential of the ES cells, the differentiated cultures contain a mixture of neural as well as non-neural tissue. These aspects of the ES cultures, and the ethics related to destruction of potential life when the ES cultures are established, greatly complicate the use of this cell type. In this thesis, we have expanded neural stem cells derived from various region of the developing mouse brain using two different neural stem cell culture systems, the neurosphere cultures and the neural stem cell (NS cell) cultures.

Definitions of a neural stem cell

What is a neural stem cell? Reynolds and Weiss were the first to introduce a neural stem cell assay and thus among the first to define the requirements for neural stem cells. They defined the neural stem cell to be (1) a proliferative cell that can (2) self-renew, thus generate more of its own kind, and (3) generate a large number of progeny including all major cell types of the CNS, i.e. being multipotent (Reynolds and Weiss, 1996). This is a quite rigorous definition of a stem cell adapted from the hematopoietic system where the bone marrow stem cells can repopulate the entire system. However, the CNS consists of thousands of different subtypes of cells and is a solid tissue unlike the circulating blood. Proving that a stem cell can give rise to a broad range of cell types at all levels of the CNS is thus quite problematic and, in practice, impossible. Instead, the formation of the three major cell types of the CNS: neurons, astrocytes and oligodendrocytes became the standard read-out of multipotency *in vitro* (Reynolds and Weiss, 1996). Later, neural stem cells were more broadly defined as progenitor cells that can self-renew and produce differentiated progeny (Temple, 2001). For simplicity, I will refer to the cultured fetal neural progenitors, as neural stem cell cultures despite that they consist of a mixture of cells some of which can fulfill the rigorous and broader definitions of stem cells, as well as progenitors with limited potential for cell division.

Neural stem cells in the developing CNS

Neural stem and progenitor cells can be isolated from all levels of the developing CNS. The CNS develops from a tube structure, the neural tube, where the hollow part of the tube forms the ventricular system (Gilbert, 1997). During development cells with stem cell properties are situated along the ventricular wall in the proliferative ventricular zone. In some regions of the CNS, such as the forebrain, these cells are located also in a secondary proliferative zone, the subventricular zone, located immediately adjacent to the ventricular zone (Smart, 1961; Takahashi et al., 1993). To isolate the neural stem cells, the region of

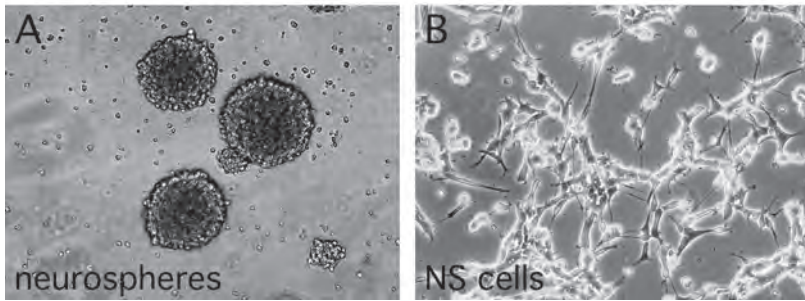


Figure 3. Examples of neural stem cells expanded as free-floating neurospheres (A) or in monolayered NS cell cultures (B).

interest is dissected and dissociated into a single cell suspension. The dissociated cells are then exposed to mitogen-stimulation, which promote the growth of the neural stem cells as well as more committed progenitors (Reynolds et al., 1992; Ray et al., 1993; Reynolds and Weiss, 1996). The most common method for expansion of neural stem cells is the neurosphere culture system introduced by Reynolds et al. in 1992.

The neurosphere culture system

Various methods with slight variations in protocol exist for establishing and expanding neurosphere cultures. In general, the starting population of cells is usually plated as a single cell suspension on uncoated plastic in serum-free medium, supplemented with a hormone mix (N2 and/or B27) and one of the two mitogens basic fibroblast growth factor (bFGF) or epidermal growth factor (EGF), or both (Vescovi et al., 1993; Morshead et al., 1994; Gritti et al., 1996; Reynolds and Weiss, 1996; Tropepe et al., 1999). The cells divide under these conditions and form free-floating spherical structures, neurospheres, which can be passaged into a single cell suspension and replated for the formation of new neurospheres (figure 3A). The neurosphere culture system was the first culture system to unequivocally demonstrate the presence of cells in the fetal and adult brain with characteristics of neural stem cells (Reynolds et al., 1992; Reynolds and Weiss, 1996) and is an extremely useful tool to analyze proliferation, self-renewal capacity, and multipotency of neural stem cells (Gritti et al., 1996; Tropepe et al., 1999; Uchida et al., 2000; Rietze et al., 2001; Jensen and Parmar, 2006).

In addition to the function as a neural stem cells assay, comparative studies of the developing brain and neurosphere cultures have shown that the neurosphere-expanded cells in several aspects behave like their *in vivo* correlates: The proliferative capacity and

differentiation potential of the cells after exposures to different external factors varies in a manner that is reflective of the developmental stage of the donor (Trobepe et al., 1999; Irvin et al., 2003). Further, the expression of a number of developmental control genes is maintained in a regional specific manner in the neurosphere cultures after several passages and the neurosphere-derived cells partially maintain the potential to generate the neuronal subtypes characteristic for their region of origin (Zappone et al., 2000; Hitoshi et al., 2002a; Ostenfeld et al., 2002; Parmar et al., 2002; Klein et al., 2005). Due to their relevance for treatment of patients with Parkinson's disease much effort has been put into generating mesDA neurons from neurosphere-expanded ventral midbrain cells. However, the potential for mesDA neuron differentiation does not appear to be maintained in the neurosphere culture system (Caldwell and Svendsen, 1998; Ostenfeld et al., 2002; Roybon et al., 2005; Andersson et al., 2007).

Unfortunately, the neurosphere culture system is not stable over time and the capacity to form neurons declines with the number of passages (Fricker et al., 1999; Morshead et al., 2002; Suslov et al., 2002; Parmar et al., 2003). Whether this is because progenitors with capacity to form neurons are outnumbered by non-neurogenic cells and thus progressively lost, or whether the cells within the neurospheres change their properties over time, is not clear. Furthermore, the neurospheres are highly heterogeneous in nature and only a small percentage of cells within each sphere hold the neurosphere-forming capacity (Reynolds and Weiss, 1996) and even fewer fulfill the stringent criteria of neural stem cells (Reynolds and Rietze, 2005). In fact, each neurosphere contains cells at various stages of differentiation, including stem cells, proliferating neural progenitors and immature neurons and astrocytes (Suslov et al., 2002; Parmar et al., 2003). This heterogeneity makes it hard to study any specific event or cell in isolation and studies using neurospheres should be seen and interpreted as studies on a mixed population of cells and not as studies of neural stem cells. Finally, while some researchers have been successful in obtaining a considerable number of neurons after transplantation of neurosphere-expanded cells into the two neurogenic areas of the brain, the hippocampus and the subependymal layer of the striatum (Flax et al., 1998; Fricker et al., 1999; Englund et al., 2002), only poor yields of neurons have been achieved when cells are engrafted into non-neurogenic areas such as the striatum (Svendsen et al., 1997; Winkler et al., 1998; Eriksson et al., 2003; Vroemen et al., 2003). The engrafted cells survive, but differentiate into glial cells, astrocytes and oligodendrocytes, rather than neurons.

The decline in neuron formation upon multiple passages and the poor yield of neurons after transplantation to the brain limit the use of the neurosphere cultures as a cell source for cell replacement therapy.

The NS cell culture system

In 2005, Conti and colleagues introduced a new culture system, the neural stem cell (NS cell) cultures (Conti et al., 2005). Like the neurosphere-expanded cells, the NS cells are grown in defined serum-free conditions in the presence of bFGF and EGF. However, rather than being expanded as free-floating aggregates of cells, the cells in the NS cell cultures are plated on an adhesive surface that allow them to attach and form a monolayer culture (figure 3B). Under these conditions a homogenous population of dividing radial glia-like neural stem cells are generated. For decades radial glia were believed mainly to function as support for radial migration of immature neurons out of the ventricular zone (Rakic, 1972; Levitt and Rakic, 1980), but in the early 2000s the radial glial cells were identified to function as neural stem and progenitor cells of the cerebral cortex (Malatesta et al., 2000; Noctor et al., 2001; Noctor et al., 2002), and later at all levels of the CNS (Anthony et al., 2004). The homogenous NS cell cultures can be extensively expanded (passaged more than 100 times, with 3-4 fold increase in cell number per passage) without changing their growth properties or their potential to form neurons (Conti et al., 2005). Clonal analysis has shown that the NS cells retain their self-renewal capacity and upon growth factor removal can generate both neurons and astrocytes. Later, the NS cell cultures were shown also to harbor the potential for oligodendrocyte formation (Glaser et al., 2007).

The NS cell cultures were initially generated from mouse ES cells, but cells from the developing human and mouse forebrain were also shown to be expandable in the NS cell culture system (Conti et al., 2005). A separate study applied the protocol to adult neural tissue (Pollard et al., 2006). However, these initial studies of the NS cell cultures did not address the question of to what extent neural stem cells from various region of the developing brain maintain their regional specification upon expansion, nor did they study to what extent NS cell cultures can generate subtype specific neurons after differentiation in culture or after transplantation to the brain.

Aims of the present thesis

The work of this thesis can be divided into two parts. In the first part (paper I and II) we have studied the role of the proneural gene *Ngn2* in mesDA neuron development and specification. In the second part (paper III and IV) we have investigated to what extent neural progenitors from the developing brain maintain their regional specification after growth-factor-stimulated expansion in culture. In the second part we have broadened our analysis to include not only cells from the ventral midbrain but also forebrain progenitors with focus on the cells that give rise to the projection neurons of the striatum.

Specific goals:

In part I:

- 1) To analyze the expression of *Ngn2* in the ventral midbrain during the formation of the mesDA neurons (paper I)
- 2) To following the fate of the *Ngn2*-expressing cells and study their potential for mesDA neuron formation (paper I)
- 3) To study the role of *Ngn2* in mesDA neuron development and specification through the analysis of mice lacking the *Ngn2* gene (paper II)

In part II:

- 4) To investigate if forebrain-derived neural progenitors retain the potential for striatal projection neuron formation after expansion in the neurosphere culture system (paper III)
- 5) To establish NS cell cultures from various regions of the developing brain and study the maintenance of regional specification (paper IV)
- 6) To study if mesDA neuron progenitors can be expanded in the two alternative neural stem cell culture systems (paper IV)

Results

Ngn2 expression in the developing ventral midbrain (paper I)

Immunohistochemical analysis of the expression pattern of Ngn2 showed that Ngn2 expression is spatially and temporally well correlated with the generation of DA neurons in the ventral midbrain. The Ngn2 protein can be detected in the ventricular zone in the medial part of the ventral midbrain as early as E10.5, *i.e.*, at a time point shortly before the first TH-expressing DA neurons start to appear. At E11.5, the Ngn2-expressing cells are found in the ventricular zone immediately overlying the newly formed TH-expressing DA neurons (figure 4A-B). At E15.5 after the last DA neurons are born, only single Ngn2-expressing cells were detected at the most caudal part of the ventral midbrain, consistent with the known rostral-to-caudal gradient of DA neurogenesis (Bayer et al., 1995).

Adjacent sections stained for Ngn2 and the proliferative marker Ki67, confirmed that the Ngn2-expressing cells are contained within the dorso-ventral boundary of the Ki67-positive proliferative ventricular zone (figure 4C-D). However, double staining with Ngn2 and Nurr1 showed that a small fraction of the most ventral located Ngn2-positive cells co-express the postmitotic DA neuron marker (paper II). When the Ngn2 expression was compared to that of Aldh1, an early DA neuron progenitor marker demarcating the DA neuron domain, we found that most of the Ngn2-expressing cells are located within the Aldh1-positive domain, and that the caudal boundary of Aldh1 expression also marks the caudal limit of Ngn2 expression. Notably, at E11.5 Ngn2 expression extends laterally, outside the Aldh1-expressing area (figure 4 C,E).

This expression pattern suggests a role for Ngn2 in the formation of the mesDA neurons.

Isolation of Ngn2-expressing cells and their progeny (paper I)

To study the fate of the Ngn2-expressing cells, we took advantage of a genetically modified mouse where the *Ngn2* gene is replaced by the gene coding for (enhanced) green fluorescence protein (*GFP*). In heterozygous mice carrying one *Ngn2* allele and one allele for *GFP* (*Ngn2-GFP* +/-) the native fluorescence of the GFP protein, expressed from the *Ngn2* locus, allowed us to isolate the Ngn2-expressing cells by fluorescence activated cell sorting (FACS). In the

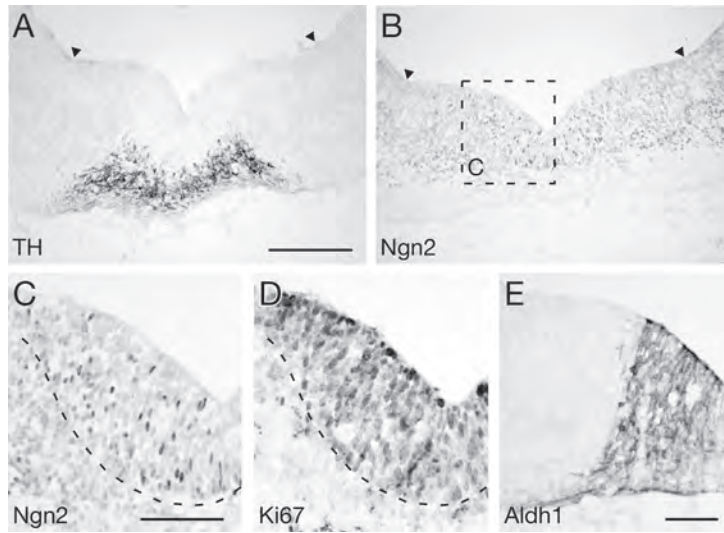


Figure 4. Ngn2-expressing cells (B,C) are located within the Ki67-positive ventricular zone (D) immediate dorsal to the TH-positive DA neurons (A). ALDH expression (E), which demarcates the DA neuron domain, shows that Ngn2 expression extend lateral to this (C,E). Scale bars: A-B, 1mm; C-E, 400µm.

ventral midbrain of *Ngn2*-GFP +/- embryos the expression of GFP correlates very nicely with that of Ngn2, however, high levels of GFP can also be detected in the Nurr1-positive/ Ngn2-negative intermediate zone. Even some TH-positive DA neurons contain low levels of the GFP protein. The maintenance of GFP labeling can be explained by higher stability of the GFP protein compared with that of Ngn2, and enables short-time fate-mapping of the Ngn2-expressing cells, showing that DA neurons are derived from this population of cell.

When cells from E12.5 ventral midbrain were sorted into GFP-negative and GFP-positive fractions and subsequently allowed to form neurons in culture, TH-positive DA neurons were exclusively generated from the GFP-positive fraction. The same result was obtained when the cells were transplanted into the striatum of newborn rats and the grafts analyzed 4 weeks later. The GFP-negative cell grafts contain barely any TH-positive neurons; all TH-positive neurons were located in the GFP-positive cell grafts. By contrast serotonergic (from contaminating hindbrain precursors), GABAergic and cholinergic neurons occurred almost exclusively in the GFP-negative cell grafts. Furthermore, the GFP-positive cell grafts did not show any expression of the astrocyte-marker GFAP and lacked the intensive labeling with the mouse specific M2M6 antibody mixture characteristic for glial cells, suggesting that the GFP-positive cell grafts did not contain this type of cells.

These results show that the mesDA neuron precursors indeed are confined to the population of *Ngn2*-expressing cells and their immediate progress and that other neuronal subtypes of the ventral midbrain such as GABAergic and cholinergic neurons are omitted from this population at E12.5.

Development and specification of mesDA neurons in the *Ngn2* mutant mice (paper II)

Reduction of mesDA neurons in the absence of Ngn2

We next studied the phenotype of mice lacking the *Ngn2* gene, which were obtained by crossing heterozygous *Ngn2*-GFP +/- mice to generate embryos where both alleles of the *Ngn2* gene were replaced with that of *GFP*. At E11.5 only single TH-positive cells were seen in 4 out of 7 *Ngn2* mutant embryos and at E13.5 the number of TH-positive neurons was approximately 1/10 of that seen in wild-type embryos (figure 5A-B). The TH-positive cells present in the *Ngn2* mutant were primarily located in thin stripes at the lateral edges of the DA neuron domain, whereas the medial part of the DA domain lacked TH-positive cells. Between E15.5 and E17.5, however, more TH-positive neurons had been generated and the discrepancy between wild-type and *Ngn2* mutant embryos with respect to both number and distribution of the TH-positive cells was less pronounced (figure 5C-D). At birth and postnatal day 18 (P18) the distribution of TH-positive cells was similar in wild-type and mutant mice, however, the number of TH-positive cells was markedly reduced both in VTA and SN (figure 5E-F). When the number of TH-positive cells was quantified at P18 the quantification revealed a 70% loss of mesDA neurons in the *Ngn2* mutant in both the VTA and SN.

To ensure that what we had observed was a true loss of mesDA neurons and not only a reduction in the expression of TH, we stained for two additional markers for DA neurons, aromatic amino acid decarboxylase (AADC) and vesicular monoamine transporter (VMAT2). Both markers showed the same reduction in cell numbers as TH (figure 5G-H), indicating an actual loss of DA neurons rather than a selective down-regulation of the *TH* gene itself. The number of Isl1- and Brn3a-positive cells marking two non-DA neuron nuclei of the ventral midbrain (Wallen et al., 1999; Agarwala et al., 2001), however, was not affected in the *Ngn2* mutant. Thus, the loss of *Ngn2* function within the ventral midbrain specifically affects the formation of DA neurons.

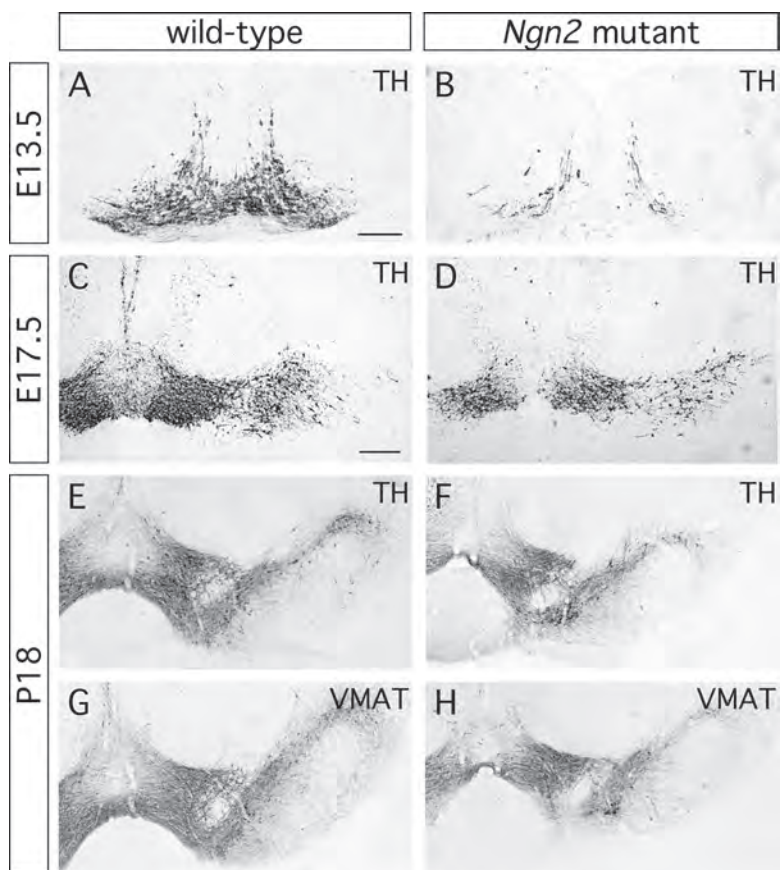


Figure 5. TH expression reveals a dramatic reduction of DA neurons at early- and mid-stages of DA neuron development in the *Ngn2* mutant embryos compared to wild type (A-B). Later in development, the DA neuron phenotype is partially rescued, and the discrepancy between wild-type and *Ngn2* mutant embryos with respect to number and distribution of the TH-positive cells is less pronounced (C-F). Same reduction in DA neuron number is detected with VMAT2, another DA neuron marker (E-H).

Correct specification of the remaining DA neurons

The initial lack of cells in the medial part of the DA domain could indicate that the DA neurons formed were not correctly specified into SN neurons laterally and VTA neurons medially. The TH-positive cells were correctly located in the midbrain after birth and the SN and VTA were equally affected. Additionally, the distribution of TH-positive fibers in the striatum and the adjacent limbic and cortical forebrain areas in the *Ngn2* mutant did not differ from that seen in wild-type littermates, suggesting that the DA neurons remaining in

the mutant SN and VTA were projecting to their appropriate target areas. Finally, Girk2 and calbindin, the two best-described markers of SN and VTA DA neurons (Liang et al., 1996; Schein et al., 1998; Thompson et al., 2005), respectively, were correctly expressed in the mesDA neurons of the *Ngn2* mutant. These results indicate that the mesDA neurons formed in the absence of *Ngn2* are correctly specified with respect to their specification into SN and VTA DA neurons.

In the ventral spinal cord *Ngn2* is required for the correct expression of a number of homeodomain transcription factors involved in neuronal subtype differentiation (Scardigli et al., 2001). However, the mesDA neurons of the mutant mice expressed the homeodomain factors *Pitx3* and *Engrailed1/2* to the same extent as in wild type. Together these data indicate that the mesDA neurons formed in *Ngn2* mutant mice are correctly specified.

The mechanism of *Ngn2* function in mesDA neuron development (paper II)

Further analysis of the *Ngn2* mutant showed a reduction in not only mature DA neurons but also in *Nurr1*-expressing DA neuron precursors situated in the intermediate zone. Similar to TH, we detected *Nurr1*-positive cells only at the lateral edge of the presumptive DA domain. Staining against cell nuclei (DAPI) revealed a dramatic reduction of cells in the mantle zone in the medial section of the DA domain, together with an accumulation of cells in the ventricular zone at E13.5. The presence of cells in the medial section was also visualized by the GFP expression from the *Ngn2* locus.

On closer examination, we noticed a displacement of GFP-positive cells towards the ventricle and a tendency of these cells to maintain contact with the ventricular surface in the mutant. These GFP-positive cells co-labeled with the radial glial marker GLAST (Shibata et al., 1997; Anthony et al., 2004) suggesting that the absence of *Ngn2* leads to an increase in the number of radial glial neural progenitor cells. However, the number of dividing cells within the medial section was not increased in the mutant, as detected by Ki67 and incorporation of the thymidine analog bromo-deoxyuridine (BrdU). This indicates that, despite the increase in radial glia, the accumulating cells in the medial section of the *Ngn2* mutant are postmitotic. At the same time, none of the accumulating cells expressed PSA-NCAM or GFAP, which speaks against the presence of neurons or astrocytes, respectively.

The expression of TH showed an initial loss of DA neurons followed by a partial recovery between E15.5 and E17.5. To investigate if the later-formed neurons were generated from a retained pool of dividing cells, we exposed embryos to BrdU at E15.5 and E16.5, i.e. at time-points when mesDA neurogenesis is complete in wild-type mice. At birth, no BrdU/TH doubled labeled mesDA neurons were observed in either wild-type or *Ngn2* mutant mice. The

increase in mesDA neurons between E15.5 and E17.5, therefore, is unlikely to be explained by a prolonged neurogenesis, beyond E15.5.

Together, these results suggest that in the absence of Ngn2 most precursors within the mesDA domain are initially arrested in their differentiation at an early postmitotic stage when they have not yet acquired the characteristics of neuronal precursors. A fraction of these cells appear to maintain radial glial characteristics. Eventually, some of these precursors appear to be released from this arrest.

Regional specification of neurosphere-expanded precursors (paper III)

Generation of striatal projection neurons from expanded forebrain cultures

Previous studies from our laboratory had shown that neural progenitors from the lateral ganglionic eminences (LGE), the ventral part of the forebrain known to give rise to olfactory bulb interneurons and projection neurons of the striatum (Deacon et al., 1994; Olsson et al., 1995; Olsson et al., 1998; Wichterle et al., 2001), only partially maintain their native differentiation potential after expansion in neurosphere cultures (Parmar et al., 2002). The neurosphere-expanded LGE cells generate neurons with characteristics of olfactory bulb interneurons but not striatal projection neurons (Parmar et al., 2002). This suggests that either the progenitors of striatal projection neurons are not expandable in culture, or the required developmental cues for striatal neuron differentiation are not present in the standard differentiation paradigm.

To test the latter hypothesis, neurosphere-expanded cells were differentiated in coculture with primary cells directly isolated from the developing LGE, as these primary cultures are known to generate striatal projection neurons (Ivkovic and Ehrlich, 1999; Toresson et al., 1999). Neurosphere-expanded LGE cells, differentiated in coculture with primary cells, expressed both the dopamine and cAMP-regulated phosphoprotein (DARPP-32) and Isl1, factors expressed by the striatal projection neurons and their precursors, respectively (Anderson and Reiner, 1991; Toresson et al., 2000; Toresson and Campbell, 2001). Thus the neurosphere-expanded LGE cells do harbor the potential to generate neurons with the characteristics of striatal projection neurons.

Genetic evidence suggests that both striatal projection neurons and olfactory bulb interneurons are derived from Gsh2-expressing progenitors of the LGE ventricular zone (Corbin et al., 2000; Toresson et al., 2000; Toresson and Campbell, 2001; Yun et al., 2001;

Yun et al., 2003). The expression of *Gsh2* is maintained in the neurosphere-expanded LGE cells, and in order to examine if the potential for striatal neuron differentiation depends on the presence of *Gsh2*-expressing progenitors, we made use of a genetically modified mouse lacking *Gsh2* (Szucsik et al., 1997). The neurospheres generated from the *Gsh2* mutant showed no major changes in growth or neuron formation when compared to wild-type-derived cultures. However, neither *Isl1* nor *DARPP-32* expression could be detected in cells derived from the *Gsh2* mutant after differentiation in coculture with primary LGE cells. Thus, in the absence of *Gsh2* the potential to generate neurons with characteristics of striatal projection neurons is compromised in the neurosphere-expanded LGE cultures, as is the case in the forebrain during development (Corbin et al., 2000; Toresson et al., 2000; Toresson and Campbell, 2001; Yun et al., 2001; Yun et al., 2003).

Nature of the inducing signal present in the cocultures

To investigate the nature of the developmental cues directing the striatal fate we tested: (1) the effect of specific candidate proteins, (2) whether the developmental cues in the primary cultures were contact-dependent, and (3) the importance of region of origin of the primary cells.

Factors such as retinoic acid (RA), brain-derived neurotrophic factor (BDNF), SHH, and neurotrophin-3 (NT3) have been shown to increase the number of *Isl1* and/or *DARPP-32*-expressing cells in tissue slices and primary cultures (Roelink et al., 1995; Ivkovic et al., 1997; Kohtz et al., 1998; Dutton et al., 1999b, a; Ivkovic and Ehrlich, 1999; Toresson et al., 1999; Kohtz et al., 2001). These factors alone or in combination, however, were insufficient to promote *Isl1* or *DARPP-32* expression in the differentiated LGE-derived neurosphere cultures. We were also unsuccessful in promoting a striatal neuron fate when the neurospheres were differentiated in conditioned medium from primary LGE cells, or differentiated in coculture with, but physically separated from, the primary cells. Finally, only primary cells isolated from the LGE, and to a lesser extent the neighboring medial ganglionic eminences (MGE), but not those isolated from the developing cortex or ventral midbrain, were found to provide the factors necessary to guide striatal projection neuron differentiation.

Taken together, these data indicate that the factors necessary to guide striatal projection neuron differentiation are provided by the primary cells isolated from the LGE in a contact-dependent and region-specific manner.

Regional specification in the NS cell culture system (paper IV)

Expansion of fetal neural progenitors in the NS cell culture system

Neurospheres had been used in our laboratory for many years as the standard method of expanding neural progenitors from the developing brain. However, because of the decline in neuronal yield upon passage (Fricker et al., 1999; Morshead et al., 2002; Suslov et al., 2002; Parmar et al., 2003) and the general poor neuronal differentiation after transplantation to the brain (Svendsen et al., 1997; Winkler et al., 1998; Eriksson et al., 2003; Vroemen et al., 2003), we decided to apply the new NS cell culture system to our fetal tissue source.

We successfully established NS cell cultures from three subregions of the developing mouse brain: cerebral cortex, ganglionic eminences (GE)(at a stage, E11.5, where LGE and MGE cannot be distinguished) and ventral midbrain (VM). The NS cell cultures from all three regions showed stable growth over an extensive number of passages (currently above passage 100). As described in the original paper, the cultures were highly homogeneous in their morphology and the vast majority of the cells expressed the radial glial markers GLAST (Shibata et al., 1997), and brain lipid-binding protein (BLBP)(Feng et al., 1994), and the neural progenitor markers nestin (Lendahl et al., 1990) and Sox2 (Rex et al., 1997). Conversely, the expression of GFAP and β -III-tubulin was negligible, indicating that under growth-factor-stimulation the cultures did not contain differentiated astrocytes or neurons, respectively. When we applied the published differentiation protocol to our fetal NS cell cultures we experienced extensive cell death, and consequently obtained only a small number of neurons at the end of the differentiation period. However, by optimizing the differentiation protocol we managed to limited the cell death and consistently obtained 30-50% neurons independent of the region of origin. The neurons formed have a mature morphology and express the two neuronal markers tested β -III-tubulin and Map2. This potential for neuronal differentiation does not decline with passages, as observed in cultures passaged more than 60 times.

Regional differences across the region of origin

There were clear regional as well as temporal differences in the behavior of the cells during the establishing phase of the NS cell cultures. Cells from the E10.5 and E11.5 ventral midbrain easily attached and formed homogenous NS cell cultures, whereas cells from the E15 cortex were more prone to generate free-floating spheres, a feature also present in the established cultures when grown at high density. Because of lack of attachment we were unsuccessful in generating NS cell cultures from cortex before E15 and from LGE at E12.5

and E13.5. Despite being morphologically alike, the cortical NS cells also appeared with smaller soma and longer and thinner processes than GE- and VM-derived NS cells.

Staining for regional markers during the expansion phase, showed that the expression of most regional markers are lost in the NS cell cultures. The cortical expression of Pax6, however, is both maintained in and restricted to the cortical-derived NS cell cultures. After differentiation differences exist between the different regions of origin with respect to the morphology of the neurons formed and their expression of regional markers, but the types of neurons generated are not always reflective of the region of origin. As with the neurosphere cultures DA neurons were not generated from the VM-derived NS cell cultures upon differentiation, although both differentiated VM- and GE-derived NS cell cultures express Nurr1.

Thus although lacking the expression of many classical regional markers, the NS cell cultures show some differences across the three regions of origin with respect to cell adhesion, regional marker expression and neuronal subtype differentiation.

Growth-factor-stimulated expansion of mesDA neuron progenitors (paper IV)

As DA neurons are neither formed in the neurosphere nor in the NS cell culture system, we next studied if progenitors with the characteristic of DA neuron progenitors are expanded in the current culture conditions at all. In this experiment cells were isolated from E10.5 ventral midbrain, to ensure that the starting material contained dividing DA neuron progenitors. Directly after dissection a significant fraction of the cells co-expressed the homeodomain transcription factors Lmx1a and Foxa2, the latter expressed by the floor plate cells and in the cell populations lateral to the DA neuron domain (Ferri et al., 2007; Ono et al., 2007). However, already in the unpassaged neurospheres and NS cells the number of positive cells was dramatically reduced. After the first passage both factors were undetectable in both culture systems. Additionally, in both culture system the vast majority of the cells expressed the radial glial and neural progenitor markers GLAST, BLBP and nestin before the first passage.

These results suggest that cells with DA neuron progenitor characteristic are lost in the two culture systems already at the first passage, even when isolated at an early stage of DA neuron development.

Discussion

Discussion

In the first part of this thesis we show that *Ngn2* is expressed in a pattern correlating with the generation of mesDA neurons and that cells with the potential for generating DA neurons in culture and after transplantation to the brain are contained within the *Ngn2*-expressing cells and their immediate progeny. Analysis of mice lacking the *Ngn2* gene showed that *Ngn2* is required for proper development of the mesDA neurons. In its absence, the mesDA neurons are correctly specified, although, their numbers dramatically reduced. In the *Ngn2* mutant embryos the mesDA neuron precursors are retained at an undifferentiated stage where they have not yet acquired a neuronal fate. In the second part, we show that regional differences persist in fetal neural stem cells expanded as neurospheres or in the new NS cell culture system. Our analysis, furthermore, showed that the environment of the standard culture system is insufficient in revealing the full differentiation potential of the expanded cells. For example, neurons with characteristics of striatal projection neurons are only formed from neurosphere-expanded cells when they are differentiated in contact with cells directly isolated from the developing striatum. We also show that the NS cell cultures offer several advantages over the neurosphere culture system when it comes to extensive expansion of fetal neural stem cells with sustained potential for generating a high yield of neurons upon differentiation. Finally, we show that both in the neurosphere and in the NS cell culture system cells isolated from the developing ventral midbrain lose their mesDA neuron precursor characteristics upon expansion.

***Ngn2* in mesDA neuron development**

Ngn2 in the molecular pathway of mesDA neuron specification

A number of developmental genes, such as *En1/2*, *Lmx1b*, *Pitx3* and *Nurr1*, are expressed in the DA neuron domain during development, and has been shown to be of major importance for the formation of the mesDA neuron system (Zetterstrom et al., 1997; Smidt et al., 2000; Simon et al., 2001; van den Munckhof et al., 2003). For the most part, however, these genes are expressed at postmitotic stages and are important for the terminal differentiation, maintenance, or survival of the mesDA neurons. When we published our analysis on *Ngn2*, it was the first example of a gene expressed in the ventricular zone progenitors of the ventral midbrain, where loss of function caused impaired mesDA neurogenesis without affecting other types of neurons within the ventral midbrain. After our study, the study on *Lmx1a*

and *Msx1* was published, identifying *Lmx1a* as a mesDA neuron determinant and *Msx1* as a downstream target of *Lmx1a* important for the timing of mesDA neuron neurogenesis (Andersson et al., 2006). Interestingly, *Msx1* up-regulates the expression of *Ngn2*, and in mice lacking the *Msx1* gene the number of both *Ngn2*- and *Nurr1*-expressing cells is reduced to 60%, suggesting that these three genes act in the same molecular cascade (figure 6).

Specific requirement for Ngn2 in mesDA neuron formation

In parallel with our analysis of *Ngn2* expression, Kele et al. 2006 performed a similar analysis including the expression of two additional bHLH transcription factors, *Mash1* and *Ngn1*. Their analysis showed that *Mash1*, but not *Ngn1*, is expressed in the DA neuron domain of the ventral midbrain (Kele et al., 2006). Of notice, *Mash1* is expressed in the ventricular zone of the entire midbrain, in contrast to *Ngn2* that shows a much more restricted expression pattern (Nakatani et al., 2007). Furthermore, Kele et al. showed that the partial rescue of the DA neuron phenotype between E15.5 and E17.5 depends on the expression of *Mash1*, as this rescue does not occur in mice lacking the genes for both *Ngn2* and *Mash1*. On the other hand, *Mash1* is not required for mesDA neuron formation as this process is unaffected in *Mash1* mutant mice (Kele et al., 2006).

Our analysis showed that the mesDA neurons formed in the *Ngn2* mutant are correctly specified both with respect to their expression of homeodomain transcription factors and their specification into SN versus VTA DA neurons. Yet, mice expressing *Mash1* from the *Ngn2* locus still lack about 50% of the mesDA neurons (Kele et al., 2006), showing that *Mash1* cannot fully substitute for *Ngn2*. *Ngn2* does, therefore, appear to have a unique role in mesDA neuron formation in addition to its proneural activity. The *Ngn2*-knock-out-*Mash1*-knock-in mice were not studied after birth, and it is therefore unknown if the mesDA neurons generated in these mice are correctly specified. A more detailed analysis needs to be performed to reveal how the activity of *Ngn2* and *Mash1* differ in the differentiation and specification of the mesDA neurons.

Mechanism of Ngn2 function in the development of the mesDA neurons

The results from our FACS sorting experiments where the *Ngn2*-GFP-positive and -negative cells were separated indicate that precursors expressing *Ngn2* are committed to a neuronal fate as no glial cells could be detected in cell grafts from the GFP-positive cell fraction (the cells expressing *Ngn2* or their immediate progeny). All *Ngn2*-expressing precursors, however,

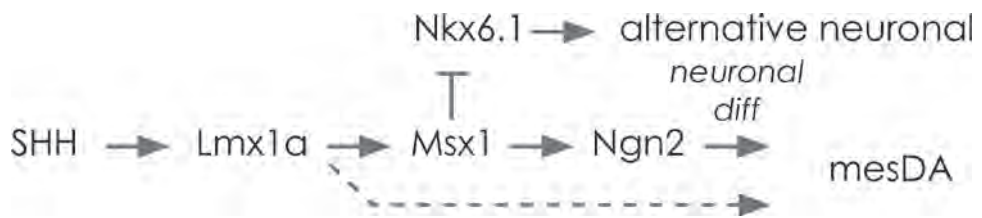


Figure 6. Schematic presentation of *Ngn2* in the molecular pathway of *Lmx1a* and *Msx1* in mesDA neuron development.

are not fully committed to a DA neuron fate, as only a small fraction of the cells within the graft become DA neurons. That *Ngn2* is involved in promoting neuronal differentiation and commitment of precursors to a neuronal fate is supported by the loss of *NeuroD* expression, a neuronal determinant (Lee et al., 1995), in the DA neuron domain of the *Ngn2* mutant.

In a simple model of neurogenesis, an increased number of dividing cells would be a logical consequence of the delayed neuronal differentiation in the *Ngn2* mutant. We detected an increase in the number of cells along the ventricle in the DA neuron domain and a tendency for these to maintain the expression of the radial glia marker GLAST. However, we did not detect an increase in the number of dividing cells. In agreement with these results, no increase has been detected in the number of proliferating cells in the cortex of the *Ngn2* mutant (Britz et al., 2006) and the number of dividing cells is reduced in the forebrain of the *Mash1* mutant (Casarosa et al., 1999; Horton et al., 1999). In the *Mash1* mutant the reduction was explained by a disruption of the Notch signaling pathway. Notch keeps progenitors in an undifferentiated radial glial state (Gaiano et al., 2000; Hitoshi et al., 2002b) partially by upregulating the expression of the *Hes* genes, which repress the expression of the proneural genes (Sasai et al., 1992; Ishibashi et al., 1995; Jarriault et al., 1995). Contrary, the proneural genes activate the expression of Delta-like, a ligand of Notch (Bettenhausen et al., 1995; Ma et al., 1996)(figure 7). This signaling loop is important for the balance between proliferation and differentiation. The differentiating cells stimulate the Notch pathway in the neighboring cells and thus prevent them from differentiating, creating a salt-and-pepper-pattern of immature progenitors and committed neuronal precursors. Kele et al. showed a loss of Delta-like1 and *Hes5* expression in the *Ngn2* mutant at E11.5 (Kele et al., 2006), together with the lack of increased number of dividing cells, this indicates that the salt-and-pepper-pattern of precursors is disrupted in the DA neuron domain in the absence of *Ngn2*.

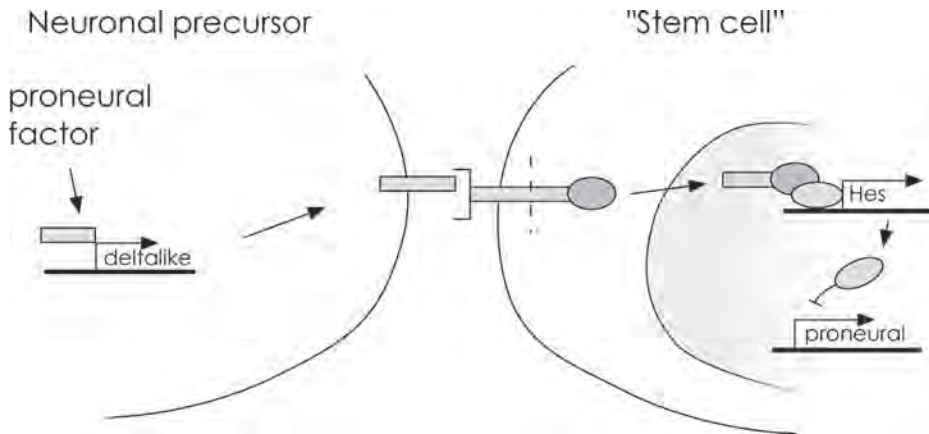


Figure 7. Schematic presentation of the Notch-signaling pathway in immature and differentiating neural precursors.

Thus, Ngn2 is likely to work downstream of Lmx1a and Msx1 in the specification of mesDA neurons and is specifically required for the DA neuron precursors to adopt a neuronal fate. In the absence of Ngn2 the number of mesDA neurons are dramatically reduced and the natural balance between differentiating and undifferentiated precursors in the ventricular zone of the DA domain is disrupted.

Mitogen-stimulated expansion of fetal neural stem cells

Regional specification of cultured fetal neural stem cell

Previous studies from our and other groups have shown that fetal neural stem cells partially maintain their regional specification after expansion in the neurosphere culture system (Yamamoto et al., 2001; Hitoshi et al., 2002a; Ostenfeld et al., 2002; Parmar et al., 2002). If this is true for the NS cell culture system, however, had not been studied. Our results show that clear differences exist between NS cell cultures isolated from different regions of origin. As for neurospheres (Ostenfeld et al., 2002), differences exist in the growth characteristics of the NS cell cultures with respect to cell attachment and cell morphology across different regions of origin. Additionally, differences were detected in the expression of regional markers during expansion and in the neuronal subtypes formed after differentiation. These differences in gene protein expression, however, are few and the neuronal subtypes formed do not necessary reflect the region of origin. Thus, although the NS cell cultures have

been suggested to consist of only unspecified immature symmetric dividing stem cells (Conti et al., 2005), our data indicate that the fetal-derived NS cells retain regional differences also after long-term expansion.

Neurosphere-expanded LGE cells were known to maintain the potential to generate olfactory bulb interneurons, but appeared to lose the potential for striatal neuron differentiation (Parmar et al., 2002). Interestingly, our analysis of neurosphere-expanded cells from the LGE showed that these cells to a greater extent than previously reported retain their native differentiation potential. When differentiated in coculture with primary cells directly isolated from the LGE, the neurosphere-expanded cells generated cells and neurons expressing Isl1 and DARPP-32, two proteins expressed in immature and mature striatal projection neurons, respectively (Anderson and Reiner, 1991; Toresson et al., 2000; Toresson and Campbell, 2001). Thus, the neurosphere-expanded LGE cells do retain the potential for striatal neuron differentiation. Additionally, we showed that this potential depends on the presence of Gsh2-expressing ventricular-zone-like progenitors in the expanded cultures. The developmental cues provided by the primary cells were shown to be contact-mediated and region-specific. These results draw attention to the fact that the environment the cells encounter during differentiation has a major impact on the differentiation potential revealed and that simple culture systems are unlikely to reveal the full differentiation potential of a cell.

Neurosphere cultures versus NS cell cultures

Some of the drawbacks of the neurosphere cultures have been the relatively limited yield of neurons upon differentiation, the instability of the system, and the heterogeneous cell composition of the individual neurospheres (Jensen and Parmar, 2006). In our study on NS cells, we show that the NS cell culture system is an efficient system for expansion of progenitors derived from different regions of the developing brain. The NS cell cultures are highly homogenous consisting of radial glia-like neural progenitors. Upon differentiation, these cultures, independent of region of origin, consistently give rise to 30-50% neurons, which is significant higher than what is obtained from neurosphere-expanded cells. Furthermore, this potential for neuronal differentiation is maintained even after more than 50 passages (equals more than 10^{12} -fold expansion), showing that the NS cell culture system is stable over time. For comparison, most neurosphere studies are conducted at passage 8, or lower.

The regional analysis of the NS cell cultures showed that the NS cells retain some regional differences, but whether they retain it to the same extent as in the neurosphere cultures

cannot be determined from our analysis. It is not unlikely that the homogenous population of immature cells in the NS cell cultures increases the probability of losing regional specification compared to the heterogeneous neurosphere cultures where there is a mixture of immature and mature cell types. However, a comparative and more comprehensive analysis would be required to draw any conclusions on this issue, including studies of differentiation in the presence of developmental factors, in coculture with primary cells, or on tissue slices.

Nevertheless, the NS cell culture system appears superior of the neurosphere culture system for expansion of fetal neural stem cells with respect to homogeneity, stability of the system, and the differentiation potential of the expanded cells.

Expansion of mesDA neuron progenitors

Neural progenitors from the ventral midbrain are known to be expandable in the neurosphere culture system, but the multi-passaged cells show limited ability to generate DA neurons (Caldwell and Svendsen, 1998; Yan et al., 2001; Roybon et al., 2005; Chung et al., 2006; Andersson et al., 2007). We show that ventral midbrain progenitors can be expanded as NS cell cultures and give rise to 30-40% neurons in cultures after prolonged expansion. However, the NS cells are, as previously reported for neurosphere-expanded cells, unable to generate DA neurons under standard differentiation conditions. Furthermore, our analysis of the expression of *Lmx1a* and *Foxa2* in unpassaged and single-passaged neurospheres and NS cell cultures showed that cells with DA neuron progenitor characteristics are lost already at the first passage in both culture systems. These results can explain the limited success in generating DA neurons from multi-passaged cultures, and underlines that alternative strategies for expansion of mesDA neuron progenitors need to be developed.

If we take a more critical look at the mitogens used, the EGF receptor is undetectable in the ventricular zone cells at early stages of the mesDA neuron development (Kornblum et al., 1997) and in the rat, cells first become EGF responsive after E12 (equivalent to E10.5 in mice) (Mytilineou et al., 1992; Bouvier and Mytilineou, 1995). However, at E10.5, when we isolate cells for expansion, the cells are bFGF responsive. Unfortunately, bFGF is known to promote EGF-responsiveness (Ciccolini and Svendsen, 1998; Santa-Olalla and Covarrubias, 1999), indicating that the cells we expand in our culture systems are likely to have the characteristics of the later-stage expandable progenitors. Therefore, in order to expand the mesDA neuron progenitors present in the E10.5 ventricular zone one might have to use alternative mitogens. Three FGFs (FGF8, 17, and 18) are expressed at the mid-hindbrain

boundary, and signaling through their receptors is important for the proliferation of the early mesDA neuron progenitors during development (Saarimäki-Vire et al., 2007). Additionally, *Wnt1* mutant mice show a dramatic reduction in the number of proliferating cells in the ventral midbrain (McMahon et al., 1992; Prakash et al., 2006). Finally, SHH not only has a ventralizing effect but also a mitogenic effect (Agarwala et al., 2001). FGF8 and Wnt1 have been shown to increase proliferation of mesDA neuron progenitors in short-term expanded cultures (Studer et al., 2000; Castelo-Branco et al., 2003). However, if any of these proteins can maintain dividing mesDA neuron progenitors in a multi-passaged culture system is as yet unexplored.

Cells for cell replacement therapy

Transplantable DA neuron precursors from neural stem cell cultures

How can the results of this thesis have any implication for the development of cells for cell replacement therapies? First, our findings on the role of Ngn2 in midbrain development, together with the recent findings from other groups, have contributed to a better and more detailed understanding of the specification and differentiation of the mesDA neurons. This knowledge can be used to develop improved protocols for direction of mesDA neuron differentiation from expanded neural stem cells. ES cells, which can give rise to all cell types in the embryo, are particularly responsive to treatment with different types of specifying factors, soluble as nuclear, and have successfully been directed into spinal cord motoneurons (Wichterle et al., 2002), cortical pyramidal (Bibel et al., 2004) and DA neurons (Kawasaki et al., 2000; Lee et al., 2000). Today, three main protocols exist for generating DA neurons from ES cells, all of which include SHH and FGF8 in the culture medium (Lee et al., 2000; Barberi et al., 2003; Ying et al., 2003). However, contaminating undifferentiated tumorigenic ES cells remain a serious problem after transplantation (Bjorklund et al., 2002; Morizane et al., 2006). Secondly, even though DA neurons can be generated from ES cells, few studies have shown that they successfully can generate bona fide mesDA neurons (Andersson et al., 2006; Rodriguez-Gomez et al., 2007), which can be of major importance for ability of the transplanted cells to reinnervate the striatum, and thus have major impact on the functional outcome after DA neuron transplantation. Increased knowledge on mesDA neuron development will help to refine the existing protocols, both with respect to the specification of the DA neurons formed and possibly also for synchronization of the differentiation process to limit the number of undifferentiated ES cells and increase the yield of transplantable neural precursors at the time of grafting. One strategy to eliminate the undifferentiated tumorigenic ES cells is to isolate the DA neuron precursors by FACS. Our transplantation study showed

that the *Ngn2*-GFP-expressing cells are committed to a neuronal fate and are sufficiently immature to survive the isolation and transplantation procedure, and could thus provide a useful tool for isolation of non-tumorigenic transplantable precursors from differentiated ES cells. With respect to neural stem cells isolated from the fetal ventral midbrain, our research shows that cells with the characteristic of mesDA neuron progenitors are lost upon EGF- and bFGF-stimulated expansion in both cultures system tested. Additionally, expanded fetal neural stem cells appear less responsive to the native developmental factors (Yan et al., 2001; Roybon et al., 2005; Andersson et al., 2007). Therefore, the usefulness of expanded fetal neural stem cells as a source of mesDA neurons will most likely depend on the development of optimal culture systems for long-term expansion of mesDA neuron progenitors.

Fetal neural stem cells in cell replacement therapy

The coculture differentiation paradigm used in this thesis provides a model for studies of striatal neuron differentiation from growth-factor-expanded neural stem cells. In the past, studies addressing similar questions for primary cells have successfully been addressed in transplantation studies (Gaiano and Fishell, 1998; Campbell and Olsson, 2000). However, grafting experiments with expanded fetal cells have been less informative because of the limited neuronal yield after transplantation of neurosphere-expanded cells to the brain (Winkler et al., 1998; Eriksson et al., 2003). This limitation is not seen in the coculture system. Thus, the coculture paradigm can be used as a simple, easy manipulative and timesaving alternative to transplantation for studies on striatal neuron differentiation from growth-factor-expanded fetal neural stem cells. Such studies could in the longer perspective have implication for the development of new therapies for disorders such as Huntington's disease and stroke, where there is a loss of striatal projection neurons.

The low yield of neurons after transplantation of neurosphere-expanded fetal neural stem cells to the CNS has been a returning problem (Winkler et al., 1998; Eriksson et al., 2003), and has limited their usefulness as a cell source for cell replacement therapy. ES-derived NS cells have been reported to generate 40% neurons after transplantation to the adult brain (Conti et al., 2005), but the study did not include a description of the neuronal morphologies or any functional data. More thoroughly analyses, therefore, need to be performed to determine if the NS cell cultures are suitable as a source for cell replacement therapy.

Thus, both ES-derived and fetal neural stem cells harbor the potential to serve as a source of transplantable precursors. It is, therefore, important to continue increasing our knowledge

on neuronal subtype specification and applying this on different culture systems. The new knowledge should also continuously be applied to human tissue to as efficient as possible move towards the final goal of bringing cell replacement therapies of neurodegenerative diseases to the clinic.

Methods and Materials

Methods and Materials

Mouse lines and viral vectors

All animal procedures were conducted in accordance with the guidelines set by the Ethical Committee for use of laboratory animals at Lund University. In all four papers NRMI mice were used as the wild-type strain.

Ngn2-GFP mice (paper I and II): Heterozygous *Ngn2-GFP* mice (Seibt et al., 2003) were maintained on a CD1/129 background and crossed to produce homozygous embryos or mice. For genotyping see paper I and II.

CMV-GFP mice (paper III): Mice expressing GFP under the cytomegalovirus (CMV)- β -acting promoter (Okabe et al., 1997) were bred on a NMRI background.

VSV-G pseudotyped retroviral vectors: In paper II and III a VSV-G pseudotyped retroviral vector expressing GFP under the CMMPLTR modified from (Ory et al., 1996) was used to transfect cells, as a control or to identify neurosphere-expanded cells, respectively. In addition a *Ngn2-IRES-GFP* vector (Falk et al., 2002) was used in paper II to drive ectopic expression of *Ngn2*.

Dissection

Procedure: Following either cervical dislocation or lethal exposure to CO₂, embryos were removed from the uterine horns of timed-pregnant mice (time of vaginal plug was considered as E0.5) and placed in ice-cold PBS. The embryos were stored on ice and dissection performed in cold Leibovitz's L-15 medium (Gibco). After dissection and removal of meninges the tissue was mechanically dissociated into a single-cell suspension.

Dissections: Dissections were performed at different embryonic stages depending of the subregion of interest. In forebrain dissections the brains were dissected out and cortex cut open along the dorsal midline exposing the ventral part of the forebrain enclosing the GE at E11.5 and LGE and MGE at E13.5. Cortex was isolated at E13.5 (paper III) and E15 (paper IV). For the ventral midbrain dissections [E10.5 (paper IV), E11.5 (paper II and IV) and E12.5 (paper I and III)] the mesencephalic part of neural tube was cut out of the embryo and the ventral part dissected (figure 8).

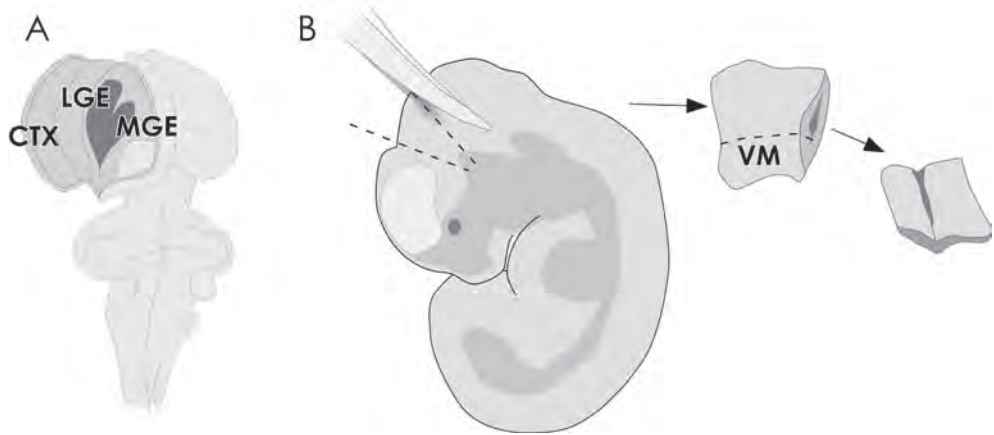


Figure 8. Schematic representation of fore- (A) and midbrain (B) dissections. CTX: cortex.

***In vitro* expansion of fetal derived neural progenitors**

Neurospheres (paper III & IV): Neurospheres were obtained by plating the cells on uncoated plastic at 100,000 cells/ml in basic medium [DMEM/F12 (Gibco) with N2 hormone mix (25µg/ml insulin, 100µg/ml apo transferrin, 20nM progesterone, 60µM putrescine, 30nM selenium chloride (Reynolds et al., 1992)), 2nM L-glutamine, 100µg/ml Pen/Strep, 15mM HEPES (only in paper III) and 1.25% sodium bicarbonate (all from Sigma)] supplemented with 10ng/ml bFGF and 20ng/ml EGF (both from R&D systems). The neurospheres were passaged weekly by mechanical dissociation and replated at 50,000 cells/ml. Experiments were done on passage 5-8 neurospheres.

NS cell cultures (paper IV): To establish the NS cell cultures, neurospheres were generated as described above. Six-seven days after plating, spheres were collected and allowed to sediment. The medium was replaced with NSA-N2 medium [NSA medium (Euromed, Euroclone) with N2 hormone mix, 2nM L-glutamine, 100µg/ml Pen/Strep] supplemented with 20ng/ml bFGF and 20ng/ml EGF and the spheres replated on 0.1-0.2% gelatin or in Iwaki T25 flasks. After 4-21 days the spheres had attached and cells migrated out to form a monolayer of bipolar NS cells. At this point the cells were passaged. Cells were detached by incubation with 1ml accutase for 1-2 min followed by knocking on the flask to generate a single cell suspension. Cells were spun 3 min at 1200rpm and replated in 5ml NSA-N2 medium with growth factors at a density of approx 2.5×10^6 cells/T25. Under these conditions cells were split every 2-3 day, 1:3-1:6.

***In vitro* differentiation**

Primary cultures: As a standard, primary cells were differentiated for 5-7 days at a density of 120 cells/cm² on poly-L-lysine-coated (PLL) chamber slides in basic medium supplemented with 1% fetal bovine serum (FBS). To support attachment and DA neuron survival PLL was replaced with Matrigel (BD Biosciences), N2 with B27 (Gibco) and the medium further supplemented with 10ng/ml glial cell lines-derived neurotrophic factor (GDNF, Biosource), and 100μM ascorbic acid (paper II).

Neurosphere cultures (paper III): The neurosphere cultures were differentiated by plating the neurospheres at a density equivalent to 60,000 cells/cm² on PLL-coated chamber slides in basic medium containing 1% FBS. The cultures were allowed to differentiate over 3-10 days. When differentiated in coculture with primary cells, the primary cells were plated at 120,000 cells/cm² and allowed to attach for 24 hours before applying the neurospheres (60,000 cells/cm²). To identify the neurosphere-expanded cells these were either isolated from mice with ubiquitous expression of GFP (Okabe et al., 1997) or transduced with a GFP-expressing viral vector prior to coculturing.

NS cell cultures (paper IV): In the optimized differentiation protocol, cells were plated at 100,000 cells/cm² in NSA-N2 supplemented with B27 and 10ng/ml bFGF on laminin-coated plastic (10μg/ml, Sigma) for differentiation. After 3 days of differentiation the cells were dissociated and replated on laminin-coated plastic at a density of approx 10,000 cells/cm² in a medium compositioned of NSA-N2:Neurobasal (Gibco)-B27:DMEM-B27 (1:1:1) supplemented with 10ng/ml bFGF, 10ng/ml brain-derived neurotrophic factor (BDNF, R&D systems), 10ng/ml GDNF and 100μM ascorbic acid. Differentiation was continued for a total of 10, 14 and 21 days.

Fluorescence activated cell sorting (FACS)

In paper I cells from *Ngn2*-GFP heterozygous embryos were FACS sorted based on fluorescence intensity. The cell suspensions for FACS were prepared by mechanical dissociation in PBS-Ca²⁺/Mg²⁺ (Gibco) with 1mM EDTA and 0.5% bovine serum albumin at a concentration of approximately 3.0x10⁶ cells/μl. To identify and eliminate dead cells, 7-aminoactinomycin-D (7AAD, 10μl/ml, Sigma) was included. Gate settings for GFP^{neg} were determined using wild-type tissue. An 'unsorted' cell preparation was generated for transplantation by passing wild-type cells through the FACS and gating for live cells.

Table 1. Antibodies used in this thesis.

Antibody	Company	Dilution	Protocol
rabbit α -AADC	Chemicon	1:1000	standard
rabbit anti-Aldh11	R.Lindahl	1:500	standard
mouse α - β III	Promega	1:1000	standard
mouse α - β III	Sigma	1:500	standard
rabbit α - β III	Covance	1:2000	standard
mouse α - β gal	Promega	1:500	standard
rabbit α - β gal	5'-3' Inc	1:500	standard
rabbit α -BLBP	Chemicon	1:5000	standard
rat α -BrdU	Oxford Biotechnology	1:100/1:500*	**/**
mouse α -Bm3a#	Santa Cruz	1:50	antigen retrieval
rabbit α -CalbindinD28k	Chemicon	1:1000	antigen retrieval
rabbit α -ChAT	Chemicon	1:1000	standard
mouse α -CNPase	Sigma	1:100	standard
mouse α -DARPP	P. Greengard	1:20 000	standard
mouse α -En1	Hybridomas Bank	1:50	antigen retrieval
rabbit α -En1/2	A. Joyner	1:1000	standard
rabbit α -Er81	T. Jessel/S Morton	1:2000	standard
goat α -Foxa2 (HNF3 β)	Santa Cruz	1:600	standard
rabbit α -GABA	Sigma	1:250/1:1500*	standard
rabbit α -GABA	Incstar	1:5000	##
mouse α -GAD65	BDPharMingen	1:1000	standard
rabbit α -GFAP	DAKO	1:1000	standard
chicken α -GFP	Chemicon	1:5000	standard
rabbit α -Girk2	Alomone labs	1:80	antigen retrieval
guinea pig α -Glast	Chemicon	1:1000	###
rabbit α -5HT	Incstar	1:5000/1:10000	*standard
mouse α -Isl1	Hybridomas Bank	1:100(0.37ug/ml)	citrate buffer
rabbit α -Isl1	Abcam	1:100	standard
rabbit α -Isl1/2	T. Edlund	1:400	standard
rat α -mouseKi67	DAKO	1:50	antigen retrieval
rabbit α -Lmx1a	M. German	1:10 000	standard
rat α -M2	C. Lagenaur	1:50	standard
rat α -M6	C. Lagenaur	1:50	standard
mouse α -MAP2	Sigma	1:250	standard
mouse α -Nestin	BD Bioscience	1:500	standard
mouse α -Ngn2	D. Andersson	1:20	standard
mouse α -Ngn2	M. Nakafuku	1:1000	standard
mouse α -Nkx2.1(TTF1)	Abcam	1:200	standard
mouse α -Nkx6.1	Hybridomas Bank	1:100(0.27ug/ml)	standard
rabbit α -Nurr1	Santa Cruz	1:1000	standard
rabbit α -Otx2	Chemicon	1:4000	standard
mouse α -Parvalbumin	Sigma	1:2000	standard
rabbit α -Pax6	BioSite	1:500	standard
rabbit α -Pbx1-3	Santa Cruz	1:400	standard
mouse α -PSA-NCAM	Chemicon	1:200	standard
rabbit α -Pitx3	P.H. Burbach	1:400	standard
mouse α -RC2	Hybridomas Bank	1:50	standard
mouse α -Sox2	R&D	1:50	antigen retrieval
rabbit α -Tbr1	Chemicon	1:10 000	standard
rabbit α -TH	Pel-Freeze Biological	1:1000	standard
mouse α -TH	Chemicon	1:4000	standard
rabbit α -vGLUT1	SySy	1:2000	standard
rabbit α -VMAT	Chemicon	1:1000	standard

* *in vivo/in vitro*

** For immunohistochemical staining DNA was denaturated in 1M HCl at 65°C for 30min prior to preincubation.

*** Immunocytochemistry was done according to Caldwell et al., 2004. Cells were permeabilize with ice-cold MeOH for 20min at -20°C. DNA denaturated with 1N HCl for 20min at 37°C. The cells were neutralize by washing twice with 0.1M borate buffer prior to pre-incubation.

The blocking solution used for mouse α -Bm3A is: 10% normal serum, 1% milk, 1mg/ml BSA in 0.02M KPBS.

Fixation with 4%PFA and 0.4%glutaraldehyde.

Only works with bioatynylated secondary antibody followed by streptavidin.

Transplantation

The transplantation in paper I was performed in P3 Sprague-Dawley rats as described by (Nikkhah et al., 2000). The pups were deeply anesthetized by hypothermia and mounted in a mouse stereotaxic frame in a flat skull position. Injections were performed with a 5µl Hamilton syringe fitted with a thin glass capillary to limit damage at the injection site. The cells were resuspended at a concentration of 5×10^4 cells/µl in HBSS- $\text{Ca}^{2+}/\text{Mg}^{2+}$ (Gibco) and 1µl cell suspension was delivered over 2 min to the right striatum. The capillary was left in place for additional 2 min to allow the cells to sediment. Injection coordinates were: 0.7mm anterior and 1.9mm lateral to the Bregma, and 2.9mm below the dura surface.

Tissue processing for immunochemical analysis

Embryonic/Neonatal: The embryonic heads were immersion fixed in 4% PFA (in 0.01M phosphate buffer) overnight at 4°C. At P0 the brains were removed from the skull prior to fixation. The tissue was subsequently cryoprotected overnight in 30% (E11.5-E13.5) or 25% (E15.5-P0) sucrose and sectioned on a cryostat (12-16µm, coronal sections, 10 series)

Adult: P18 mice and 4-week old rats received lethal doses of pentobarbitone and were trans-cardinally perfused with 0.9% saline (1 min of 30-40ml/min) followed by 4% PFA (7 min of 30-40ml/min). The brains were post-fixed for 2 hours, cryoprotected overnight in 25% sucrose, and sectioned on a freezing-microtome (35µm, coronal sections, 12 series).

Cells: Cultures were fixed in ice-cold 4% PFA for 15 min at room temperature.

Immunohistochemical and immunocytochemical procedures

The standard procedure: Sections (on glass and free-floating) and culture slides were pre-incubated for 1 hour in blocking solution containing 2-5% normal serum and 0.25% TritonX-100 (Amersco) in 0.02M KPBS. Primary antibodies diluted in blocking solution were applied overnight at room temperature or at 4°C. After rinses, biotinylated (Vector diluted 1:200 in blocking solution) or fluorophore-conjugated secondary antibodies (Jackson Laboratories diluted 1:400 in blocking solution, Molecular Probes 1:500) were applied for 1-2 hours at room temperature. Biotinylated secondary antibodies were followed either by incubation with streptavidin-horseradish peroxidase complex (ABC elite kit, Vectastain) for 1 hour and subsequent exposure to di-amino-benzidine (DAB, 0.5 mg/ml; Sigma)

or by fluorophore-conjugated streptavidin for 1 hour (Cy3-streptavidin, 1:400, Jackson Laboratories). For DAB staining endogenous peroxidase activity was quenched for 15 min with 3% H_2O_2 prior to pre-incubation. For primary antibody dilutions see table 1. Cell nuclei were visualized by staining with DAPI (1:1000; Sigma). Fluorescence stainings were mounted with anti-fading medium PVA-DABCO (recipe from Dr. Peterson, Salk Institute, USA).

Antigen retrieval: Antigen retrieval was performed by boiling in 10mM citrate buffer (pH 6.0) prior to staining with a subset of antibodies (see table 1).

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