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Sektionen för Neurovetenskap
Institutionen för Experimentell Medicinsk Vetenskap

Generation of midbrain dopaminergic neurons *in vivo* and *in vitro*:
the role of *Neurogenin2*

Akademisk avhandling
av
Elin Andersson

Som med vederbörligt tillstånd av Medicinska Fakulteten vid Lunds Universitet för avläggande av doktorsexamen i medicinsk vetenskap kommer att offentligen försvaras i Segerfalksalen, Wallenberg Neurocentrum, Lund

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Abstract Parkinsons disease (PD) is a neurodegenerative disorder where dopaminergic neurons of the substantia nigra (SNc) in the mesencephalon are progressively eliminated. The ensuing loss of dopaminergic innervation of the basal ganglia manifests itself as severe motor deficits in PD patients. Clinical trials have shown that cell replacement therapy, where dopaminergic neuroblasts derived from fetal ventral mesencephalon (VM) are transplanted to the striatum, may be an alternative to pharmacological treatment of PD patients. The limited access and ethical concerns with using fetal tissue have prompted the use of stem cells as a renewable and limitless source of dopaminergic neurons. However, the mechanisms of specification of mesDA neurons *in vivo* need to be elucidated for identification and generation of mesencephalic dopaminergic (mesDA) neurons from stem cells *in vitro*.
In this thesis I have identified expression of the proneural gene *Neurogenin2* (*Ngn2*) in a restricted pattern in the embryonic VM during mesDA neurogenesis. The protein was expressed in the progenitor population in the ventricular zone but not in mature neurons in the mantle zone. When isolating the Ngn2-expressing cells and their direct descendants by FACS from an *Ngn2-GFP*-KI mouse, I found that the *Ngn2*-GFP-positive cell fraction contained dopaminergic neurons, in contrast to *Ngn2*-GFP-negative cells. This shows that Ngn2 label early mesDA neuron precursors. Furthermore, when I analysed the Ngn2 knockout mutants, I found that they displayed an early loss of mesDA neurons that was partially maintained at postnatal stages, showing that Ngn2 has a role in the generation of the mesDA neurons. No other neuronal subtype in the VM was affected suggesting that this role for Ngn2 is specific for the mesDA neurons.
Using embryonic mouse tissue obtained at the stage of mesDA genesis, I was able to generate cultures of neural stem and progenitor cells, so called neurosphere cultures, that were neurogenic and maintained a ventral midbrain character over several passages. Although the neurospheres did not spontaneously give rise to dopaminergic neurons when differentiated, TH-positive cells were detected when Nurr1 was over-expressed in the cultures. The frequency with which this occurred, and the morphology of the TH-positive cells, differed from the results obtained when over-expressing Nurr1 in forebrain-derived expanded cells. This suggests that neurosphere expanded cells derived from VM specifically contain progenitors that can generate dopaminergic neurons under certain conditions. When over-expressing Ngn2 together with Nurr1 TH-positive cells were generated that displayed a mature neuronal morphology. Furthermore, I found that they expressed other dopaminergic markers which were not seen when either Nurr1 or Ngn2 were over-expressed alone. This suggests that Nurr1 and Ngn2 interact to specify a more mature dopaminergic phenotype.
The results in this thesis have identified a new cellular marker of mesDA progenitors in the developing embryo and also provided new insight into the development of mesDA neurons.

Key words: stem cell, progenitor cell, neurospheres, bHLH, transcription factors, proneural genes, neuronal differentiation, TH, mesencephalon, development, neuronal specification, dopamine, Parkinson's, cell replacement

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Generation of midbrain dopaminergic neurons *in vivo* and *in vitro*:

the role of *Neurogenin2*

Elin Andersson, M.Sc

LUND
UNIVERSITY

Cover: TH-positive cells (red) with mature neuronal morphology and elaborate arborisation are generated from expanded fetal midbrain cells transduced simultaneously with retroviral constructs containing Nurr1 and Neurogenin2 (front). TH-positive cells with more immature morphology are generated after transduction with Nurr1 only (back). Cells also label for reporter gene GFP (green) which show that they are derived from transduced cells.

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I made this!

My soul, sit thou a patient looker-on;
Judge not the play before the play is done:
Her plot hath many changes; every day
Speaks a new scene; ***the last act crowns the play.***

Francis Quarles,
Epigram, Respice Finem

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I. Lachlan H. Thompson*, Elin Andersson*, Josephine B. Jensen, Perrine Barraud, Francois Guillemot, Malin Parmar, and Anders Björklund
Neurogenin2 identifies a transplantable dopamine neuron precursor in the developing ventral mesencephalon
* equal contribution

Submitted to Experimental Neurology

II. Elin Andersson*, Josephine B. Jensen*, Malin Parmar, Francois Guillemot, and Anders Björklund
Development of the mesencephalic dopaminergic neuron system is compromised in the absence of Neurogenin2
* equal contribution

Accepted in Development

III. Elin KI Andersson, Dwain K Irvin, Jessica Ahlsiö, Emeli Nilsson, and Malin Parmar
Ngn2 and Nurr1 facilitates dopaminergic neuron differentiation from neurosphere expanded ventral mesencephalic cells

Submitted to Molecular and Cellular Neuroscience

Abbreviations

bFGF	Basic fibroblast growth factor
bHLH	Basic helix loop helix
BMP	Bone morphogenetic protein
BrdU	5-bromo-2-deoxyuridine
cDNA	Complementary DNA
CKO	Conditional knock-out
CNS	Central nervous system
DA	Dopamine
DAPI	4'-6-Diamidino-2-phenylindole
E	Embryonic day
EGF	Epidermal growth factor
ES	Embryonic stem (cell)
FACS	Fluorescence activated cell sorting
FGF8	Fibroblast growth factor 8
GABA	Gamma-aminobutyric acid
GFP	Green fluorescent protein
HD	Homeodomain (protein)
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
IHC	Immunohistochemistry
IZ	Intermediate zone
L-DOPA	levodopa, 3,4-dihydroxy-L-phenylalanine
MesDA	Mesencephalic dopamine (neuron)
MHO	Mid-hindbrain organizer
MZ	Mantle zone
PCR	Polymerase chain reaction
PD	Parkinson's disease
PNS	Peripheral nervous system
RRF	Retrorubral field
SHH	Sonic hedghog
SNc	Substantia nigra parscompacta
SVZ	Sub-ventricular zone
TH	Tyrosine hydroxylase
TUNEL	Terminal deoxynucleotidyl Transferase Biotin-dUTP Nick End Labeling
VM	Ventral midbrain
VTA	Ventral tegmental area
VZ	Ventricular zone

Summary

Summary

Parkinsons disease (PD) is a neurodegenerative disorder where dopaminergic neurons of the substantia nigra (SNc) in the mesencephalon are progressively eliminated. The ensuing loss of dopaminergic innervation of the basal ganglia manifests itself as severe motor deficits in PD patients. Clinical trials have shown that cell replacement therapy, where dopaminergic neuroblasts derived from fetal ventral mesencephalon (VM) are transplanted to the striatum, may be an alternative to pharmacological treatment of PD patients. The limited access and ethical concerns with using fetal tissue have prompted the use of stem cells as a renewable and limitless source of dopaminergic neurons. However, the mechanisms of specification of mesDA neurons *in vivo* need to be elucidated for identification and generation of mesencephalic dopaminergic (mesDA) neurons from stem cells *in vitro*.

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The results in this thesis have identified a new cellular marker of mesDA progenitors in the developing embryo and also provided new insight into the development of mesDA neurons.

Populärvetenskaplig sammanfattning

I hjärnan finns många olika typer av nervceller. De använder sig av olika signalsubstanser, kallade *neurotransmittorer*, för att kommunicera med andra nervceller. En viss typ av nervceller använder neurotransmittorn *dopamin*. Dopaminceller finns på många ställen i hjärnan men de flesta ligger i mellanhjärnan i ett par olika cellgrupper som var och en skickar signaler till sina specifika områden i andra delar av hjärnan. En av dessa cellgrupper kallas *substantia nigra* och signalerar till ställen som styr en människas motorik. Hos patienter med Parkinsons sjukdom, dör cellerna i denna grupp och då försvinner även dopaminsignalerna till de delar som styr motoriska förmågor. Därför har Parkinson-patienter typiska symptom, som problem med motoriken och svårighet att sätta igång rörelser. För att lindra dessa symptom kan Parkinson-patienter ta medicin som ska ersätta dopaminet. Man har också testat andra behandlingsmetoder som går ut på att ersätta dopamincellerna inuti hjärnan. Genom att ta dopaminceller från fostervävnad och transplantera till hjärnan har man lyckats återskapa dopaminsignalleringen utan mediciner. Tyvärr kan denna teknik ännu inte tillämpas på många patienter eftersom det är svårt att få tag på tillräckligt mycket vävnad. Man har därför börjat undersöka hur man kan generera dopaminceller på annat sätt. En metod är att använda stamceller, celler som kan förökas i kultur och som kan utvecklas till vilka sorters celler som helst. För att få stamcellerna att bli dopaminceller så måste man veta vad det är som gör att just den sortens nervceller bildas. Vi måste förstå vilka de bakomliggande faktorerna är som styr cellutvecklingen mot dopaminceller.

I min avhandling har jag undersökt vilka signaler och gener som är viktiga för att dopaminceller ska bildas. För att ta reda på det har jag tittat på dopaminceller under fosterutvecklingen. I mina studier använde jag möss som en modell för vad som händer i människan. Jag fann att en gen, *Neurogenin2*, var påslagen (uttryckt) i precis de celler som skulle bli dopaminceller hos mössfoster. När jag sedan undersökte muterade möss där denna gen var borttagen såg jag att dopamincellerna i mellanhjärnan inte bildades som de skulle. Detta visar att *Neurogenin2* är viktig för bildandet av dopaminceller. Jag försökte också påverka odlade stamceller att utvecklas till dopaminceller genom att se till att *Neurogenin2* uttrycktes i cellerna. När jag uttryckte *Neurogenin2* tillsammans med en annan gen, *Nurr1*, som också är viktig för att det ska bli dopaminceller, gav det bättre resultat än att använda dem var och en för sig och jag såg att det bildades dopaminceller i cellkulturerna.

Med resultaten som presenteras i den här avhandlingen har vi kommit ännu en bit på väg för att veta hur dopaminceller genereras. Mina resultat kan användas bl.a för att identifiera celler som ska bli dopaminceller. I ett längre perspektiv kan mina resultat bidra till att man kan generera dopaminceller från stamceller och därmed ge de patienter som lider av Parkinsons sjukdom en alternativ behandling.

Introduction

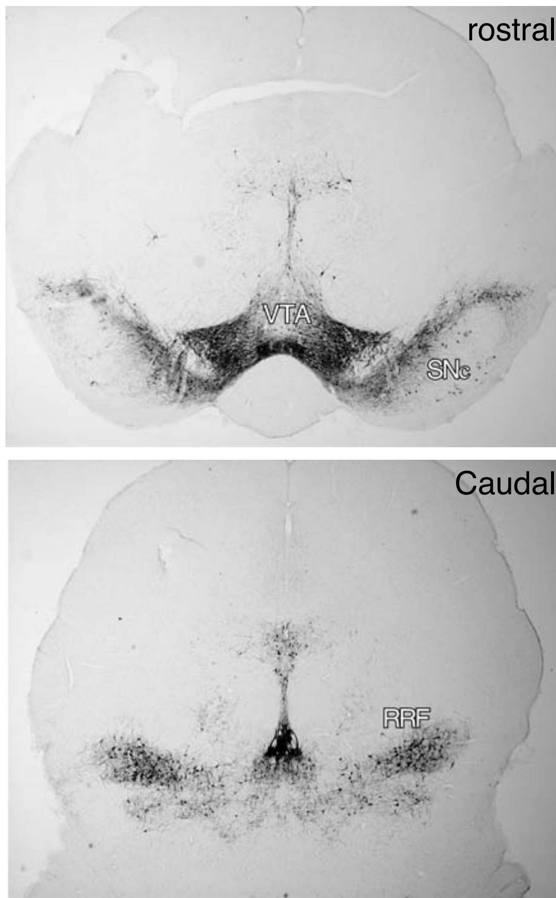
Introduction

Dopamine is a neurotransmitter employed by specific neurons in the central nervous system (CNS). Dopaminergic neurons are involved in neural processes as diverse as neuroendocrine hormonal release, cognition, emotion, reward and initiation of motor responses, however it is for their role in the neurodegenerative disorder Parkinson's disease (PD) that so much interest has been placed upon this subtype of CNS neuron. The neuropathology of PD is a gradual loss of mesencephalic (midbrain) dopaminergic neurons and their innervation of the basal ganglia in the ventral forebrain (for review see Lang and Lozano, 1998a; Lang and Lozano, 1998b). It is predominantly the dopaminergic neurons in the substantia nigra, one of the nuclei in the midbrain, that are affected in PD. Attempts to replace lost dopaminergic transmission in the basal ganglia by transplantation of immature dopaminergic neuroblasts from fetal tissue have proved to be a viable approach, in animal models of PD but also in PD patients (Lindvall et al., 1990; for review see Dunnett and Bjorklund, 1999; Winkler et al., 2005). Since cell replacement therapy requires large numbers of transplantable dopaminergic neurons and the yield from fetal tissue is limited, stem cells have been suggested and explored as an option to meet this need. Advances within the field of stem cell research in early 1990s saw the emergence of protocols how to grow and maintain neural stem cells in culture (Reynolds et al., 1992; Reynolds and Weiss, 1992; Reynolds and Weiss, 1996). This fuelled research on the specification of dopaminergic neurons during embryogenesis and how this could be applied to the *in vitro* generation of dopaminergic neurons from stem cells.
The aim of this thesis work has been to further elucidate mechanisms and events important for the development of the midbrain dopaminergic neurons *in vivo* and explore ways to generate this kind of neuron *in vitro*.

Dopaminergic neurons in the brain

Location of dopaminergic neurons in the brain

The dopaminergic neurons in the brain are organized into ten nuclei ranging from the caudalmost cell group A8, the retro-rubral field, to the rostralmost A17, a group of amacrine interneurons in the retina (Björklund and Lindvall, 1984; Dahlstrom and Fuxe, 1964). Small groups of dopaminergic neurons are located in e.g the olfactory bulb and the diencephalon (part of forebrain), however, the vast majority of dopaminergic neurons, around 75%, reside in nuclei in the ventral mesencephalon (VM): the substantia nigra pars compacta (SNc, A9), the ventral tegmental area (VTA, A10) and the retro-rubral field (RRF, A8) (Björklund and Lindvall, 1984; Dahlstrom and Fuxe, 1964) (Fig 1a). The dopaminergic neurons of the mesencephalic nuclei (the mesDA neurons) are often investigated together since they develop from the same progenitor location.



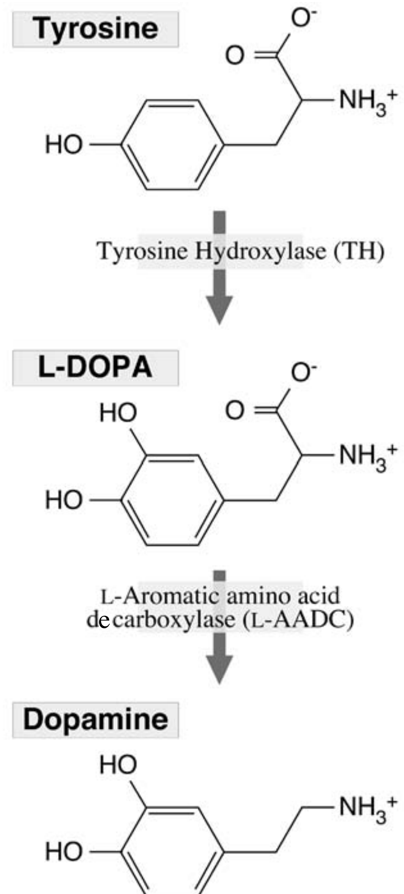


Fig. 1A) Coronal section of P18 mouse midbrain showing the localization and distribution of the midbrain dopaminergic nuclei ventral tegmental area (VTA), substantia nigra (SNc) and retro-rubral field (RRF) B) Enzymatic pathway of dopamine synthesis

Projections and functions of midbrain dopaminergic neurons

The dopaminergic neurons of the midbrain nuclei have distinct functions in the brain and consequently innervate separate structures. The dopaminergic neurons whose cellbodies are located in the SNc, and which are the neurons most affected in PD, project to the dorso-lateral striatum and caudate putamen forming the so-called nigrostriatal pathway (Ungerstedt, 1971). The nigrostriatal pathway modulates the output of these basal ganglia structures that, together with cortical areas control initiation of voluntary movement, posture etc. Since the nigrostriatal innervation is lost in PD patients, they have characteristic symptoms of motor dysfunction such as rigidity and slowness of movements that is accompanied by tremor (Lang and Lozano, 1998a; Lang and Lozano, 1998b). The VTA neurons innervate limbic areas in the ventro-medial

striatum (mesolimbic system) and pre-frontal cortex (mesocortical system) and are involved in emotion, cognitive processes and reward behaviours (Björklund and Lindvall, 1984; Ungerstedt, 1971). The RRF neurons send axons along the same pathways as VTA and SN.

Identification of dopaminergic neurons
Dopaminergic neurons are generally identified by their expression of the rate-limiting enzyme in the dopamine pathway, tyrosine hydroxylase (TH). TH modifies the aminoacid tyrosine to dopamine precursor L-DOPA, which is in turn converted to dopamine by aromatic aminoacid decarboxylase (AADC) (Fig 1b). Dopaminergic neurons share expression of proteins involved in production, storage and release of dopamine, such as TH, AADC, dopamine transporter (DAT) and vesicular monoamine transporter (VMAT2). Other proteins are differently expressed in different dopaminergic nuclei. These proteins are likely to play a role in the function of the particular dopaminergic neuron subtype but can be used simply as markers to distinguish them. The mesDA neurons have very similar expression profiles, more so during development than at adult stages. They can obviously be identified by location and projections in intact adult tissue, and their morphologies differ slightly (Thompson et al., 2005). At adult stages, mesDA neurons in SNc and VTA can also be distinguished by presence of the markers Girk2 and calbindin, respectively (Liang et al., 1996; Schein et al., 1998; Thompson et al., 2005). Additionally, progenitor marker Aldh1 (see below) is maintained preferentially in SNc neurons in adult (McCaffery and Drager, 1994). However, since mesDA neurons develop from the same cells, at approximately the same time, and no early marker has been reported to be expressed specifically by either, it is at present not possible to distinguish them during development.

Development of mesDA neurons

Patterning of the midbrain
Development of the CNS is initiated by the formation of neural ectoderm, the so-called *neural plate*. The neural plate will invaginate into a neural fold and subsequently close along the dorsal midline forming the neural tube (*neurulation*). The neural tube consists at this stage of one layer of dividing cells, the *neuroepithelial* cells. They will divide to give rise to all cells of the CNS. A rostro-caudal and dorsal-ventral patterning of the neural tube is established early which provides positional information to the dividing neuroepithelial cells and ensures that the neurons generated in a specific position is of the correct subtype. Local organizing centers are involved in defining such developmental compartments by secreting factors that influence surrounding tissue (Jessell and Sanes, 2000b; for review of patterning of the neural tube see Lumsden and Krumlauf, 1996).
MesDA neurons develop in close proximity to two organizing centers, the floorplate and the isthmus which control the dorso-ventral and anterior-posterior patterning, respectively. Floorplate cells were shown to ectopically induce dopaminergic neurons in dorsal midbrain (Hynes et al., 1995b). This inductive effect of floorplate cells is mediated by the protein Sonic hedgehog, SHH (Hynes et al., 1995a; Wang et al., 1995; Ye et al., 1998) which is secreted by the

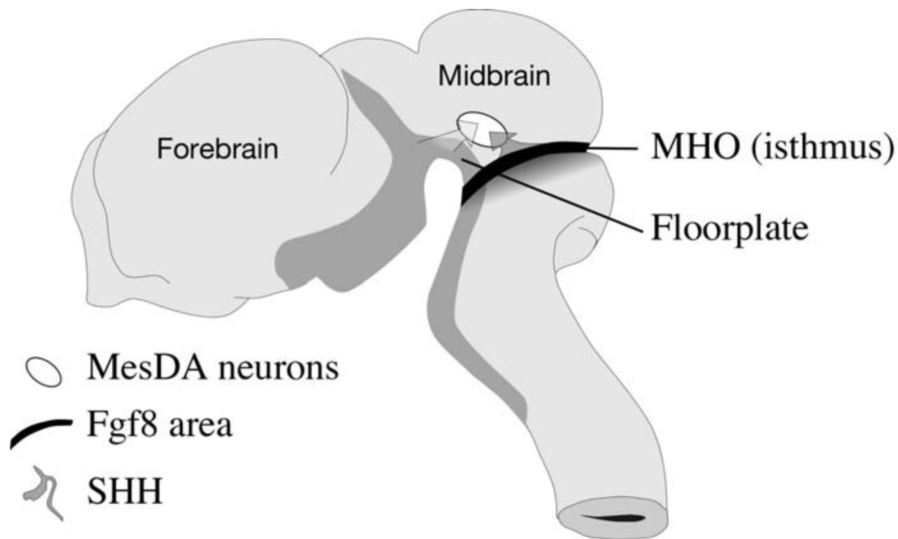


Fig. 2 Schematic drawing of a developing mouse brain, viewed from the side. MesDA neurons are located in the ventral midbrain, above the mesencephalic flexure. They develop at the site where signaling molecules FGF8, secreted from the mid-hindbrain organizer (MHO), and SHH from floorplate cells intersect.

floorplate cells all along the ventral neural tube (Fig 2). SHH is instrumental in defining ventral cell identities throughout the neuraxis, but its effect is most studied in the spinal cord (for review see Jessell, 2000). Interestingly, forebrain dopaminergic neurons are also generated under the influence of SHH (Ye et al., 1998) implicating SHH as a crucial factor for dopaminergic neuron development.
The isthmus, or the mid-hindbrain organizer (MHO) as it is also called, is a constriction of the neural tube separating the midbrain and the hindbrain. The MHO is established at the site of interaction between two transcription factors: Otx2, expressed in the early rostral neural tube that will later develop into the forebrain and midbrain, and Gbx2 whose expression domain covers the presumptive hindbrain and spinal cord. Otx2 and Gbx2 interact by suppressing expression of the other to position the MHO (Broccoli et al., 1999; Wurst and Bally-Cuif, 2001). The MHO can be shifted caudally by ectopic expression of Otx2 in the rostral hindbrain or rostrally by loss of Otx alleles, which expands the Gbx2 expressing domain. Shifting the MHO caudally and rostrally increases and decreases the number of mesDA neurons, respectively (Broccoli et al., 1999; Brodski et al., 2003). The secreted factor Fibroblast growth factor 8 (FGF8) is responsible for the patterning effects of the MHO and FGF8 soaked beads were shown to induce an ectopic midbrain (Crossley et al., 1996). Dopaminergic neurons develop in the ventral neural tube just rostral to the MHO at a site where SHH and FGF8 signals intersect, indicating that the combined action of SHH and FGF8 controls the precise location of mesDA neurons and is essential for their formation (Ye et al., 1998) (Fig 2).

A number of transcription factors, Lmx1b, Pax2, Pax5, En1 and 2 and secreted signalling molecule Wnt1 are expressed in or around the isthmus following its establishment (reviewed by Joyner, 1996; Liu and Joyner, 2001; Wurst and Bally-Cuif, 2001). A few of these, En1, En2 and Lmx1b will continue to be expressed in maturing mesDA neurons and will then exercise functions other than general patterning of the mid-hindbrain region (see below).

Neurogenesis of mesDA neurons

Immunohistochemistry (IHC) for TH has shown that dopaminergic neurons start to appear at embryonic day E11 in mice (Foster et al., 1988) and E12.5 in rats (Specht et al., 1981). However, depending on detection method and staging of embryos TH-positive cells have been reported as early as E9.5 in mice (Di Porzio et al., 1990). Labelling studies in rats showed that mesDA neurons incorporated [^{3}H]thymidine during E11-E15 with a peak at E13 (Altman and Bayer, 1981). Similar studies on mice showed that most mesDA neurons are born on E12 (E11.5 if the morning of the plug is E0.5), however neurons of the SNc and RRF are born slightly earlier than VTA neurons (Bayer et al., 1995). Neurogenesis of SNc neurons take place between E10-E13 with a peak at E11-E12 and neurogenesis of VTA neurons is ongoing from E10 to at least E14 with the majority being born at E12-13. Within the SNc and VTA there is also an anterior-posterior, lateral-medial gradient such that neurons in posterior parts of the nuclei are born later than neurons in the anterior parts and lateral regions contain more early generated neurons than medial parts (Bayer et al., 1995).

Origin and organization of mesDA neurons

Dopaminergic precursors are generated from the proliferative ventricular zone (VZ) overlying the ventral midline (Fig 3a). They are generated just anterior of the isthmus, as previously mentioned. As they become postmitotic they start to express TH and migrate ventrally along radial glia cells expressing vimentin and tenascin (Kawano et al., 1995; Shults et al., 1990) (Fig 3a). It is thought that the TH-positive cells migrate in this fashion until they reach the ventral pial surface in the mantle zone (MZ) when they instead follow tangentially oriented axons and migrate laterally to form the RRF, the SNc and the VTA (Kawano et al., 1995). However, since AADC, which is expressed 2 days prior to TH in dopaminergic precursors (Teitelman et al., 1983), is found lateral to the mesDA neuron domain it has been suggested that some precursors, that give rise to lateral SNc neurons, are generated from a more lateral progenitor population and migrate perpendicular to the ventricle (Hanaway et al., 1971; Smidt et al., 2004).
The beginnings of mesDA nuclei are evident at E17.5 in mice, however the system continues to develop during the weeks after birth. For example, we have noted that the expression pattern of Girk2 is not fully developed until three weeks after birth.

Genes involved in development of mesDA neurons

Many genes have been shown to be expressed in the mesDA neurons and are important for different aspects of maintenance and development of these neurons. These include Aldh1, Nurr1, Pitx3, Lmx1b, En1 and En2 (Fig 3b).

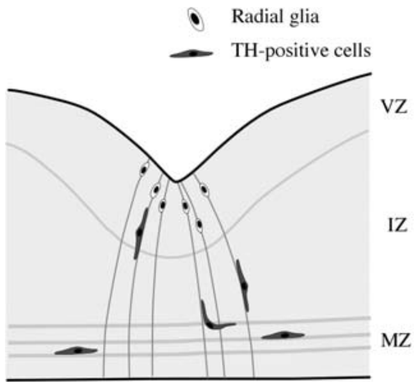
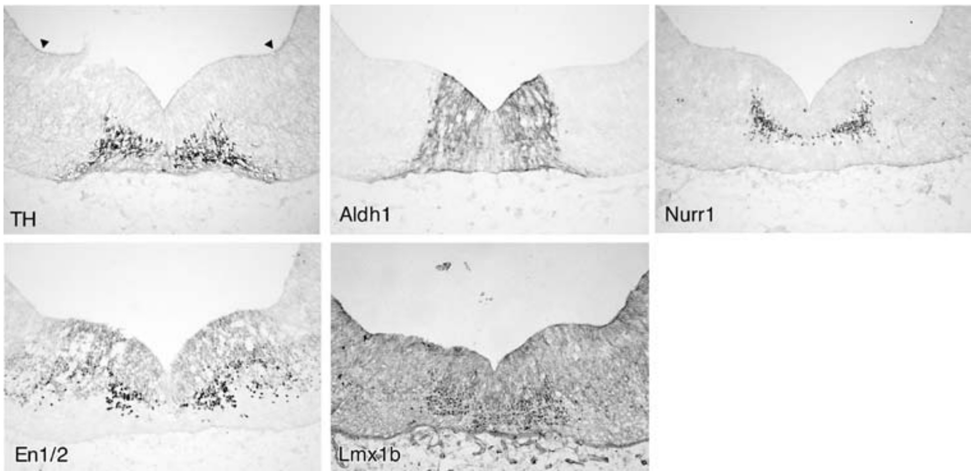


Fig. 3A) Schematic drawing of a coronal section of the VM during mesDA neurogenesis. Dividing progenitors of mesDA neurons are located in the ventricular zone (VZ). As they become postmitotic they start migrating ventrally along radial glia through the intermediate zone (IZ). When the mesDA precursors reach the mantle zone (MZ) above the pial surface, they start migrating laterally along horizontal fibers.
B) Coronal sections through the VM of an E11.5 mouse embryo. Developing dopaminergic neurons are identified by the expression of TH. They are first seen at around E11.5 in the MZ. Aldh1 is expressed in the mesDA progenitors in the VZ and also in more mature mesDA precursors in the IZ and MZ. The postmitotic mesDA precursors also express other markers such as Nurr1, En1/2 and Lmx1b. Black arrowheads mark the lateral sulcus which separates the VM from DM and was used as a morphological landmark for VM dissections.



Aldh1

Aldehyde dehydrogenase, Aldh1 (also known as AHD-2) is an enzyme in the retinoic acid pathway, converting retinaldehyde to retinoic acid (Lindahl and Evces, 1984). It is expressed as early as E9.5 in the midbrain. At this early stage the expression pattern coincides with SHH in a narrow wedge encompassing the ventral midline and presumably labels early proliferating dopaminergic precursors (Wallen et al., 1999). Immunostainings at E11.5 show that Aldh1 at this stage is expressed in both the ventricular zone and budding mantle zone, where it co-localizes with TH-expressing neurons. It is thus a marker for both dopaminergic precursors and postmitotic mesDA neurons. At post-natal stages Aldh1 is mainly confined to the mesDA

neurons of the SNc (McCaffery and Drager, 1994). The function of Aldh1 in the development and/or maintenance of dopaminergic neurons is as yet unclear as no knockout mice or over-expression data for the gene have been presented.

Nurr1

Nurr1 (Nr4a2) is an orphan nuclear receptor transcription factor (no ligand identified as yet) and widely used as a marker for dopaminergic precursors. It is one of the first genes to be expressed in postmitotic mesDA neuron precursors, the protein expression is evident in midbrain from E10.5 in mice, preceding the expression of TH by about a day (Zetterstrom et al., 1997). Nurr1 expression remains in the adult mesDA neurons (Backman et al., 1999; Zetterstrom et al., 1996). It is also expressed in other dopaminergic neurons such as the olfactory bulb A16 neurons (Backman et al., 1999). In addition, it is expressed in cells that are not dopaminergic, both during development and in adult tissue (Zetterstrom et al., 1996). Nurr1 has been shown to bind to the promoter region of the *Th* gene (Iwawaki et al., 2000; Sakurada et al., 1999) and is essential for the neurotransmitter phenotype of dopaminergic neurons, but does not seem to be involved in neurogenesis and other aspects of mesDA neuron specification (Castillo et al., 1998; Saucedo-Cardenas et al., 1998; Smits et al., 2003). In Nurr1 knockout mice, dopaminergic Aldh1-positive precursors are formed and differentiate to express transcription factor En and mesDA specific marker Pitx3 (see below) but do not express TH (Castillo et al., 1998; Saucedo-Cardenas et al., 1998; Wallen et al., 1999; Zetterstrom et al., 1997). Nurr1 mutants also lack expression of other proteins connected to the neurotransmitter phenotype, DAT and VMAT, and show lower levels of AADC (Castillo et al., 1998; Smits et al., 2003). The role of Nurr1 is specific for the mesDA neurons as other DA neurons, that also express Nurr1 during development, were not affected in the Nurr1 mutants (Castillo et al., 1998; Saucedo-Cardenas et al., 1998; Zetterstrom et al., 1997). The dopaminergic precursors (expressing Pitx3 and Aldh1) that are formed in Nurr1 mutants, are eventually lost and an increase of apoptotic TUNEL stained cells is seen, demonstrating that Nurr1 is necessary also for the survival of the dopaminergic neurons (Saucedo-Cardenas et al., 1998; Wallen et al., 1999).

En1/2

Engrailed 1 and 2 (*En1* and *En2*) are homologous homeobox transcription factors that have largely overlapping expression patterns, in particular in the VM (Davis and Joyner, 1988; Simon et al., 2001). En1 and 2 are expressed early in the mesencephalic region (E8, Davis and Joyner, 1988) and also in the adult mesDA neurons, in the case of En2 at high levels only by a subset of the neurons (Simon et al., 2001). En1 and 2 are also expressed by cells in the hindbrain and dorsal mesencephalon but are not expressed by other dopaminergic neurons. En1 single mutants have severe deletions in the cerebellum and inferior colliculus reflecting the normal expression in the dorsal mesencephalon, whereas En2 mutants only have minor defects in cerebellar foliation (Joyner et al., 1991; Millen et al., 1994; Wurst et al., 1994). The mesDA neurons appear intact in the single mutants par for a minor alteration in cell density of the VTA in the En1-/- mutant (Simon et al., 2001). Thus, in En1 and En2 single mutants, the related genes appear to compensate for eachother with respect to their function in dopaminergic

neurons. On the contrary, in En1-/-,En2-/- double mutants, TH-positive cells are formed but the expression domain is smaller and by E14 they are completely lost (Simon et al., 2001). The Engrailed genes are thus essential for the survival of dopaminergic neurons and have also been implicated in the regulation of α-synuclein (Simon et al., 2001).

Lmx1b

Lmx1b is a member of the LIM homeodomain protein family. Similar to En1 and En2, Lmx1b have an early expression pattern in the midbrain. Lmx1b is first expressed in neural tissue at E7.5 and the expression is also seen in adult SNc and VTA neurons (Smidt et al., 2000). Lmx1b is thus expressed in the mesencephalic region before postmitotic mesDA markers Nurr1, Pitx3 and TH and could be considered to be a marker of both a dopaminergic precursor cell and a mature mesDA neuron. However, it is not a specific precursor marker as it is not restricted to the part of VM that give rise to mesDA neurons. There is also some evidence that the early Lmx1b expression is downregulated in the dividing progenitors prior to mesDA neurogenesis and subsequently upregulated in post-mitotic mesDA neurons (unpublished data). In the Lmx1b knockout mutant TH-positive cells are formed but are reduced in number. The TH-positive cells that are formed express Nurr1 but not Pitx3, pointing to a link between Lmx1b and Pitx3. The TH-positive cells in the Lmx1b mutant remain up to E16, after which TH-expression is lost (Smidt et al., 2000). This indicates that Lmx1b or its downstream targets are necessary for the development of certain mesDA neurons and the survival of the others.

Pitx3

Pitx3 is a paired-like homeobox transcription factor that is expressed exclusively in the mesDA neurons in the CNS. Pitx3 mRNA is apparent at around E11.5 and overlaps completely with TH at E12.5 (Smidt et al., 1997). The expression continues in the developing mesDA neurons into adulthood. All SNc and VTA neurons in adults show expression of Pitx3 (Smidt et al., 2004; Smidt et al., 1997; Zhao et al., 2004) although some studies report that only around 50% of the mesDA neurons are Pitx3-positive (van den Munckhof et al., 2003).

Several studies on the Aphakia mouse, shown to be a Pitx3 mutant (Rieger et al., 2001; Semina et al., 2000), have revealed a near complete loss of dopaminergic neurons in the SNc specifically, while VTA neurons were less affected. The innervation of the striatum was also partially lost in the aphakia mice (Hwang et al., 2003; Nunes et al., 2003; Smidt et al., 2004; van den Munckhof et al., 2003). Some studies reported an effect on the morphology of the VTA neurons in the mutants (Hwang et al., 2003; Smidt et al., 2004) but the neurotransmitter phenotype of these cells was intact and they expressed other mesDA related genes such as En1/2, Lmx1b and Nurr1.

The loss of TH-expressing neurons in Pitx3 mutants is seen as early as E12.5 (Maxwell et al., 2005; Smidt et al., 2004). Maxwell et al used a *Pitx3-GFP* knock-in mouse, where the homozygotes are functional knockouts, instead of the Aphakia mice. Using GFP (green fluorescent protein) to track progenitors in the knockout mutant they reported equal numbers of *Pitx3*-GFP positive cells in heterozygotes and mutants during embryonic stages (E12.5 and onwards), indicating that Pitx3 is not essential for the generation of mesDA precursors. However,

as there were significantly fewer TH-positive cells among the GFP-expressing precursors in the mutants, Pitx3 is likely to be involved in the regulation of *Th* gene in a subset of dopaminergic neurons. A responsive element for Pitx3 on the TH promoter has also been shown which supports this notion (Cazorla et al., 2000; Lebel et al., 2001). In addition, there were subgroups of mesDA precursors in the heterozygotes that expressed either only *Pitx3*-GFP or TH at E12.5. The *Pitx3*-GFP+/TH- cells were predominately located in a ventrolateral position where the SNc will form. This suggests that mesDA neurons may develop from distinct precursors that express Pitx3 and TH in slightly different sequence and that SNc neurons are derived from precursors that express Pitx3 prior to TH which fail to survive when Pitx3 is missing.
The induction of Pitx3-expressing precursors in the Nurr1 mutants showed that its expression is independent of Nurr1 (Saucedo-Cardenas et al., 1998; Wallen et al., 1999). The loss of Pitx3 expression but not Nurr1 expression in Lmx1b mutants indicates that Pitx3 form an independent pathway together with Lmx1b in mesDA neuron development (Smidt et al., 2000).

Cell replacement therapy

In PD patients there is a progressive loss of dopaminergic neurons and thus lowered transmission of DA in the striatum. Although recent studies show that new neurons can be generated in the adult brain in response to injury (Arvidsson et al., 2002) there is still debate whether or not re-generation of dopaminergic neurons occurs in the adult SNc (Frielingsdorf et al., 2004; Zhao et al., 2003). It does seem clear however that the entire nigrostriatal innervation would be difficult to recreate in adult individuals from potential newly formed dopaminergic neurons. Cell replacement therapy, whereby developing dopaminergic neuroblasts from fetal VM tissue are placed in the striatum at the site of dopamine transmission, have instead emerged as a possible strategy to alleviate motor symptoms (reviewed in Dunnett and Bjorklund, 1999; Winkler et al., 2005). Several studies in rodent models showed that cells in grafted tissue were spontaneously active and established functional synaptic contacts thus restoring dopamine transmission (reviewed in Dunnett et al., 2000). This has prompted research on generating an optimal cell preparation for transplantation purposes. Stem cells, which can be expanded indefinitely and have the potential to differentiate into any cell type would be an ideal source. However, much work remains to identify the optimal stem cell and find ways to expand it without loosing its potential.

Stem cells

Stem cells – definitions and concepts

Stem cells have a unique ability for proliferation and subsequent differentiation to specialized cells that make them ideal for use in replacing damaged or lost tissue of the body. The theoretical stem cell is per definition a cell that can self-renew (i.e give rise to more of itself), proliferate indefinitely and differentiate to any given celltype. Stem cells are classified by

their differentiation potential as *totipotent*, being able to give rise to all cell types, *pluripotent*, capable of generating cells derived from all three germ layers or *multipotent*, giving rise to many cell types usually of one particular lineage (Kirschstein and Skirboll, 2001).
Most stem cells are studied by isolating putative stem cell fractions and characterize them *in vitro*. There is an obstacle in reconciling the theoretical stem cells with its *in vitro* counterpart. Self-renewal can be evaluated by *clonal assays*, where the progeny of one single cell is shown to contain cells that can repeatedly give rise to new cells with the same properties as the original cell (Reynolds and Weiss, 1996). Proliferation capacity can be somewhat determined in culture however the longevity of it must by necessity be limited to "long-term". Many thus prefer to label putative stem cell preparations as *progenitor* cells, which refer to cells with a more restricted potential than true stem cells (McKay, 1997). The term *precursor* is also used in connection with stem and progenitor cells and signifies any cell that is earlier in the developmental pathway than another defined cell (McKay, 1997) without saying anything about the "stemness" of it.
It is also necessary to distinguish between the *fate* of stem cells *in vivo*, within its normal context, and the *potential* of cultured stem cells (Gaiano and Fishell, 1998). The potential of an *in vitro* stem cell can be evaluated by differentiation *in vitro* or by introducing it to its normal environment, usually by transplantation. Assessment of the full differential potential of a stem cell may be difficult if the signals necessary for differentiation to a certain subtype are not known or not provided.
However, it is also possible to manipulate stem cells *in vitro* and expose them to circumstances that they do not normally encounter. In this context it can be valuable to distinguish between the *capacity* of a stem cell, which is an intrinsic differentiation potential and what it will differentiate into under normal circumstances *in vitro* or when transplanted into its original environment, and the *capability* of stem cell, which reflects what it can become under certain exaggerated conditions (Kirschstein and Skirboll, 2001).

Neural stem cells

Cells with at least some of the cardinal features of stem cells have been demonstrated in many different organs in the body, and in recent years also in the brain. However, most of these are restricted in their developmental potential to generate only cells of the organ where they originate (Kirschstein and Skirboll, 2001). *Neural stem cell* is the definition of a cell that can generate all three main neural celltypes: neurons, astrocytes or oligodendrocytes and/or is a cell that is derived from the nervous system (Gage, 2000). Embryonic stem cells (*ES cells*) is one of two stem cell preparation that can definitely be classified as pluripotent (the other being embryonic germ cells). ES cells are derived from the inner cell mass of the blastocyst (the very early embryo) and are subsequently cultured *in vitro* (Kirschstein and Skirboll, 2001). Factors and protocols that direct ES cells towards a neural fate have been reported, both for human and murine ES cells (Bain et al., 1995; Okabe et al., 1996; Perrier et al., 2004; Strubing et al., 1995).
Neural stem cells can be derived from both fetal and adult tissue and is stimulated to proliferate *in vitro* in response to various growthfactors such as epidermal growth factor (EGF) and basic

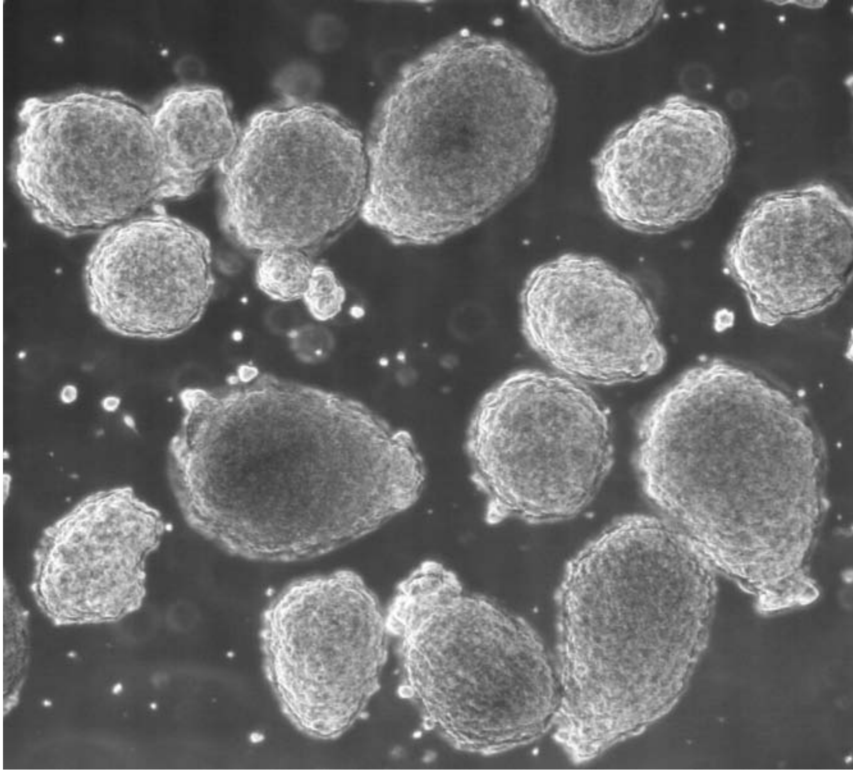


Fig. 4 Neurospheres derived from mouse E11.5 VM tissue

fibroblast growth factor (bFGF) (for review see Gage, 2000; Martinez-Serrano et al., 2001; McKay, 1997). However, there is some debate what this cell is and where it is located at different developmental stages. It has been suggested that neuroepithelial cells in the ventricular zone corresponds to an early *fetal neural stem* cell that is characterized by its responsiveness to bFGF. These early stem cells will in some regions give rise to subventricular zone (SVZ) cells where another, EGF-responsive stem cell appears during late embryogenesis and remain in neurogenic zones in the adult tissue from which it can be isolated as an *adult neural stem* cell (Pevny and Rao, 2003). Alternatively, radial glia cells have been proposed to be the stem cells of the fetal brain and that SVZ cells in adult animals are derived from this population (Alvarez-Buylla et al., 2001). This controversy points to a major obstacle when studying neural stem cells, namely the lack of markers to positively identify the neural stem cells. A few markers such as nestin, *Sox* genes and Musashi have been suggested to label murine neural stem cells (Aubert et al., 2003; Lendahl et al., 1990; Sakakibara et al., 1996). However, although they label cells with the characteristics of neural stem cells they also label more restricted cell types (Barraud et al., 2005).

Neurospheres as a way to expand neural stem cells

Many of the problems in connection with characterizing neural stem cells lies in the difficulty in maintaining them in culture as a pure population. The *neurosphere assay* was initially presented as way to isolate a neural stem cell that self-renewed to form more of itself and whose progeny could give rise to all the celltypes of the CNS, neurons, astrocytes and oligodendrocytes (Reynolds and Weiss, 1992; Reynolds and Weiss, 1996). Neurosphere cultures have since been generated from several subregions of both adult and fetal neural tissue and used to expand neural stem cells. Neural tissue is dissociated into single cells, suspended in defined medium with added mitogens and seeded out in low attachment vessels. After a few days, the cells have divided to form free-floating spheres (Fig 4). However, the neurospheres contain a very heterogenous population of cells of which the self-renewing stem cells only make up a small fraction (Reynolds and Weiss, 1996). It also appears as though other cells with more limited properties than a neural stem cell have sphereforming capacity (Reynolds and Rietze, 2005).

It has been shown that culture conditions affect the characteristics of the expanded cells (reviewed in Gage, 2000; Lillien, 1998; Martinez-Serrano et al., 2001). Factors like choice of mitogen, additions to the culture medium or cell density may alter the properties of the cells or select for a specific cell to be expanded. Selection for a specific cell assumes that there are differences between neural stem (or progenitor) cells. Indeed, many studies have shown that fetal neural stem/progenitor cells display region specific characteristics (Hitoshi et al., 2002; Horiguchi et al., 2004; Klein et al., 2005; Parmar et al., 2002; Zappone et al., 2000) indicating that this is the case.

Expansion of midbrain neural stem and progenitor cells

Early studies attempting to expand neural stem cells from mesencephalic tissue showed that EGF was mitogenic for progenitors in embryonic rat VM (Mytilineou et al., 1992) and that mesencephalic tissue from mice as young as E10 could be induced to proliferate with bFGF and serum (Kilpatrick and Bartlett, 1993). More specifically, bFGF was shown to prolong division of dopaminergic precursors and delay the differentiation of TH-positive cells in attached primary cultures from E12 rat embryos (Bouvier and Mytilineou, 1995). Subsequent studies established neurosphere cultures from E14-E16 rat mesencephalon using EGF (Ptak et al., 1995; Svendsen et al., 1995) or EGF/bFGF combined (Caldwell and Svendsen, 1998). The cells could be passaged and remained mitotically active over long time but only a small number differentiated to TH-positive neurons (Caldwell and Svendsen, 1998; Ptak et al., 1995). However, specific inducing protocols showed that expanded neurospheres had the capability to develop into dopaminergic neurons under certain circumstances (Carvey et al., 2001; Ling et al., 1998; Storch et al., 2001; Storch et al., 2003).

Attached culture methods proved successful in expanding dopaminergic precursors from rat fetal tissue, however these cultures were not expanded long-term (Studer et al., 1998). Similar protocol has also been used to expand dopaminergic precursors from human fetal mesencephalic tissue (Sanchez-Pernaute, 2001).

Aims of this thesis

The overall aim of this thesis work has been to further elucidate mechanisms and events important for the development of the midbrain dopaminergic neurons *in vivo* and explore ways to generate this kind of neurons *in vitro*. These issues have been investigated in the papers included in this thesis in the following way:

- Identify and isolate early dopaminergic precursors – **Paper I**
- Identify new factors involved in the development of dopaminergic neurons – **Paper II**
- Expand neural stem and progenitor cells with a potential to differentiate into mesDA neurons – **Paper III**

Results

Results and comments

Identifying early midbrain neural progenitors

One of the aims of this thesis was to identify genes that labelled early precursors in the dopaminergic neuron lineage to gain more insight into how dopaminergic neurons are formed. In order to do this, I evaluated the midbrain expression of several proteins known to be involved in development in the forebrain. The most relevant are pictured in Figure 5. Many of them did not present a pattern that was suggestive of being involved in dopaminergic neuron development but were instead expressed in either lateral or intermediate VM domains (Fig 5). However, pre-B-cell homeodomain protein PBX was found to be located in the same domain as TH at E11.5 and onwards (see Fig 5 and also **paper I**). I also evaluated the embryonic expression of FA-1 or Dlk-1, a protein previously reported to be expressed by SNc and VTA neurons in the adult rat (Jensen et al., 2001). I found that its expression correlated well with TH also during embryonic stages (Fig 5). These results identified two new markers of mesDA neurons.

Sox-genes are differentially expressed in the midbrain

Before the start of this thesis Aldh1 was the only marker known to be expressed specifically in early mesDA progenitors, however Aldh1 also labels more mature neurons (McCaffery and Drager, 1994). We wanted to identify genes that labelled mitotic dopaminergic progenitors to be able to isolate cells for expansion in culture and possibly follow the cells in different expansion protocols.

The transcription factor Sox1 had previously been shown to be one of the earliest markers for neural ectoderm (Pevny et al., 1998; Wood and Episkopou, 1999). It was reported to be expressed by neuroepithelial cells throughout the early neural tube, and associated with dividing neural cells. When I analysed the midbrain expression in a transgenic mouse expressing GFP under the *Sox1* promoter (Aubert et al., 2003), I found that both *Sox1*-GFP and the Sox1 protein was conspicuously absent from the medial part of the midbrain where progenitors of the mesDA neurons would be located (Fig 5). However, it was expressed profusely in the dorsal midbrain. The dorsolateral expression extended ventrally over the lateral sulcus which separates the VM from the DM, such that a narrow Sox1 domain was present at the lateral edges of the defined VM, coinciding with expression of e.g Pax6 and Meis2 (Fig 5). I subsequently found that Sox2, closely related to Sox1 and expressed in early neuroectoderm as well as in VZ from neurulation (Cheung et al., 2000; Wood and Episkopou, 1999), is expressed in the VZ throughout the midbrain, including the medial region of the ventral midbrain. This suggests that it labels also dopaminergic mitotic progenitors (unpublished results). The *Sox* genes are thus, despite extensive overlap in other parts of the nervous system, differentially expressed in the midbrain during the period of mesDA neuron development.

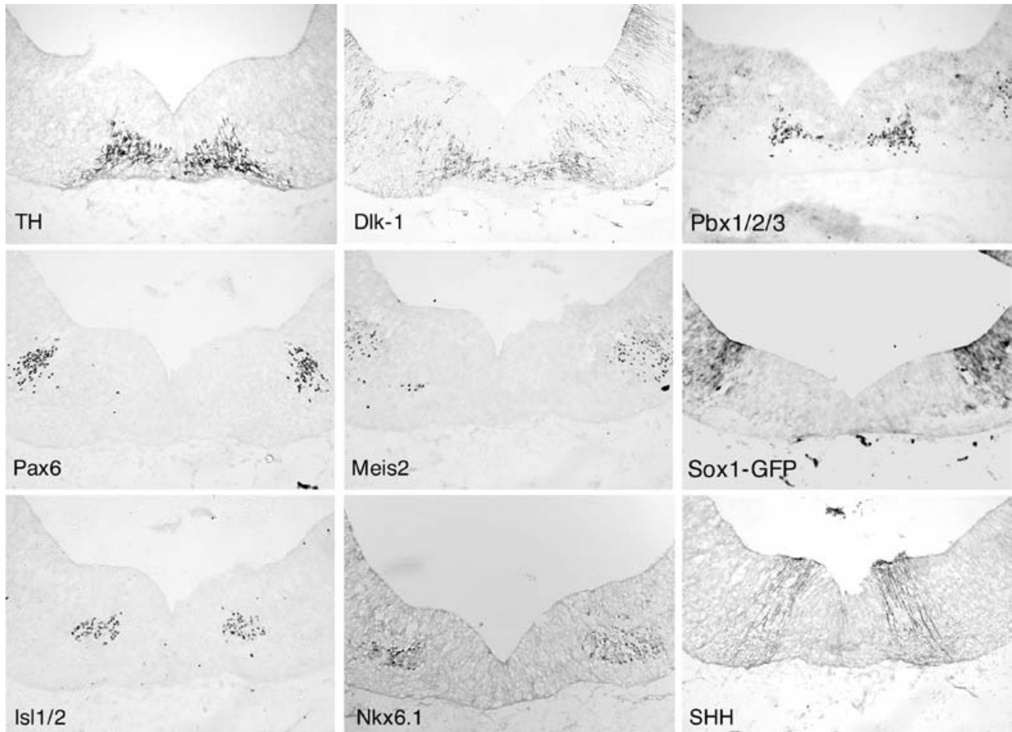


Fig. 5 Coronal sections through the VM of E11.5 mouse. Dlk-1 and Pbx1/2/3 were found to co-localize with TH in developing mesDA neurons. Other markers were found to be expressed in either a lateral domain spanning the lateral sulcus (Pax6, Meis2 and Sox1) or in an intermediate domain (Isl1/2 and Nkx6.1). Curiously the protein expression of SHH appeared to be outside of the medial domain where dopaminergic neurons are generated.

Neurogenin2 labels progenitor cells in the ventral midbrain (Paper I and II)

One group of transcription factors that had not previously been implicated in dopaminergic neuron development was basic helix loop helix (bHLH) factors. bHLH transcription factors are active in determination and differentiation in many tissues including muscle and nerve. In nervous tissue their main function is to select neuronal progenitors and activate genetic programmes for a generic neuronal phenotype (therefore also called proneural genes) however they have also been implicated in subtype specification of certain neurons.

The most common proneural genes are *Mash1* and *Neurogenins*. They are related to two gene families that control neural determination in separate subclasses of neurons in drosophila, the achaete-scute family (Mouse achaete-scute homolog1) and atonal family, respectively. As in drosophila they are expressed in complementary and mostly non-overlapping regions of the peripheral nervous system, PNS and CNS suggesting they define distinct progenitor populations (reviewed in Bertrand et al., 2002).

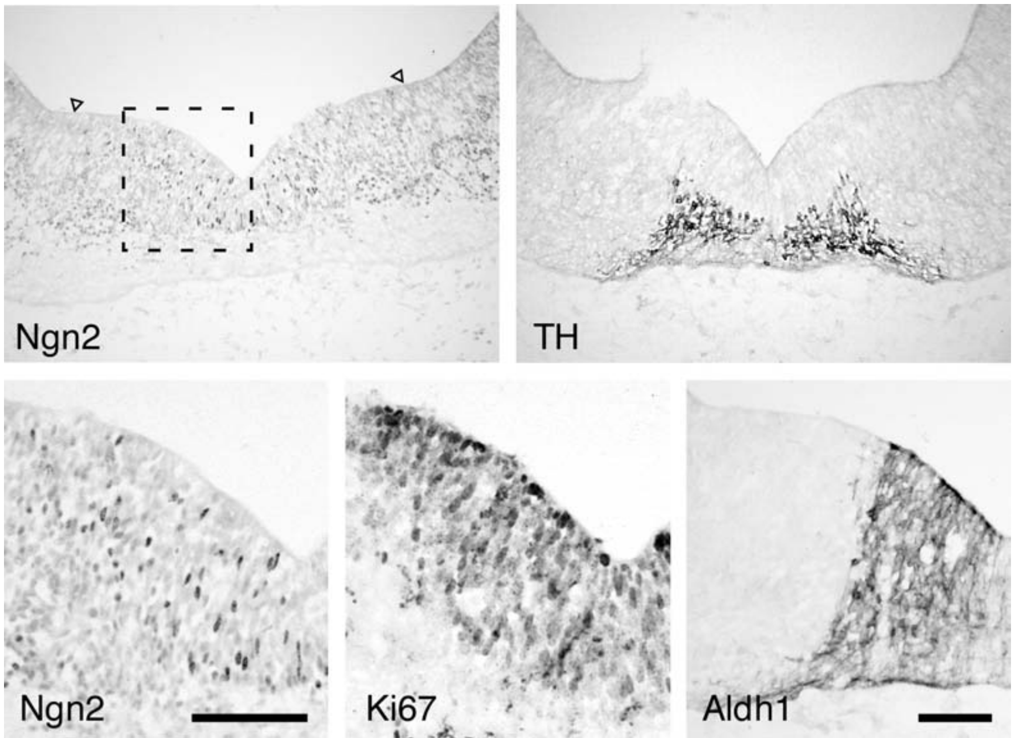


Fig. 6 Coronal sections through the VM at E11.5. Proneural gene Neurogenin2 (Ngn2) is expressed in a restricted pattern in the VM, correlating with the developing TH-positive cells. Ngn2-positive cells are contained within the VZ as determined by staining for Ki67, a marker for dividing cells, in parallel sections. The expression domain of Ngn2 extends lateral to the expression domain of Aldh1 (also in parallel sections), suggesting that it also labels progenitors outside the mesDA domain. The expression of Ngn2 does not overlap with Sox1 expression in the lateral domain of the VM (Sox1 expression limit indicated by open arrowheads). Scale bars: 400 μm

I screened for expression for proneural genes in the VM and found that Neurogenin2 (Ngn2) was expressed in a pattern that was suggestive of it labeling mitotic dopaminergic neuron precursors. The temporal and spatial expression of Ngn2 and also its expression in comparison to dopaminergic neurons were investigated in detail in **Paper I**. Mitotic nuclei in the VZ undergo a process called interkinetic nuclear migration, i.e they move perpendicular to the ventricular surface depending on where in the cell cycle the cell is. This gives the VZ an impression of being multilayered, termed a pseudo-stratified layer (Jessell and Sanes, 2000a). Ngn2 labeled nuclei that displayed this sort of pattern with strongly labeled and more weakly labeled cells arranged at various distances from the ventricular surface. Parallel sections were stained for the marker Ki67, which labels dividing cells, to determine the extent of the VZ. From this I concluded that the Ngn2 labeled cells were confined to the VZ (Fig 6). Subsequent investigations showed

that although Ngn2 staining appear confined to the VZ, single examples of Nurr1/Ngn2 double stained cells could be detected (**Paper II**). Nurr1 is one of the first proteins to be expressed in postmitotic dopaminergic precursors (see Introduction). If these cells represent postmitotic cells where expression of Ngn2 remain or if they are mitotic cells where Nurr1 is expressed prematurely is at present uncertain, as is the significance of this finding.
The expression of Ngn2 was present in the VM as early as E10.5, before TH-positive cells were detected. At E11.5, when the first TH-positive neurons appeared in the MZ, the Ngn2-positive cells were found to be located in the VZ directly above them. When comparing the expression domain of Ngn2 to that of Aldh1, it extended further lateral of the midline but not as far as the Sox1 expression domain (Fig 6). This quite restricted expression pattern suggested that Ngn2 labeled dopaminergic progenitors and possibly some other progenitor population lateral to them. The caudal limit of Aldh1 coincided with the end of midline expression of Ngn2, however Ngn2 expression domain extended further rostrally into the diencephalon.
At subsequent stages, the expression domain of Ngn2 was restricted more and more towards the midline and included fewer cells, concurrent with fewer cells being committed to a dopaminergic fate (Bayer et al., 1995). Additionally, expression of Ngn2 was first abolished in the rostral part of the mesDA domain, in accordance with the rostrocaudal shift of dopaminergic neurogenesis previously reported (Bayer et al., 1995). Altogether this suggested that Ngn2 labeled a mesDA progenitor cell.

Fate of Ngn2 expression cells in the midbrain

Fate mapping of the Ngn2-positive cells reveal that they are mesDA precursors (Paper I)
The correlation between the spatial and temporal dynamics of Ngn2 staining and dopaminergic neurogenesis was compelling, however it did not prove that Ngn2 was labeling a precursor of mesDA neurons. In order to further elucidate the fate of the ventral midbrain Ngn2-positive cell we made use of a transgenic mouse that had GFP knocked into the *Ngn2* locus (*Ngn2-GFP*-KI, Seibt et al., 2003). We found that GFP expressed from this locus remained in the cells after expression of Ngn2 was downregulated. Thus we could trace the cells that had expressed Ngn2 by their continued expression of GFP. As would be expected, fatemapping of the cells was restricted in time according to the turnover of GFP protein and the detection limit of the antibody for GFP.
At E12.5, Nurr1-positive postmitotic mesDA precursors in the intermediate zone (IZ) co-expressed high levels of GFP and further differentiated, TH-positive cells in the MZ expressed a low level of GFP. This showed that cells expressing Ngn2 will differentiate into mesDA neurons *in vivo*. The high level of GFP in the IZ was an interesting finding. It was clear from GFP IHC that cells in the IZ expressed GFP at a higher level than cells in the VZ (ie cells that also expressed Ngn2). This could be interpreted in many ways, however the most likely explanation is that expression of GFP is slightly lagging and that it reflected very recent events in the dynamics of Ngn2 expression. It is feasible that while the expression of Ngn2 is tightly regulated and the protein is broken down quickly, GFP has a longer half-life and remain. This

high level of GFP in the IZ in cells that have just exited the cell cycle, suggested that Ngn2 is highly upregulated just before or as cells were leaving the cell cycle.

Ngn2-expressing precursors also give rise to other types of neurons (Paper I and II)
We also saw GFP-positive cells lateral to the Nurr1- and TH-positive cells indicating that Ngn2-expressing precursors will give rise to other cell types. The lateral extent of the GFP expression at E12.5 correlated well with the lateral border of Ngn2 expression seen at E11.5 although expression of the Ngn2 itself at this timepoint was more restricted. This suggests that a precursor population expressing Ngn2 and giving rise to a celltype lateral to the dopaminergic neurons was proliferating at E11.5 but no longer at E12.5. Isl-positive neurons of the oculomotor nuclei are located in this lateral region but did not express GFP at E12.5 (**Paper I**). However, when analysed at E11.5 they showed weak GFP expression (**Paper II**). Ngn2-expressing precursors are thus also likley to contribute to oculomotor neurons.

The role of Ngn2 in mesDA neuron development

Absence of Ngn2 results in a dramatic loss of mesDA neurons (Paper II)
Since Ngn2 was expressed by early progenitors in the midbrain, and among them dopaminergic neuron progenitors, we wanted to investigate its role in the development of the dopaminergic neurons. The homozygous *Ngn2-GFP*-KI mice are functional knockout mutants as the coding regions of both alleles are replaced by GFP.
Analysis at the embryonic stages when the dopaminergic neurons are normally generated (E11.5-E13.5), revealed that only a minor fraction of the TH-positive cells were generated in the homozygous knockout mutants. These TH-positive cells were consistently located at the lateral edges of the expected dopaminergic domain but appeared by all accounts to be normal (Fig 7). In postnatal (P0 and P18) mutants the remaining dopaminergic cells were distributed equally between the SN and VTA and shown to project to the correct areas in the forebrain. They also displayed the expected expression of subtype specific markers. The difference between wildtype and mutant mice regarding the number of dopaminergic neurons was less at these postnatal stages however there was still 60-70% reduction of mesDA neurons in the mutant (Fig 7). Interestingly only the dopaminergic neurons, and not the Isl-positive cholinergic neurons, were affected.

Ngn2 is important for the medial mesDA neuron precursors specifically (Paper II)
The loss of dopaminergic neurons in the mesencephalic nuclei appeared to result from an inability of precursors in a medial section of the mesDA domain to develop properly. By the GFP expression and DAPI staining of cell nuclei we could see that cells were generated also in this sector, however they remained close to the ventricle and did not migrate out into the MZ. They did not express NeuroD (a neural differentiation factor downstream of Ngn2), Nurr1 or Pitx3. BrdU and Ki67 stainings showed on the other hand that they were not proliferating. We concluded that Ngn2 is not essential for cell-cycle exit but that in its absence the precursor

cells are arrested from migration and expression of more mature neuronal markers as well as all dopaminergic specific markers are absent. However, they did not express astrocytic markers which indicate that they did not acquire an alternative fate.

Over-expression of Ngn2 does not induce dopaminergic differentiation (Paper II)
In order to determine if Ngn2 by itself promoted a dopaminergic fate we overexpressed Ngn2 in dorsal mesencephalic primary cells and also in primary VM cells. This did not increase the number of TH-positive cells in either culture however it increased the number of β-III-tubulin -positive cells. Thus, Ngn2 is important for the generation of a subset of mesDA neurons but is not on its own sufficient to specify cells to become dopaminergic.

Potential of the *Ngn2*-GFP positive cells

***In vitro* differentiation (Paper I)**
Since GFP was expressed under the *Ngn2* promoter in the *Ngn2-GFP*-KI mice it was possible to isolate Ngn2-positive cells and their immediate descendants by fluorescence activated cell sorting (FACS) and determine their developmental potential *ex vivo* (outside the brain). As mentioned before we had noticed varying intensities of GFP expression among the cells generated from Ngn2-positive cells. Cells in the VZ, where Ngn2-positive cells are located, exhibited a relatively low GFP expression while the direct descendants, the Nurr1-positive cells of the IZ, showed high level of GFP expression. In order to determine their differentiation potential we separated GFP-positive cells from E12.5 VM into GFP^{high} and GFP^{low}, which corresponded approximately to IZ cells and VZ+MZ cells respectively. The cells were differentiated *in vitro* with minimal serum content. The results confirmed that *Ngn2*-GFP positive cells are precursors to mesDA neurons as only the GFP-positive fractions contained TH-positive cells. However, it also showed that most TH-positive cells that developed *in vitro* were postmitotic at the time of plating, as they rarely incorporated BrdU. This suggested that Ngn2-positive cells from the VZ will not develop into TH-expressing cells *in vitro*, when removed from the surrounding cells and possibly that they are not yet intrinsically specified.

***In vivo* differentiation (Paper I)**
We also wanted to determine the potential of the *Ngn2*-GFP-positive cells to survive and differentiate in a putatively more suitable environment. We therefore transplanted GFP-positive and GFP-negative cells separately to the striatum of newborn rats. The yield of *Ngn2*-GFP cells was too low to further separate the GFP-positive cells into GFP^{high} and GFP^{low} for transplantation. Four weeks after transplantation the grafts were assessed by IHC. By transplanting the mouse-derived cells to neonatal rats it was possible to distinguish them by murine specific antigens. The results showed that, as *in vitro*, mouse derived TH-positive cells were all found in animals which had recieved grafts from the GFP-positive fraction. These grafts were also enriched in TH-positive cells compared to grafts derived from non-sorted control cells transplanted at the same density. By contrast, all other neuronal types detected; serotonergic, GABAergic and

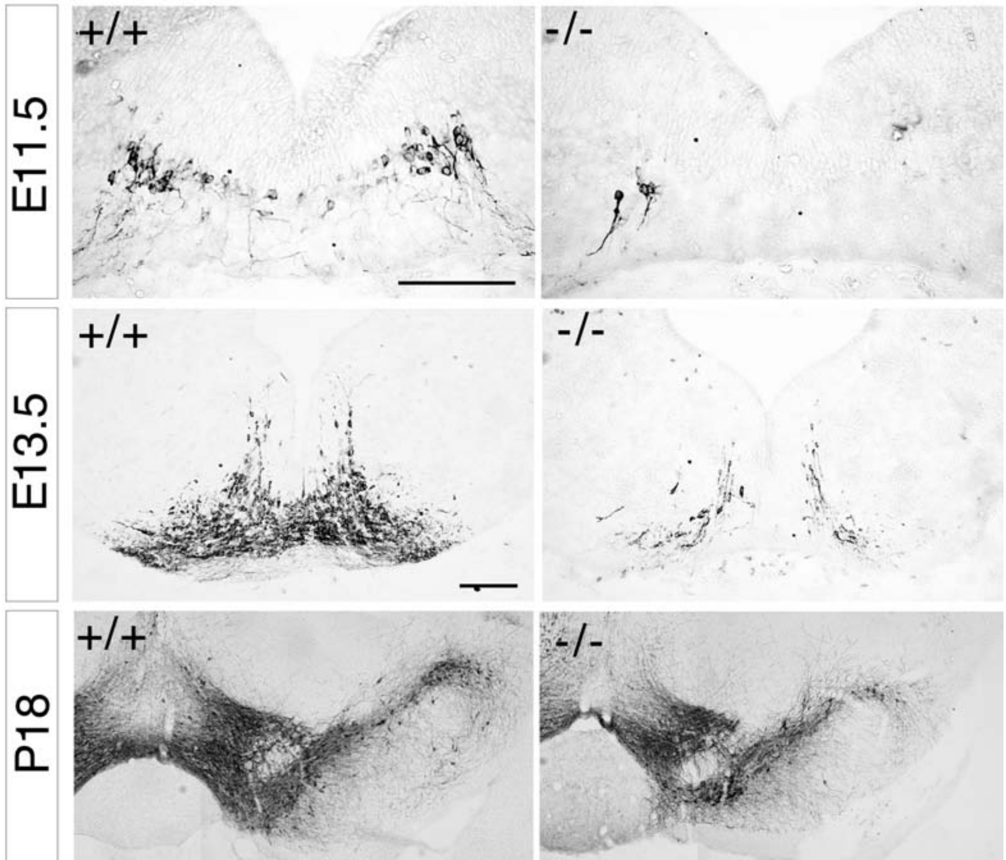


Fig. 7 Coronal sections through midbrain of embryonic and young mice, wt (+/+) and Ngn2-KO (-/-) respectively. The majority of TH expressing cells is lost in Ngn2-KO mice. At embryonic stages (E11.5-E13.5) when the mesDA neurons are normally generated, only a few cells are seen, located at the lateral edge of the mesDA neuron domain. The number of TH-positive cells in the knockout increase during later embryonic development, however there are still around 60-70% fewer TH-positive cells at postnatal stages (P18).

cholinergic were almost exclusively found in grafts derived from the GFP-negative fraction. Interestingly, although both types of grafts contained many neurons, virtually no glia was found in the GFP-positive fraction. They were however abundant in the GFP-negative fraction and the unsorted control tissue. This suggests that *Ngn2*-GFP positive cells isolated from the VM at E12.5 are marked for a neuronal fate and some of the cells more specifically for a dopaminergic neuronal fate. Notably, GFP^{low} cells (which were also present among the GFP-positive cells), although not specified enough to develop into dopaminergic neurons *in vitro* nevertheless appear specified to become neurons, assuming they survive in the grafts.

Expanding neural stem and progenitor cells from the ventral midbrain

Neurosphere cultures (Paper III)

One of the aims of this thesis was to expand neural stem and progenitor cells that had a potential to differentiate into dopaminergic neurons. Previous studies had indicated that neural stem and progenitor cells are regionally specified and retain some of this specification after expansion *in vitro* (Hitoshi et al., 2002; Horiguchi et al., 2004; Klein et al., 2005; Parmar et al., 2002; Zappone et al., 2000). To increase the possibility of generating mesDA neurons I therefore isolated tissue from E11.5 mouse VM, the time and region where these neurons are generated. Other studies have used mesencephalic tissue of later stages with limited success in generating mesDA neurons (Caldwell and Svendsen, 1998). Dopaminergic neuron progenitors are present and dividing also at earlier stages than E11.5, as shown in [^{3}H] thymidine labelling studies (Bayer et al., 1995). However, I found that E10.5 tissue was difficult to dissect free of contaminating meninges and the yield of cells was limited, and for these reasons avoided as starting material.

The cells were plated in neurosphere medium with growthfactors EGF and bFGF and rapidly formed neurospheres. The neurospheres could be passaged and yielded new spheres even after 10 weeks in culture. The spheres needed to be passaged once a week, and this interval between passages did not change over the 10 weeks suggesting that the division rate remained the same over time. The number of plated cells expanded on average 8-fold once the cultures were established. Although the cells formed spheres with the same approximate size and displayed similar growth characteristics as forebrain cultures there were tangible differences. Midbrain derived spheres were denser and required more force to separate into single cells at passage. Moreover, they attached more easily to plastic surfaces, both during the expansion and proliferation phase. This may be attributed to denser extra-cellular matrix however this was not assessed.

Differentiation potential of progenitors from ventral midbrain expanded as neurospheres (Paper III)

When neurospheres were differentiated by removing growth factors, adding a low concentration of serum to the medium and plated on a permissive surface, the expanded cells gave rise to neurons, oligodendrocytes and astrocytes. I did not grow cells at clonal densities and therefore can not be certain that single cells within the neurospheres were multipotent, however the cultures as whole gave rise to neurons and oligodendrocytes with a frequency of approximately 5% and 1% respectively, even after several passages. The majority of the remaining cells were astrocytes judging by their morphology and immunoreactivity for GFAP. The cultures were neurogenic for several passages, however at passage 10 or later, the cultures generated only GFAP-positive cells when differentiated. This suggests that the potential of the expanded cells changed over time or that a glia restricted progenitor was preferentially expanded.

Neurospheres from E11.5 ventral midbrain are regionally specified (Paper III)

As mentioned above, neural stem and progenitors maintain some of their region character *in vitro* and the progeny matches the subtypes generated in their region of origin. However, all neural phenotypes generated in a certain region do not arise spontaneously from corresponding stem cell preparation, but can require additional factors (Hitoshi et al., 2002; Jensen et al., 2004; Klein et al., 2005). I found that neural stem or progenitor cells in the neurosphere cultures maintained a VM molecular identity, at least on the mRNA level, but that neurons with a dopaminergic phenotype were not generated by standard *in vitro* differentiation. Positional information provided by surrounding cells and other extrinsic cues necessary for certain cellfates are largely lost in neurosphere culture (Jensen et al., 2004). Our attempts to provide some of the extrinsic cues present in the developing VM were not successful which indicate that either we did not provide the correct factors or the concentrations and/or combinations were inadequate. An alternative is that the progenitor cells that were expanded with our protocol could not respond to these cues.

However, over-expression of Nurr1 generated TH-positive cells that also expressed other mesDA neuron markers. The frequency with which this occurred and the phenotype of the TH-positive cells generated was specific for VM progenitors and demonstrated that VM neurosphere cultures are particularly equipped to respond to this stimulus compared to forebrain derived neurospheres. Although not verified by other stainings it is also possible that the Nurr1-induced TH-positive cells generated from forebrain derived neural progenitors were olfactory glomerular dopaminergic neurons as these also express Nurr1 during development (Backman et al., 1999).

Over-expression of Ngn2 and Nurr1 together generate a more complete mesDA phenotype (Paper III)

When Ngn2 was over-expressed together with Nurr1 in the neurosphere expanded cells, this generated morphologically mature TH-positive cells. Some of these TH-positive cells also expressed other dopaminergic markers such as VMAT2, Dlk-1, Pitx3 and En1/2. Over-expression of Ngn2 alone did not induce the expression of either of these markers. Thus, although Ngn2 on its own did not appear to affect the differentiation of cells towards a dopaminergic phenotype, it induced a change possibly to make the cells more receptive to either Nurr1 over-expression or to changes induced by Nurr1. Nurr1 and Pitx3/En1/2 are thought belong to separate developmental pathways as En1/2 and Pitx3-positive cells are found in Nurr1-knockout mice at early stages. It is worth noticing that both En1/2 and Pitx3 appear to have been initiated by Nurr1 and Ngn2 co-transduction but not by transduction of either factor independently.

Discussion

Discussion

Due to the findings in this thesis one more gene, *Neurogenin2* (*Ngn2*), can be added to the list of markers expressed in the midbrain dopaminergic neuron lineage. Importantly, this is the first gene so far to be expressed in the proliferative progenitors but not in the more differentiated cells and which is selectively, although not exclusively expressed by mesDA neuron precursors in the ventral midbrain.
Ngn2 is not just a marker for mesDA neuron progenitors, although it may be used as such, but also plays a role in the development of dopaminergic neurons. Its precise role in the sequence of events leading to generation of dopaminergic neurons has not been fully elucidated yet.
Drawing from the results of the respective studies I will attempt to assemble a more complete picture of progenitor cells in the midbrain and present some theories of the role of Ngn2 in the neurogenesis of mesDA neurons

Phenotype of the mitotic progenitor in the ventral midbrain
Members of the *Sox* gene family are detected in mitotic cells in VZ of most the CNS (Bylund et al., 2003; Cheung et al., 2000, Pevny et al.,1998) and have been shown to label cells in the telencephalon that display functional properties of stem cells (Barraud et al., 2005; Zappone et al., 2000). As mentioned in the results section, we found that Sox1 was not expressed in the medial part of the VM from E11.5 and onwards, however Sox2 expression is present in the VZ at the time when dopaminergic neurons are generated (unpublished results). GLAST (glutamate transporterEAAT1), which labels radial glia cells in the forebrain (Shibata et al., 1997), was also found to be expressed in the VM at this time (**Paper II** and unpublished results). Radial glia are shown to give rise to the vast majority of neurons in the CNS and are suggested to act as fetal progenitor cells (Anthony et al., 2004; Malatesta et al., 2003; Noctor et al., 2001). One can thus hypothesize that a neural progenitor in the medial VM, which will give rise to dopaminergic neurons, is likely to express GLAST, Sox2 or both.

Dynamic expression of Ngn2 within the VZ
We found that proneural gene *Ngn2* have an expression pattern during embryogenesis consistent with it being expressed in a mitotic progenitor in the VM. Ngn2 is expressed in scattered nuclei in the VZ, and low level of GFP is also seen in the VZ of *Ngn2-GFP*-KI mice (**Paper I and Paper II**). We could not perform double labeling of Ngn2 and Sox2 for technical reasons, however Ngn2 is most likely expressed by the neural progenitors of the VM that also express Sox2. Moreover , we saw co-expression of nestin and Ngn2 (**Paper I**). In other studies Ngn2 has consistently been reported to be expressed in VZ and SVZ and also to label for BrdU showing that it is expressed in cells that are dividing (Gradwohl et al., 1996; Sommer et al., 1996).
Close inspection of *Ngn2 in situ* hybridisation suggested that *Ngn2* mRNA has its highest expression at the ventral edge of the VZ, close to the postmitotic IZ. Although this is not as clear from the protein expression, which shows Ngn2-positive cells throughout the thickness

of VZ, Ngn2 may be expressed at low levels (too low for detection by IHC) by the majority of VZ cells and be upregulated during last cell cycle. In *Ngn2-GFP*-KI mice the GFP expression, which is slightly lagging compared to Ngn2 protein expression (presumably due to longer half-life of the protein), show the highest expression in the IZ cells, which also suggest a surge in Ngn2 expression prior to cell cycle exit (**Paper I and II**).

According to the established model for neurogenesis, mitotic progenitors initially express proneural genes at low levels while they are not yet committed to differentiation (Bertrand et al., 2002; Bylund et al., 2003; Kintner, 2002) Positive feedback loops result in high levels of proneural genes in a subpopulation of progenitor cells, concurrent with the cells being irreversibly committed to differentiation. The initial upregulation of proneural gene expression is the result of lateral inhibition where Notch signalling repress proneural expression and reduce levels of notch ligand in some cells, thereby enabling higher levels of proneural gene expression in the progenitors selected for neuronal fate. However, other positive feedback mechanisms are needed to increase and/or maintain the elevated levels of proneural gene expression (Bertrand et al., 2002; Kintner, 2002). Interestingly, in the Ngn2 knockout mutants we detected cells with high expression of GFP that maintained contact with the ventricle, similar to radial glia, and which were expressing GLAST (**Paper II**). This indicates both that the neural progenitor of the VZ normally experiences an upregulation of Ngn2 and that this neural progenitor is a GLAST-positive radial glia-like cell. It is notable that the GFP in the *Ngn2* locus was still upregulated despite the lack of Ngn2 suggesting that Ngn2 protein activity is not necessary for the regulatory feedback loops.

Possible interaction of Ngn2 with Sox2 in neurogenesis of ventral midbrain cells

Proneural genes function on several levels of neurogenesis. Progenitors are selected for a neuronal fate by an upregulation of proneural genes, which promotes cell cycle exit and start developmental programmes for neuronal differentiation (Bertrand et al., 2002; Kintner, 2002). Bylund et al (2003) showed that activity of Sox proteins in neural progenitors in chick spinal cord maintains them in an undifferentiated state and that high levels of Ngn2 protein most likely mediate neurogenesis by downregulating Sox1-3 expression.

We saw that a lack of Ngn2 caused an accumulation of cells arrested in differentiation in the medial part of the mesDA neuron domain in the Ngn2 knockout mutants (**Paper II**). Although we did not ascertain the expression of Sox2 in the mutants it is likely that the lack of Ngn2 enabled continued Sox2 expression in these progenitors. Maintenance of some progenitor character such as contact to the ventricle and expression of GLAST may thus be a result of this prolonged Sox2 expression.

Interestingly, the lack of Ngn2 did not greatly affect cell cycle exit of the progenitor cells in the VM, as we did not see an increase in proliferating cells (**Paper II**). Several studies connect over-expression of Ngn2 with premature cell cycle exit (Lo et al., 2002; Mizuguchi et al., 2001). What drives the cells out of cell cycle in the VM remains unclear.

Ngn2 involved in acquiring full neuronal phenotype of mesDA neuron precursors

Proneural genes regulate the expression of general neuronal character at neurogenesis and over-expression of these genes often leads to increased neuronal differentiation both *in vivo* and in cultured cells (Farah et al., 2000; Mizuguchi et al., 2001; Sun et al., 2001). We also found that over-expression of Ngn2 increased the number of β-III-tubulin positive neurons in both primary cells from the dorsal mesencephalon (**Paper II**) and in neural progenitor cells expanded as neurospheres (**Paper III**).

Specific members of the bHLH transcription factor family act as neuronal differentiation genes downstream of earlier expressed proneural genes (Bertrand et al., 2002). The Ngns have previously been shown to co-localize with, and in many areas precede, the expression of NeuroD, a neuronal differentiation gene (Sommer et al., 1996). In the VM we found that NeuroD was expressed by cells bordering the IZ, suggesting that NeuroD is a downstream effector of Ngn2. The lack of NeuroD coupled with the loss of all neuronal markers in the accumulated cells confirmed that NeuroD act as neuronal differentiation factor regulated directly or indirectly by Ngn2 and the absence of Ngn2 (and thus NeuroD) prevent further neuronal differentiation of the newly postmitotic cells. It is worth noticing that NeuroD expression was seen in the dorsal midbrain and regions lateral to the mesDA neuron domain also in the Ngn2 mutants, suggesting that other proneural genes control expression of NeuroD in other regions of the midbrain. We also saw β-III-tubulin positive cells that did not express TH in the MZ of the Ngn2 mutants, suggesting that neurogenesis is maintained for other types of neurons that are thus not dependent on either Ngn2 or NeuroD (**Paper II**).

Does Ngn2 have a role in both differentiation and specification of mesDA neurons?

Although the major role of proneural genes such as *Ngn2* is to regulate genes for a generic neuronal phenotype, they have also been implicated in specification of neuronal subtype. Proneural gene *Mash1* specifies noradrenergic phenotype both in the PNS (Hirsch et al., 1998; Lo et al., 1998) and in the CNS (Pattyn et al., 2000), GABAergic interneurons in the ventral forebrain (Fode et al., 2000) and serotonergic neurons in the hindbrain (Pattyn et al., 2004). Ngn2 on the other hand is involved in the specification of motor neurons in the spinal cord (Scardigli et al., 2001) and glutamatergic neurons in the dorsal forebrain (Fode et al., 2000).

Loss of Ngn2 both affects the neuronal differentiation of the medial VM progenitors and abolishes the expression of genes signifying a dopamineric phenotype (**Paper II**). However, whether Ngn2 is involved in specifying the dopaminergic phenotype in VM precursors remains to be determined. The cells were arrested in an undifferentiated state, which could leave them unable to respond to differentiation cues without Ngn2 being involved in the phenotypic specification per se. A similar block in neuronal differentiation is seen for precursors of cranial ganglia sensory neurons in the Ngn2 mutants (Fode et al., 1998). However, expression of subtype specific homeodomain proteins that regulates the neurotransmittor phenotype of these cells is unaffected, in contrast to our results.

Substituting the proneural function of Ngn2 by another proneural gene may reveal if the role of Ngn2 for the generation of dopaminergic neurons goes beyond its proneural function and if so, how specific this role is for Ngn2. In a separate study to ours, Kele et al (submitted) have used

$Ngn2^{Mash1/Mash1}$ mice, where the coding sequence for Ngn2 is replaced by the coding sequence for Mash1 in both alleles, to study the contribution of Ngn2 to neuronal differentiation and subtype specification in the VM. They found that Mash1 in the Ngn2 locus increase the number of postmitotic Nurr1-positive precursors but that it could not completely rescue the loss of dopaminergic neurons (Kele et al, submitted). This suggests that Ngn2 do indeed have a specific role in the specification of mesDA neurons.

Possible mechanisms for Ngn2 fate specification

It is clear that over-expression of Ngn2 does not induce a dopaminergic phenotype in expanded neural progenitors (**Paper III**) or primary mesencephalic cultures (**Paper II** and unpublished results). However, this finding is not surprising and Ngn2 may nevertheless have a role in specification of dopaminergic neurons. Many gain of function studies have shown that while Mash1 have the capacity to re-specify progenitors, *Neurogenin* genes, when involved in specification, generally function as permissive factors rather than instructive (Parras et al., 2002). Both Ngn2 and Mash1 are expressed in several areas of the brain that give rise to many different neuronal subtypes. To generate specific subtypes, local co-factors are thought to act together with the proneural genes. For example, Ngn2 interacts with Olig2, another bHLH family member, to specify motor neurons in the spinal cord (Mizuguchi et al., 2001). Mash1 cannot replace Ngn2 to generate these neurons, which shows that Ngn2 is specific in this role (Parras et al., 2002). *Neurogenin* genes are thought to be more dependent on cell context than Mash1 and it is hypothesized that the co-factors needed are very localized, thus explaining that Ngns are rarely instructive when over-expressed. As an example, Ngn1 can induce sensory neuron markers when misexpressed in chick but only if the local concentration of extrinsic factor BMP2 is adequate (Lo et al., 2002).

If Ngn2 is involved in the specification of dopaminergic neurons it is likely there is a co-factor acting together with Ngn2 also in the midbrain. A plausible contender is extrinsic factor SHH, with similar dorso-ventral patterning function as BMPs and known to be essential for dopaminergic neuron development in the midbrain (Jessell, 2000; see also introduction). SHH mRNA expression is lost in neural progenitor cultures (**Paper III**) and presumably not present in high enough concentration in the dorsal midbrain, which could explain why over-expression of Ngn2 does not induce a dopaminergic phenotype in these cultures (**Paper II**). However, a recent study has showed that SHH responsive cells only contribute to dopaminergic neurons before E9.0 (Zervas et al., 2004). This makes SHH unlikely as a co-factor in the Ngn2-positive cells seen at E11.5 and onwards.

Interaction of Ngn2 and homeodomain proteins

Another option for co-factor(s) possibly acting together with Ngn2 to specify a dopaminergic fate is homeobox transcription factors. Mash1 has been shown to interact with homeodomain (HD) protein Phox2b to induce other, lineage specific homeobox transcription factors and determine noradrenergic phenotype (Hirsch et al., 1998; Lo et al., 1998). Ngn2 is part of specifying the phenotypic identity of ventral spinal cord motorneurons, but is unable to promote motor neuron development in chick neural tube without the presence of HD proteins Isl1 and Lhx3 (Lee and Pfaff, 2003).

Homeobox transcription factors play several roles in the specification of neuronal subtypes. In the spinal cord early HD proteins are expressed in response to the opposing patterning action of SHH ventrally and BMPs dorsally, to set up discrete progenitor domains (for review see Jessell, 2000; Lee and Jessell, 1999). Later expressed HD proteins function to specify the identity of newborn neurons from the different domains (Tanabe et al., 1998). Evidence suggests that activation of proneural genes such as *Ngn2* is coordinated with the activation of these subtype specifying homeobox transcription factors, not least since proneural genes also in some cases display specification functions. In the spinal cord motorneurons, a direct transcriptional interaction between Ngn2 and HD proteins Isl1 and Lhx3 provide the mechanism for integrating these developmental programmes (Lee and Pfaff, 2003).
A homeobox transcription factor that could function as a co-factor for Ngn2 would need to be expressed in the progenitor cells in the VZ together with Ngn2. The homeobox transcription factors known to be involved in mesDA neuron development are mainly expressed in the postmitotic neurons. Lmx1b is expressed early in the neural tube, but is not present at high levels in the VZ at E11.5 (Fig 3). En1/2 antibody staining shows a weak expression in the VZ (**Paper I**) but is mainly confined to postmitotic cells (**Paper I** and Simon et al., 2001). However, the possibility exist that there is an as yet unidentified homeobox transcription factor expressed by the progenitors in the VM that could act together with Ngn2 to specify dopaminergic neurons. A very recent study has revealed the presence of two homeobox genes in the VZ at the time of dopaminergic neurogenesis, *Lmx1a* and *Msx1*, which were hitherto unknown to be expressed in the ventral midbrain. The study also shows that they are essential for the generation of dopaminergic neurons. Lmx1a induce the expression of Msx1 while, interestingly, Msx1 is shown to induce expression of Ngn2 at the ventral midline (Andersson et al, *in press*). Thus Ngn2 acts in the presence of these HD proteins to determine neuronal fate and possibly specify some aspect of the dopaminergic phenotype. However, any direct interaction such as in spinal motorneurons remains to be determined.

Induction of mesDA phenotype *in vitro* by over-expression of Ngn2 and Nurr1

An interesting finding which gives more clues about the role of Ngn2 in dopaminergic neuron development is that we managed to get TH-positive neurons that also expressed other dopaminergic markers from expanded fetal tissue, but only when over-expressing Nurr1 and Ngn2 together in the neural progenitors (**Paper III**). TH-positive cells seen when over-expressing Nurr1 alone were immature and did not express En1/2 or Pitx3. This indicates again that Ngn2 is in fact involved in the specification of mesDA neurons. However, if Ngn2, alone or together with a co-factor, induce the expression of specific dopaminergic transcription factors such as En1/2 and Pitx3 one would expect expression of these markers also in cultures where only Ngn2 is overexpressed. Interaction with Nurr1 or its downstream targets seems plausible, yet loss of Nurr1 has been shown to not affect initial expression of Pitx3 or En1/2 (Wallen et al., 1999).

What about the lateral population?

A curious finding in the Ngn2 mutant is that neurogenesis of some mesDA neurons is unaffected. A lateral population of mesDA neurons is formed on time, and the cells express NeuroD, Nurr1, Pitx3 and TH in the expected manner and sequence (**Paper II**). This would suggest that Ngn2 is not involved in the neurogenesis of a subtype of the dopamine neurons or that another gene can compensate for its function in these particular progenitors. Assessment of presence and/or localization of other proneural genes in medial versus lateral mesDA progenitors may explain why this lateral population is spared in the Ngn2 mutants.

Neurogenin1 (Ngn1) is reported to have a weak expression in the rostral midbrain (Sommer et al., 1996) and could possibly assume the function of Ngn2 in the lateral population. Ngn1 and Ngn2 have overlapping expression in the spinal cord (Sommer et al., 1996) and presence of Ngn1 enables expression of general neuronal markers in ventral spinal cord interneuron and motorneuron precursors in the Ngn2 mutants. However, Ngn1 cannot compensate for the role of Ngn2 in expression of the correct subtype specific HD proteins (Scardigli et al., 2001). If Ngn1 compensates for the lack of Ngn2 in the lateral population of dopaminergic neurons in the VM it must assume both the differentiation and specification functions of Ngn2 in these precursors. Alternatively, Ngn2 has only a differentiation function at least in these lateral progenitors.

Another option for a compensating proneural gene is Mash1, which is expressed broadly in the ventral midbrain, including both the lateral and medial mesDA neuron domains (Vernay et al., 2005; **Paper I** and unpublished observation). Mash1 is known to specify GABAergic neurons in the forebrain (Fode et al., 2000), a cell type that is abundant in the adult VM. Even if the normal function of Mash1 does not involve specifying the dopaminergic phenotype it is nevertheless possible that it acquires this function when Ngn2 is absent. For example, Mash1 is upregulated in dorsal telencephalic progenitors in the absence of Ngn2 (Fode et al., 2000). However, this ectopic expression of Mash1 re-specifies the neurons produced from the dorsal telencephalon (Fode et al., 2000), which is apparently not the case in the VM (**Paper II**).

It is also interesting to note that while Ngn2-positive progenitors give rise to Isl-positive motorneurons lateral to the mesDA domain these neurons are unaffected by the lack of Ngn2 (**Paper II**). If there is a rescue mechanism for these neurons or if Ngn2 is redundant for their formation remains to be determined.

Induction of Ngn2 in ventral midbrain

A study by Verney et al (2005) recently revealed a role for late expressed Otx2 in regulating Ngn2 and Mash1 in the midbrain (as opposed to early expressed Otx2 which function to establish the position of the MHO; Broccoli et al., 1999). In Otx2 conditional knock-out mice (CKO), where the expression of Otx2 is downregulated in nestin-expressing cells from the age of E10.5 and completely abolished by the age of E12.5, the expression of Ngn2 and Mash1 is reduced in the ventral midbrain already at E11.5. The Otx2 CKO display a phenotype similar to what we see in the Ngn2 mutant mice, with dopaminergic neurons lost predominantly around the ventral midline, although the reduction of TH-positive neurons in these Otx2 CKO is not as severe as what we have found in the Ngn2 mutants (**Paper II**). In the ventral spinal cord, early expressed HD protein Pax6 have been shown to directly regulate Ngn2 (Scardigli et al., 2003)

and both Ngn2 and fellow bHLH transcription factor Olig2 are lost in motorneuron progenitors in the Pax6 mutant (Mizuguchi et al., 2001). Whether Otx2 act in a role similar to Pax6 in the spinal cord to directly regulate Ngn2 is not yet clear. Msx1, which is also shown to induce expression of Ngn2 (Andersson et al, *in press*) is likely to act in the context of Otx2.

Why is it so hard to get a dopaminergic neuron *in vitro*?

Many attempts have been made to generate mesDA neurons from expandable neural stem and progenitors and few successful approaches have been reported. The question thus arise whether the difficulty lies in providing the correct induction for a dopaminergic phenotype in an otherwise responsive progenitor population or if there is a specific dopaminergic progenitor population that is hard to expand.

Previous studies have identified several key factors in the dopaminergic neuron development, such as SHH, FGF8, Lmx1b, Nurr1, En1/2 and Pitx3 (see Introduction). Our results showed that Ngn2 is required for proper generation of a subset of dopaminergic neurons *in vivo* and over-expression of Ngn2 together with Nurr1 in expanded neural progenitors can give rise to mesDA neurons with some of the distinct features of mesDA neurons (**Paper III**). The recent results regarding new HD proteins in the developing dopaminergic progenitors reveal that there are other cell-intrinsic factors previously unknown that are both sufficient and required to generate dopaminergic neurons (Andersson et al, *in press*). It is also clear that the nature of the progenitor is essential. To generate a dopaminergic phenotype, only progenitors with a ventral midbrain character are relevant (**Paper III** and Andersson et al, *in press*). Judging from our results and others, it seems clear that the emergence of a mesDAphenotype requires a complex interaction of extrinsic signals at precise concentrations and cell-intrinsic factors.

Nonetheless, certain culture conditions may be more suited to preserve the expression of cell-intrinsic factors and perhaps also to expand specialized cells that provide the extrinsic signals. We tried to optimize the culture conditions by adding B27 in the medium, which is reported to be beneficial for dopaminergic neuron development of cultured cells (Kim et al., 2003; Svendsen et al., 1995) and removing HEPES that inhibits differentiation of TH-positive cells in ES cell cultures (Lee et al., 2000). We also explored several different growth factors but none of the options appeared superior in generating dopaminergic neurons and only the use of EGF/bFGF combined resulted in long-term expansion (**Paper III**). In separate experiments attached cultures were established using EGF/bFGF but these cultures were also unable to robustly and spontaneously give rise to clearly dopaminergic neurons (unpublished results).

An aspect of culture variables we did not explore was using hypoxic conditions that are more similar to the *in vivo* situation. Lowered oxygen (<5% O_2) during incubation is reported to alter the proliferation and differentiation of mesencephalic precursor cells *in vitro*. Both neurogenic potential and dopaminergic yield are improved over cultures grown in standard oxygen levels and the effect is specific for midbrain precursors (Milosevic et al., 2005; Storch et al., 2001; Studer et al., 2000). Lowered oxygen is hypothesized to preserve an immature, multipotent phenotype of the mesencephalic progenitor. High oxygen levels also affect the survival of progenitors and/or more mature cells possibly by increasing formation of free radicals to which midbrain dopaminergic neurons appear particularly sensitive (Milosevic et al., 2005).

Are we expanding the correct progenitor?

Although we did see cells capable of responding to Nurr1 and Ngn2 and forming dopaminergic neuron in our expanded cultures the frequency of such cells was low.

This raised a question if we were expanding the correct progenitor in neurosphere cultures. Using heterozygous *Ngn2*-GFP embryos as starting material for neurospheres we attempted to follow the progenitor cells in culture by assessing the expression of *Ngn2*-GFP. However, we and others (F. Guillemot, personal communication), have experienced that *Ngn2*-GFP expression is lost after dissociation and plating, and in our experiments the expression of *Ngn2*-GFP was not significantly upregulated even when cells were differentiated (unpublished results). Both upregulation and maintenance of proneural genes is the result of positive feedback loops, initially dependent on cell to cell signalling (Bertrand et al., 2002; Kintner, 2002). Lack of cell contact at plating contact could possibly hinder the upregulation of *Ngn2*-GFP.

We also explored the possibility that another subpopulation of neural progenitor cells were preferentially expanded under the conditions used. A study in this lab found that *Sox1*-GFP-positive cells derived from the forebrain are enriched in neurosphere forming cells and that *Sox1*-GFP negative cells did not form neurospheres when plated (Barraud et al., 2005), We had found, as previously mentioned, that Sox1 expression was absent from medial VM (Fig 5). However, the subdissected VM pieces used to start neurosphere cultures included the lateral *Sox1*-GFP-positive region at the edges (see Material and methods). This raised the question whether this fraction was preferentially expanded in neurosphere cultures. Sub-dissected VM from *Sox1*-GFP mice were sorted by FACS, and the GFP^{pos} and GFP^{neg} fractions were plated. Interestingly, only the GFP-positive cells formed neurospheres (unpublished data). However, an explanation for this could be that *Sox1*-GFP positive cells are more resistant to the FACS procedure than medial progenitors. Furthermore, when VM tissuepieces were dissected into medial and lateral fractions, and plated (without sorting), both subregions were found to form neurospheres with similar expansion characteristics (unpublished data). Gene expression data also indicated that cells expanded as neurospheres had a ventral midbrain character, and mRNA expression of both Ngn2 and Aldh1 was also seen (**Paper III**). Taken together this shows that neural stem and progenitors from the VM can be expanded in neurosphere cultures however the proportion of different midbrain progenitors within the spheres are unknown and the proportion of mesDA progenitors is likely to be low.

Material and methods

Material and methods

Experimental animals

In this thesis wild type NMRI mice were used as a standard for histological analysis (Paper I) and establishment of tissue cultures (Paper III).
Featured in Paper I and II is a transgenic mouse strain where the first exon of the proneural gene *Ngn2* is replaced with the coding sequence for green fluorescent protein (Ngn2-KI-GFP). The mouse was generated by homologous recombination in ES cells, on a129Sv/CD1 background (Seibt et al., 2003) and maintained on the same background in house. Heterozygous mice (Ngn2+/-) were used as reporter mice to detect and isolate the cells expressing Ngn2 and their progeny (paper I and II). Homozygous *Ngn2*-GFP mice are functional knockouts (Ngn2-/-) and used to study the effect of deleting Ngn2 (Paper II). For generation of heterozygote embryos used for cell sorting (Paper I) Ngn2+/- males were mated with wild-type NMRI females. The embryos were genotyped by GFP-fluorescence and the expected 50/50 ratio was generally found. For Paper II wildtype, heterozygote and homozygote littermates were collected from Ngn2+/- x Ngn2+/- matings and genotyped by PCR (see below).
In Paper III a transgenic mouse strain expressing GFP under the *β-actin* promoter was used (Okabe et al., 1997). We also utilized a transgenic mouse expressing GFP under the *Sox1* promoter (Aubert et al., 2003) in some unpublished studies.
For generation of embryos, adult females (>6 weeks) were mated with adult males overnight and checked for vaginal plug before noon the following day. This day was considered embryonic day (E) 0.5. At the day of collection of embryos, the females were euthanized by CO_2 exposure before noon to ensure that repeated experiments generated embryos of similar developmental stage. For paper II we also used neonatal and P18 pups littermates. The size of the litters were reduced to <5 pups on P0 to increase survival of Ngn2-/-.

PCR genotyping

Ngn2 wildtype allele was detected by primers Ngn2KI5 and Ngn2KI3 generating a 813 bp product. For genotyping of the mutant allele primers Ngn2KI5 and Ngn2KImutant3 generated a 440 bp product. The primer sequences were
Ngn2KI5 5'-GGA CAT TCC CGG ACA CAC AC-3'
Ngn2KI3 5'- AGA TGT AAT TGT GGG CGA AG-3'
Ngn2KImutant3 5'-GCA TCA CCT TCA CCC TCT CC-3'

Histological analysis

Preparation of tissue

Embryos were immersion fixed in 4% PFA overnight at 8° and rinsed in PBS. P0 pups were decapitated, the brain removed and placed in 4% PFA overnight. P18 mice and adult rats received lethal doses of pentobarbitone and were perfused trans-cardially with 0.9% saline

followed by 4% PFA. The brains were then dissected out and post-fixed for 2 hours. All tissue was cryo-protected in sucrose before being sectioned, young embryos (E10.5-E13.5) in 30% sucrose and older embryos (E15.5-E17.5), P0 and adult brain in 25% sucrose. Embryos and P0 brains were mounted in OCT compound and sectioned on a cryostat. Coronal and saggittal sections, 12 μm (E10.5-E13.5), 14 μm (E15.5-E17.5) and 16 μm (P0) thick were mounted on Super FrostPlus glass-slides in series of 10. The slides were stored in –20°. P18 mice and adult brains were cut on a sliding microtome at a thickness of 30 μm and 40 μm respectively, in series of 5. Sections were stored in freezing media in –20°.

Immunohistochemistry

Sections mounted on slides were air-dried to ensure that the sections remained on the glass. Slides and free-floating sections were rinsed in KPBS 3x10 min to remove OCT compund and freezing medium respectively. Depending on type of immunostaining and antibody one or more of the following pre-treatments were performed on the tissue:

Quenching – Sections were incubated with 3% H_2O_2 and 10% methanol in KPBS for 10 min to exhaust endogenous peroxidase in the tissue. This was performed if the peroxidase-based ABC-kit (Vectastain) was used for antibody detection.

Antigen retrieval – Sections were boiled in 10 mM citrate buffer, pH 6 for 20 min. Alternatively, DNA was denatured in 1M HCl at 65°C for 30 min.

Pre-incubation – As a standard, sections were incubated with a blocking solution made up of 2-5% normal serum (from the species the secondary antibody was raised in) and 0.25% triton-X in KPBS. Some antibodies (see table 1) required special blocking solution consisting of 1% dry-milk/10% normal serum/1mg/ml BSA in KPBS. Sections were pre-incubated for an hour.

Following these pre-treatments, primary antibodies, diluted in blocking solution, were incubated with the sections overnight at room temperature (for exceptions see table 2). Slide-mounted sections were kept in humified chamber. The following day the sections were rinsed with KPBS 3x10 min and incubated with secondary antibody for two hours, in the dark in cases where fluorescent secondary antibodies were used. Biotinlyated secondary antibodies were followed by incubation with streptavidin-horseradish peroxidase complex (ABC elite kit, Vectastain) for 1-1.5 hours. Addition of Di-amino-benzydine, DAB (0.5 mg/ml, Sigma) and H_2O_2 to the sections visualized the antibody binding.

To visualize nuclei in fluorescent immunostained tissue, DAPI (1:1000, Sigma) were added to the final rinse in KPBS. Slides were subsequently coverslipped in PVA-DAPCO, an anti-fading mounting media. DAB-stained slides were dehydrated in increasing ethanol and xylen baths and coverslipped with DBX.

In situ hybridization

A subcloned fragment of Ngn2 cDNA (F. Guillemot) was used as a template to generate an antisense digoxigenin (DIG)-labelled cRNA probe. 1 μg of DIG-labeled probe and 50 μg yeast tRNA was added per ml hybridization solution. After proteinase K treatment, postfixation and dehydration in ethanol the slides were incubated overnight at 55°C in a sealed humified

chamber. Following hybridization the slides were washed in 2X SSC/50% formamide at 65°C for 30 min and RNAse treated (20 mg/ml) at 37°C for 30 min. The slides were washed twice with 2X SSC/50% formamide, at 65°C for 20 min each, then 2X SSC and 0.1X SSC at 37°C for 15 min each and finally PBT (PBS + 0.1% Tween-20) for 15 min. Colour reaction was performed using BM purple (Boehringer Mannheim).

Tissue culture

Dissection

Embryos were kept in ice-cold PBS. Sub-dissection of the VM from the embryos were performed in L15 medium (Gibco). The VM was restricted rostrally by the diencephalon, caudally by the mid-hindbrain constriction and contained within the sulcus separating alar and basal plates (see Fig 3). All meninges were carefully removed from the neural tube. Tissue pieces were kept in L15 on ice before being processed further.

Neurosphere cultures

Tissue pieces were incubated with DMEM (Gibco) containing 0.05%DNAse/0.1%trypsin for 15 min at 37°C. The trypsin solution was inactivated with DMEM containing 10% fetal bovine serum (FBS) and removed. The tissue pieces were dissociated mechanically to a single cell suspension in neurosphere culture medium. Cell number was estimated by diluting cell suspension 1:10 in Tryphan Blue (Sigma). Viable, non-stained cells were counted in a Bürker chamber.

The cells were plated in tissue culture flasks in neurosphere medium with 10 ng/ml bFGF (R&D systems), 20 ng/ml EGF (R&D systems) and 40 U heparin/ml (Sigma) at a concentration of 100 000 - 500 000 cells/ml.

Neurospheres were passaged once a week and replated in proliferation medium at 100 000 cells/ml. Alternatively the cells were cryo-preserved in freezing medium and stored at –150°C. When re-established the cells were plated at 2X normal cell density.

Differentiation of neurospheres

Neurospheres were differentiated on plastic 4-well slides that had been coated by PLL (4 μg/cm^2, slides incubated for an hour RT, then rinsed and let to dry) and directly prior to plating, were coated with laminin (50 μg/ml, incubated on slides 2 hours in 37°C). Whole spheres were re-suspended in basic neurosphere medium without growthfactors but with added 1% FBS. 0.625 ml of neurospheres were re-suspended in 1 ml differentiation medium and added to each 4-well chamber (≈625 000 cells /cm^2).

Primary cultures and co-cultures

Subdissected VM were mechanically dissociated and plated at a density of 120 000 cells/cm^2 on PLL coated plastic chamberslides (Paper 1) or 150 000 cell/cm^2 on matrigel (BD Biosciences) coated plastic chamberslides (Paper II). The cells were processed further for BrdU labeling and viral transduction, see separate sections.

For co-cultures, primary cells from E11.5 and E12.5 VM were plated in differentiation medium on PLL coated plastic slides (150 000 cells/cm^2). Expanded neurosphere cells were added to the wells 24 hours later (≈ 400 000 cells/cm^2) and the slides were maintained under differentiating conditions for 5-7 days.

Immunocytochemistry

Cells were fixed in 4% PFA or 4% PFA/0.4% Glutaraldehyd (for GABA staining) for 15 min and rinsed in PBS or KPBS for 3x10 min. A pre-incubation for 1 hour with 10% normal serum and 0.1% triton-X in KPBS was always performed before applying primary antibodies. Cells were incubated with primary antibody overnight at room temperature, followed by gentle rinses with KPBS, 3x10 min. Appropriate Cy2-, Cy3- and Cy5-conjugated secondary antibodies (1:200, Jackson lab) were applied and left on for 2 hours, room temperature. Double and triple stainings were performed simultaneously. For BrdU staining the cells were permeabilized with –20°C methanol for 20 min then treated with 1M HCl for 20 min at 37°C. The acid was neutralized with 0.1 borate buffer. DAPI nuclear stain (1:1000, Sigma) was included to visualize all cells for subsequent cell counts. Slides were coverslipped using PVA-DAPCO mounting medium and kept covered at 8°C.

RT-PCR

mRNA was isolated from freshly dissected tissue as well as proliferating neurospheres using the kit RNAqueous™-4PCR from Ambion. In brief, proliferating spheres and freshly dissected tissue were dissociated in the supplied Lysis/Binding solution. The samples were immediately transferred to –80°C if they were not processed further directly after collection.

The supplied protocol was followed using a centrifuge to draw solutions through the filter cartridge. Suggested maximum volumes, centrifugation times and speed were used throughout. The RNA was eluted with 40 μl + 20 μl Elution solution.

The isolated RNA was DNAse treated twice to remove remaining DNA. RNAse free DNAse1 (Ambion) and DNAse buffer were incubated with each sample at 37°, 1 hr. The DNAse was inactivated with DNAse inactivation reagent, supplied with the kit and the sample transferred to a fresh tube. Heat treatment, 95° for 4 min, was followed by another round of DNAse treatment and inactivation.

The samples were analyzed on agarose gel to ensure that the integrity of RNA was intact. PCR using primers for the housekeeping gene *Gpdh* established that the samples were free of genomic DNA.

cDNA was synthesized from the samples using the SUPERSCRIPT II for RT-PCR protocol (Invitrogen). Approximately 1 μg of total RNA was used per 20 μl reaction.

To enable a semi-quantative analysis of the samples, PCR for *Gpdh* was performed on each sample comparing amplified products after increasing number of cycles on an agarose gel. Based on the amount of product after 20, 25, 30 and 35 cycles respectively the cDNA from each sample was diluted to approximately the same concentration and thereafter used for PCR analysis. Full list of PCR primers and programs are found in table 1.

FACS procedure

Sub-dissected VM from E12.5 Ngn2+/- or wild-type embryos was dissociated mechanically in PBS without Ca^{2+} and Mg^{2+} (Gibco) with 1 mM EDTA and 0.5% BSA. The cells were diluted to approximately 3 x 10^6 cells/ml. To exclude dead cells and cell debris at sorting, 7-aminoactinomycin-D (7AAD, Sigma) was added at 10 μl/ml. Cells were sorted using DIVA flow cytometer (Becton-Dickson) equipped with 488 nm argon and 633 nm helium-neon lasers. Gates for GFP-negative cells were set using wild-type cells while gates for GFP^{high} and GFP^{low} were chosen arbitrarily.

Transplantation

Unsorted, GFP^{neg} or GFP^{pos} cells were transplanted to neonatal rats under deep hypothermic anaesthesia. 50 000 cells were grafted unilaterally into the striatum of each animal. Injection co-ordinates (mm) were 0.7 anterior and 1.9 lateral to bregma, and 2.9 below the dural surface. The rats were allowed to survive for 4 weeks before processed for IHC (as described above).

BrdU labeling of embryonic tissue and primary cells

Pregnant females were injected i.p with BrdU dissolved in 37°C PBS (8 mg/ml) such that they received 50 mg BrdU/g body weight. BrdU was allowed to incorporate for 45 min before the embryos were collected.
FACSorted GFP^{high}, GFP^{low}, GFP^{neg} primary cells were maintained either under proliferating conditions for two days, with BrdU present in the culture medium (0.2 μM BrdU, Sigma) and subsequently differentiated during 5 days, or plated in differentiation medium and maintained for 7 days.

Retroviral transduction

Primary cells (Paper II) and neurosphere expanded cells (Paper III) were transduced with VSV-G pseudotyped retrovirus with IRES2-eGFP, Ngn2-IRES2-eGFP and Nurr1-IRES2-eGFP vectors respectively. All viral vectors were derived from Moloney Murine Leukemia virus, murine stem cell based (Clontech). The Ngn2-IRES2-GFP construct was received from Dr. Jonas Frisén and the cDNA for Nurr1 was received from Dr. Thomas Perlmann. The viral vectors were packaged using a packaging cell line (Ory et al., 1996) and had a titre of around 10^8 TU/ml
Neurosphere expanded cells were passaged and seeded out in proliferation medium (passage 3). 24 hours later the cells were spun down and incubated with retrovirus in a small volume for maximum exposure to the viral particles. Proteamine sulfate was added at 4 mg/ml. The ratio was 1 TU/cell. Cells were resuspended in proliferating medium after four hours and plated out in non-coated 4-well plastic slides (100 000 cells/ml). The cultures were treated as neurosphere cultures and fresh medium (100 μl/well) and growth factors were added every other day. After

7 days total of expansion the proliferation medium was removed and differentiation medium was added to the cells. They were differentiated for 4 days.
Primary cells were dissociated and plated out in proliferation medium. Fifteen hours later virus was added to the cells (1 TU/cell). Another 12 hours later the medium was switched to differentiation medium and the cells were left to differentiate for 5 days.

List of mediums

Neurosphere medium

To make 100 ml		Final concentration	
10 ml	DMEM/F12 10X	1X	*(Gibco no 32500-027)*
1 ml	L-Glutamine 200 mM	2 mM	*(Sigma no G5763)*
1.5 ml	$NaHCO_3$ 7.5%	1.125%	*(Sigma no S5761)*
2 ml	Glucose 30%	0.6%	*(Sigma no G7021)*
1 ml	Penicillin/Streptomycin	1000 U/ml, 100 mg/ml	*(Gibco no 5140-114)*
82.5 ml	MilliQ sterile H_2O		
2 ml	B27 (sterile)	0.2X	*(Gibco no 17504-044)*

Differentiation medium

Neurosphere medium and 1% FBS

Freezing medium

Neurosphere medium and 10% BSA, 7.5% DMSO

Table 1. PCR primers and programs

Primer sequence			Program
Aldh1:	sense	5’-ACT CTC AGC AGT GGT ACA CA-3’	A
	antisense	5’-CAG CTG ACT CTG CAG TCA TT-3’	
En1:	sense	5’-TCA AGA CTG ACT CAC AGC AAC CCC-3’	D
	antisense	5’-CTT TGT CCT GAA CCG TGG TGG TAG-3’	
Lmx1b:	sense	5’-AGA CAT TGG CAG CAG AGA CA-3’	D**
	antisense	5’-CTG AGG GAG GTA TCA CTA TC-3’	
Ngn2:	sense	5’-CGT CAA TAC TGA GAC TCT GC-3’	A*
	antisense	5’-ATC TTC GTG AGC TTG GCA TC-3’	
Nurr1:	sense	5’-TAA AAG GCC GGA GAG GTC GTC-3’	A*
	antisense	5’-CTC TCT TGG GTT CCT TGA GCC-3’	
Pbx1:	sense	5’-GTG AGA GAA GTC AGA GCT CA-3’	A
	antisense	5’-ACT GGA AGG AGA TCT ACA GG-3’	
Shh:	sense	5’-GCA CCC CAA AAA GCT GAC CC –3’	E
	antisense	5’-CGT GGT GAT GTC CAC TGC TCG-3’	
Smo:	sense	5’-CTG CCT AGA AGA GCT GTG TA-3’	C
	antisense	5’-TCA CTG CAG TTT TAA GGT GC-3’	
TH:	sense	5’-TTC CGT GTG TTT CAG TGC AC-3’	D
	antisense	5’-TCC AGT ACA TCA ATG GCC AG-3’	
Gpdh:	sense	5’-ACC ACA GTC CAT GCC ATC-3’	B
	antisense	5’-TCC ACC ACC CTG TTG CTG TA-3’	

Program:
A: 94°, 5 min; 94°, 1 min; 55°, 1 min, 72°, 1 min
B: 94°, 5 min; 94°, 1 min; 56°, 1 min, 72°, 1 min
C: 94°, 5 min; 94°, 1 min; 57°, 1 min; 72°, 1 min
D: 94°, 5 min; 94°, 1 min; 60°, 1 min; 72°, 1 min
E: 94°, 2 min; 94°, 30 s; 58°, 30 s; 72°, 1 min

* 0.5 mM MgCl and 5% DMSO in PCR buffer
** 0.5mM MgCl

Table 2. List of antibodies

Antibody	Dilution	Source	Species	Special treatments
AADC	1:1000	Chemicon	rabbit	1
Aldh1	1.500	R. Lindahl	rabbit	
β-III-tubulin	1:333	Sigma	mouse	
BrdU	1:100	Oxford Biotechnology	rat	2a, 2b
Brn3a	1:50	Santa Cruz	mouse	1, 4
Calbindin D28	1:1000	Chemicon	rabbit	1
ChAT	1:1000	Chemicon	rabbit	
CNPase	1:100	Sigma	mouse	
Dlk-1	1:5000	CH Jensen	rabbit	
En1/2	1:500/1:1000	A Joyner	rabbit	
GABA	1:250/1:2000	Sigma	rabbit	(tissue/cells)
GFAP	1:1000	DAKO	rabbit	
GFP	1:5000	Chemicon	chicken	
Girk2	1:80	Alomone Labs	rabbit	1
Isl1	1:100	Hybridoma	mouse	1
Isl1	1:100	Abcam	rabbit	
Isl1/2	1:500	H & T Edlund	rabbit	
Ki67	1:100	Dianova	rabbit	
Ki67	1:50	DAKO	rat	1
M2M6	1:50	Hybridoma	rat	
Nestin	1:10/1:1000	BD Biosciences	rat	adult /embryonic tissue
NeuroD	1:400	Santa Cruz	goat	
Ngn2	1:1000	M Nakafuku	rabbit	
Ngn2	1:20	D. Anderson	mouse	1
Nurr1	1.1000	Santa Cruz	rabbit	
Parvalbumin	1:2000	Sigma	mouse	
Pbx1/2/3	1:400	Santa Cruz	rabbit	
Pitx3	1:400/1:1000	P Burbach	rabbit	1 (tissue/cells)
PSA-NCAM	1:200	Chemicon	mouse	
TH	1:1000	Chemicon	mouse	
TH	1:1000	Pelfreeze	rabbit	
VMAT2	1:1000	Chemicon	rabbit	1
5-HT	1:10000	Incstar	rabbit	

1. Antigen retrieval with 10 mM citrate buffer 20 min
2. Antigen retrieval
    a) For sections: antigen retrieval with 1M HCl 30 min (65°)
    b) For cells: MeOH 20 min (-20°), 2M HCl 20 min (37°), 0.1M 2x10 min (RT)
3. Pre-incubation with milk/serum/BSA solution

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