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REGIONAL CONTROL OF CELL FATE DETERMINATION AND NEUROGENESIS IN THE DEVELOPING CNS

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Stockholm 2008

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Cover picture depicts an E11.5 dissected mouse brain showing Lmx1a mRNA
expression in the ventral midbrain.

ISBN 978-91-7409-215-8

Wisdom comes with winters.

(Oscar Wilde)

ABSTRACT

The development of a functional central nervous system relies on the generation of distinct neuronal subtypes in a spatially and temporally defined order. The spatial organisation is achieved at early developmental time points when neural progenitor cells encounter fields of secreted morphogenic signalling molecules along the anterioposterior and dorsoventral axes of the embryo. Translation of these gradients into distinct expression patterns of determinant genes leads to the establishment of molecularly defined progenitor domains, each producing a specific type of neuron. The process of neurogenesis through which progenitor cells differentiate into maturing neurons is tightly regulated. Proneural genes promote neurogenesis, whereas Notch signalling counteracts this activity to ensure a balance between the numbers of progenitor cells and neurons. One of the challenges in the field of developmental neuroscience, and the main subject of this thesis, is to unravel the molecular cascades that underlie the differentiation programmes of distinct types of neurons.

In **paper I and II** we identify key components of the midbrain dopaminergic (mDA) and hindbrain serotonergic (5-HT) differentiation pathways. mDA and 5-HT neurons are clinically relevant cell types as the degeneration of mDA cells is the major hallmark of Parkinsons's disease, and dysregulation of 5-HT homeostasis has been associated with a number of disorders including autism, schizophrenia, and drug addiction. In paper I we propose that the transcription factors Lmx1a and Msx1/2 are important for the acquisition of the mDA cell fate by suppressing alternative cell fates, promoting the progression of neurogenesis, and inducing expression of mDA specific marker genes. Moreover, we find that Lmx1a has the ability to direct differentiating embryonic stem cells into mDA neurons, an approach that may be instrumental in the development of cell replacement strategies for the treatment of patients with Parkinson's disease. In Paper II we identify the transcription factor Lmx1b as an early postmitotic marker of 5-HT neurons. We provide evidence that Lmx1b acts as an intermediate determinant in the serotonergic differentiation programme downstream of the progenitor marker Nkx2.2 but upstream of neurotransmitter expression. In **paper III** we construct a comprehensive human atlas of the developmental expression of molecules that have previously been implicated in neuronal and glial patterning, specification and differentiation in common model organisms. We find that the majority of the developmentally important genes found in model organisms show a conserved expression pattern in human suggesting preserved molecular mechanisms, thus validating the use of model organisms to understand human development and disease. Nevertheless, a few deviations were observed, emphasising the importance of such comparisons. In **paper IV** we investigate the control and functional rationale behind the regional expression of the Notch ligands, Dll1 and Jag1, in the developing spinal cord. We find that the patterning genes which govern cell fate determination also delimit the expression of these Notch ligands into distinct progenitor domains. Furthermore, a similar expression control of the Notch-modifying Fringe genes prevents Notch signalling across borders between $Dll1^+$ and $Jag1^+$ domains. We surmise that these two levels of signalling regulation ensure a domain specific control of neurogenesis which may be important to make sure that the correct numbers of each neuronal subtype are generated in the developing spinal cord.

LIST OF PUBLICATIONS

This thesis is based on the following articles, which will be referred to in the text by their roman numerals:

I Andersson E*., **Tryggvason (Marklund) U***., Deng Q*., Friling S., Alekseenko Z., Robert B., Perlmann T and Ericson J. (2006) Identification of Intrinsic Determinants of Midbrain Dopamine Neurons. Cell 124 (2), 393-405.

II Ding YQ., **Marklund U**., Yuan W., Yin J., Wegman L., Ericson J., Deneris E., Johnson RL and Chen ZF. (2003) Lmx1b is Essential for the Development of Serotonergic Neurons. Nature Neuroscience 6 (9), 933-8.

III **Marklund U**., Alekseenko Z*., Andersson E*., Falci S., Kjældgaard A., Perlmann T., Sundström E and Ericson J. A Comprehensive Analysis of Cell Fate Determining Genes in the Developing Human Neural Tube. Manuscript

IV **Marklund U**., Hansson E.M., Sundström E., Hrabé de Angelis M., Przemeck G.K., Lendahl U., Muhr J and Ericson J. Domain Specific Control of Neurogenesis Achieved through the Patterned Regulation of Delta1 and Jagged1 Expression. Manuscript

* These authors contributed equally

CONTENTS

LIST OF SELECTED ABBREVIATIONS

Proteins

Ascl = Mash:	Achaete-scute complex homolog
BMP:	Bone Morphogenetic Protein
Dll:	Delta-like
Gbx:	Gastrulation Brain Homeobox
Hes:	Hairy and Enhancer of Split
Hh:	Hedgehog
Hox:	Homeo box
Jag = Ser:	Jagged; Serrate
Ldb:	LIM domain binding
Lfng:	Lunatic fringe
Lmx:	LIM homeobox transcription factor
Mfng:	Manic fringe
Msx:	Msh-like homeobox
Nurr1 = Nr4A2:	nuclear receptor subfamily 4, group A, member 1
Otx:	Orthodenticle homolog
RA:	Retinoic Acid
RBP-J = CSL:	**C**BF/RBP-J – **S**uppressor of hairless- **L**ag1
Shh:	Sonic Hedgehog
TGFβ:	Transforming Growth Factor β
TH:	Tyrosine Hydroxylase
Wnt:	Wingless-related MMTV integration site

Others

5-HT = serotonin:	5-hydroxytryptophan
AP:	anterioposterior
bHLH:	basic Helix-Loop-Helix
CNS:	Central Nervous System
DV:	dorsoventral
ES cell:	Embryonic Stem cell
HD:	Homeodomain
HH:	Hamburger-Hamilton
IN:	Interneuron
IsO:	Isthmic Organiser
k/i:	Knock-In
k/o:	Knock-Out
LR:	Left-Right
mDA:	mesencephalic/midbrain Dopamine
MHB:	Midbrain-Hindbrain-Boundary
MN:	Motor Neuron
NG-switch:	Neurogenic-Gliogenic switch
PM:	Paraxial Mesoderm
SEP:	Sperm Entry Point
SN:	Substantia Nigra
VAD:	Vitamin A Deficient
VTA:	Ventral Tegemental Area

DEFINITIONS:

Amniote:	mammals and avians, but not frog and fish
Neuronal/Neural Progenitor:	mitotic uni- or multipotent stem cell residing in the neuroectoderm
Neuronal/Neural Precursor:	early postmitotic immature neuron
Node:	organiser in the anterior tip of the primitive streak through which cells invaginate during gastrulation
Primitive Streak:	a structure consisting of thickening of cells along the future AP axis during gastrulation
$\mathbf{X^{Yk/i}}$:	gene Y is expressed under the regulatory sequences of gene X
$\mathbf{X^{k/o};X^{Yk/i}}$**:**	gene X is replaced by gene Y
$\mathbf{X^{Yk/o}}$**:**	gene Y is conditionally knocked out in cells expressing gene X

<u>Cells that are:</u>

Competent:	have the ability to respond to inducing signals
Induced:	have received inducing signals but need sustained signalling to convert to a certain fate
Specified:	have received the inducing signals and can by means of cell intrinsic mechanisms (under neutral conditions) differentiate to a certain cell type, but can still respond to signals that repress this fate
Committed:	will progress to a certain fate even in the presence of counteracting signals
Differentiated:	are postmitotic and have taken basic cell fate decisions

INTRODUCTION

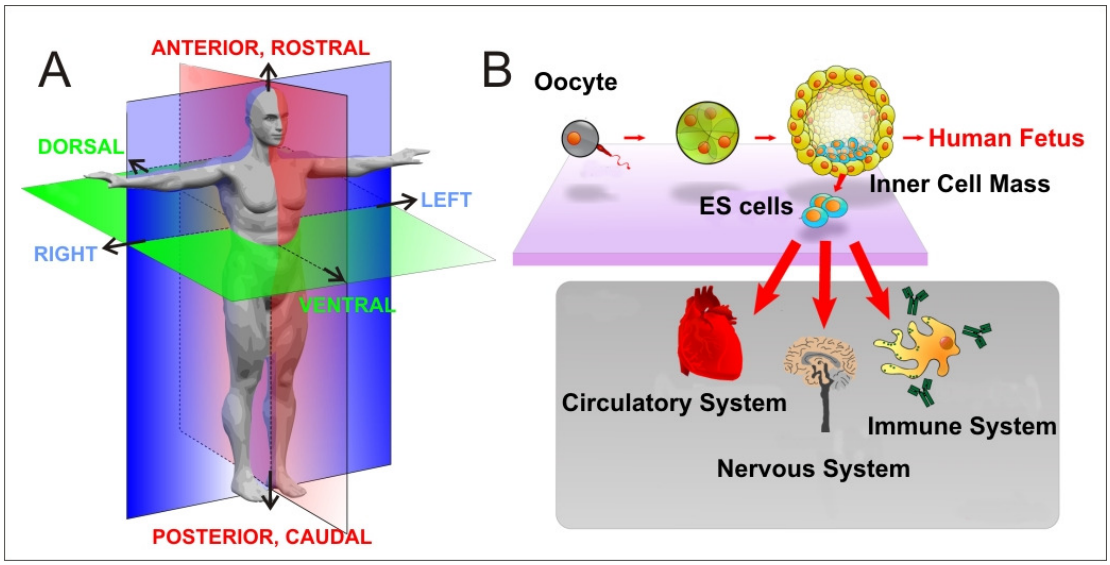
The fundamental question in developmental biology deals with how a single fertilised egg cell can give rise to an entire organism, consisting of such an extensive diversity of different cells arranged in a very precise complex pattern. Ultimately the blueprint of this pattern is contained within the double-stranded DNA helix that is the makeup of our genome. Since the genome of nearly every cell is identical, cells do not differ as a result of different genetic information but rather because they are instructed to express only a subset of this information. During development, the genes that a cell expresses depend both on the cell's past and its present environment. In Nordic mythology, the fate of a person's life was believed to be set at birth by the three Norns representing destiny as it twined with the flow of time. Urd, Verdandi and Skuld were each associated with past, present and future and their fate setting ability relied on their talent to interweave the threads of destiny. Similarly, differentiating neural cells are rather peculiar in that they undergo a distinct set of stages analogous to development, birth and maturation, with each step ultimately determined by the selected activity of particular threads of the genome. At a very early phase of development, a group of embryonic cells is demarcated to form the prospective central nervous system (CNS). The neural cells within this area have a broad developmental potential but positional instructions restrict their choices over time. Throughout these developmental stages, cells divide extensively but eventually exit the cell cycle and start maturing into functional neurons, a process considered to be the birth of neurons. It is believed that the collection of genes expressed at the moment of birth determines the fate of that cell, i.e. the type of neuron it becomes. Consequently, at birth or shortly thereafter, the basic destiny of a neuron becomes fixed and it then matures according to the guidelines of the expressed genes. Nevertheless, similar to us humans, neurons are influenced by their environment throughout their existence. An intriguing mission in developmental neuroscience today and the main focus of this thesis is to unravel the molecular cascades that underlie the differentiation paths of distinct types of neurons.

BASIC CONCEPTS DURING EMBRYOGENESIS

The complex events leading to the formation of a body can be pinned down to four fundamental cellular processes: proliferation, movement, specialisation and cell-cell interactions. These cellular activities must be finely orchestrated in order to achieve the first major organisation, the body plan, which is the backbone for subsequent development in all multicellular organisms. The cells within the body plan receive a sense of identity through their particular position along the grids of the three axes. The first axis to be generated is along the length of the body - anterioposterior (AP), followed by the establishment of stomach/back - dorsoventral (DV) and left-right (LR) axes (*Box1A*).

In amniotes, the first sets of divisions of a fertilised egg create basically two cell layers (*Box1B*), known as the inner cell mass (ICM) in mouse (*Box1B*) or epiblast in chick, and the trophectoderm in mouse or hypoblast in chick. These layers will give rise to the embryo proper and extraembryonic tissues, e.g. the placenta, respectively. At this early stage, germ cells are singled out from the ICM/epiblast with the remaining cells undergoing dramatic rearrangements into a three-layered structure in a process called gastrulation. Cells remaining at the outer surface form the ectoderm which is the anlage for both skin and the nervous system. Other cells are brought inside the embryo to form the endoderm - the precursor of the gut, lung and liver for example. A third group of cells ends up in between the ectoderm and endoderm, forming the mesoderm which gives rise to muscles and bone (Gilbert et al., 2006).

How do cells then acquire their basic cell lineage identity? Important throughout development is the ability of cells to communicate with one another. This allows cells to receive information about their positions both in the basic body plan and relative to each other and to adjust accordingly to restrict themselves to the appropriate cellular fate. Interestingly, certain clusters of cells, so called organisers, have specialised signalling abilities, and function to coordinate and diversify surrounding cells. One such example, the node, is a funnel-shaped structure through which cells destined to become mesoderm and endoderm invaginate through during gastrulation (Gilbert, 2006). The positional information provided by the node and other organising structures is delivered in the form of proteins that are either secreted or presented on the extracellular part of the cell membrane. Such messengers impose distinct signalling cascades in the responding cell that usually results in the regulation of gene transcription. Interestingly, only seven basic signalling pathways (Hedgehog (Hh), TGFβ, Notch, Wnt, Receptor Tyrosine Kinase (RTK), JAK/STAT and nuclear receptor) operate during embryogenesis, despite the incredible complexity of this process (Barolo and Posakony, 2002). Some of these molecules can function as morphogens which are extracellular proteins secreted from a defined source creating a



Box 1: (A) Schematic drawing of a human showing the three axes of the Central Nervous System*. The dorsoventral (DV) axis extends along the back-to-stomach direction. The left-right (LR) axis is important for the correct distribution of organs and the brain displays a clear LR asymmetery. The axis along the head-to-tail direction can be referred to as the anterioposterior (AP) or rostrocaudal (RC) axis. Note that this axis is curved along the brain from the backhead to the front. Thus, the anteriormost position resides in the forehead. Picture was modified from: http://en.wikipedia.org/wiki/Image:Human_anatomy_planes .svg*

(B) Embryonic Stem (ES) cells can differentiate into multiple cell types in vitro. *ES cells are pluripotent stem cells derived from the ICM which may be coaxed to differentiate into cell types of all three germ layers in vitro. Significant efforts are placed into developing protocols for the efficient production of clinically relevant cell types that could be used in future cell replacement treatments for human diseases including Parkinson's disease and diabetes. In addition, human ES cell-derived specific cell lines can be used as platforms for drug screening, toxicity evaluation and disease modelling (Menendez et al., 2006). Picture was modified from: http://en.wikipedia.org/wiki/Image: Stem_cells_diagram.png*

gradient or morphogenic field in the surrounding tissue. The transciptional response to a morphogen depends both on the precise concentration received and the previously imposed positional identity. Due to these interpretational aspects, a single morphogen can instruct cells to multiple fates.

No matter how fascinating each aspect of embryogenesis is, each process is very complex so one must constrain oneself and focus on defined questions. We have chosen to try to understand control mechanisms for specification and neurogenesis of neuronal subtypes in the context of DV patterning and the role of Sonic hedgehog (Shh) and Notch signalling in these processes. As a preface for the thesis I will describe the delineation of the neuronal fate from a fertilised egg, the basic model of patterning along the axes and how neuronal birth is controlled. Then mechanisms behind specification of two clinically relevant cell types, serotonergic and dopaminergic neurons, will be discussed.

FROM GERM CELL TO NEURONAL PROGENITOR

Establishment of Polarity

In many classical model organisms the fertilisation of the zygote is thought to originate polarisation or pre-patterning, which is the backbone for the axis formation in the basic body plan. In frog it has long been established, and in mouse it has been suggested in recent years, that the sperm entry point (SEP) actually provides the first positional information affecting timing and pattern of the first sets of cleavages, which in turn has key roles in establishing polarity in the embryo (Moon and Kimelman, 1998; Pitrowska et al., 2001). More specifically it appears as if the SEP in mouse correlates to the future border between epiblast and trophectodermal cells and thereby imposes directionality in the early embryo. However, theories of pre-patterning have been disputed and even if it is present it is uncertain whether it actually translates into the definitive axes of the embryo (Rivera-Perez, 2007). At embryonic day (E) 5.5 definitive polarity in mouse can be identified by the uneven distribution of marker proteins in the extraembryonic tissue which may be important to impose the **AP axis** of the embryo proper (Thomas et al., 1998; Takaoka et al., 2006). The origin of the **DV axis** is less explored in mammals but a theory for DV formation in chick was formulated already two decades ago (Stern and Canning, 1988; Gilbert, 2006). This model argues that the axis is based on the difference in pH between the two sides of the early epiblast sheet, such that the side facing the albumin (egg white) becomes dorsal, whereas the subgerminal side (a cavity separating the epiblast from the yolk) will form the ventral structures (Stern and Canning, 1988). Also in mammals, the ICM cells closest to the internal cavity (blastocyst fluid) will form the ventralmost tissues (Gilbert, 2006). Nevertheless, a molecular mechanism for the establishment of the DV axis remains to be elucidated. As development proceeds, a mesodermally derived organiser, the notochord, informs cells of their positions along the DV axis of the neural tube, which will be described in greater detail in "*Consolidation of the axes in the developing neuroectoderm assigns neuronal subtype fates"*. In contrast, the DV axis in frog is set by the SEP (Gilbert, 2006). The **LR axis** is established relatively late, at E8 in mouse, and depends on the pre-existing AP and DV axes (Takaoka et al., 2007).

Establishment of Neuroectoderm

As discussed in the previous paragraph, AP polarisation is a relatively early event and probably precedes the establishment of the three germ layers, which classically was believed to be set at gastrulation. Although it may appear as if cells are determined to a certain basal lineage (ecto-, endo- or mesoderm) during gastrulation, studies now indicate that in birds, the ectoderm, particularly the neuroectoderm, has started to be

initiated at earlier stages and that a major function of gastrulation is to organise the cell types into appropriate positions to form a functional body (Wilson et al., 2000; 2001).

A number of contradicting views on neural induction have arisen from the study of different model organisms. It is therefore difficult to ascertain whether the analogous processes are distinct in different organisms, or whether the differences found simply reflects the experimental constraints set by each organism. In any case, common to all vertebrates studied is that neural tissue is delineated from the ectodermal germ layer. In fish and frogs, a long prevailing view has stated that ectoderm by default is specified as neural and that signals are required to induce the epidermal fate. More specifically, it has been shown that BMPs of mesodermal origin induce epidermis but that neural fate is retained in ectoderm exposed to BMP antagonists (Stern, 2006). In chick, however, inductive signals are also indeed required for neural fate, arguing against the idea that it would simply be the default state. In evidence are the findings that BMP antagonists are not sufficient to induce, and BMPs cannot block, the neural fate (Streit et al., 1998). Instead, FGF has been identified as having dual roles in the acquisition of neuronal fate. First, it is required to activate the pathway necessary for the progression of neuronal fate and second, it represses BMP. Conversely, the acquisition of epidermal fate requires BMP to initiate the lineage specific differentiation pathway, and Wnt to suppress the activity of neural-inducing activities of FGF (Wilson et al., 2000; 2001). FGF and Wnt have been assigned important roles also in frog supporting the idea that neural induction in different organisms may be achieved through common mechanisms (Wilson and Edlund, 2001; Stern, 2006).

To determine the time of neural induction in chick, explants were isolated from different stages, cultured *in vitro*, and examined for definitive neuronal markers. Using this method it was shown that neural induction by FGF commenced already *in utero* (stage Ayal-Giladi Kochav IX) and that epiblast cells were committed to the neural fate (i.e. was no longer responsive to epidermal-inducing cues) at late gastrula stages (stage Hamburger-Hamilton (HH)4). The exact time point for specification (i.e. when cells under neutral conditions (by cell intrinsic means) would choose to become neural) has not been set but may actually just coincide with the onset of the earliest known definitive specific neural marker Sox2 (SRY box containing gene 2) (HH4) (Wilson et al., 2000; Rex et al., 1997). Sox2 has been shown to be a key factor for cells to commit to the neuronal differentiation program (Wegner and Stolt, 2005) and analysis of the Sox2 enhancer regions has revealed the presence of binding sites for components downstream of FGF and Wnt signalling, although the activity of these appears to be exclusively associated with Sox2 expression at post-gastrula stages (Takemoto et al., 2006). Recent investigation of an enhancer element active at early stages unveiled intricate regulatory events concluding in Sox2 expression (Papanayotou et al., 2008). According to this study, at early stages, FGF signalling activates epiblast expression of two coiled/coil domain proteins, Geminin and ERNI (Streit et al., 2000). Geminin

promotes Sox2 expression by disrupting binding between the chromatin-remodelling protein, Brm, and the transcriptional repressor HP1α, whereas ERNI counteracts the activity of Geminin. At later stages (HH3-4), BERT expression is enhanced in prospective neuroectoderm leading to suppression of ERNI, thus releasing the repression of Sox2 expression exerted by HP1α (Papanayotou et al., 2008). As upregulation of BERT expression appears to be the triggering event for Sox2 induction and hence commitment to a neural programme, it will be interesting to understand how BERT expression is controlled. Importantly, Sox2 expression is not limited to neuroectoderm but is found also in the epiblast and in some placodal and neural crest derived lineages (Kamachi et al., 1998; Avilion et al., 2003; Wakamatsu et al., 2004), indicating that it may be involved in maintenance of progenitors in a context dependent manner. So far, very few components in the early cascades leading to the establishment cells of a neural identity distinguishing it from the meso- and endodermal lineages, have been identified. Microarray analysis of embryonic stem (ES) cell derived cells representing different germ layers (*Box1B*) may aid in unravelling a defined "neural transcriptional profile".

Early AP Regionalisation of Neuroectoderm

Several lines of evidence suggest that induction of neuroectoderm commences at pre-gastrula stages (Wilson et al., 2000; Streit et al., 2000) and that neuronal fate is consolidated at late gastrula stages in chick (Wilson et al., 2000). Interestingly, explants from the presumptive posterior neural plate taken at early gastrula stages are devoid of posterior markers and express a marker indicative of anterior fate, Otx2 (Muhr et al., 1999). This implies that the default neuroectodermal fate is anterior and that additional signals are required to posteriorise the tissue. The caudal paraxial mesoderm (PM) has been observed to have posteriorising activity (Itasaki et al., 1996) and when cultured together with early explants it imposes a posterior character (Muhr et al., 1999). A large set of experiments have, furthermore, shown that PM gradually imposes midbrain and hindbrain character through its secretion of Wnt8c and Wnt11 (Nordström et al., 2002) and more caudal fates by retinoic acid (RA) signalling (Muhr et al., 1999) at late gastrula stages (HH4). Importantly, in these processes, FGF, presumably from the primitive streak (gastrula structure), is required as a permissive factor (Muhr et al., 1999; Nordström et al., 2002). Recently, these regulatory events have been further scrutinised to reveal that Wnt and FGF initially impose rostral hindbrain and caudal spinal cord identity in a concentration-dependant manner, establishing a positional context for RA and FGF to subsequently modify intermediate parts into caudal hindbrain and rostral spinal cord (Nordström et al., 2006). Thus, FGF, Wnt, and RA conspire to induce the earliest known transcriptional profile of forebrain (Otx2), midbrain (Otx2, En1), rostral hindbrain (Gbx2), caudal hindbrain (Gbx2, Hoxb4), rostral spinal cord (Hoxb8) and caudal spinal cord (CdxB/C, Hoxc9) (Nordström et al.,

2002; 2006). These marker genes are crucial positional determinants that are needed for further maturation and diversification of each part to occur. However as each structure has stabilised these marker proteins may become dispensable and are in some cases downregulated.

Consolidation of the Axes in the Developing Neuroectoderm Assigns Neuronal Subtype Fates

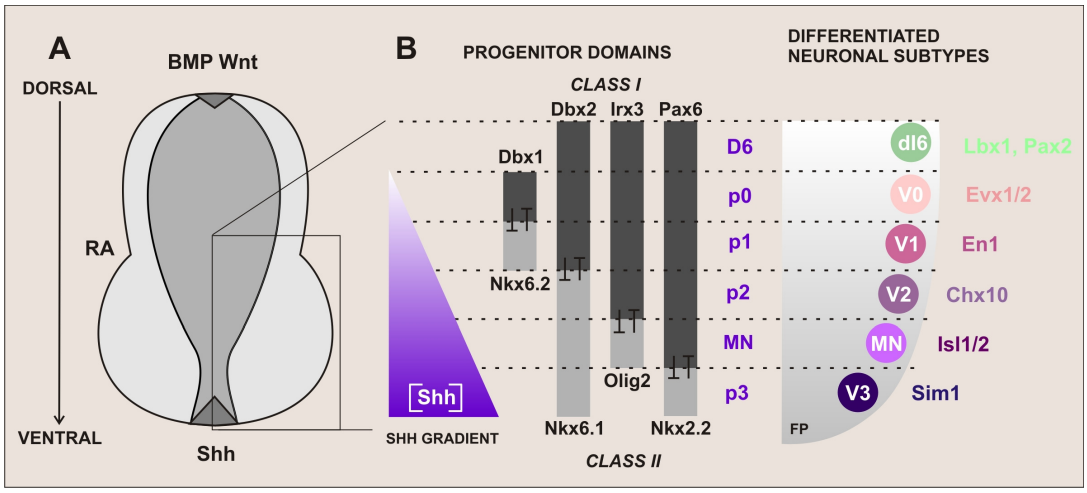
At the time of induction, the neuroectoderm has, as discussed above, an anterior character which is modified by signalling molecules at posterior positions. These findings indicate that although the embryo as a whole has established an AP axis, the axis is not implemented in every cell and individual cells still need continuous guidance to appreciate their positional value within the developing body. In fact, from the point of neuroectodermal commitment to neurogenesis, neural progenitors are flexible and require appropriate positional information in order to commit to the appropriate neuronal or glial cell type fate. This process of positional guidance I will refer to as patterning in the rest of the thesis.

Patterning along the DV axis

Gastrulation, though dispensible for the initiating neural induction *per se*, is the process in which the neuroectoderm is refined, shaped and put into the appropriate context within the embryo. During gastulation the developing neural tissue is positioned along the midline of the AP axis forming a morphologically distinct structure, the neural plate. In a process called neurulation, the neural plate of amniotes curves up to form a U-shaped structure which eventually closes at the top to form a hollow tube - the neural tube (Gilbert, 2006). As a consequence, a very distinct DV axis is set where the dorsal end positions close to the overlying epidermis while the ventral part faces the mesoderm underneath. Interestingly, signals from non-neural ventral, dorsal and intermediate tissues are crucial for the patterning, i.e. diversification, of cells along the neural tube (*Box2A*) (Pierani et al., 1999; Gilbert, 2006). The key ventralising tissue is the notochord, a slender rod of cells that forms in all vertebrates and is thought to develop from the node. Dorsal fates are induced by signals from the epidermal lineage and paraxial mesoderm influences the intermediate cell fates. The spinal cord is believed to be the least complex part of the CNS and has therefore been selected as a model for the rest of the neural tube. Increasing evidence suggests that lessons learnt from the spinal cord can indeed be applied to more anterior parts like the hindbrain and midbrain which I will go through in more detail in *"The Life Journeys of Dopaminergic and Serotonergic cells"*. However, below I will first describe the prevailing model of CNS patterning based on studies in the spinal cord.

Ventral Patterning:

The notochord is essential to impose ventral fates by directly signalling to the ventral neural tube at early stages and, indirectly, by converting the ventralmost region of the neural tube into another signalling centre, the floor plate (Placzek and Briscoe, 2005). The key molecule in these processes is Shh, which initiates the patterning of the early ventral neural tube, induces the floor plate and then serves as the main morphogen secreted from the floor plate. The importance of the floor plate for ventral fates was demonstrated by surgical removal or ectopic positioning which led to loss or gain of ventral cell fates, respectively (Ericson et al., 1992). That Shh is the essential mediator in this ventralisation process was shown by classical explant experiments (Ericson et al., 1995; 1997). Moreover, in the absence of Shh, the ventral spinal cord acquires dorsal patterning characteristics and fails to generate the ventralmost cell types (Chiang et al., 1996). Based on a series of elegant *in vivo* and *in vitro* assays, a mechanistic model for ventral spinal cord patterning with Shh as a key player was formulated in the late 1990's (reviewed in Jessell, 2000). In this model (*Box2B*), the Shh gradient is translated into distinct transcriptional outputs, which in turn dictate neuronal fate. Shh is required, at different concentration thresholds, for the induction of a group of transcription factors, denoted the class II proteins. These transcription factors typically contain homeodomains (HD) in their DNA-binding motifs, but also the basic helix-loop-helix (bHLH) protein, Olig2, belongs to this group. Another set of HD proteins, the class I proteins, were initially believed to be direct negative targets of Shh signalling but recent data suggest that the repression exerted by Shh is indirect, via class II proteins (Pachikara et al., 2007). Importantly, class I and class II proteins couple up to form cross-repressive pairs resulting in further refinement of the expression patterns first induced by Shh. Ultimately, the combined activity of Shh and the reciprocal inhibition between transcription factors establishes five major ventral progenitor domains (p0-p3; pMN), each of which is defined by the combinatorial expression of a distinct set of transcription factors. When progenitor cells exit the cell cycle, these expression codes are deciphered into specific cellular programmes of differentiation, i.e. five major neuronal subtypes (V0-V3; MN) (*Box2B*). Within the expression codes, certain transcription factors function as key determinants and these have been identified through loss- and gain-of-function experiments (Jessell, 2000; Poh et al., 2003). For example, forced expression of Olig2 re-programmes progenitor cells at ectopic DV positions into somatic motor neurons (sMN) (Novitch et al., 2001). Conversely, absence of Olig2 results in failure to induce the sMN-specific differentiation programme and consequently in mouse $Olig2^{-/-}$ mutants, no sMNs are formed. Instead, Irx3, which is the cross-repressive counterpart to Olig2, is ventrally expanded and cells are re-specified to become V2 interneurons (IN) (Briscoe et al., 2000; Lu et al., 2002, Takebayashi et al., 2002).



Box 2: Patterning of the spinal cord. *(A) Schematic drawing of morphogenic activities from the roof plate (BMP, Wnt), the paraxial mesoderm (RA) and the floor plate (Shh). (B) Ventral/Intermediate patterning. Shh regulate the expression of HD- or bHLH-containing patterning genes in a concentration dependent manner. Cross-repression between pairs of class I and class II patterning proteins restrict and stabilise the expression patterns. The final combinatory expression profiles of the patterning genes define five ventral and six dorsal major domains, each of which give rise to a specific neuronal subtype.*

Postmitotic neuronal subtypes are distinguished from one another by the specific expression of marker proteins, which in some cases are important components in the distinct differentiation pathways and may control neuronal maturation, neurotransmitter choice, migration, and axonal projection patterns.

The mechanism whereby the intricate pattern of repressor proteins specifies cell fate is not known but a "model of de-repression" has been postulated. This argues that enhancer regions of effector genes for each subtype have binding sites only for those proteins expressed at positions where these effector genes are not supposed to be expressed. Consequently, they are allowed to be expressed only in domains that lack expression of those repressors (Muhr et al., 2001).

Importantly, it has been realised that some of the major progenitor domains produce several distinct neuronal descendants. The basis for this is either sub-patterning within the progenitor domain (Pierani et al, 1999) or diversification at the cellular level occurring just prior to birth or in the early postmitotic state (Del Barrio et al., 2007). It is well-known that time is an important regulator of cell fate diversification within a single domain. This is made possible due to that progenitors retain plasticity and therefore may change their fate according to temporal differences in gene expression within a domain. Consequently, changes within one domain may contribute to the sequential generation of multiple cell types (Zhou et al., 2001; Pattyn et al., 2003; Jacob et al., 2008; Ohsawa and Kageyama, 2008).

Dorsal Patterning

The basic logic for patterning of the ventral half is generally applicable to the dorsal portion of the spinal cord. Also here non-neural tissue, in this case constituted by

cells in the interface between the neural plate and epidermis proper, function as the major polarising agent which converts the dorsalmost neural plate cells into a dorsal counterpart of the floor plate, rationally denoted the roof plate (Chizhikov and Millen, 2005). The abundance of BMP and Wnt proteins secreted both from epidermal ectoderm and the roof plate motivated investigation of their potential roles for the dorsal patterning (*Box2A*). BMP signalling was shown to be a key pathway for the roof plate, both for its induction and for its ability to instruct dorsal neuronal fates in a concentration dependent manner (Liem et al., 1997; Timmer et al., 2002; Millen et al., 2004; Chizhikov and Millen, 2005; Liu et al., 2004). Initial studies in chick failed to reveal any role in dorsal patterning for Wnts and suggested that they would mainly act as proliferative agents. However, in mouse, important roles for Wnts in the specification of the dorsalmost IN was more recently demonstrated by both loss- and gain-of-function experiments (Chichikov and Millen, 2005). Since the instructive abilities of Wnts were shown in mouse and not in chicken, Wnts may show a species difference in their function. In the dorsal spinal cord, six major neuronal subtypes (dI1-6) are formed from six progenitor domains (dP1-6) at early stages, but two of the domains (dP4 and 5) generate additional neuronal types (dILa and dILb) at later stages (Helms and Johnson, 2003). Cross-repressive interactions appear to be an important aspect in the establishment of the dorsal domains but in contrast to in the ventral positions, bHLH proteins are major determinant transcription factors (Muller et al., 2005; Gowan et al., 2001; Parras et al., 2002; Helms et al., 2005).

Intermediate Patterning

In addition to being polarised by signals emanating from the very extreme ends of the DV axis, the spinal cord also displays further patterning complexities by integrating positional information from intermediate positions, namely the PM (Pierani et al., 1999). The intermediate patterning signal, shown to be RA, is required for the specification of V0 and V1 IN by the induction of their HD containing determinant genes, Dbx1 and Dbx2 (*Box2A*). The expression of these determinants persists in $Shh^{-/-}$ mutant embryos showing that Shh is dispensable for their expression (Pierani et al., 1999). Furthermore, as high levels of both Shh and BMP suppress Dbx1 and 2, the consolidation of their normal expression pattern at intermediate positions can probably be explained by the conspired action of RA, Shh and BMP. Interestingly, the analysis of the vitamin A deficient (VAD) quail model showed that Shh expression along with other ventral patterning genes expanded dorsally (Wilson et al., 2004) in the absence of RA, indicating that RA also plays a role in regulating either expression, spreading or the interpretation of Shh.

Patterning along the AP axis

As described, the neuroectoderm is demarcated into distinct compartments along the AP axis even before it has transformed into a neural tube. Subsequent refined patterning within caudal hindbrain and spinal cord is somewhat different from that of the midbrain and rostral hindbrain (Nordström et al., 2006) and I will therefore discuss these areas separately.

Caudal Hindbrain and Spinal cord

RA, secreted from PM, is required for the early induction of prospective caudal hindbrain and rostral spinal cord (Nordström et al., 2002; 2006). Moreover, due to the teratogenic effects (disturbance of the basic body plan) exerted by RA in several model organisms as well as in humans, it was established early on as the pre-eminent signal for the regionalisation of the neural tube along the AP axis (Glover et al., 2006). It was also realised that the patterning ability of RA relies on its ability to regulate the HD-containing Hox genes. Interestingly, in insects the paralogs to the Hox-genes dictate the patterns of segmentation. Although the amniote body is not segmented, the hindbrain shows features of subdivision in that it is compartmentalised into eight rhombomeres (R), which is controlled by the combinatorial expression of Hox-genes. No or little intermingling of cells occurs between the boundaries of the rhombomeres and each rhombomere produces a distinct set of neuronal progeny (Glover et al., 2006).

The Hox gene transcription factors are organised into clusters on four chromosomes (Hoxa-d) and are regulated in a 3' to 5' direction so that the 3' genes are expressed first in the anterior parts while more 5' genes appear progressively later in the posterior neural plate, a phenomenon called co-linearity (Glover et al., 2006). Thus along the spinal cord the Hox clusters show a nested expression pattern where 3' genes extend more anterior than the 5' genes. This pattern is made possible since the neural tube is progressively formed in a rostral to caudal direction. The formation of the spinal cord through its whole extent is tightly controlled by the caudalmost part, the stem zone, which by producing FGF maintains cells in a stem cell like state characterised by rapid proliferation (Diez del Corral and Storey, 2004; Delfino-Machin et al., 2005). Alongside the developing neural tube, there is gradual maturation of PM into somites (the precursors of the backbone and muscles) which by secreting RA counteracts the effects imposed by FGF. It has been suggested that for establishment of the caudalmost parts of the spinal cord, prolonged exposure to FGF is required not only to ensure that enough cells are maintained in the stem zone but also to make certain that the 5' most Hox genes eventually are induced to be expressed. In this process, RA would induce the 3' most Hox genes in the beginning of the spinal cord formation but prevent further transcription of 5' Hox genes by suppressing FGF. Later formed (caudal) neural tube has instead experienced longer exposure to FGF and can therefore turn on the 5' most

Hox genes leading to the distinct characteristics of the caudalmost part of the spinal cord (Diez del Corral and Storey, 2004).

In the fully extended spinal cord, RA is produced at equal levels throughout the axis from the PM. The rostral boundary of the PM coincides with the caudal hindbrain. Since enzymes with RA catabolising activity (Cyps) are expressed primarily in the rostral hindbrain, the hindbrain exhibits a caudal to rostral gradient of RA activity (Glover et al., 2006). It is thought that the RA gradient regulates the differential Hox gene expression in the caudal half of the hindbrain and indeed in VAD rat embryos, the Hox pattern is disturbed and these parts fail to undertake their normal characteristics (White et al., 2000). In addition, several RA signalling components are expressed in a patterned manner indicating that the gradient of RA may be interpreted differently in each rhombomere (Glover et al., 2006). How Cyps and RA related molecules are regulated in a patterned fashion along the hindbrain to ensure the right interpretation and distribution of RA is currently unknown.

Hox gene transcription has also been shown to be regulated by RA-independent means. Kreisler and Krox-20 are differentially expressed in the hindbrain at early stages and can act directly on Hox gene expression (Deschamps et al., 1999; Cordes et al., 2001), and the prolonged expression of Hoxb1 in R4 is dependent on the HD proteins Nkx6.1 and Nkx6.2 (Pattyn et al., 2003). In addition, Hox genes may regulate one another and some display auto-regulatory loops (Maconochie et al., 1997; Gould et al., 1997; Deschamps et al., 1999).

Midbrain and Rostral Hindbrain

As mentioned above, the rostral part of the hindbrain counteracts RA action by expressing catabolitic enzymes and is furthermore essentially normal in VAD quail/rat embryos. Thus, it is plausible to assume that signals other than RA from the PM control the development of the rostral neural tube. In line with this, neither midbrain nor R1 display Hox gene expression. As discussed above, early demarcation and partition of prospective midbrain and rostral hindbrain is believed to be set up by graded Wnt-signalling. The molecular subdivision of prospective midbrain from hindbrain is crucial and has great impact for the subsequent development. Here the juxtaposed expression of anterior Otx2 and posterior Gbx2 initiate a genetic cascade leading up to the formation of the so called isthmic organiser (IsO). Early components of the specification programme include Pax2, Lmx1b and En1 and they conclude in the expression of the important signalling molecules Wnt1 and FGF8 (Hidalgo-Sanchez et al., 2005). The expression of these markers is initially dynamic but eventually stabilises in a distinct pattern ensuring that the IsO coincides with the mid-hindbrain boundary (MHB) (Hildalgo-Sanchez et al., 2005). Similar to DV patterning molecules, Otx2 and Gbx2 have the ability to repress each other (cross-repressive pair), an activity important to stabilise and refine the MHB. However, in addition to this, by unknown mechanisms,

Otx2-Gbx2 confrontation induces the IsO. Thus, misexpression of Gbx2 in the midbrain has two consequences, first, Otx2 is repressed and second, an ectopic IsO is formed creating a new MHB territory within the midbrain (Millet et al., 1999; Katahira et al., 2000). Importantly, FGF8 is sufficient to induce ectopic midbrain and rostral hindbrain characteristics when applied in the diencephalon, midbrain and rostral hindbrain, while caudal hindbrain only responds by inducing rostral hindbrain and is not competent anymore to form ectopic midbrain (Hidalgo-Sanchez et al, 2005). Furthermore, FGF8 signalling regulates both proliferation and the AP polarity in the developing midbrain (Lee et al., 1997; Crossley et al., 1996).

Wnt1 appears to be crucial not for the induction but for the maintenance of FGF8 and En1, which are required for a proper midbrain/R1 development (Wurst et al., 1994). Thus, in the absence of Wnt1 the IsO is not properly induced and most of the midbrain and the rostral hindbrain (cerebellum) fail to be established (McMahon et al., 1992). Although the prospective midbrain and rostral hindbrain are demarcated by the early Wnt gradient (Nordström et al., 2002), the formation of the IsO is crucial both for the maintenance and further cellular diversification of this area. In the third chapter *"The Life Journeys of Serotonergic and Dopaminergic cells"*, I will describe those patterning mechanisms that specify two distinct types of neurons, the midbrain dopaminergic and hindbrain serotonergic cells.

FROM NEURONAL PROGENITOR TO THE BIRTH OF A NEURON OR GLIA

Throughout early development, prospective neurons are maintained as proliferative progenitors. An extensive multiplication of cells is crucial given the enormous growth the embryo must undertake. Furthermore, premature exit from the mitotic stage would lead to that neuronal progenitors would initiate neuronal differentiation programmes according to the wrong developmental context. Therefore the process of neurogenesis must be suppressed until the right developmental stage is reached. At neurogenic stages, cells are singled out to start the programme of neurogenesis which coordinates cell cycle exit with acquisition of basic neuronal features (pan-neuronal) and subtype specific characteristics, dictated by the distinct transcriptional profile at the moment of birth. The neurogenesis is strictly regulated by the balance between promoting (proneural genes, Sox4, Sox11, Sox21) versus counteracting (Sox1-3, Notch (*Box3A*)) proteins (see below).

Proneural Genes Promote Neurogenesis

Already in the 1970's it was realised that the activity of the achaete-scute gene complex was coupled to neurogenesis in fly (Garcia-Bellido and Santamaria, 1978). Together with the atonal gene (Jarman et al., 1993), achaete-scute genes were therefore collectively denoted as "proneural genes". The same clusters are present in vertebrates and gene products Ascl1, Math1 and Neurogenin (Ngn)1-3 were identified as the main

proneural genes by loss- and gain-of-function experiments (Kageyama et al., 2005). Proneural genes are transcription factor of the bHLH family, which appear to activate a selected plethora of genes by binding to the E-box sequence as heterodimers with E-proteins. Due to this ability, proneural genes activate a genetic programme that controls multiple steps of the neurogenesis including precursor selection (Castro et al., 2006), cell cycle exit (Doe, 2008), migration (Ge et al., 2006; Heng et al., 2008), pan-neuronal (Sandberg et al., 2005; Bergsland et al., 2006), and subtype-specific differentiation. That proneural genes can convey neuronal subtype characteristics in fly was established already in the early 1990´s (Jarman et al., 1993) but was realised relatively recently in mammals (Parras et al., 2002). One clear example of a proneural gene with roles in specification is Ngn2 which in MN precursors collaborates with LIM3 and Isl1 to activate the expression of the sMN marker gene, HB9 (Lee and Pfaff, 2003, Ma et al., 2008). This activity can not be compensated by Ascl1 as a conversion of fate from MN to V2 occurs in $Ngn2^{Ascl1k/i}$ transgenic embryos, in which Ascl1 is expressed under the regulatory sequences of Ngn2 (Parras et al., 2002). The cunning genetic trick to replace Ascl1 with Ngn2 and *vice versa* was instrumental to understand their respective roles also in the generation of serotonergic and dopaminergic cells (Pattyn et al., 2004; Kele et al., 2006, Andersson et al., 2006; see "*The Life Journeys of Dopaminergic and Serotonergic cells*"). In the dorsal spinal cord, as mentioned, several neuronal bHLH genes function not only as proneural genes but also as pre-eminent determinants (Nakada et al., 2004; Helms et al., 2005).

Hes Counteracts Neurogenesis

The molecular basis underlying the maintenance of neural mitotic progenitor cells in the early commited neuroectoderm is not known. At E7.5 in mouse, however, the bHLH proteins Hes1 and Hes3 are readily expressed along the entire neural plate. That these factors are key guardians of the progenitor pool at this stage has been suggested from the effects seen in Hes1;Hes3;Hes5 knock-out (k/o) mice where cells underwent premature neurogenesis already at E8.5 (Hatakeyama et al., 2004; Hatakeyama and Kageyama, 2006). Throughout the early stages, neuroepithelial cells divide symmetrically to produce two equal daughter cells. Slightly later, some cells start asymmetrical division, which results in the generation of one differentiating neuron and one new progenitor cell. Symmetric division may also give rise to two neurons at this stage (Wilcock et al., 2007). Upon neurogenic phases the neuroepithelial cells convert into radial glia, which can be monitored by their change in appearance and properties. A gradual change in Hes expression is also seen; Hes1 and Hes5 are manifested whereas Hes3 is downregulated (Hatakeyama and Kageyama, 2006; Mori et al., 2005; Temple, 2001).

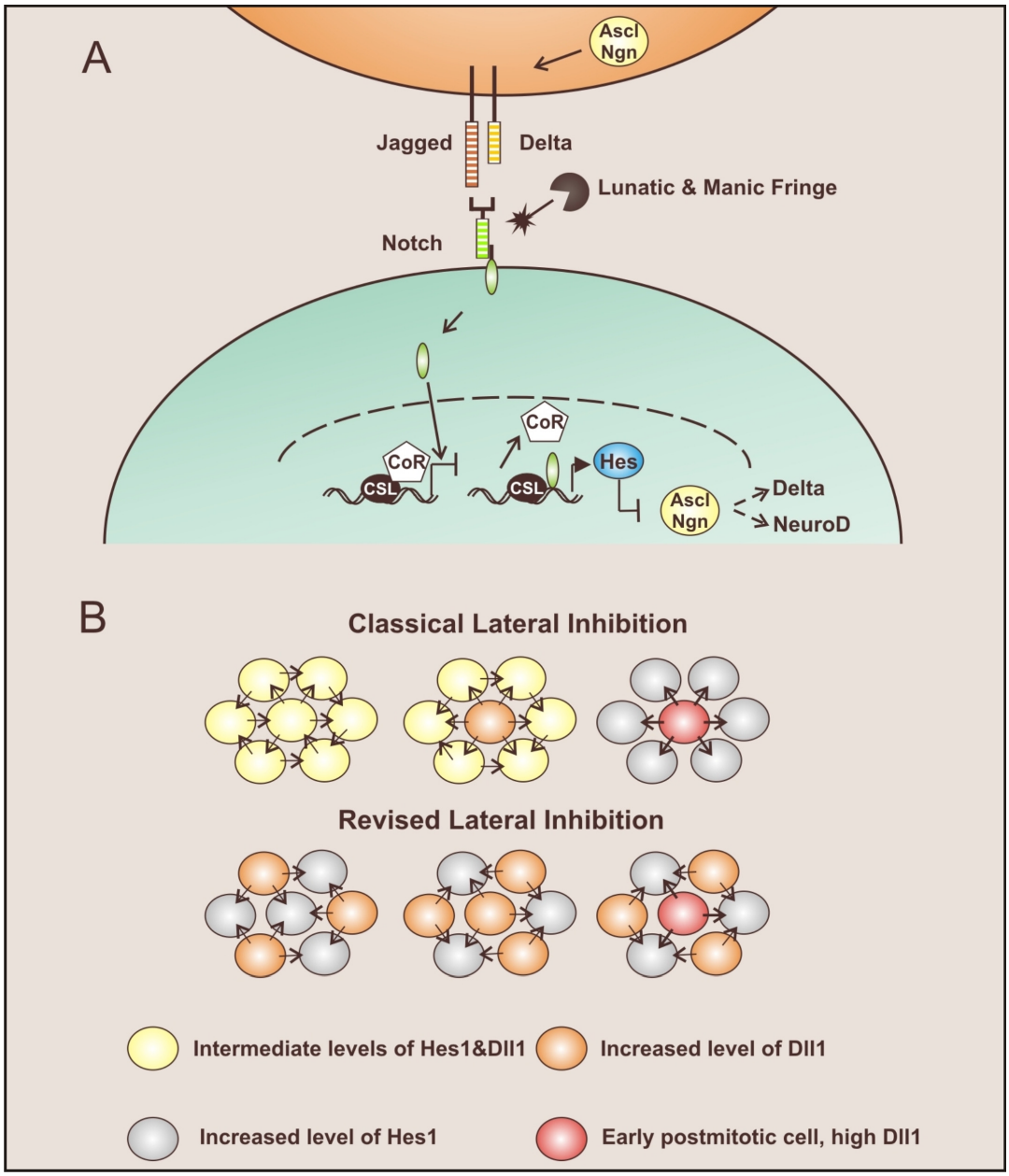
Hes genes are known to be the principle effector genes in the Notch signalling pathway (*Box3A*) (Louvi and Artavanis-Tsakonas, 2006). Curiously, Notch component

expression (Hatakeyama et al., 2004; Hatakeyama and Kageyama, 2006) and Notch signalling activity (Del Monte et al., 2007) correlate to the time point when neural progenitor cells become neurogenic (start expressing proneural genes) (E8.5 anteriorly). The early expression of Hes1 and Hes3 is thus Notch-independent and has instead been suggested to be controlled by LIF (Leukaemia Inhibitory Factor) signalling (Hitoshi et al., 2004). It is clear from the analysis of Notch1$^{-/-}$ mutant embryos that the maintenance of the progenitor pool is dependent on Notch signalling (de la Pompa et al., 1997). In such mutants, neuronal differentiation markers, Ascl1 and NeuroD were shown to be upregulated at E9. Furthermore, in Hes1$^{-/-}$;Hes5$^{-/-}$ mutant embryos at E9.5-E10.5 there was a gradual loss of radial glia markers, a subsequent upregulation of proneural genes, and a premature and accelerated neurogenesis (Hatakeyama et al., 2004). Taken together, these data suggest that Notch-dependent expression of Hes1 and Hes5 functions to ensure that neurogenesis occurs at the right time and pace in the developing neural tube. The premature and increased expression of proneural genes in the mutants indicates that one way that Notch signalling may suppress neurogenesis is by direct inhibition of proneural gene expression (Chen et al., 1997; Kageyama., et al 2007; Louvi and Artavanis-Tsakonas, 2006; Holmberg et al., 2008).

Progenitor Maintenance versus Neurogenesis

Classical Lateral Inhibition

That Hes genes can inhibit expression and function of proneural genes (*Box3A*) is one of the key features in the mechanism known as "lateral inhibition" (*Box3B*) which was first described in insects to determine neural versus epidermal lineages (Doe and Goodman, 1985; Heitzler and Simpson., 1991) and was later found to apply in many other contexts such as in the neurogenesis of the vertebrate CNS (Yoon and Gaiano, 2005; Louvi and Artavanis-Tsakonas, 2006). According to the classical model, lateral inhibition occurs in a homogenous cell population when one of the cells starts to express more Notch ligands than the surrounding cells. This imbalance could be due to stochastic variation or influence from extrinsic pathways such as Rel/NFκB, EGF or Wnt (Bash et al., 1999; Tsuda et al., 2002; Hofmann et al., 2004). The consequential increased Notch signalling in adjacent cells results in suppression of proneural function/expression and an accompanied reduction of Notch ligand expression (Castro et al., 2006; Kageyama et al., 2005). Thus, receiving cells will have a decreased ability to signal back to the first cell. In this way an initial small difference between Notch ligands and receptors in a cell population will be amplified and eventually lead to an on- or off-state of Notch signalling. In the vertebrate nervous system, the off-state will allow differentiation whereas the on-state maintains cells as proliferative progenitors (*Box3B*).



Box 3: (A) Basic components in the canonical Notch signalling pathway. *In mouse four ligands can activate Notch; Jagged (Jag)1,2 and Delta (Dll) 1,4. Binding to Notch (1-4) triggers the cleavage of the intracellular portions of the receptor. This liberated part is translocated to the nucleus where it converts the CSL(RBP-J) repressor into an activator. Downstream target genes include members of the Hes and Hey families. Hes1 and 5 can suppress the transcription of proneural genes (Ascl/Ngn). In cases of maintained proneural expression, neuronal markers (e.g NeuroD) and Notch ligands are upregulated. Lunatic and Manic fringe are glycosyltransferases which can alter Notch ability to respond to its ligands. When Notch is modified, Dll1 signalling is potentiated whereas Jag1 mediated signalling is suppressed. (B)* ***Different views on lateral inhibition****. In the classical model, lateral inhibition occurs in an initially homogenous population when one of the cells starts to produce more ligand in relation to the others. Such imbalance is further potentiated as the increased Notch signal in the surrounding cells leads to lower levels of ligands and thus a decreased ability to evoke a Notch signal in the first cell. Ultimately, the first cell is devoid of Notch signal and is able to go through neurogenesis, whereas the surrounding cells are maintained in a mitotic stage. Increasing evidence suggest that the levels of Notch components and proneural genes display an oscillatory pattern during the different phases of the cell cycle (compare first and second time point). In a revised model for lateral inhibition, this rapid cyclic expression of Notch receptors would preclude the possibility to accumulate substantial differences of ligand expression between cells. The initial selection for neurogenesis is therefore likely to depend on mechanisms distinct from lateral inhibition. Nevertheless, lateral inhibition is important to maintain cells in the progenitor zone by signalling from maturing cell to mitotic cells or by reciprocal signalling in between the progenitor cells (third time point).*

Notch Signalling Oscillation & Lateral Inhibition

That lateral inhibition would be the mechanism underlying the selection of neuronal progenitors for neurogenesis in the developing mammalian CNS has recently been challenged (*Box3B*). Three independent studies showed this year that Hes1, Delta1(Delta-like1;Dll1), and Ngn2 are expressed in an oscillatory manner in vertebrate neural progenitors in the retina, brain and spinal cord (Shimojo et al., 2008; Del Bene et al., 2008; Cisneros et al., 2008). According to one study Hes1 is downregulated in the G1-phase, allowing for high level expression of Dll1 and Ngn2. In the S-phase such expression patterns were found to be inversed (Shimojo et al., 2008). The other reports also provide evidence for a coupling between Notch signalling and the cell cycle but suggest that high Notch signalling is associated with mitosis rather than the S-phase (Del Bene et al., 2008; Cisneros et al., 2008). Regardless of the exact pattern, this oscillatory expression of Ngn2 may be advantageous for the proliferation at early stages, as it induces Dll1, enabling Notch signalling in adjacent cells, apparently without promoting neurogenesis. It is speculated that high static levels of proneural genes would be required to commence neurogenesis. How this oscillation is broken to induce stable Ngn2 expression required for neurogenesis is not known but may involve external factors that can prevent Hes1 levels to rise. Another possibility could be an asymmetric distribution of the Notch inhibitor Numb, which would ensure a maintained proneural gene expression in certain daughter cells (Cayouette and Raff, 2002; Johnson, 2003; Shen et al., 2002). Alternatively, the numbers of oscillation cycles may determine the timing of neurogenesis by the gradual accumulation of proneural target genes (possibly BM88/CEND1) (Politis et al., 2007). One suggested current view (Kageyama et al., 2008) on mammalian neurogenesis is thus that the initial neuronal precursor selection operates relatively independent on lateral inhibition. Nevertheless, lateral inhibition is required for progenitor maintenance by reciprocal signalling between neural progenitors and from early postmitotic neurons to neural progenitors (*Box3B*).

Sustained Hes1 Expression in Non-Neurogenic Regions of the CNS

The oscillation of Hes genes appears to be crucial for cells to remain proliferative. Low expression of Hes1 promotes cell cycle progression while persistent and high levels instead inhibit the transition from the G1-phase (Baek et al., 2006). High levels of Hes genes are therefore characteristic of non-neurogenic regions of the CNS which act as organisers or boundaries, such as the floor plate, roof plate, dentate gyrus and the isthmus (Baek et al., 2006; Imayoshi et al., 2008). The mechanisms that control sustained versus oscillatory expression of Hes1 are not known, but Id proteins and the JAK/STAT signalling has been suggested as possible regulators (Bai et al., 2007; Yoshiura et al., 2007; Kamakura et al., 2004; Shimojo et al., 2008).

Sox Genes Regulate Several Steps during Neurogenesis

As described earlier, Sox2 is the earliest known specific marker for neuroectoderm. The other two members of the SoxB1 family, Sox1 and Sox3, are also expressed in the developing neural tube and have likewise been shown to be essential to maintain neural cells in a proliferative state (Bylund et al., 2003; Graham et al., 2003). Their mechanisms of action appear to involve interference with proneural protein function (Bylund et al., 2003), in contrast to Notch-mediated inhibition of neurogenesis which is based on suppressed expression of proneural genes and the E-protein, E47 (Kageyama et al., 2007; Holmberg et al., 2008). Moreover, SoxB1 genes control the progenitor state in a wider context than Notch signalling, as Notch signalling fails to inhibit neurogensis in the absence of SoxB1 genes (Holmberg et al., 2008).

In contrast to the SoxB1 proteins, other members of the Sox gene family have been implicated in promoting the progression of neurogenesis. Sox21 has been shown to be upregulated by proneural genes and promote neurogenesis by counteracting the expression of the SoxB1 genes (Sandberg et al., 2005). Sox4 and Sox11 have also been demonstrated to act downstream of proneural genes to establish pan-neuronal properties, curiously, without promoting cell cycle exit (Bergsland et al., 2006). Thus, exit from the cell cycle, and onset of neuronal marker expression, are two features during neurogenesis that are controlled by separate molecular pathways downstream of proneural genes.

From Neural Progenitor to Glia

The neuroectoderm does not only generate neurons; when the main neurogenic period is completed, a gliogenic stage commences in which oligodendrocytes and astrocytes are produced. Thus, the process of neurogenesis must comprise mechanisms that suppress gliogenesis. Studies aimed to unravel mechanism that underlies the temporally defined neurogenic-to-gliogenic switch (ng-switch) have focused specifically on either spinal cord or the cortex (Richardson et al., 2006; Millen and Gauthier, 2007). The ng-switch appears to be dependent on a cohort of signalling activities that are finely orchestrated to coordinate both the inhibition of neurogenesis and the activation of gliogenesis.

JAK/STAT pathway

In the cortex, an increased JAK/STAT signal appears to be crucial for the commencement of gliogenesis (Sun et al., 2001). As described earlier, proneural genes are essential for the conversion of neuronal progenitors into differentiating neurons. In addition to this function, it has now become increasingly clear that Ngn1 actively suppresses gliogenesis by interfering with JAK/STAT signalling (Sun et al., 2001). Thus, suppression of proneural genes may be a mean to de-repress JAK/STAT

signalling and Notch, BMP and Wnt signalling have all been suggested to serve this function (Hirabayashi et al., 2004; Miller and Gautier, 2007). Notch may also promote gliogenesis by promoting the JAK/STAT pathway in more direct means (Kamakura et al., 2004), but since Notch1 mutant embryos die before gliogenesis commences any *in vivo* function of Notch is hard to pinpoint. However, Nestin-mediated depletion of the Notch target CSL (RBP-J) have negative effects on gliogenesis (Taylor et al., 2007) and forced expression of Notch blocks neurogenesis and results in an excess of oligodendrocytes (Rowitch, 2004). Notably, the accelerated oligodendrogenesis occurs at the normal stage, indicating that Notch is unable to set the timing for the ng-switch. Contrary to the current view on proneural genes, it has been recently shown that the proneural gene Ascl1 promotes both specification and maturation of oligodendrocytes, providing the first evidence for roles of proneural genes in non-neuronal cells in the CNS (Parras et al., 2007; Sugimori et al., 2007; 2008; Battiste et al., 2007).

Other processes that feed positively into the JAK/STAT status include a positive autoregulatory loop (He et al., 2005) and secretion of the JAK/STAT ligand CT-1, from newly formed neurons. The latter would support a model in which the first-born cells, the neurons, instruct the remaining precursors to generate a second cell type, the astrocytes (Miller and Gauthier, 2007).

Intrinsic Determinants

Independent on the JAK/STAT line of research, three important cell-intrinsic determinants for glia have been found, Sox9 (Stolt et al., 2003), NF1A (Deneen et al., 2006), and COUP-TFI/II (Naka et al., 2008). NF1A is interesting in that it appears to have dual functions. First, it is required to commence the ng-switch and second it promotes the astrocytic fate if not counteracted by the oligodendrocyte marker Olig2 (Deneen et al., 2006). Interestingly, just prior to gliogenesis, Hes5 expression becomes dependent on NF1A rather than Notch, and these cells may from that moment be specified to a glial fate (Deneen et al., 2006). This Hes expression may be indicative of an astroglia fate as Hes genes have been shown to antagonise the oligodendrocyte marker Olig2 (Miller and Gauthier, 2007). It will in the future be interesting to understand how JAK/STAT and intrinsic gliogenic determinants conspire to specify glia. One recent study provided a link between glial STAT target availability and the transcription factor COUP-TFI/II (Naka et al., 2008). This transcription factor appears to be essential but not sufficient for the ng-switch.

Patterning of Glia

Similar to neurons, it is becoming increasingly evident that glia can be categorised into different subtypes. That diversification of different types of astrocytes is regulated by means of patterning was recently shown (Hochstim et al., 2008). Oligodendrocyte generation is also restricted to specific progenitor domains although

the relevance of which is not clear today. Oligodendrogenesis commences from a ventral domain and has recently been shown to ensue from dorsal positions at later time-points (Vallstedt et al., 2005; Cai et al., 2005; Richardson et al., 2006). Ventral oligodendrogenesis commences from $Olig2^+$ progenitors both in mouse and chick. In the latter, however, a dorsal expansion of Nkx2.2 into the Olig2 domain occurs just prior to the switch, thus creating a unique subdomain ($Nkx2.2^+/Olig2^+$) in which proneural expression is suppressed allowing for oligodendrocytes to be generated (Zhou et al., 2001).

THE LIFE JOURNEYS OF DOPAMINERGIC AND SEROTONERGIC CELLS

At spinal cord levels, basic principles underlying patterning are well studied and several major downstream determinants that initiate subtype specific programmes have been identified (Jessell, 2000). It was anticipated, and has to some extent been confirmed, that the basic mechanisms for patterning can be applied essentially along the entire extent of the neural tube. However, the greater morphological complexity and the formation of other neural subtypes in the brain suggest that the mechanisms found in the spinal cord are modified at more anterior positions. Thus, as described in *"Patterning along the AP axis"*, patterning along both DV and AP axes of the hindbrain/midbrain area rely on the relatively late actions of the IsO as well as on signals that are common with the spinal cord. Below I will describe the extrinsic and intrinsic cues found to operate to ensure the correct spatial and temporal generation of two clinically important neurons, the midbrain dopaminergic cells and hindbrain serotonergic cells.

Dopaminergic Cells in the Ventral Midbrain

Definition, Location and Function

Midbrain Dopaminergic (mDA) cells constitute a subclass of cathecholaminergic cells and utilise, as the name implies, dopamine as neurotransmitter. Dopamine is formed from two enzymatic modification of tyrosine. The first step executed by tyrosine hydroxylase (TH) results in L-DOPA, and the second modification by L-Aromatic amino acid decarboxylase (L-AADC) produces dopamine. TH and L-AADC are therefore indicative markers of DA cells, although they are present in all cathecholaminergic cells. However, in these (noradrenergic and adrenergic cells), dopamine is further converted into other neurotransmitters. Thus, the DA transcriptional signature is also defined by the absence of the enzymes (DBH and Phenylethanolamine N-methyltransferase) that can further modify dopamine (Goridis and Rohrer, 2002).

The most prominent DA cells reside in the tegmentum and are subdivided into two main locations, the substantia nigra (SN, A9 nucleus) and the ventral tegmental

area (VTA, A10 nucleus) (Björklund and Lindvall, 1984). Both components reside in the ventralmost area of the midbrain, but, whereas SN is positioned ventrolaterally, VTA has a medial location. As the anatomical subdivisions imply, mDA cells within these two nuclei project to different brain areas and display distinct functions. VTA DA cells connect with the ventral striatum and prefrontal cortex to form the mesocorticolimbic pathway which is involved in reward behaviour and motivation (Perrone-Capano and Di Portzio, 2000; Nestler and Carlezon, 2006). SN DA cells project to the striatum, forming the nigrostriatal pathway that is important most notably for motor control (Groenewegen, 2003). Importantly, the selective degeneration of SN DA cells accounts for the major pathology of Parkinson's disease (Blandini et al., 2000). Future promises to treat this devastating disease with cell replacement strategies motivate studies aimed towards gaining a molecular understanding of the formation of SN DA cells (see "*Results and Discussion, Paper I*").

The Transcription Profile of Ventral mDA neurons

In addition to TH and L-AADC that are common to all DA cells, multiple transcription factors have been found to specifically mark and regulate the maturation and survival of postmitotic midbrain DA cells. The orphan nuclear receptor **Nurr1** is one of the earliest markers of mDA cells and its functions are essential for maturation and maintenance of these cells (Perlmann and Wallen-Mackenzie, 2004). Most notably, Nurr1 is required for the onset of TH, vesicular monoamine transporter 2 (VMAT2), dopamine transporter (DAT) and Ret proto-oncogene (Ret) expression (Wallen et al., 1999; 2001; Smits et al., 2003). In mutants lacking Nurr1, mDA cells are born but are completely degenerated by the time of birth possibly as a consequence of the failure to acquire a proper phenotype (Wallen et al., 2001). The loss of mDA cell could also be attributed to Nurr1's ability to promote survival in response to RXR ligands when heterodimerized with RXR (Wallen-Mackenzie et al., 2003). The paired HD transcription factor **Pitx3** can be found in all ventral mDA cells, although at variable levels and with temporally distinct onsets in different subpopulations (Maxwell et al., 2005). Despite the ubiquitous expression, loss of Pitx3 leads to a selective deficiency, where SN TH cells are almost totally degenerated whereas half of the VTA TH cells are spared (Smits et al., 2006). The molecular basis for the selective vulnerability is not known, but microarray comparisons between VTA and SN cells have identified differentially expressed genes that may hold the answer to this riddle and also why SN DA cells are affected the most in patients with Parkinson's disease (Jacobs et al., 2006). **En1/2** demarcates the midbrain/R1 region at early stages, but is again upregulated in postmitotic mDA neurons and is considered to be one important marker of mDA cells (Simon et al., 2004). In En1/2 mutants, mDA neurons are born in reduced numbers but mature in a phenotypically correct way. However, shortly after the normal onset of the En1/2 genes, at E14, there is a complete loss of all mDA neurons indicating that En1/2

has anti-apoptotic functions (Alberi et al., 2004; Simon et al., 2001). Another early onset gene with a prolonged expression into the postmitotic stage is **Lmx1b**. This LIM-HD transcription factor appears to control the onset of Pitx3 relative to TH and is crucial for survival as all mDA neurons are lost after E16 (Smidt et al., 2000) in $Lmx1b^{-/-}$ mutant embryos. Furthermore, substantially fewer cells are intitially generated in these mutants but the gross effects of the IsO hampers a detailed understanding of the role of Lmx1b (more discussion in "*Results and Discussion, Paper I*"). **Forkhead box (Fox)A2** (previously known as HNF3β) can be found in the floor plate along the entire neural tube whereas the expression of **FoxA1** (previously known as HNF3α) is limited to the midbrain (Sasaki and Hogan, 1993). Misexpression of FoxA1/2 transforms neuronal progenitors into floor plate cells at ectopic positions in the dorsal neural tube (Sasaki and Hogan, 1994, own unpublished observation). Additional functions of these proteins in the midbrain are however implied as FoxA2 has a broader expression domain than Shh and is also maintained in postmitotic ventral midbrain neurons. A careful analysis of the gene dosage effects of FoxA1 and 2 revealed that high levels of FoxA1/2 is crucial in order for early postmitotic $Nurr1^{+}$ cells to mature and commence expression of TH and AADC (Ferri et al., 2007). An independent long-term study showed, in addition, that FoxA2 heterozygous mice develop Parkinsonian-like symptoms and a correlative selective degeneration of SN DA cells (Kittappa et al., 2007).

Taken together, TH, L-AADC, Lmx1b, En1/2, Pitx3 and Nurr1 and FoxA1/2 are crucial components in the postmitotic mDA differentiation pathway. However, the initiation of the mDA specific programme is triggered already in mitotic cells that in turn have been made competent by pre-patterning activities (explained in "*Patterning along the AP axis*"). What is then the molecular code for competence, the triggering extrinsic factors, and the initial road-marks along the intrinsic dopaminergic differentiation pathway?

Extrinsic Cues Act on Competent Ventral Midbrain Cells to Initiate mDA Generation

As described in "*Early AP Regionalisation of the Neuroectoderm*", pre-patterning of the neural plate into the prospective midbrain occurs already at mid-to-late gastrula. Later, cues from the roof plate, floor plate and isthmus must be fed into the rudimentary differentiation programme in order for this area to flourish into a functionally correct midbrain encompassing a variety of unique neuronal subtypes. What is then the nature of the extrinsic cues that ensures the formation of mDA cells? As hinted from the ventral location of mDA cells close to the IsO, **Shh** and **FGF8** play important roles in the induction of these cells, as shown in the mid 1990's (Wang et al., 1995; Ye et al., 1998). These studies relied on loss- and gain-of-function experiments in *ex vivo* experimental setups. In these, Shh was shown to induce ectopic mDA cells at lateral midbrain positions and FGF8 was capable to impinge the mDA fate in ventral

forebrain tissue. Although not directly tested in these studies it is likely that relatively high concentration of Shh is required to induce mDA cells and that gradually lower amounts specify more lateral cell types such as MN and IN (our unpublished observations). Intermediate levels of FGF8 may be sufficient and optimal for mDA induction as mDA cells are generated in greater abundance at rostral positions in the midbrain and possibly also in the caudal diencephalon (Smits et al., 2006). Moreover, misexpression studies have revealed that Otx2 is suppressed by high levels of FGF8 (Martinez et al., 1999)

More recently, **TGFβ** and **Wnt1** have been suggested to be implicated in mDA cell generation. Components of the TGFβ pathway are found in the developing ventral midbrain (Farkas et al., 2003; our unpublished observation). Neutralisation of TGFβ before mDA generation in chick results in a significant reduction in the number of mDA cells (Farkas et al., 2003). Furthermore it has been observed that fewer mDA cells are generated in the TGFβ2-3 double k/o (Roussa et al., 2006). A more careful analysis of the progenitor identity and the neurogenic status in both mentioned experiments is needed to reveal what step(s) during mDA neurogenesis require or are stimulated by TGFβ. A number of studies have provided strong evidence for the involvement of Wnt1 in the generation of mDA cells. The first indication came from the aforementioned analysis of the $Wnt1^{-/-}$ mutant embryos where the number of mDA was severly reduced (Wurst et al., 1994). It was also observed that induction of ectopic mDA cells in the forebrain by FGF8 was preceded by rapid Wnt1 expression (Martinez et al., 1999; Crossley, 1996), although not seen by Ye et al., 1998. This induction of Wnt1 appears to be pivotal as FGF8 is unable to induce mDA cells in the forebrain of $Wnt1^{-/-}$ mutant embryos (Prakash et al., 2006). In the same study the authors analysed a transgene where Wnt1 was transcribed under the En1 promoter ($En1^{wnt1k/i}$) resulting in an expansion of the ventral Wnt1 expression caudally into the hindbrain. As a consequence, the authors report that the hindbrain floor plate converts into a midbrain-like neurogenic region generating mDA cells from ectopic Otx2 expressing mitotic progenitors. From these data it would appear as if Otx2 mediates the induction by Wnt1. The importance of Otx2 for the generation of mDA cells has also been demonstrated in $Nestin^{Otx2k/o}$ mutants. In these embryos, Otx2 was ablated from E10.5 leading to an overall decrease in the number of mDA cells although this may be attributed to a reduced proliferation and failure to induce proneural genes (Vernay et al., 2005). Nkx2.2 is normally confined to lateral progenitors giving rise to IN, but in $Nestin^{Otx2k/o}$ mutants its expression domain was ventrally expanded. Ventral $Nkx2.2^{+}Otx2^{-}$ cells are normally only found caudally where they in the hindbrain give rise to serotonergic (5-HT) cells (Briscoe et al., 1999). Indeed, in these mutants, ectopic 5-HT neurons were generated adjacent to the diminished mDA cell population. A stronger effect on the generation of mDA cells was seen in $En1^{Otx2k/o}$ mutants where Otx2 ablation commenced already at E9 (Puelles et al., 2004) and the Nkx2.2

expansion extended into the domain that normally generates mDA cells. To test if the ectopic Nkx2.2 constituted the block of mDA cell fate, Nkx2.2$^{-/-}$ mice were crossed into the En1$^{Otx2k/o}$ line. In these embryos, the mDA cell generation was partly rescued, indicating that one important function of Otx2 is to suppress Nkx2.2, which would otherwise override the DA cell specification programme (Prakash et al., 2006). It is still likely that Otx2 is a crucial competence factor needed to translate FGF8/Shh signals into the initiation of the mDA fate programme, which probably occurs before E9.

Elucidation of the exact function(s) of the various pre-patterning factors and growth factors operating during mDA specification is challenging as most of them appear to play important role in proliferation, neurogenesis and survival. In addition, exact levels, positions and maintenance of the expression of the discussed proteins appear to be fine-tuned in regulatory loops. Any distinct role in cell fate determination may therefore be masked by gross morphological changes, increased apoptosis or failure to maintain expression of other genes than the one of interest. Nonetheless, determination of the Otx2/Gbx2 boundary at the correct position is no doubt crucial for a normal development of mDA cells to occur and it is clear that Shh, Wnt1 and FGF8 are all essential players in this process. The interactions between the intrinsic and extrinsic factors in the ventral midbrain are interpreted and converted into the mDA cell specific programme of differentiation. Paper I will discuss the identification of two important transciption factors acting already in progenitor cells. In addition to those, Lmx1b has been found to be expressed at early stages in the mitotic mDA lineage (Wallen et al., 1999; Smidt et al., 2000), which will be discussed in greater detail in "*Results and Discussion*" in relation to Paper I.

Proneural Genes and mDA Differentiation

Differentiating mDA progenitors upregulate both **Ascl1** and **Ngn2** (Kele et al., 2006). These proneural genes have been reported in other regions of the CNS to have instructive as well as pan-neuronal properties and their respective functions were therefore scrutinised using various mouse mutants (Parras et al., 2002; Pattyn et al., 2004). Analysis of the single k/o embryos for Ascl1 or Ngn2 revealed that only Ngn2 appeared to be required for proper neurogenesis to occur. In Ngn2$^{-/-}$, differentiation of mDA cells was severely compromised at early stages (Kele et al., 2006) but a partial recovery resulted in that 30-50% of the normal numbers of mDA neurons were seen postnatally (Andersson et al., 2006). Moreover, the authors reported that many of the generated postmitotic neurons stalled in their differentiation at the Nurr1^{+} stage and failed to turn on TH at the appropriate stage, indicating that Ngn2 regulates both the timing for neurogenesis and progression of the early postmitotic differentiation. To investigate whether the phenotype reflects a specific function for Ngn2, Ascl1 was expressed under the regulatory sequences of Ngn2 (Ngn2$^{k/o}$; Ngn2$^{Ascl1k/i}$). Using this line, it was found that Ascl1 could only to a mild extent ameliorate the Ngn2$^{-/-}$

phenotype, suggesting that Ngn2 indeed possessess unique regulatory functions in the mDA lineage. This was further supported by the fact that the generation of MNs and red nucleus cells were unaffected in the Ngn2 mutant, despite the presence of Ngn2 during the normal differentiation of these cell types (Andersson et al., 2006).

Serotonergic Cells in the Hindbrain

Definition, Location and Function

Serotonin (5-HT) is like dopamine produced in a two-step enzymatic reaction. In this, tryptophan is first converted by tryptophan hydroxylase (TPH) and then by L-AADC to produce 5-HT. Interestingly, 5-HT is not only utilised as a neurotransmitter in the CNS and Peripheral Nervous System (PNS) but is also secreted from the gut and functions as a hormone in all vascularised tissues (Alenina et al., 2006). Within the CNS, most serotonergic cells are generated in the ventral hindbrain where they cluster to become the most abundant and important component of the raphe nuclei. Based on their distribution and main projections, the raphe nuclei are annotated into two main groups, the rostral (B9-4) and the caudal (B3-B1) (Dahlström and Fuxe, 1964; Steinbusch, 1981). The rostral group is generated from R1-3 and the caudal from R5-8, leaving a gap in R4 where no 5-HT neurons are formed (in mouse and human; Pattyn et al., 2003; Hornung, 2003; "*Results and Discussion, Paper III*"). Rostral groups can be further subdivided into the dorsal raphe nuclei (B7, B6), median raphe nuclei (B8, B5), which are located in the hindbrain, and the B9 group, which is finally positioned within the midbrain (Hensler, 2006). The rostral raphe nuclei connect to most areas of the brain, whereas the projections of the caudal subdivision are limited to the caudal brainstem and the spinal cord. As a whole, the raphe nuclei are involved in a multitude of central body functions such as mood, sleep and appetite. As a consequence, malfunction of raphe nuclei contributes to human diseases of the mood and mind. Schizophrenia, depression, and aggression are all disorders that can be ameliorated by treatments targeting the serotonin pathway (Hensler, 2006). An increased understanding of mechanisms for serotonergic specification and differentiation may be instrumental in the development of ES cell-based engineering of 5-HT neurons. Such *in vitro* systems could be used as platforms for drug screening or for the generation of cells to replace 5-HT neurons in psychiatric and neurodegenerative diseases with a loss in serotonergic tone.

Extrinsic and Intrinsic Cues Important for 5-HT Neuron Specification

The functional and spatial subdivision of the raphe nuclei is probably a reflection of the different mechanism whereby rostral and caudal hindbrain is generated and patterned along the AP axis at early developmental stages. In previous chapters I have

described that the rostral hindbrain characteristics are induced by Wnt and FGF and that later actions of RA converts parts of this into the caudal hindbrain (Nordström, 2002;2006). RA is also instrumental in setting up the Hox code which further subdivides the hindbrain into 7-8 rhombomeric units. As the rostral hindbrain is mostly unaffected in VAD rats (White et al., 2000), other molecules than RA may function to ensure the patterning of R1-4. The rostral hindbrain is in close vicinity to IsO and it is clear from analysis of Wnt1 and En1 mutant embryos (Wurst et al., 1994; Liu and Joyner, 2001) that the development of R1 is indeed dependent upon a functional IsO. Moreover, using explant experiments it was shown that FGF8 is absolutely required for the generation of 5-HT neurons in the rostral hindbrain but not in the caudal parts (Ye et al., 1998). Like in the case of mDA neurons, Shh has also been found to be both required and sufficient for the induction of 5-HT neurons. That the same signals (FGF8, Shh) stimulate the generation of both serotonergic and dopaminergic cells indicate that their mode of action is context dependent. It is plausible to assume that expression of either Otx2 or Gbx2 mediates the differential response to these factors in the midbrain and hindbrain, respectively. It was observed that early exposure of FGF4 to midbrain explants, changes the interpretation to FGF8 and Shh so that 5-HT neurons were formed (Ye et al., 1998). These results indicate that FGF4 acts by suppression of Otx2 and that it may be instrumental in setting the MHB.

Although 5-HT neurons with very different functions are generated at distinct positions along the AP axis of the hindbrain, the general 5-HT differentiation programme appears to be governed by one key determinant induced by Shh; **Nkx2.2** (Briscoe et al., 1999). In Nkx2.2$^{-/-}$ mutants, all serotonergic constitutes of the raphe nuclei are extinguished, except for those in the dorsal raphe nuclei, where the homologoue Nkx2.9 may have a compensatory function. Curiously, the generation of 5-HT neurons from the Nkx2.2 domain is preceded by an earlier neurogenic wave producing visceral motor neurons (vMN) (Pattyn et al., 2003). This is made possible by the fact that at early stages, Nkx2.2 proteins are accompanied by the expression of the vMN determinant Paired like homeobox (Phox)2b (Pattyn et al., 2000), which actively suppresses the serotonergic differentiation programme. The generation of serotonergic cells may therefore be achieved by the gradual onset of a suppressor of Phox2b and a recent study suggests that FoxA2 fulfils the requirements of being this repressor (Jacob et al., 2007). However, there are important exceptions to the rule; no temporal switch from vMN to 5-HT neuron production occurs in R1 and R4 (Cordes et al., 2001; Pattyn et al., 2003). In R1, vMN are never produced, probably due to the absence of Phox2b expression. In contrast, only vMN and no 5-HT neurons are produced in R4, owing to a prolonged expression of Phox2b. The upstream determinant for this rhombomere specific mode of neuronal production is, not surprisingly, a Hox gene, Hoxb1 (Pattyn et al., 2003). Hoxb1 expression appears to allow a maintained expression of Phox2b, possibly through suppression of FoxA2. Interestingly, the HD proteins Nkx6.1 and

Nkx6.2, which are also expressed in the Nkx2.2 domain along the entire hindbrain, are required for a prolonged expression of Hoxb1 in R4. Consequently, whereas the vMN production was replaced by early production of 5-HT neurons in the R4 of Hoxb1$^{-/-}$ and Phox2b$^{-/-}$ mutant embryos, in Nkx61$^{-/-}$;6.2$^{-/-}$ mutants, R4 acquired the "canonical mode of neurogenesis" where vMN production was followed by generation of 5-HT neurons (Pattyn et al., 2003). This is an evident example of where molecules involved in DV patterning can modulate the expression of an AP patterning gene resulting in a unique regulation of neuronal differentiation. The importance of Nkx6 proteins for the specification of 5-HT neurons appear to differ between mouse and chick. In mouse, 5-HT neurons are born along the entire hindbrain in the absence of Nkx6 proteins (Pattyn et al., 2003). In contrast, specific downregulation of Nkx6.1 with morpholinos in chick was shown to suppress the formation of 5-HT neurons (Craven et al., 2003), indicating that Nkx6.1 may be equally important as Nkx2.2 for the initiation of the serotonergic differentiation programme in chick.

Both vMN and 5-HT neurons turn on **Ascl1** as they enter the neurogenic programme. Despite this, it was realised by the analysis of Ascl1$^{-/-}$ mutant embryos that Ascl1 is actually only required for the generation of 5-HT neurons and is dispensible for vMN formation (Pattyn et al., 2004). By replacing the coding sequence of Ascl1 with that of Ngn2 (Ascl$^{k/o}$; Ascl1$^{Ngn21k/i}$) it was furthermore shown that Ascl1 has a subtype specifying role, as cells could go through neurogenesis but failed to intiate the serotonergic differentiation programme in such mutants. It is of interest to note that Ascl1 is also required for the formation of 5-HT neurons in the enteric nervous system (Blaugrund et al., 1996) and the thyroid gland (Lanigan et al., 1998), indicating that Ascl1 may be an essential and general determinant of the generic serotonergic phenotype. Note also that differentiation of 5-HT neurons depend on Ascl1 expression while proper formation of mDA neurons requires Ngn2.

The Transcription Profile of Raphe Nuclei Serotonergic Cells

Mostly through loss-of-function studies, a number of postmitotically expressed transcription factors have been demonstrated to play important roles in the maturation and survival of 5-HT neurons. The ETS domain protein Plasmacytoma expressed transcript 1 (**Pet1**), is specifically expressed in the hindbrain serotonergic lineage just prior to production of 5-HT (Hendricks et al., 1999). In Pet1$^{-/-}$ mutant mice, the number of 5-HT neurons was diminished by 70% in all raphe nuclei (Hendricks et al., 2003). However, the remaining cells appeared to migrate, cluster into raphe nuclei, and send appropriate axonal projections. A closer examination of their transcription profile revealed a lower expression of TPH and serotonin transporter (SERT). Pet1 binding sites have indeed been found in the enhancer regions of these genes (Hendricks et al., 1999) suggesting that the regulation may be direct. The basis to why only a subset of the precursors fail to mature and function in the absence of Pet1 is not clear but may be

due to an uncharacterised heterogeneity within the 5-HT populations. As a consequence of the overall lower levels of 5-HT, Pet1$^{-/-}$ mutant mice displayed heightened anxiety-like behaviour and aggression (Hendricks et al., 2003).

Pet1 does not constitute the beginning of the postmitotic 5-HT programme. Two factors with even earlier postmitotic expression are **GATA2** and **GATA3**. In chick, GATA2 expression is initiated before GATA3, but the two proteins form a positive regulatory loop later (Craven et al., 2003). Since GATA2$^{-/-}$ mutant embryos die early, explants were grown to assess the necessity of this factor for serotonergic differentiation. In these explants, no markers of the postmitotic serotonergic lineage except for GATA3 were initiated, indicating that GATA2 may be essential to execute the programme started by Nkx2.2. In support of this, ectopic GATA2 had the ability to induce ectopic 5-HT neurons in chick R1 without prior induction of Nkx2.2. In contrast, GATA3 did not possess similar potency to induce 5-HT neurons (Craven et al, 2003) and appeared to be more crucial for the establishment of neurotransmitter phenotype in the caudal raphe nuclei (van Doorninck et al., 1999; Pattyn et al. 2003). In GATA3 mutants (rescued with a noradrenergic agonist (Lim et al., 2000)), 80% of the neurons in the caudal raphe nuclei failed to turn on 5-HT and 30% in the rostral parts. In the rostral nucleus, there appears to be some heterogeneity in respect to GATA3 as only 50% of the 5-HT neurons co-express GATA3 (van Doorninck et al., 1999).

In addition to the abovementioned transcription factors, **Lmx1b** plays important roles in the establishment of the serotonergic phenotype which will be discussed in "*Results and Discussion, Paper II*".

No major differences have hitherto been found in the transcription profile between 5-HT neurons in different nuclei although each nucleus innervates very distinct parts of the CNS. Future microarray analysis may shed light on how the various AP cues are translated into diversifying the serotonergic system into the functionally distinct nuclei.

AIMS

The research presented in this thesis aimed towards gaining a better understanding of molecular mechanisms for cell fate specification and neurogenesis in the developing CNS. Specific questions addressed were:

- What are the key intrinsic determinants in the cell fate programme of ventral midbrain dopamine cells? Can such determinants be applied in the development of cell replacement strategies for the treatment of Parkinson's disease?

- What is the function of Lmx1b in the early acquisition of the hindbrain serotonergic phenotype?

- Are the molecular mechanisms for cell fate determination found in model organisms relevant during human development of the CNS?

- What is the role of the regional expression of Notch ligands in regulation of neurogenesis and boundary formation in the developing spinal cord?

RESULTS & DISCUSSION

IDENTIFICATION OF INTRINSIC DETERMINANTS OF MIDBRAIN DOPAMINE NEURONS (PAPER I)

Tegmental dopamine neurons are formed in the ventral midbrain at the intersection between the morphogenic fields of FGF8 secreted from the isthmus and Shh produced from the floor plate. The activities of these signalling pathways are believed to induce the mDA specification cascade by feeding into the prepatterned prospective midbrain programme. The molecular signature of the rudimentary midbrain has been long known to encompass Otx2 and recent data suggest that TGFβ and Wnt1 are important signalling mediators downstream of FGF8 and Shh. Moreover, postmitotic markers important for phenotypic maturation and survival have been identified. Despite this, little was known about the molecular constitute of the mDA specification programme acting downstream of Otx2/FGF8/Shh but upstream of mDA-specific lineage markers such as Nurr1 and Pitx3. Lmx1b had been shown to be expressed in ventral midbrain progenitor cells but in $Lmx1b^{-/-}$ mutant embryos, mDA cells are born, although with a slightly altered phenotype (Smits et al., 2000). Moreover, as the IsO is affected in these mutants it is difficult to ascertain weather the reduced numbers of mDA neurons are a direct effect of the absence of Lmx1b or just due to the gross overall defects in the midbrain (Guo et al., 2007). In Paper I our aim was to identify transcription factors that would fulfil the requirements of serving as determinants during differentiation of mDA neurons. By relying on previous findings that cellular determinants often encompass HD DNA binding domains and are specifically expressed in dividing progenitors we devised strategies to find HD proteins in the early developing ventral midbrain. We identified HD transcription factors that were specifically expressed in mDA progenitor cells, LIM homeobox gene 1a (Lmx1a) and msh-like homeobox gene 1 and 2 (Msx1/2). Using *ex vivo* explant techniques we first showed that these proteins were induced as a response to Shh in lateral midbrain, but not in hindbrain or forebrain tissue (our unpublished data), showing that their expression is regulated downstream of Shh exclusively in a midbrain context.

The Functions of Lmx1a

To assess the function of Lmx1a we deployed loss- and gain-of-function experiments in chick embryos. Reduction of Lmx1a expression by RNA interference resulted in a concomitant loss of Msx1/2 and a block of mDA neuron generation. Conversely, forced expression of Lmx1a at lateral positions in the developing midbrain led to ectopic mDA neuron induction at the expense of other neuronal subtypes. This conversion of cell fate indicated that the progenitor code had been changed by Lmx1a and indeed we could detect ectopic Msx1 expression and downregulation of the

presumed MN determinant Nkx6.1. We noted though that the efficiency of Lmx1a to induce the mDA cell fate was diminished in a ventral to lateral direction. Lmx1a was totally unable to induce mDA neurons in the dorsal half, indicating that the activity of Lmx1a is context dependent. In support for this, Lmx1a could not either induce mDA cells in the hindbrain (our unpublished observation). Thus, in chick, Lmx1a is both sufficient and required for the generation of mDA neuron in the ventral midbrain.

Next we considered whether Lmx1a would be potent to induce mDA neuron specification in differentiating mouse (m) ES cells (*Box1B*). To this end we transfected mES cells with a construct in which Lmx1a would be induced in neuroectodermal cells under a Nestin enhancer/promoter region (NesE) and differentiated these cells as monolayer cultures in the presence of Shh and FGF8. In these cultures, DA cells with a midbrain profile (Nurr1, Ptx3, DAT, En1/2, Lmx1a and Lmx1b, but not GABA) were induced very efficiently, again suggesting that Lmx1a acts as a potent determinant in the mDA differentiation pathway. Importantly, Msx1/2 expression was induced in the neuroectodermal stage prior to the neurogenesis of mDA neurons. The ES cell experiments have been elaborated after the completion of this study and will be discussed in "*Efficient Generation of Mesencephalic Dopamine Neurons by Lmx1a Expression in Embryonic Stem Cells*".

The Functions of Msx1/2

As mentioned above, Msx1/2 was induced upon forced Lmx1a expression both *in vivo* in chick and *in vitro* in differentiated mES cells. Furthermore, as Msx1/2 expression is initiated 12 h after that of Lmx1a and was downregulated in the absence of Lmx1a in RNA interference experiments, it seems plausible to assume that Lmx1a acts upstream of Msx1 in the mDA differentiation cascade. Is then the function of Lmx1a executed strictly through Msx1 or do these proteins sub-serve different functions during the differentiation of mDA neurons? From a number of experiments we could conclude that Lmx1a and Msx1 indeed induce parallel parts of the mDA differentiation programme. That Lmx1a has unique functions independent of Msx1/2 was shown in misexpression experiments where Msx1/2 was unable to induce mDA neurons ectopically in the chick lateral midbrain. Thus, Lmx1a is likely to induce dopamine specific markers such as Nurr1 independently of Msx1/2. However, examination of the progenitor profile shortly after misexpression of either Msx1 or Lmx1a suggested that Msx1 but not Lmx1a could suppress Nkx6.1. Furthermore, in $Msx1^{-/-}$ mutants, Nkx6.1 showed increased expression levels in the mDA domain. One important function of Msx1/2 may therefore be to inhibit alternative programmes of differentiation which in turn would ensure a correct execution of the mDA specific differentiation cascade. A second function of Msx1/2 was implied in transgenic embryos in which Msx1 expression was regulated under the Shh enhancer region (Shh-Msx1). In such transgenic embryos, mDA cells were prematurely induced to

differentiate and similar effects were seen in differentiation of mES cell transfected with NesE-Msx1. Furthermore, in $Msx1^{-/-}$ mice the mDA neurogenesis was significantly reduced (by 40%). Together these results indicate that Msx1/2 is involved in setting the timing for neurogenesis possibly by inducing Ngn2 expression.

The mDA Neuron Differentiation Programme - Anno 2008

In recent years, several studies have substantially contributed to the understanding of the molecular mechanisms for mDA specification and differentiation (Ang, 2006; Prakash and Wurst, 2006) However, future research must emphasize into fitting the different molecular pieces together in order to get a comprehensive picture of the mDA fate jigsaw puzzle (*Box4*).

Upstream of Lmx1a

The earliest known component of the pre-patterning profile believed to be required for initiation of the mDA programme is Otx2. Two sets of recent experiments indicate that Otx2 is required and sufficient for the induction of Lmx1a expression. In one report, analysis of $En1^{Otx2k/o}$ embryos, showed that Lmx1a and Msx1/2 expression was lost in the absence of Otx2 (Omodei et al., 2008), indicating that Otx2 may constitute the competence needed to induce the mDA cell fate in response to regional extrinsic factors such as Shh. Furthermore, in a second study, ectopic Otx2 expression in the hindbrain and spinal cord was shown to be sufficient to convert the floor plate into a neurogenic domain (Ono et al., 2007). Interestingly, as a response to Otx2, Lmx1a was induced and the whole mDA cell fate programme commenced as indicated by TH and Nurr1 expression in the neuronal descendants. Importantly, that Otx2 induces cells with an mDA phenotype, indicates that the mDA cell fate programme may be independent of signals from the isthmus, which may instead mainly serve to stabilise the Otx2 expression and support survival of cells in the midbrain. This would also explain the expression pattern of Lmx1a which extends into the diencephalon which is probably not reached by signals from the isthmus. The study also investigated the effects of ectopic expression of Lmx1a together with Ascl1 and showed that Lmx1a is not able even with neurogenic support to induce the mDA programme in a caudal context. Taken together this shows that Otx2 is important to convert the midbrain floor plate into a neurogenic region, and also that it somehow sets the molecular context in which Lmx1a can function to induce the mDA programme. One observation that would undermine the importance of Otx2 was made by Prakash et al., 2005. They report that the negative effect on mDA neurogenesis seen in $En1^{Otx2k/o}$could be rescued by the loss of Nkx2.2 function ($En1^{Otx2k/o}$; $Nkx2.2^{-/-}$). This rescue was paralleled by the re-establishment of Wnt1 expression, although the levels appeared to be reduced. The function of Wnt1 is hard to deduce as possible effects on Lmx1a/Msx1/2 expression have not been determined in either the above mentioned line nor in the $En1^{Wnt1k/i}$

embryos. It is formally possible that Wnt1 substitutes to induce Lmx1a and thus rescues the DA programme in $En1^{Otx2k/o}$; $Nkx2.2^{-/-}$. Another alternative would be that Otx1 to some extent compensates for the loss of Otx2 in these mutants.

That Lmx1a is not induced until E9 despite that both Shh and Otx2 are present much earlier, suggests that these factors conspire to induce a secondary wave of either intrinsic and/or extrinsic factors that would act directly to induce Lmx1a expression. Candidate extrinsic molecules in this process are Wnt1, TGFβ and BMP that are probably expressed in the relevant region at time of Lmx1a induction (our unpublished data). Future scrutiny of the enhancer regions of Lmx1a is likely to reveal more information on this matter.

Several lines of evidence also indicate that Lmx1b may be an intrinsic factor involved in the induction of Lmx1a. In the chick roof plate Lmx1b functions upstream of Lmx1a (Chizhikov and Millen, 2004) and misexpression of Lmx1b in chicken midbrain induces Lmx1a in progenitors (our unpublished data). Despite this we believe that Lmx1b is a less specific determinant of the mDA cell fate as 1) its expression domain encompasses the progenitor zone of the MNs and red nucleus cells at early stages 2) forced expression of Lmx1b in mES cells does not equally efficiently induce the mDA cell fate as Lmx1a 3) mDA neurons fail to be formed when Lmx1a is suppressed in chicken ventral midbrain despite continuous expression of Lmx1b and 4) it is in contrast to Lmx1a also expressed in the floor plate of the hindbrain and spinal cord (Paper II). Nonetheless, in mouse, Lmx1a and Lmx1b may serve partially redundant functions as Lmx1a mutants (Dreher mutant; Millen et al., 2004) only loose about half of the mDA population (Ono et al., 2007; our unpublished data).

Downstream of Lmx1a

What molecular events ensue in mDA progenitors once Lmx1a is turned on? One unresolved question stems from the fact that the anterior extent of the expression of Lmx1a/Msx1/2 does not match the mDA producing domain. We have not mapped the extent carefully but surmise that it coincides with the rostral limit of Lmx1b which is at prosomere (P)4 of the diencephalon (Asbreuk et al., 2002). At these rostral positions, Lmx1a and Msx1/2 are expressed in progenitors generating $Nurr1^{+}/Lmx1b^{+}/TH^{-}/Isl1/2^{-}$ neurons (our unpublished observation). In addition, Lmx1a has important functions in determining the roof plate (Millen et al., 2004) and is expressed in a subset of spinal cord neurons (our unpublished data), the cerebellum and choriod plexus (Failli et al., 2002). Thus, as mDA neurons are only found in the midbrain, the molecular context in these other positions does not permit Lmx1a to induce mDA neurons but is amenable for Lmx1a to participate in alternative differentiation paths. The ability of Lmx1a to induce mDA neurons is thus restricted both on the AP and DV axes. This could be accounted for by the need for cofactors which are only present in the ventral half of the midbrain. Alternatively, or in addition,

other differentiation programmes may override the ability of Lmx1 to induce mDA neurons in the dorsal and rostral Lmx1a expression domains.

The diverse activities of Lmx1a may be explained by its structural domains. Lmx1a is a transcriptional activator and a member of the Lhx transcription factor family which encompasses a LIM domain in addition to a HD motif. In contrast to the HD domain, the LIM domain lacks the ability to bind DNA and is believed instead to function as a modular protein-binding interface to mediate protein-protein interactions (Kadrmas and Beckerle, 2004). The transcriptional activity of the HD motif of Lhx proteins is often dependent on the binding of Ldb1 (LIM domain binding 1) proteins to the LIM domain. Ldb1 dimers facilitate the formation of homo- and heterodimers of Lhx proteins, which in turn may enhance binding to DNA or bring distant sites into proximity. In this way Ldb1 proteins may mediate the synergistic activation of multiple promoters (Matthews and Visvader, 2003). It has elegantly been shown that activation of the sMN marker HB9 is dependent on the Ldb1-mediated assembly of heterodimers between the Lhx proteins Lhx3 and Isl1 (Thaler et al., 2002). Furthermore it has been discovered that the activity of the complex relied on interaction with the proneural gene Ngn2, providing a direct link between a proneural gene and the differentiation of a specific neuronal subtype (Ma et al., 2008). Absence of Isl1 or presence of Ascl1 in this protein complex, led to a failure to induce HB9 expression and allowed for the V2 IN differentiation programme to commence. Hence, by virtue of its LIM domain, Lmx1a can probably participate in a plethora of large protein-complexes and the specification pathway executed by Lmx1a would then ultimately depend on the specific binding partners. The exact complex with which Lmx1a may initiate the mDA differentiation pathway is not known. In frog, LIM-HD and Otx-HD proteins synergise in the presence of Ldb1 to activate certain promoters (Bach, 2000). Therefore, it will be interesting in the future to unravel whether Lmx1a, in analogy with mentioned cases, synergises with Otx2 or Ngn2 during cell fate determination in the midbrain. Furthermore, as Lmx1a is maintained in postmitotic neurons, it may play active roles in the maturation of mDA cells through the assembly with other transcription factors such as Lmx1b or Pitx3. Indeed, Ldb1 is present in both SN and VTA and has in other contexts been shown to mediate interaction between Pitx and Lhx proteins (Bach et al., 2000).

Downstream of Msx1/2

The biochemical properties of the HD proteins Msx1 and Msx2 are very similar and both function as transcriptional repressors (Catron et al., 1996).

Repression of Alternative Fates

Upon forced expression of Msx1, Nkx6.1 is promptly downregulated, indicating that Nkx6.1 may be a direct downstream target of Msx1. Conversely, we found no evidence for Nkx6.1 mediated repression of Msx1/2 since the expression of Msx1/2

was not affected in a Shh-Nkx6.1 transgene (our unpublished data) nor in a chick electroporation assay (our unpublished data). Thus, Msx1/2 and Nkx6.1 do not form a classical cross-repressive pair. As Msx1/2 appears to be activated downstream of Lmx1a and not directly by Shh, demarcation of the Lmx1a domain may be enough to delimit Msx1/2 expression and so there would be no need for Nkx6.1 to repress Msx1/2 in the pMN domain. Conversely, since Lmx1a appears to be unable to repress Nkx6.1, Msx1/2 would be required to suppress Nkx6.1 in the mDA progenitor domain. We cannot exclude the possibility that Nkx6.1 also may be suppressed by partly redundant as to now unidentified proteins.

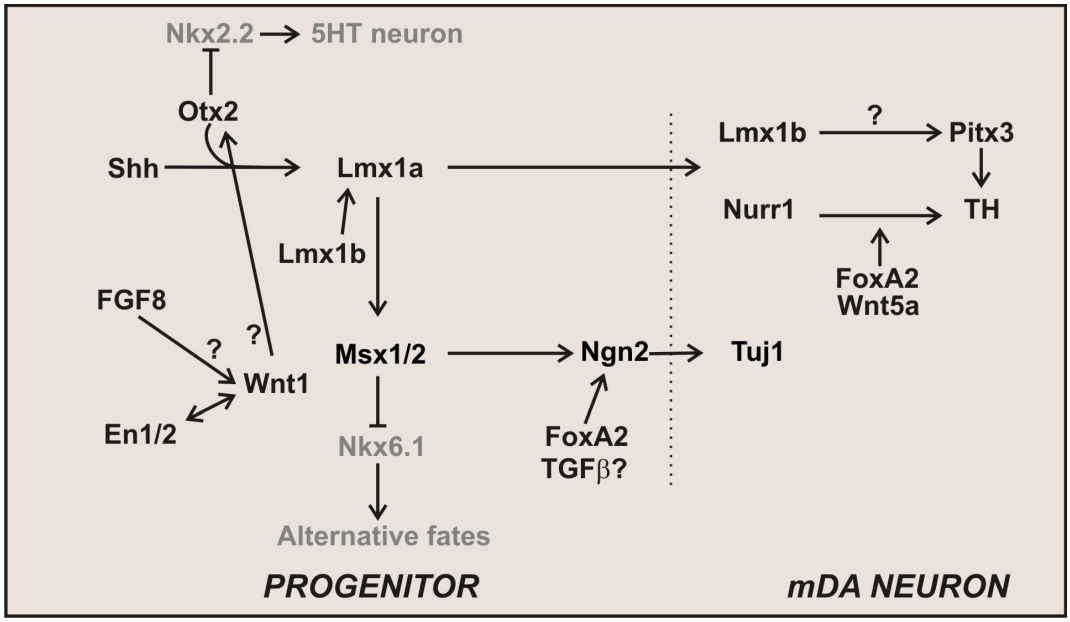
The early demarcation of the mDA and pMN domains may be based on Shh levels. Prior to the onset of Lmx1a at E9, Nkx6.1 is expressed throughout the midline. However, as Msx1/2 expression is gradually intitated starting from E9.5, Nkx6.1 expression retracts and eventually the two domains stabilise side by side. It should be noted that a similar progressive change in Olig2 expression is seen in the spinal cord. It is initially expressed at ventralmost positions but as the repressor Nkx2.2 is turned on slightly later, the expression of Olig2 retracts dorsally (Dessaud et al, 2007). In this case a prolonged high level of Shh is needed for Nkx2.2 expression, which may also hold true for the induction of Lmx1a and Msx1/2. Thus, the lateral extent of the $Lmx1a^{+}Msx1^{+}$ domain is likely to be set by the border of high levels of Shh around E9.5.

Neurogenesis

At E10.5 the Shh domain broadens to encompass more lateral progenitor domains and it eventually retracts from the mDA domain around E11.5. In Shh-Msx1 transgenes, retraction of Shh occurs prematurely, paralleled with the earlier onset of mDA production, indicating that repression of Shh may be achieved by Msx1/2 and may be a prerequisite for mDA neurogenesis. How the conversion from a floor plate to a neurogenic region is achieved in the midbrain is still an enigma, but, as previously discussed, factors downstream of Otx2 appear to mediate the switch. Msx1/2 may be involved but is not sufficient as misexpression of Msx1 in the caudal neural tube in Shh-Msx1 transgenes or by chick electoporation cannot induce neurogenesis (our unpublished data).

The neurogenic effect of Msx1 observed in the Shh-Msx1 transgene and in mES cell experiments contradicts previous studies where Msx1/2 has been associated with suppression of neurogenesis by virtue of its negative effects on proneural gene expression (Liu et al., 2004) and upregulation of cyclin D1 expression (Hu et al., 2001). We believe that the effect Msx1/2 has on neurogenesis depends on context and levels of expression. We observed that both in chick electroporation and mES cell assays, high levels of Msx1 result in suppression of differentiation or cell death (our unpublished data), whereas low levels seem to impose upregulation of proneural genes.

That low-level activities of Msx1/2 would govern mDA generation are supported by the fact that the endogenous levels of Msx1/2 are very low in both chick and mouse compared to that in the dorsal neural tube (our unpublished observation). The molecular mechanisms for how Msx1/2 can exert these level-dependent opposing activities remain to be determined.



Box 4: **Components implicated in the midbrain dopaminergic programme of differentiation.** Note that arrows indicate that a genetic connection has been reported by loss- and/or gain-of-function experiments and does not reflect a direct interaction in most cases.

EFFICIENT GENERATION OF MESENCEPHALIC DOPAMINE NEURONS BY LMX1A EXPRESSION IN EMBRYONIC STEM CELLS (FRILING AND ANDERSSON, ET AL., MANUSCRIPT IN PREPARATION)

In addition to provide insights into basic developmental concepts for cellular specification, studies on the development of mDA neurons may be instrumental in future cell replacement strategies to treat patients with Parkinson's disease. Parkinson's disease is the second most common neurodegenerative disease of the elderly and affects about 1% of the population above 65 years (Hirtz et al., 2007). Major hallmarks of the disease are tremor, hypokinesia (difficulties in initiating movement), bradykinesia (slowness of movements), and rigidity. Current treatments in use or in trial (Schapira et al., 2006, Dostrovsky et al., 2006) aim towards: 1) replacement of dopamine (L-dopa, dopamine agonists), 2) replacement of mDA cells (foetal ventral mDA implants) 3) neuroprotection of DA cells (MAO inhibitors, GDNF) or 4) direct relief of motor inhibition (Deep Brain Stimulation (DBS), adenosine receptor antagonists, NMDA receptor antagonists). These approaches suffer from the following limitations: 1) re-emergence of symptoms and side effects (Hauser et al., 2006); 2) limitation in cell material supply and quality as aborted foetuses are used, ethical issues

and immunological responses (Correia et al., 2005); 3) marginal effects on DA cell survival (Chen and Le, 2006); 4) being an invasive and expensive technique, limited knowledge of the exact mechanisms and possible detrimental side-effects in the case of chemical treatments (Dostrovsky et al., 2002; Schapira et al., 2006). Thus, none of the current methods satisfy the need for a long-term alleviation of motor symptoms. An optimal treatment would irreversibly restore the function of the nigrostriatal motor function by regeneration or replacement of defective cells. In recent years, the increasing success of steering ES cells towards desired fates (Aejaz et al., 2007; Suter and Krause, 2008), have elicited hopes for an ES-cell based replacement treatment for Parkinson's disease. Numerous trials using foetal midbrain implants have included more than 350 patients of which many have had a relief of their symptoms (Lindvall and Bjorklund, 2004; Snyder and Olanow, 2005; Li et al., 2008). These trials although not only successful (Freed et al., 2001; Olanow et al., 2003), serve as a "proof of principle" that cellular replacements could be a conceivable treatment for Parkinson's disease. However, the limited supply of cells, the varying quality and ethical issues posed by the use of foetal tissue, makes it necessary to base future treatment on alternative sources. Established neural stem cell lines derived either from foetuses or differentiated ES cells exhibit limited potency (Kim et al, 2006; Ko et al, 2007). Instead, pluripotent cells including ES cells, induced pluripotent stem cells (iPS) and embryonic germ stem cells (GSC) constitute the most promising options to be used in cell therapy approaches today (Morizane et al., 2008; Nakagawa et al., 2008; Cibelli et al., 2002).

Since ES cells have the potential to become essentially any type of cell in the body (Gilbert, 2006) strategies must be developed to restrict the cells into the desired developmental pathway. Several protocols have been devised in which mouse and human ES cells are efficiently converted to neurectodermal tissue (Kawasaki et al., 2000; Lee et al., 2000; Ying and Smith, 2003; Nat et al., 2007). These formulations are largely based on empirical efforts but the neural induction can be modified in predictable ways; addition of BMP inhibitors has for example positive effects (Sonntag et al., 2007) in accordance to mechanisms for neural induction described in "*Establishment of Neuroectoderm*". Supplements that confer a growth advantage to neural cells is typically used, as serum has negative effects on neural induction (possibly due to presence of BMP) (Dhara and Stice, 2008). Moreover, lessons from developmental biology have been applied to pattern neural progenitors by FGF8 and Shh to promote mDA cell fates (Lee et al., 2000; Kawasaki et al., 2000). However, in current ES cell differentiation protocols mDA cells constitute only a fraction of the neurons generated and the identity of remaining cells is often unclear. Thus strategies must be devised to increase the efficiency of mDA differentiation and also to extract these desired cells from the heterogeneous population of cells resulting from ES cell differentiation.

Engineering of mDA neuron-producing ES Cell Lines using Lmx1a.

In Paper I, we showed that forced Lmx1a expression in neuroectodermal progenitor cells generated from mES cells, steers cells into the midbrain dopaminergic lineage. These experiments were based on transient expression of Lmx1a and were performed as "proof of principle" of the usefulness of Lmx1a in the production of mDA cells. In this follow-up study (Friling, Andersson et al., manuscript in preparation) we have created a stable mES cell line in which Lmx1a is expressed upon neuroectodermal differentiation under a Nestin promoter/enhancer region (NesE-Lmx1a). Examination of the neuronal composite following differentiation of the NesE-Lmx1a line revealed that more than 70% of the neurons were TH^+ and that the majority of these co-expressed markers indicative of midbrain DA neurons. DA neurons corresponding to both SN ($Girk2^+$) and VTA ($Calbindin^+$) (Thompson et al., 2005) populations were present in the cultures. Moreover, in addition to exhibit the correct molecular markers for *bona fide* mDA cells, TH cells differentiated from the NesE-Lmx1a line also displayed electrophysiological profiles that were strikingly similar to the properties of native DA cells. To test if the strategy was applicable to generate human mDA cells, Lmx1a expression was introduced into human (h) ES cells using lenti-viral transduction (Hamaguchi et al., 2000). Similar to in mES cells, Lmx1a expression in differentiating cells (Nat et al., 2007) promoted the formation of mDA cells with a midbrain phenotype from hES cells. Thus, forced expression of Lmx1a in differentiating human and mouse ES cells enrich for mDA cells in their neural progeny. As the majority of the neurons appear to closely resemble *in vivo* mDA cells, these lines have great potential already now to be useful in drug screening and in the future as material in the development of cell replacement therapies for Parkinson's disease.

Transplantation of NesE-Lmx1a Derived mDA Cells

Next, we wanted to assess the survival and innervation efficiency of NesE-Lmx1a derived mDA cells *in vivo*. To this end, the NesE-Lmx1a line was differentiated 3-4 days and injected into the striatum of hemi-lesioned 6-hydroxy dopamine (6-OHDA) neonatal rats (Cunningham and McKay, 1993; Schwarting and Huston, 1996). 3-4 weeks following the transplantation, rats were sacrificed and analysed. The implants had survived in about 50% of the animals and were rich in TH^+ neurons. Also *in vivo* the cells expressed correct mDA postmitotic marker genes and they projected preferentially to dorso-lateral striatal regions, which are the targets of SN mDA neurons. A recurrent problem arising in most attempts of transplanting ES-based cells into animal models of Parkinson's disease (Thinyane et al., 2005), including our own, is an uncontrolled overgrowth of the non-DA cell content of the transplants. Strategies must therefore be devised to enrich for desired cells and eliminate the tumour forming cells prior to transplantation (Pruszak et al., 2007). We have conducted several types of

cell sorting approaches, including Magnetic Cell Separation (MACS) and Flourescent Activated Cell Sorting (FACS). Such efforts limited the occurrence of overgrowth but at the expense of the survival of grafted cells. Nevertheless, we believe that with optimisation of cell sorting protocols and culture conditions, this obstacle may be overcome. Mouse and human ES cell lines expressing Lmx1a are very potent to generate DA cells that are indistinguishable from *bona fide* primary mDA neurons and may thus directly be of use *in vitro* drug screening efforts and to decipher the mDA differentiation molecular pathway by microarray analysis.

LMX1B IS ESSENTIAL FOR THE DEVELOPMENT OF SEROTONERGIC NEURONS (PAPER II)

Serotonergic cells (5-HT neurons) are important components of the Raphe nuclei and the reticular formation located in the brainstem. Although the exact function of 5-HT neurons is not known, the fact that changes in 5-HT homeostasis has been associated with the physiopathology of syndromes including anxiety disorders, drug addiction and autism highly motivates investigation of the machinery underlying serotonergic specification.

The Role of Lmx1b in the Differentiation of 5-HT neurons

5-HT neurons are generated at most AP levels in the ventral hindbrain. Shh has been shown to be pivotal for the early initiation of the serotonergic specification programme by inducing the HD containing protein Nkx2.2 in dividing progenitor cells (Briscoe et al., 1999). Shortly after withdrawal from the cell cycle, differentiating cells turn on the expression of serotonergic markers, GATA2, GATA3, Pet1 and later the serotonin transporter (Sert) and 5-HT (serotonin). In paper II we found that the LIM HD transcription factor, Lmx1b, is expressed in early postmitotic serotonergic precursor cells. Analysis of $Lmx1b^{-/-}$ mutant embryos revealed that loss of Lmx1b did not affect the expression of Nkx2.2, but that later post-mitotic markers Pet1 and 5-HT were lacking suggesting that Lmx1b is pivotal for full maturation of serotonergic cells. The expression of GATA3, another early differentiation marker, seemed to be dependent on Lmx1b in the caudal but not rostral nuclei; GATA3 expression was unaffected in the rostral nuclei but almost completely lost at caudal positions. Furthermore, in $Nkx2.2^{-/-}$ mutant embryos, Lmx1b expression failed to be induced along with all other 5-HT specific markers (except for in the dorsal raphe nucleus). In summary, in Paper II Lmx1b was identified as an intermediate determinant of the serotonergic differentiation programme downstream of the progenitor marker Nkx2.2 but upstream of more definitive subtype specific events like onset of neurotransmitter expression.

The 5-HT neuron Differentiation Programme - Anno 2008

The exact roles of Lmx1b during the maturation of 5-HT precursor cells have been revisited since the publication of Paper II.

The Acquisition of a 5-HT Phenotype

Cheng et al. demonstrated that Lmx1b is important for the maintenance of Pet1 expression rather than its induction (Cheng et al., 2003), as Pet1 expression could be detected between E11.5 and E14 in $Lmx1b^{-/-}$ mutant embryos. This transient expression of Pet1 was missed by us due to that our analysis focused on stages when the expression already had been downregulated. It was furthermore shown that forced expression of Lmx1b, Pet1 and Nkx2.2 was sufficient to induce 5-HT in the spinal cord, but that neither Lmx1b nor Pet1 could execute this function alone (even in the presence of Nkx2.2). This suggests that Pet1 and Lmx1b act in parallel pathways to induce the serotonergic neurotransmitter phenotype. Since a substantial number of functional 5-HT neurons are present in adult $Pet1^{-/-}$ mice, it appears as if Pet1 is not totally indespensible for the expression of 5-HT. In contrast, Lmx1b seems to be required for 5-HT neuron formation, as a conditional depletion of Lmx1b expression in $Pet1^{+}$ neurons, resulted in that cells that initially induced 5-HT failed to maintain the expression (Zhao et al., 2006). As a consequence no functional 5-HT neurons were found in the adult raphe nuclei in such mice. Tracing of $Lmx1b^{-/-}$ cells demonstrated that the reduced numbers of $5\text{-}HT^{+}$ cells at embryonic stages were mainly due to differentiation blockage rather than cell loss. However, since the cell density of the raphe nuclei was dramatically reduced, the authors conclude that most prospective 5-HT neurons were eliminated at postnatal stages. It cannot be ruled out that the drastic effects could partly be attributed to the gradual loss of Pet1 expression (as a cause of the diminishing levels of Lmx1b), making it hard to completely distinguish the functions of each protein.

Other Functions of Lmx1b

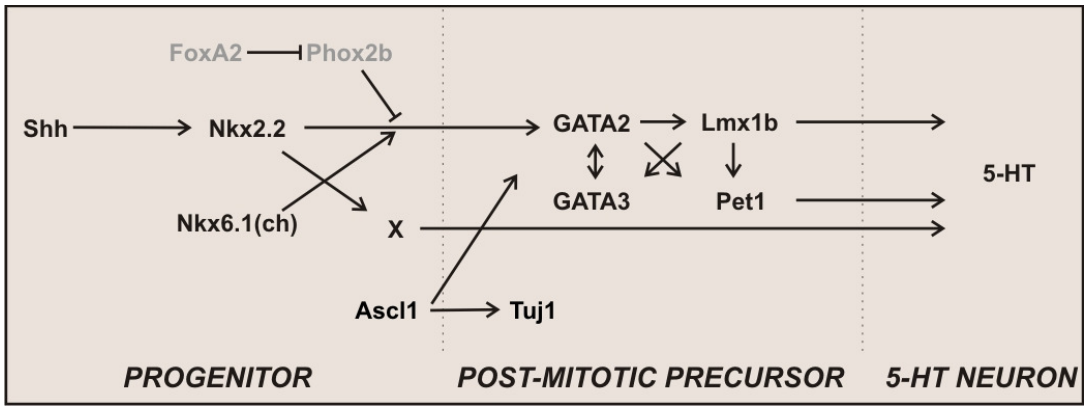
The early expression of Lmx1b in the 5-HT lineage, suggests that it may be involved in additional aspects of serotonergic cell maturation other than in neurotransmitter expression. No obvious migration defects where seen in the conditional $Pet1^{Lmx1bk/o}$ mutant. In contrast, we observed in Paper II that the $Lmx1b^{-/-}$ cells exhibited aberrant migration patterns, indicating an early function for Lmx1b to specify the migration routes.

Considering the close ontogeny of mDA cells and raphe nuclei 5-HT neurons, it would be interesting to assess whether Lmx1b exerts partly similar functions in these neurons. Indeed in both types of neurons, loss of Lmx1b leads to the failure to induce the vesicular monoamine transporter VMAT2 (Cheng et al., 2003). The protein structure of Lmx1b contains a LIM domain and similar to suggested in the

differentiation of mDA cells, Lmx1b may participate in large protein complexes supported by Ldb1.

Upstream of Lmx1b and Pet1

The fact that both Pet1 and Lmx1b are lacking in Nkx2.2$^{-/-}$ mutant embryos, positions Nkx2.2 in the top of the serotonergic specification hierarchy. However as Nkx2.2 is expressed throughout the neural tube and participates in the specification of many other cell types, a hindbrain specific activity of unknown factor(s) must be fed into the specification programme executed by Nkx2.2. It is possible that Hox proteins could constitute such contributing factors, although 5-HT neurons arise from R1 which lack Hox gene expression. Moreover, as forced expression of Pet1 and Lmx1b fails to induce 5-HT expression at ectopic positions, additional factor(s) must be induced by Nkx2.2 that contributes to the full serotonergic programme. Surprisingly, the function of GATA2 has not been assessed in this respect although it has been previously shown to be essential for serotonergic specification (Craven et al., 2003, see "*The Transcription Profile of Raphe Nuclei Serotonergic Cells*"). GATA3 seems like a more unlikely candidate at least in the rostral hindbrain, as the rostral raphe nuclei are essentially normal in GATA3$^{-/-}$ mutant embryos. The current view of the molecular cascade leading up to the formation of 5-HT neurons is summarized in *Box5*.



Box 5: Components implicated in the 5-HT differentiation programme in the hindbrain. Note that arrows indicate that a genetic connection has been reported by loss- and/or gain-of-function experiments and does not reflect a direct interaction in most cases. In a few instances the connection was limited to a certain AP axis and species; Lmx1b is only crucial for GATA3 expression in the caudal hindbrain and GATA2 can only induce Lmx1b, Pet1 and 5-HT in R1 in chick. Ch = chick only, X= unknown factor.

A COMPREHENSIVE ANALYSIS OF CELL FATE DETERMINING GENES IN THE DEVELOPING HUMAN NEURAL TUBE (PAPER III)

Despite the apparent disparate body features of different metazoan organisms, increasing evidence suggests that their basic body plan originates from a common ancestor. With the discovery of the importance of Hox orthologs for the formation of the AP axis in diverse animals such as insects (Gilbert et al., 1996) frogs (Carrasco et al., 1984) and amniotes (Gaunt, 1994; Burke et al., 1995), it has become clear that the

way nature sustains basic developmental elements is by preserving orthologous gene expression. The eye is an example of a structure that initially was believed to be analogous in different animals. However, in all studied animals with visual conception, orthologs of Pax6 (Gilbert et al., 1996; Ball et al., 2004) was found to be the pre-eminent master gene, demonstrating that the photoreceptors may only have emerged once during evolution. Similarly, the induction of neural tissue have, in all metazoan studied, been associated with suppression of BMP (Cornell and Ohlen, 2000; Ball et al., 2004). Although the subsequent DV patterning of neural tissue does not share upstream signalling pathways completely, a consensus of evolutionary conserved DV elements have been described in cnidarian, fish, insects and amniotes (Ball et al., 2004; Cornell and Ohlen, 2000). In this, the neural tissue is divided into three parts expressing Msh/Msx, Ind/Gsh or Vnd/Nkx, respectively. The conserved expression pattern of orthologs does not in all cases reflect a preserved function as motor neurons are produced from all these three levels in fly but only in the Nkx region in amniotes. Nevertheless, Nkx6 gene has been shown to be a motor neuron determinant in fish, fly and aminotes (Sander et al., 2000; Vallstedt et al., 2001; Cheesman et al., 2004). A good example of a gene that has a conserved function in cellular specification in several organisms is Lmx1a. Lmx1a is specifically expressed in the mDA progenitor domain in chicken, mouse (Paper I) and human (this paper) and ectopic expression of this gene *in ovo* in chick (Paper I) or during differentiation of mouse (Paper I) or human ES cells (Friling, Andersson et al, manuscript in preparation), results in reprogramming of cells into the mDA cell pathway. Thus, we surmise that it is plausible to assume that a conserved gene expression pattern amongst amniotes indicate a conserved developmental function.

Nevertheless, along with speciation, new features require modification of old themes and different molecular basis for form and function are therefore likely to be found between distinct organisms. Thus, based on current comparisons between vertebrates, human development is likely to show a substantial degree of conservation, but also distinct deviations. To get a comprehension of the development of the human CNS, we decided to construct an expression pattern map of proteins that has been described in model organisms to control neuronal diversification.

Mouse versus Chicken

The expression patterns examined in this project resembled to a large extent those previously found in mouse and chicken. However, in cases where mouse and chicken development deviated, we found almost equal chance that human would resemble one or the other. Caution should therefore be taken to assume a greater resemblance between mouse and human with reference to that both are mammals. Since the split between the avian and mammalian lineages, the chicken recombination rate has been relatively slow whereas the rodent genome has diverged considerably. Consequently,

the overall organisation of the human chromosomes is closer to that of chicken than the mouse (Burt et al, 1999). Another similarity between humans and chicken is that both gastrulate from a flat structure called the blastodisc, like most other mammals (O'Rahilley and Muller, 2006). In contrast, the mechanisms in mouse have diverged and they form a curved egg cylinder at gastrula stages (Gilbert, 2006). For the study of early human embryogenesis, the mouse model may therefore be a suboptimal choice. Chicken may be a convenient system but closer relatives such as the rabbit is probably preferable (Fisher et al, 2002) and in cases of using ES cells, primate cells would be the optimal choice.

Spinal Cord Neurogenesis and Oligodendrogenesis

Analysis of the expression patterns of proteins described to be involved in regionalisation of the DV axis into five ventral and six dorsal major progenitor domains revealed a complete conservation between human and mouse spinal cord development. Similary, based on the localised expression of neuronal subtype specific marker proteins, the eleven progenitor domains appeared to generate the same motor neurons and distinct interneurons as found in mouse. With very few exceptions, the human progenitor and postmitotic codes correlated also to those in chicken.

Oligodendrogenesis is a process that is mechanistically distinct in mouse and chicken. Generation of oligodendrogenesis occurs in chicken from $Nkx2.2^{+}Olig2^{-}$ and $Nkx2.2^{+}Olig2^{+}$ progenitors, whereas $Olig2^{+}$ $Nkx2.2^{-}$ progenitors is the major source in mouse (Zhou et al, 2001; Novitch et al., 2001; Fu et al., 2002; Rowitch, 2004). Analysis of human spinal cord at gliogenic stages revealed that similar to in chicken $Nkx2.2^{+}Olig2^{+}$ and $Nkx2.2^{+}Olig2^{-}$ progenitors appeared to produce oligodendrocytes. Curiously, a discrete $Nkx2.2^{+}Olig2^{+}$ domain could be detected already at neurogenic stages and correlated with MN production. The pMN domain produces several distinct MN subtypes (Jessell, 2000). In light of this, the separation of the human Olig2 domain into $Nkx2.2^{+}$ and $Nkx2.2^{-}$ compartments raises the possibility that the diversification of MN may partly rely on a spatial sub-patterning already within the pMN domain.

Hindbrain Neurogenesis

The vertebrate hindbrain is compartmentalised into rhombomeric units which show unique neuronal composites (Cordes, 2001). The basic organisation is similar between mouse and chicken, except for two evident differences. First, in mouse but not in chicken rhombomere R4 is devoid of 5-HT neurons. Second, sMN are produced from R5, 7 and 8 in mouse but from R5, 6 and 8 in chicken (Chandrasekhar, 2004). By using established mouse rhombomeric landmarks we found that the rhombomeric neural composition in human resembles that of mouse rather than chicken.

Along the DV axis, the regionalisation of neuronal subtypes is similar in mouse and chicken and we found no human deviations from the established patterns. Taken

together, based on our marker assortment we suggest that the mouse hindbrain serve as a useful model system for the study of human hindbrain development. It is plausible to assume that the same neuronal subtype markers can be applied in attempts to generate for example 5-HT neurons from either mouse or human ES cells.

Midbrain Neurogenesis

The most clinically relevant cell type born in the midbrain is the SN DA cell, as the specific degeneration of this subtype is the major hallmark in patients with Parkinson's disease. From studies in mouse and chicken, several important components within the mDA differentiation programme have been identified, including Lmx1a and Msx1/2 (Andersson et al., 2006), FoxA2 (Ferri et al., 2007; Kittappa et al., 2007), Lmx1b (Smidt et al., 2000), Nurr1 (Zetterstrom et al., 1996), tyrosine hydroxylase (TH) and Pitx3 (Smidt et al., 1997). We examined the expression of the above mentioned marker genes in human midbrain and compared to the patterns in mouse and chicken. Lmx1a, FoxA2 and Pitx3 displayed similar expression patterns in all three organisms. Lmx1b is in mouse downregulated in the progenitor zone during the course of mDA neurogenesis and this appeared to be the case also in human but not in chicken.

The Human Msx1/2 Expression is Similar to that in Chicken

We found that the human expression domain of Msx1/2 is weak in the very midline and stronger at the borders of the mDA progenitor domain, resembling the expression pattern in chicken but not in mouse (Paper I). It is possible that the differential expression of Msx1/2 within the mDA domain reflects a functional sub-patterning. In light of this it is interesting that the mDA domain gives rise to two types of mDA neurons which contribute to either VTA (A10) or the SN (A9) nuclei (Björklund and Lindvall, 1984). These neurons are born at largely overlapping time points and positions, but the neurogenic onset of SN cells occurs before that of the VTA and it has been suggested that SN cells show an anterior predisposition (Bayer et al., 1995; Smits et al., 2006). Whether the mDA progenitor domain is subpatterned on the DV axis is an unresolved issue. Regulatory differences within the mDA domain were observed in $Ngn2^{-/-}$ mutant embryos, where medially generated mDA cells were reduced to a greater extent than laterally formed mDA cells (Kele et al., 2006). However, no connection to these functionally distinct mDA domain sub-regions and the generation of either VTA or SN mDA cells could be established as these groups were equally affected in the mutant at later stages (Andersson et al., 2006b). We have previously shown that Msx1 have neurogenic effects and in mutant embryos for Msx1 we observed a 40% reduction in the numbers of DA cells (Paper I). Notably, the accompanied reduced levels of Ngn2 appeared to be most pronounced in midline regions (our unpublished observation), suggesting that Msx1 in mouse would be of greater importance for midline cells to launch neurogenesis. This would be in contrast

to human and chicken, where the levels of Msx1/2 are much weaker at medial positions than laterally. It would be interesting to address what functional relevance, if any, the variations in Msx1/2 expression levels play within the DA domain in chicken and human.

To investigate whether the similar expression pattern of Msx1/2 in human and chicken reflects conserved or convergent evolution, we decided to analyse the expression pattern in a third representative mammalian model organism. The marsupial *Monodelphis domestica* (*M. domestica*) is a useful model for mammalian basic features as it is situated basally in the mammalian tree subsequent to the mammalian and avian split from a common ancestor 300 million years ago (Mikkelsen et al., 2007). Curiously, in newborn *M. domestica* (kindly provided by Kathleen K. Smith at the Duke University, USA), Msx1/2 was expressed in homogenous levels throughout the DA progenitor domain thus resembling the mouse pattern (our unpublished observation). Hence, we could not provide evidence for that the Msx1/2 expression in human and chicken is ancestral and that the one in mouse is derived. Nevertheless, we cannot exclude the possibility that both mouse and *M. domestica* have acquired the homogenous Msx1/2 expression. Analysis of additional model organism would shed light on this issue.

Nurr1 and TH are Non-Specific Markers of Human mDA Cells

Examination of the expression pattern of the mDA marker Nurr1 in human ventral midbrain revealed a large anterior population of non-mDA cells with intermediate expression levels of Nurr1. Co-staining with other relevant markers suggested that these cells represent a subgroup of the LIM1/2$^+$ neurons residing laterally to the mDA nuclei. Small numbers of LIM1/2$^+$Nurr1$^+$ neurons were also observed in the anterior chick and mouse midbrain although the majority of LIM1/2$^+$ neurons were Nurr1$^-$. As the function of LIM1/2$^+$Nurr1$^+$ neurons is unknown it is difficult to speculate on the relevance for the relative increase in the numbers of these cells in human compared to mouse and chicken.

In addition to Nurr1, TH was also found to be expressed in non-mDA cells in the human midbrain. The expression coincided with Isl1/2$^+$ MN in 5.5w old embryos, but has been reported to be transient (Puelles and Verney, 1998). TH can also be seen transiently in certain neuronal populations in the mouse spinal cord (pers comm., J. Ericson) and in mouse enteric non-mDA progenitor cells (Blaugrund et al., 1996), indicating that TH may be transitorily expressed without functional relevance throughout differentiation of multiple cell types.

Nevertheless, the TH expression in human midbrain MN and Nurr1 in interneurons, disclose a significant limitation of these proteins as indicators of human mDA cells. An awareness of this unspecificity is especially important in the assessment of mDA production during differentiation of human ES cells. In line with this we could

detect significant numbers of non-mDA cells expressing TH and Nurr1 during differentiation of human ES cells. Combinatorial expression analysis of multiple mDA markers is thus required in order to obtain an accurate appreciation of the mDA production efficiency in such protocols.

Closing Remark

The present study included the majority of developmentally expressed genes which have been implicated in neuronal patterning, specification and differentiation in mouse and chicken midbrain, hindbrain and spinal cord. Since the vast majority of gene expressions analysed in this study showed evolutionary conservation between amniotes, we propose that the majority of basic developmental gene expression patterns found in the future in model organism will also be applicable to human development. However, the distinct differences found in this study motivate a constant update of the human expression pattern map along with discovery of novel marker genes.

DOMAIN SPECIFIC CONTROL OF NEUROGENESIS ACHIEVED THROUGH THE PATTERNED REGULATION OF DELTA1 AND JAGGED1 EXPRESSION (PAPER IV)

During development of the spinal cord, a number of distinct types of neurons are born and acquire phenotypes typical for their position along the DV axis of the luminal progenitor zone. The production of neurons is however not synchronized along the DV axis, meaning that cells in different progenitor domains display distinct neurogenic onsets and paces. Neural progenitors commence neurogenesis first from the MN domain and subsequently different types of interneurons are generated. This indicates that positional determinants not only specify cellular fate but may also control the time point and tempo of the neurogenesis, factors important to ensure that correct numbers of neurons are produced from each domain. It remains unclear, however, how mechanisms that govern progenitor subtype specification and differentiation are integrated.

Proneural genes, such as Ascl1 and Ngns have been shown to be essential for the conversion of progenitor cells into maturing neurons (Kageyama et al, 2005). However, to prevent excessive neurogenesis depleting the progenitor pool, proneural genes are counteracted by Notch signals. The Notch pathway (*Box3A*) includes in addition to the Notch receptor the two ligands Delta (Dll) and Jagged/Serrate (Jag), and the canonical downstream targets of the Hes gene family, that suppress proneural transcription and function (Kageyama et al., 2007). A large number of additional proteins have been shown to feed into and modify the Notch pathway. Manic and Lunatic fringe (Mfng, Lfng) are glycosyltransferases, which upon modifying the Notch receptor potentiate Dll1-mediated signalling but suppress Jag1's ability to signal via Notch (Hicks et al,

2000; Shimuzi et al., 2001). Dll1, Jag1 and Fng genes have been suggested to display patterned expressions (Lindsell et al., 1996; Henrique et al., 1995; Myat et al., 1996; Sakamoto et al., 1997; Johnston et al., 1997). This prompted us to investigate the regulatory relationship between HD proteins and the expression of the proteins affecting the Notch signalling pathway. In addition, since Notch signalling has been described to be implicated in both neurogenic control and boundary formation (Louvi and Artavanis-Tsakonas, 2006; Baek et al., 2006), we wanted to distinguish between these functional possibilities in the case of the spinal cord.

Patterning of Notch Components

In a mapping analysis we found that the expression of Jag1, Dll1, Lfng and Mfng were confined to specific progenitor domains. In the ventral half of the spinal cord Dll1, Mfng and Lfng were largely co-expressed in the p0, p2 and pMN domains, whereas high Jag1 expression was exclusively confined to the p1 domain (and dP6 in the dorsal part). Both in gain- and loss-of-function experiments we demonstrated that the HD proteins Dbx1 and Nkx6.1 delimit the expression of Dll1, Jag1, Mfng and Lfng into these specific progenitor domains. Conversely, misexpression of Dll1, Jag1 or Fng genes in chick spinal cord did not influence the establishment of the progenitor domains and nor did they affect the expression of each other. In line with this finding, HD patterning was not either affected in loss-of-function mutants for Dll1 or Jag1. Together these findings show that the HD code acts strictly upstream to set the expression patterns of Notch ligands and Fng genes. Thus, the patterned expression of Dll1, Jag1 and Fng genes does not reflect a role in the establishment or maintenance of the spinal cord progenitor domains.

Domain Specific Regulation of Neurogenesis

We next asked whether the patterned expression of Notch components instead could have a role in domain specific control of neurogenesis. Indeed, specific loss of either ligand in $Dll1^{-/-}$ and $Jag^{Ndr/Ndr}$ (Jag1 mutant) embryos resulted in failure to suppress neurogenesis from exclusive progenitor domains. $Dll1^{-/-}$ mutants showed an accelerated neurogenesis from the pMN, p2 and p0 domains, whereas $Jag1^{Ndr/Ndr}$ mutants generated V1 neurons in excess. Interestingly, we did not observe premature onsets of neurogenesis in either of the mutants, suggesting that the ligand expression only serve to control the pace and not the commencement of neurogenesis. Given the specific expression patterns of Dll1 and Jag1, these results are not surprising. However, if Notch signalling would be allowed between the boundaries of the progenitor domains, one would expect that the loss of Jag1 function in the p1 domain would reduce the Notch signalling also in the adjacent regions, leading to a slight increase in the neurogenesis also from the p2 and p0 domains. Nevertheless, in $Jag1^{Ndr/Ndr}$ mutants, neurogenesis proceeds at normal rates in the p2 and p0 domains and in Dll1 mutants

there is no neurogenic affect in the p1 domain. This would indicate that Jag1 cannot elicit Notch signals in $Dll1^+$ domains and *vice versa*. To test this, Dll1 or Jag1 were electroporated in the chicken spinal cord. Interestingly, while misexpression of Dll1 primarily reduced neurogenesis in $Dll1^+/Fng^+$ progenitors, electroporated Jag1 had the reverse activity and suppressed the generation of neurons mainly from the $Jag1^+/Fng^-$ domains. As Fng genes have been reported to enhance Dll1 mediated signalling and suppress Jag1 function, we surmised that the different activities of Jag1 and Dll1 could be attributed to the patterned expression of Fng. In line with this assumption, we showed that ectopic Mfng expression in the p1 domain increased the number of V1 interneurons, whereas enhanced Fringe in $Dll1^+$ domains led to a decrease in neurogenesis. This would suggest that Fringe suppresses Jag1 and potentiates Dll1 function, resulting in increased or decreased neurogenesis rates, respectively.

We propose a model in which the domain specific expression of Notch ligands and Fng proteins functions to make sure that the neurogenesis in each medial progenitor domain is kept independent from one another. This might be important to ensure a domain specific pace of neurogenesis that would control that accurate numbers of each neuronal subtype are ultimately produced.

Integration of Patterning Proteins and Neurogenesis

The current study is not the first demonstrating a connection between HD patterning proteins and factors involved in neurogenesis. There are several examples where patterning proteins delimit proneural expression, probably as a part of the neuronal specification (Kriks et al., 2005; Muller et al, 2005). However, the class I protein Pax6 appears to take a more direct role in regulating also the level of proneural genes. Scardigli et al have shown that Pax6 directly activates the expression of Ngn2 in regions where the expression levels are high, like in the medial spinal cord and the cortex (Scardigli et al., 2003). Curiously, it was furthermore demonstrated that although high concentrations of Pax6 promoted Ngn2 expression, such levels are incompatible with proper neurogenesis (Bel-Vialar et al., 2007). Overexpression of Pax6 resulted in Ngn2 upregulation and migration to basal positions indicative of neurogenesis. However, these cells did not upregulate Dll1 or subsequent pan-neuronal markers, indicating that high concentrations of Pax6 suppress certain features of neurogenesis. As upregulation of pan-neuronal expression has been suggested to dependent on Sox4 and Sox11 (Bergsland et al., 2006), these are likely candidates to be negatively targeted by Pax6 in the spinal cord. That the onset of neuronal production appears to be domain specific indicates that patterning genes like Pax6 feed into the general neurogenesis programme to regulate this feature.

In Paper IV we found it unlikely that HD proteins would determine the onset of the Notch ligands directly. Instead we surmise that HD proteins regulate the extent of Dll1 and Jag1 expression indirectly by influencing the target choice of proneural genes

(Castro et al., 2006). The pace of neuronal production is however more likely to be regulated by the balance between proneural proteins and Notch signalling, i.e lateral inhibition.

Regulatory relationships between HD proteins and proteins affecting the Notch pathway have also been observed in the vertebrate limb where En1 controls the expression of Radical fringe and Jag2 (Rodriguez-Esteban et al., 1997; Laufer et al., 1997), and in the *Drosophila* eye where mirror and caupolican delimit fringe expression (Cho and Choi, 1996; Dominguez and de Celis, 1998).

In our investigation a Bhlh protein, Bhlhb5 (also known as Beta3), attracted our attention since its expression profile largely coincides with that of Jag1 (Liu et al., 2007), which could indicate similar modes of regulation. Indeed, analysis of the $Dbx1^{-/-}$ and $Nkx6.1^{-/-}$ mutant embryos revealed that Bhlhb5 was affected in a similar way as Jag1 (our unpublished observation). This finding indicates that Dll1, Jag1, Mfng and Lfng probably only comprise the tip of an ice berg of proteins that are patterned downstream of HD proteins.

Roles of Notch Ligands during Development of the Spinal Cord

The main conclusion from Paper IV is that the expression of Notch ligands and Fng proteins are confined to certain progenitor domains in order to ensure a domain-specific regulation of neurogenesis. In addition to this, certain observations in the study evoked a number questions relating to other issues summarised below in five sections.

Stem Zone Maintenance:

At very early stages, Dll1-mediated Notch signalling has in chick been shown to sustain proliferation in the stem zone (Akai et al., 2005, "*Regionalisation of the Hindbrain and Spinal Cord*"). It is possible that a decreased division rate in the stem zone was a partial cause to the reduced size of $Dll1^{-/-}$ mutant embryos.

Onset of Neurogenesis

We show that in loss-of-function mutants of Dll1 and Jag1, there is no obvious premature generation of neurons. Despite this we cannot rule out that Notch signalling would not be important to suppress onset of neurogenesis as proneural genes are clearly induced at earlier stages in Notch mutants (de la Pompa et al., 1997). Notch1 may therefore be controlled by other ligands than Dll1 or Jag1 or alternatively in a Notch ligand-independent manner at early stages (Hurlbut et al., 2006). However, recent data discussed in "*Notch Signalling Oscillation and Lateral Inhibition*", indicate that the process of lateral inhibition probably is not the pre-eminent mechanism for initial precursor selection for neurogenesis and that Hes expression is Notch-independent at early stages. In light of this, loss of either Dll1 or Jag1 would not be expected to have a great impact on the neurogenic commencement.

Generation of Several Neuronal Subtypes from a Single Progenitor Domain

There are examples in the neural tube where two neuronal subtypes are generated from a common progenitor domain and Notch signalling has been demonstrated to regulate the fate choice in these cases. For example, in the distinction between V2a and V2b interneurons from the p2 domain, Dll4 mediated Notch signalling has been shown to promote the V2b fate (Del Barrio et al., 2006, Peng et al., 2007). That V2a and V2b interneurons were equally affected in the $Dll1^{-/-}$ mutants confirms that the V2a/2b distinction requires Dll4 and not Dll1.

In contrast, in the dorsal neural tube, Dll1 mediated Notch signalling has been demonstrated to single out dIL_A at the expense of dIL_B interneurons in a late Gsh1/2 progenitor domain (Mizuguchi et al., 2006). Further analysis is needed to reveal whether increased numbers of diL_B is generated in $Dll1^{-/-}$ mutant embryos.

Do Dll1 and Jag1 have Distinct Functions?

In our study we showed that Dll1 and Jag1 exhibit different activities along the DV axis and that this probably can be explained by the patterned expression of Fng genes. However, we can not rule out that Dll1 and Jag1 have distinct functions that can be separated from their different reactions to Fng modulated Notch1. In the literature there is no evidence that the Notch receptor would translate binding of the two different ligands into distinct responses. A challenging way of addressing this issue would be to replace the coding sequence of Dll1 with that of Jag1 and *vice versa* and combine these with a transgene expressing Fng gene under the Nestin promoter or a double k/o for both Mfng and Lfng. One indication that Dll1 and Jag1 would display different activities stems from the fact that the neurogenesis defect is much more severe in the $Dll1^{-/-}$ mutants than in Jag1 mutants. An alternative explanation for the milder phenotype in the Jag1 mutant could be that it may in fact be a hypomorph that retain some Notch-activating capacity.

Neurogenesis in Regions not Addressed in the Present Study

In contrast to the clear domain specific differences in Dll1 and Jag1 expression in regions addressed in our study in chick, we observed interesting deviations from the theme in other parts of the spinal cord. In addition to the strong expression of Jagged1 in the p1 and dP6 domains, weak expression was also seen in the p3 domain as well as in two unidentified domains in the dorsal spinal cord. High levels of Dll1 and Fng genes were detected in all these domains. That the neurogenesis in the p3 domain and the dorsal half was not obviously affected in either ligand mutant, suggested that Dll1 and Jag1 have interchangeable activities in these domains (our unpublished data). Also, in mouse we detected a few $Dll1^{+}$ INs within the Jag^{+} dP6 domain that could explain why no neurogenic effect was seen here in the $Dll^{-/-}$ mutant (our unpublished data).

SUMMARY AND FUTURE PERSPECTIVES:

PAPER I: In this paper we identifed Lmx1a and Msx1/2 as key transcription factors in the specification of midbrain dopamine neurons (mDA). Lmx1a is sufficient to induce mDA specific features while Msx1/2 functions to repress alternative fates and promotes general neurogenesis. It will be interesting in the future to position Lmx1a and Msx1/2 into the context of other known players and also to identify novel upstream and downstream components to these factors. This will aid in accomplishing a more comprehensive view of the DA specific differentiation programme.

PAPER II: Here we discovered Lmx1b as a postmitotic regulator of the hindbrain serotonergic differentiation programme. Analysis of mutant embryos revealed that Lmx1b operates downstream of the progenitor determinant Nkx2.2 to ascertain the correct neurotransmitter expression and migrational pattern of all hindbrain serotonergic cells. Future studies may reveal the exact epistatic relationship between Lmx1b and other serotonergic markers such as GATA2 and might moreover identify new general as well as AP-specific determinants of the serotonergic cells in the hindbrain.

PAPER III: This report aimed towards a better understanding of to which extent experimental findings in common vertebrate model organisms may be applicable to human development. To this end we gathered the expression profile of most proteins that have been reported to play a role in neuronal and glial patterning, specification or differentiation in mouse and chicken midbrain, hindbrain and spinal cord. This expression pattern map revealed that the majority of developmental expression profiles found in mouse and chicken are conserved in human embryos. Nevertheless, deviations were found in the context of oligodendrogenesis and midbrain dopaminergic marker expression, stressing the importance to make future efforts to update the expression profiling in human embryos.

PAPER IV: In this study we investigated the control of and functional rationale behind the regional expression of Notch ligands in the developing spinal cord. We found that HD proteins which determine the fate of distinct neuronal subtypes, also delimit the expression of the Notch ligands to specific progenitor domains. We provide evidence that this expression pattern ensures a domain specific control of neurogenesis which may be crucial for the correct number of cells to be generated of each neuronal subtype. The work focused on a subset of the progenitor domains and further investigation is needed to understand if also dorsal domains display cell type specific control of neurogenesis and if so how this may be achieved.

ACKNOWLEDGEMENTS

First of all, Thanks **Johan** for forcing me think not only forward, but also backwards and sideways to tackle problems, and for making me push myself just a bit further than I thought was possible. Your shiny mood, brilliance and quirky sense of humour have really helped me throughout these years.

Jonas Muhr- for being so "pilimarisk", thanks for the fruitful, fun, but LONG collaboration. **Thomas Perlmann**, for great collaboration, encouragement and for bringing organization into our lab! **Erik Sundström** for long and nice collaborations. **Urban Lendahl** for your pleasant manners and fruitful collaborations. **Ola Hermansson**, for the discovery of Cardigans ☺, for all the encouragement, help and support! I would also like to thank **Kathleen K. Smith** and **Anthony Graham** for providing newborn *M. Domestica* and valuable advice during the development of Paper III. I'm also grateful to all collaborators within the projects described in this thesis. For proof-reading the thesis I'm in serious dept to **Lizzy, Chris, Emil, José, Jonas M** and **Johan E.**

Chris, (a.k.a. the colour advisor) for being one of the most consistently pleasant persons I ever met. Also thanks for constant fun and providing information on anything from American politics to genuine gossip. You'll make a great scientist!

Joanna, for lots of fun, electro-boat trips and close friendship. Your style is impeccable!

José, for staying on sooo long and sharing frustration and despair during high input - low output periods (i.e. most of the years) and glory during the short easy-ride time periods. Thanks for all the stylistic and scientific advice and for your inspirational true dedication to science!

Lizzy, where to start. Thanks for always taking time for any problem I might have and for immediately solving them! This thesis owns a lot to you! Keep up the good wizardry! Hope we stay friends!

Mattias, for setting the standard to stay in the lab forever- with you still there I don't feel so late… Also of course for great tv-related anecdotes and help with the thesis cover. Promise to wear the Hawaii-shirt at the Party!

Qiaolin, for friendship and great collaboration. Ill never forget Shanghai! I can show you around Umeå one day…

Sanja, even though I haven't succeeded to bring you into my family (yet) you truly feel as close as family☺ You are the most loyal person I know. Thanks for all the fun (although I think you have to remind me about it)

Tony the Pony,- too many things to say about you so I'll just pick a few- thanks for the very interesting sound effects, anecdotes and jokes – also for enjoying my jokes ☺, your genuine interest for science - both your own and those of others!

Zhanna, for being super-pleasant and your excellent dedicated work

Former lab members: **Anna V** for the warm welcome to the lab, your natural ways and fun facial expressions ;) ☺☻ **Maddis** for collaboration and your enthusiasm – hope we'll get "you know what" published this millennium. **Fabrice** (Elwine and Virigine) for your nice attitude and fun times at work and elsewhere. **Mas, Peter, Alexandre** and **Pedro** for great company in the lab, **Maria L**, for increasing the family pool for a while and for all the fun last summer. **Jasmina,** my best student ever!,

A big thank you to all present and former members of Perlmann, Muhr, Lendahl, Vennström, Percipalle, Hermansson and Frisén labs. **Stina,** for great collaborations and for fun gossip sessions during the brain surgery!; **Lia**, for being so fun to dance with, great trip to Tallinn, company at F&S; **Michal,** for being incredibly helpful with FACS. It's a pity our common projects didn't become as fruitful as we had hoped; **Eva**, for being genuinely NICE; **Maria B and Banafsheh**, for being such great "party-pinglor"**, Johan H**, for helping me to turn on the microscope ☺ and your party "know-how"; **Magnus**, for introducing me to the Magnetic Fields; **Dede**-laughter is the closest distance between two people! **Emil** for turning one mouse line into two papers! and for help in a difficult situation; **Heather**, for introducing me to netball and being so easy-going. Keep up the high energy level! **Emilie**, for being so French i.e. having such a sparkling personality and being a great chef; **Raju,** for all the fun at the CMB pub and elsewhere, **Hans Christian**, for describing Umeå as something exotic; **Sarah,** for great advice during my postdoc-planning period and for being a member of "BIO-klubben".

Other friends at KI: **Alexandra**, for wonderful friendship throughout the years; **Jenny Wihlen** for being you!; **Pia**, for all the fun dinners and evenings; **Julianna** for great discussions, support, and being a good friend. Looking forward to the next conference with you! **Per-Henrik** for the nice collaboration. **Olle**, for all the help and fun times in Boston.

Matti, we are lucky to have you!; **Marie-Louise and Zdravko,** for your great attitude and excellent work; **Janet & Rosa Amanda** for your constant smiles; **Ewa,** for being understanding and helpful at all times; **Sofie** and **Sylvia** for help with the mice!; **Micke, Emilio och Jona** for wonderful computer support.

Thanks also to all the numerous **mouse** and **chick embryos**!

Kristian, lets remember the good times! **Marja-Terttu** and **Kalle** for being really nice extra parents for a while.

Jessica and Maria, my ever-lasting friends! You must visit me often!; **Smurf-Lina**. My first publication was actually written together with you. It's a pitty I cannot use "Mellanbladet" in my thesis; **Linda T, Anna G** and **Jenny N** for nice reunions; **Peter M** for being so up for it and always being pleasant; **Joan** for the music updates and for making me enthusiastic about the PhD life a bit less than a decade ago; **Michael**, great to keep in contact and I'm glad I will see you more often soon!; **Karin S** for great mancunian, umean and future friendship!

People who inspired me during undergraduate studies to find my path in science - **Helena and Thomas Edlund, Carolyn Byrne** and **Scott F. Gilbert** (for writing my Bible).

Aimee Mann, Greg Graffin, Eddie Vedder, The Postal Service and Clarice Bushnell during difficult as well as fun periods.

My family- thanks for all support throughout the years!, **My**, the cuddliest amniote ever!; **Mormor Maj-Britt** (jag hoppas jag också blir en järn-lady), **Gobben (Jakob), Jonna, Amelia, Alva and Cornelis**- thanks for all the visits that kept me away from the lab occasionally! **Mamma** (for always being so understanding, not everyone has a kindred soul in their mum), **Pappa** (your ignorance of developmental biology sparkled an interest in me ☺ so I hope this thesis will enlighten you!)

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