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A STUDY OF THE GENERATION OF DIVERSITY IN THE CENTRAL NERVOUS SYSTEM

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Dla Mojego Kochanego Karolka
(To My Beloved Grandpa)

"Mamma, not be Doctor… You be a Pumpkin Halloween"
Matthew, 3 years old

"Flawla Flawla"
Timothy, 16 months old

ABSTRACT

Developmental biology is concerned with understanding the mechanisms that govern the generation of a whole organism starting from one single cell. In the central nervous system (CNS) the development of different classes of neurons and glial cells involves both extrinsic signals and intrinsic cues that together govern the specification of different cell fates dependent on position within the CNS and the time of generation.

Different vertebrate species share many aspects of early development as well as the underlying mechanisms governing the progress of development. Therefore, a plausible assumption is that functional regions in the genome are also conserved between species. In **Paper I,** we have used a comparative genomics approach to identify Highly Conserved Non-coding Regions (HCNRs) between the human, mouse and pufferfish genomes. We find HCNRs to be statistically over represented in the proximity of transcription factors associated with spatial patterning in the developing neural tube. We show that HCNRs associated with patterning genes show an overrepresentation of binding sites for three transcription factors (Sox, Pou and Homedomain genes (SPHD)). By combining bioinformatics and large-scale expression analysis, we show that SPHD enriched HCNRs are strong predictors of CNS expression during development (83% vs. 36% of random control genes). This suggested to us that $SPHD^{+}$ HCNRs may act as CNS enhancers. Further, we isolate a putative HCNR enhancer region and show that it acts as an enhancer both *in vivo* and *in vitro*. Based on our findings, we propose a model where Sox and Pou proteins act as common activators of CNS expressed genes, while homeodomain proteins, which have been previously shown to act as repressors, act to restrict expression spatially.

While a large number of studies have provided insight into the spatial patterning mechanisms directing the generation of distinct cell types at different positions, little is known about the temporal mechanisms underlying the specification of different cell types from a common pool of progenitors in the CNS. In **Paper II,** we have addressed the question of how a seemingly homogenous population of progenitor cells in the caudal hindbrain can give rise to distinct subtypes of vagal visceral motoneurons (vMNs). We show that based on molecular marker expression we can distinguish between at least three subtypes of vMNs at early developmental time points and that each subtypes corresponds to a distinct projections pattern in the periphery. We show that these subtypes are generated sequentially and that the decision to become a specific subtype is independent of contacts with peripheral targets and cell-cell mediated interactions. Further, the homeodomain transcription factor Nkx6.1 and the orphan nuclear receptor Nurr1 are required for the specification of early born subtypes and the maturation of late born subtypes, respectively.

In **Paper III** we were concerned with the origins of oligodendrocytes in the developing spinal cord and hindbrain. Oligodendrocytes have been shown to be generated from a ventrally located domain in the spinal cord and while this ventral origin has been widely accepted, the existence of other origins remained subject to debate. We show, based on *in vitro* cultures as well as mutant analysis, that dorsal domains in the spinal cord can give rise to oligodendrocyte precursors and that these precursors have the capacity to develop to bona-fide mature oligodendrocytes based on expression of mature markers. Further we show that, at least at prenatal stages, ventrally and dorsally generated oligodendrocytes exhibit differences in expression

profiles, suggesting potential differences between these populations. Additionally, our data suggests that the decrease in BMP signaling, a known inhibitor of oligodendrogenesis, in the dorsal spinal cord over time, due to the increase in the size of the neural tube, may influence the time of induction of the dorsally generated oligodendrocyte precursors in spinal cord. Also, our data from the spinal cord and the hindbrain, show that ventral oligodendrogenesis at different anteroposterior levels is governed by different genetic programs.

LIST OF PUBLICATIONS

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

I. Bailey PJ*, **Klos JM***, Andersson E, Karlén M, Källström M, Ponjavic J, Muhr J, Lenhard B, Sandelin A, Ericson J. (2006)
A global genomic transcriptional code associated with CNS-expressed genes.
Exp Cell Res 312 :3108-19.

II. Karlén M*, **Klos-Applequist JM***, Joodmardi E, Perlmann T, Ericson J. (2011)
Sequential specification of visceral motoneuron subtypes from Nkx2.2 expressing progenitors in the hindbrain.
Manuscript.

III. Vallstedt A*, **Klos JM***, Ericson J. (2005)
Multiple dorsoventral origins of oligodendrocyte generation in the spinal cord and hindbrain.
Neuron 45: 55-67.

*These authors contributed equally to this work.

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LIST OF ABBREVIATIONS

AP	Anteroposterior
bHLH	basic Helix-loop-helix
BMP	Bone Morphogenic Protein
BrdU	Bromodeoxyuridine
CNS	Central Nervous System
DV	Dorsoventral
dOLP	Dorsally derived OLP
ESC	Embryonic Stem Cells
FGF	Fibroblast Growth Factor
FP	Floorplate
HB	Hindbrain
HCNR	Highly Conserved Non-coding Region
HD	Homeodomain
HH	Hamburger Hamilton
HMG	High-Mobility Group
Mb	Mega bases
MN	Motoneurons
MS	Multiple Sclerosis
OLP	Oligodendrocyte precursor
Pou	Pit-Oct-Unc
RL	Recurrent Laryngeal
RP	Roofplate
SC	Spinal cord
Shh	Sonic Hedgehog
SL	Superior Laryngeal
sMN	Somatic Motoneuron
Sox	Sry related HMG box
SPHD	Sox Pou Homeodomain
TFBS	Transcription Factor Bindning Sites
vMN	Visceral Motoneuron
vMN-A	Nucleus Ambiguus
vMN-D	Dorsal motor neuron complex of the 10^{th} cranial nerve

vMN-DL	Dorsal motor neuron complex of the 10^{th} cranial nerve lateral
vOLP	Ventrally derived OLP
VT	Vagal Trunk

INTRODUCTION.

While You read this thousands of nerve cells in Your brain engage in processes to exchange and store information – some cells are involved in processing the visual input from this page while some interpret the letters and give them meaning. Other nerve cells control the movements of Your eyes while you follow the lines of this text and even other cells are involved in your hand movement while turning to read the next page. Yet, other cells are involved in storing the information You have just read. The level of complexity is astonishing considering the seemingly simple task, yet without properly functioning neurons and neuronal networks, these actions could not be performed.

There are more than an estimated number of 85 billion cells in the human central nervous system (CNS) and, depending on classification criteria, up to thousands of types and subtypes of nerve cells, with different functional properties (Lange, 1975). In addition to nerve cells there are two more principal types of cells in the CNS: the astrocytes and oligodendrocytes, collectively referred to as glial cells. Glial cells are required to insulate axons, provide structural support, regulate water- and ion balance and maintain the blood-brain barrier and are important for a properly functioning nervous system. The astonishing complexity of the CNS poses one of the key questions in developmental neuroscience: what mechanisms govern the generation of the mature nervous system from a relatively small and homogenous cell population. Over the last decades much progress has been made into understanding the processes that govern the embryonic development of the CNS over space and time.

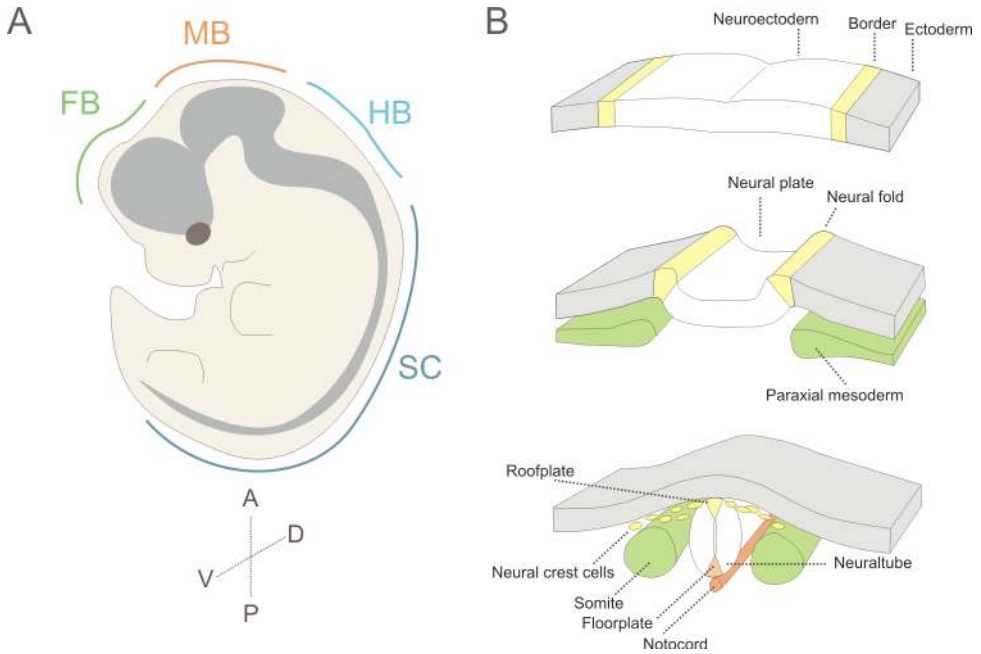

Figure 1. Illustration of the development of the CNS in vertebrates.
A) Schematic drawing of an embryo, indicating the large subdivisions of the CNS; forebrain (FB), midbrain (MB), hindbrain (HB) and spinal cord (SC). Anteroposterior (AP) and dorsoventral (DV) axes are indicated. B) The neural plate is generated from a sheet of ectodermal cells with the notochord underlying the

ventral midline. As development proceeds, the neural plate undergoes a series of folds to form the neural tube. The floorplate (FP) and the roofplate (RP) differentiate in the ventral- and dorsalmost regions of the neural tube. These then act as signaling centers involved in the establishment of progenitor cell domains, which give rise to distinct cell types. The neural tube matures into the SC. Sensory neurons, which relay sensory information from the body, are located in the dorsal part of the SC, while motoneurons and interneurons involved in controlling body movement are located ventrally.

During early vertebrate development a group of ectodermal cells are set aside to eventually make up the whole CNS. These cells form the neural plate, which undergoes multiple processes of growth and movement to form a hollow structure that is referred to as the neural tube. The neural tube consists of a population of multipotent proliferating cells, which during development obtain specific identities and give rise to both neurons as well as glial cells. The cells in the neural tube acquire identities based on their location along the anteroposterior (AP) and the dorsoventral (DV) axes and their very initial identities are set up with the help of external signaling centers. Signaling along the AP axis establishes the main division of the neural tube; the forebrain, midbrain, hindbrain and SC (Lumsden and Krumlauf, 1996), while signaling along the DV axis gives these progenitor cells a more specific identity within their AP level (Campbell, 2003; Jessell, 2000; Rallu et al., 2002). The progenitor cell populations will at later developmental stages give rise to mature neurons and glia, with properties and identities characteristic of their place of birth. In other words, patterning along the AP axis is integrated with DV patterning to give cells their specific positional identity and subsequently their cell fate by regulating intrinsic cellular programs (Briscoe and Ericson, 2001; Campbell, 2003). This can be compared to a grid – where each position within the grid is interpreted differently and the fate of the progenitor cells is dependent on their position within the grid work.

Over the last decades, evidence has emerged showing that distinct anatomical regions within the CNS, for example the floorplate (FP), roofplate (RP) and the isthmus, secrete signaling factors that play important roles in specifying regional identity with the developing CNS and that these events are largely dependent on a relatively small group of signaling families (BMPs, WNTs, TGFß, Hedgehogs, Retinoids, FGFs) (Barolo and Posakony, 2002). Programs of spatial patterning have further been shown to be integrated with pan-neuronal pathways, including Notch signaling, basic helix-loop-helix (bHLH) and Sox proteins, that control the selection of neural progenitors and their commitment to differentiation (Bertrand et al., 2002; Tumpel et al., 2009). The SC, the least complex part of the central nervous system, is commonly used as a model for the rest of the neural tube. A large amount of groundbreaking work has been performed in the SC and many of the developmental principles discovered there have been shown to be true for more anterior parts of the CNS.

One of the most illustrative examples of the process of regional specification, or so called patterning, occurs in the ventral neural tube where the external factor Sonic Hedgehog (Shh) plays an important role in imposing positional identity on the progenitor cells in the neural tube.

The notochord, which underlies the ventralmost neural tube, is the initial source of Shh and plays a role in converting the most ventral part of the neural tube

to a Shh signaling source referred to as the FP (Placzek and Briscoe, 2005). Shh has been shown to be the key mediator of ventral fates in a concentration dependent manner (Ericson et al., 1997a; Ericson et al., 1995; Ericson et al., 1992). Moreover, in the absence of Shh, the ventral SC acquires dorsal properties and fails to develop ventralmost neuronal cell types (Chiang et al., 1996; Jessell, 2000).

Shh forms a gradient in a high ventral to low dorsal manner, which is translated, into positional information by the progenitor cells. The interpretation of the gradient of Shh activity is dependent on a set of transcription factors, most of them characterized by homeodomain (HD) DNA binding motifs but also the bHLH protein Olig2. These transcription factors are divided into two groups, class I proteins that are repressed by Shh at different concentration thresholds and class II proteins that are induced by Shh. The sharp boundaries between the progenitor domains depend on cross repressive interactions between pairs of class I and class II proteins leading to the creation of 5 distinct progenitor domains (p0-p3, pMN), each with a unique expression profile (Briscoe and Ericson, 2001; Ericson et al., 1997a; Jessell, 2000; Muhr et al., 2001). As the progenitor cells exit the cell cycle each progenitor domain gives rise to a distinct kind of post mitotic neuron (V0-V3, MN), which can be identified based on the specific expression of marker proteins, e.g. Isl1/2 for MN (Ericson et al., 1992). The cell- and subtype-specific expression initiated through this process leads to the distinct differentiation pathways characteristic to each cell type and may control maturation, migration and projection patterns.

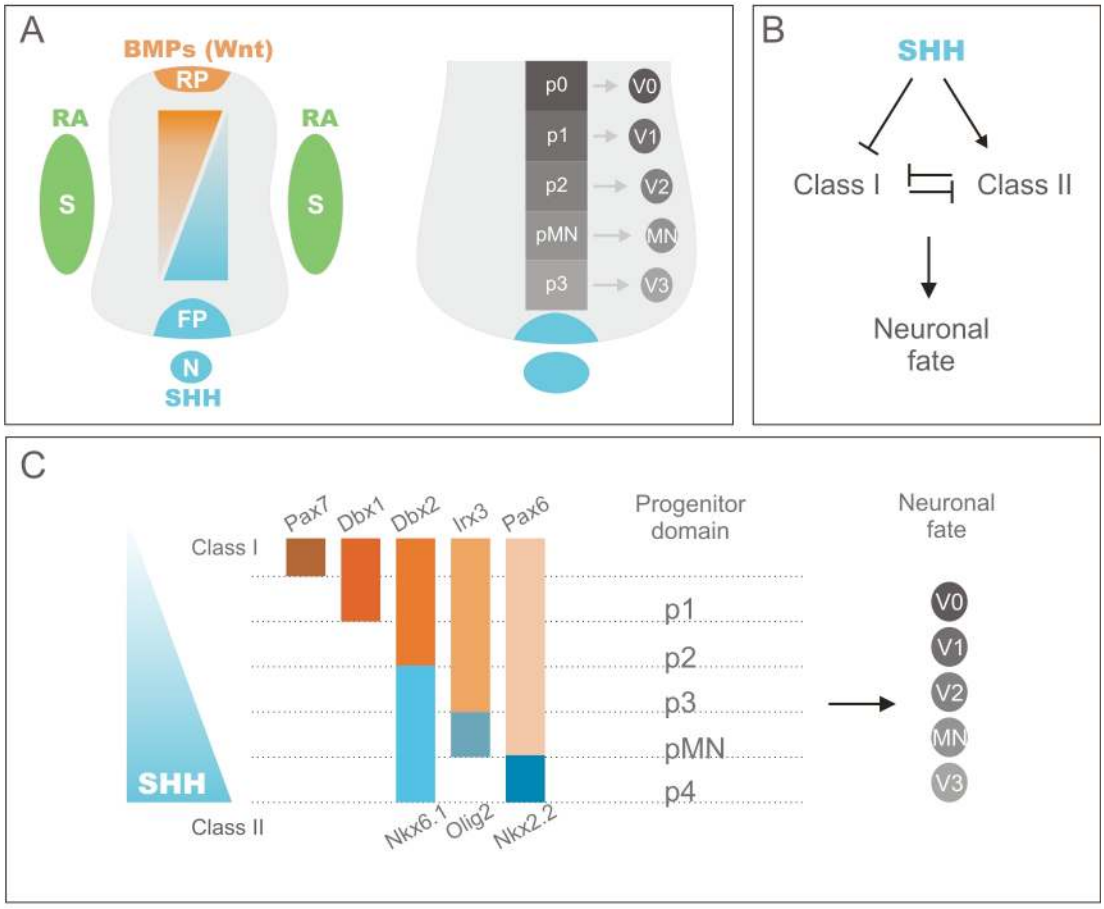

Figure 2. Schematic illustration of patterning in the ventral SC.

A) The generation of neuronal subtypes in the SC is initially dependent on signals: Shh from the FP, BMPs and WNTs the RP and retinoid acid (RA) from the somites (S). The signals from the FP and RP act as gradients in a concentration dependent manner. In the ventral SC, Shh, secreted from the floorplate acts to set up expression domains of different HD transcriptions factors in addition to the bHLH protein Olig2. Each domain will subsequently give rise to a distinct neuronal cell type. B and C) The factors are divided into those repressed by Shh (class I) and those induced by Shh (class II) at different concentrations thresholds. The sharp boundaries between the progenitor domains depend on cross-repressive interactions between pairs of class I and class II proteins leading to the creation of 5 progenitor domains (p0-p3, pMN) with distinct expression profiles. As the cells exit the cell cycle each progenitor domain gives rise to a distinct kind of post mitotic neuron (V0-V3, MN) (reviewed in (Briscoe and Ericson, 2001). (Adapted from Briscoe and Ericson, 2001).

Patterning of the dorsal SC follows the same principles as patterning of its ventral parts, with some exceptions. The cells that make up the counterpart of the ventral FP, the RP, are converted to a non-neuronal fate by signals from the overlying ectoderm (Chizhikov and Millen, 2005). Together with the overlying ectoderm the RP secretes BMPs and WNTs, which are involved in patterning of the dorsal neural tube. BMPs acts in a concentration dependent manner in the specification of progenitor cell types and WNTs have been shown to be important in the specification of dorsalmost interneurons (IN) in mouse (Chizhikov and Millen, 2004, 2005; Liem et al., 1997; Liem et al., 1995; Timmer et al., 2002).

Additionally, over time, changes dependent on factors internal or external to the progenitor domains might alter the type of cell or subtype generated from each domain (Ohsawa and Kageyama, 2008; Pattyn et al., 2003a; Pierani et al., 1999; Zhou et al., 2001). The aspect of time within developmental neuroscience allows room for the immense complexity of the CNS. The grid work has yet another dimension.

A tight and rigid control of all aspects of CNS development is crucial for a functioning system and changes in the composition of the brain - either developmental or due to a disease (e.g. Amyotrophic Lateral Sclerosis, Parkinson's disease, Multiple Sclerosis) are often detrimental to the functionality of the system.

The generation and establishment of the vast number of different types and subtypes from a small number of progenitor cells during embryonic development has been a central question throughout my PhD studies. My focus has been cell type specification in the caudal central nervous system; the SC and the hindbrain. The questions that have fascinated, and still fascinate me, are: How can a small number of cells give rise to such a variety of types and subtypes of mature cells? What processes are involved in specifying where and when a type of cell is generated? What number of cells of each type to generate? What is the switch between generating one type and another?

In the following three sections, I discuss our findings in relation to current knowledge in the field;

* A global genomic transcriptional code associated with CNS-expressed genes.

* Specification of subtype identity of visceral motoneurons of the vagal nerve.

* Oligodendrocyte generation in the spinal cord and hindbrain.

A complex organism consists of billions of cells and while all cells contain the same genetic information, all do not express the same genes. Therefore the regulation of gene expression is a key feature of life, reaching perhaps its greatest level of complexity during development as a whole organism develops from a single cell. However, despite a large body of knowledge about which genes are expressed during development, much less is known about the mechanisms underlying the regulatory logic governing development. The availability of whole genome sequences of many organisms, allowing for comparisons of DNA regions under evolutionary pressure, has facilitated insight into these processes. Although a great deal of advances has been made, interpretation of this data is complex as many regulatory sequences are poorly or not conserved between species. Interestingly, analysis of the regulatory framework of key developmental genes based on interspecies genome comparison has proved to be insightful.

The progression of development is dependent on spatial and temporal coordination of gene expression. As early vertebrate development shares a large number of key features, it is likely that the mechanisms regulating early development are conserved between species. An important aspect of comparative genomics is the choice of genomes for analysis, taking into account evolutionary relationships and genome structure.

Approximately 40% of the human and mouse genomes can be aligned at nucleotide level, however only a fraction of these alignments account for protein coding sequences. A fraction of the non-protein-coding part of the genome has been shown to exhibit extremely high levels of conservation between species (**Paper I** (Bejerano et al., 2004; Sandelin et al., 2004; Waterston et al., 2002)), however little is known about its functional properties. Several putative functional roles for these regions have been proposed, ranging from sites of chemical modification of chromatin, transcription factor binding sites and post-translational modification of RNA (**Paper I** (Carninci et al., 2001; de la Calle-Mustienes et al., 2005; Duret et al., 1993; Levy et al., 2001; Woolfe et al., 2005). Interestingly, using comparative genomics approaches, we and others have shown that clusters of highly conserved non-coding regions (HCNRs) often span the loci of developmentally expressed genes, implying important roles for these regions during embryonic development (Bejerano et al., 2005; Sandelin et al., 2004; Woolfe et al., 2005).

HCNR DEFINITION.

Previous studies have reported the existence of regions exhibiting extreme conservation levels, such as the Ultra Conserved Regions (UCRs) (Bejerano et al., 2004; Sandelin et al., 2004), Highly Conserved Non-coding Regions/Elements (HCNRs/CNEs) (de la Calle-Mustienes et al., 2005; Woolfe et al., 2005) and Conserved Non-genic Sequences (CNGs) (Dermitzakis et al., 2005). Although these regions and their definitions are largely overlapping, some differences pertaining to the number, length and level of conservation remain between them, i.e. the UCRs defined by Bejerano and co-workers exhibit 100 % conservation over at least 200 base pairs (bp) across mammalian genomes. Bejerano extracted 481 region with these properties conserved between mouse and human genomes, while Woolfe and

colleagues identified approximately 1300 elements of at least 100 bp that aligned between human and pufferfish.

In our study, we define a Highly Conserved Non-coding Region (HCNR) as showing at least 95% identity between the human and mouse genomes over a sliding window of 50 bp in length (Sandelin et al., 2004). A further requirement of HCNRs was that it overlapped with regions within the pufferfish, *Fugu rubripes*, genome. The pufferfish genome is highly compacted, carries low numbers of repeats, small intronic and intergenic regions, and it is therefore enriched for functional and regulatory regions. A HCNR fulfilling these criteria was previously referred to by us as an Ultra Conserved Region (UCR), but this nomenclature was changed as not to cause confusion between different definitions (**Paper I** (Sandelin et al., 2004)). For the purposes of this thesis, I will use the HCNR nomenclature also when referring to the UCRs described by us in the study by Sandelin et al. (Sandelin et al., 2004). Here, the term UCR will be used only when discussing regions exhibiting extreme conservation as those described by Bejerano et al. (Bejerano et al., 2004).

HCNRs IN THE PROXIMITY OF DEVELOPMENTALLY EXPRESSED TRANSCRIPTION FACTORS.

Comparative genomics has been shown to be an effective way to predict cis-regulatory sequences (Lenhard et al., 2003; Woolfe and Elgar, 2008). In a previous study, in a comparison between human, mouse and pufferfish genomes, we identified a set of 3583 HCNRs that did not overlap with any protein coding sequences (Sandelin et al., 2004). There we found that genomic positions of these HCNRs are strongly associated with the locations of genes encoding key regulators of development. An especially high correlation was observed between HCNRs and genes coding for transcription factors, in particular transcription factors characterized by a HD motif, but also for zinc finger and forkhead proteins and nuclear receptors. Therefore the majority of transcription factor classes are closer to HCNRs than to random genes – there is visible over-representation of HCNRs up to 300 kbp from HD genes and up to 150 kbp from zinc finger genes (Sandelin et al., 2004).

The median length of a HCNR was 125 bp, although some HCNRs were approximately 1000 bp in length. HCNRs were found to be present in introns, in clusters around groups of genes or in gene deserts, where no known genes were present for up to several thousands of kilobases (Sandelin et al., 2004). Interestingly, similar to other reports, we found HCNRs to be more strongly conserved between species than sequences encoding identical proteins, indicating that these regions carry important functional properties (Sandelin et al., 2004; Santini et al., 2003; Woolfe et al., 2005).

Previous studies have shown that non-coding regions exhibiting extreme levels of conservation occur more frequently in the genome than expected by chance and that such regions are significantly associated with DNA binding proteins and developmental regulators (Bejerano et al., 2004; Boffelli et al., 2004; Sironi et al., 2005; Woolfe et al., 2005).

HCNRs ASSOCIATED WITH PATTERNING GENES.

At the time of our study, a small number of large, highly conserved enhancers had been identified to act as control regions associated with important developmental genes such as *Dach, Sox9, Dlx3,* the *Hox* genes, *Wnt1, Pax9, Nkx2.9* and *Irx3* (Bagheri-Fam et al., 2001; Nobrega et al., 2003; Santini et al., 2003 and reviewed in (Woolfe et al 2008 ; Spitz et al., 2003; Sumiyama and Ruddle, 2003). Also, Shh expression in the zone of polarizing activity in the developing limb, had been shown to be dependent on an enhancer element and that mutations in this element were associated with human polydactyly disorders (Lettice et al., 2003). In the recent years, several studies have pointed to the medical importance of the genetics of gene regulation, as deletions, disruptions or mutations of regulatory regions have been implicated in several diseases e.g. Hirschprung disease, X-linked adrenal hypoplasia and cleft lip (Emison et al., 2005; Goto and Katsumata, 2009; Rahimov et al., 2008). Additionally, genome wide association studies have revealed that non-coding genomic regions are associated with disease susceptibility and pointed to that many of these regions have putative enhancer function (Visel et al., 2009b). Since many diseases are not associated with any documented mutations, it is likely that the underlying mechanisms may involve cis-regulatory region disruptions or deletions resulting in phenotypes reminiscent of hypomorphic or null alleles of the gene in question.

We analyzed the HCNRs associated with twelve genes involved in dorsoventral patterning of the neural tube. These HD patterning genes have previously been shown to function as repressors of transcription through the recruitment of the co-repressor Groucho (Briscoe and Ericson, 2001; Muhr et al., 2001) and their function has been shown to be conserved through evolution (Cornell and Ohlen, 2000). We examined a one megabase (Mb) region of sequence flanking each of the twelve patterning HD genes and found that all were associated with HCNRs (**Paper I**). Importantly, these HCNRs exhibited an overrepresentation of binding sites for Sox (S), Pou (P) and HD transcription factors (**Paper I**)[1].

Sox proteins (Sox1-3) and Pou proteins (Brn1, 2 and 4) are broadly expressed in the developing CNS. Sox1-3 have been shown to function to keep neural progenitors in an undifferentiated state (Bylund et al., 2003; Graham et al., 2003) and act together with proteins of the Pou family to activate expression of stem cell genes (Tanaka et al., 2004). The SPHD binding site enrichment found in HCNRs associated with patterning genes might therefore suggest that Sox (S) and Pou (P) proteins act as common activators of HD genes involved in DV patterning

[1] In each given HCNR, we centred a 400 bp region which was scanned with the appropriate transcription factor binding model and counted the number of S, P and HD binding sites with the 400 bp region (Paper I). The probability of any given count was compared to a background set, B. The background set was generated by randomly dividing the human genome (assembly hg 15) into 400 bp regions and performing the same evaluation. The likelihood of finding a region where B (S) ≥ S, B (P) ≥ P and B (HD) ≥ HD could then be estimated by counting the number of regions where these criteria are satisfied and divided by the total number of regions in the background set B. This is equivalent of calculating the joint cumulative distribution for S, P and HD (Paper I). By this method each evaluated HCNR obtained a S, P and HD count and a corresponding p-value (**Paper I**).

of the neural tube (**Paper I**). Supporting this hypothesis, we show that forced expression, by *in ovo* electroporation, of a dominant negative variant of Sox3 (Sox^{EnR}) repressed the expression of patterning genes in the chick SC (**Paper I**).

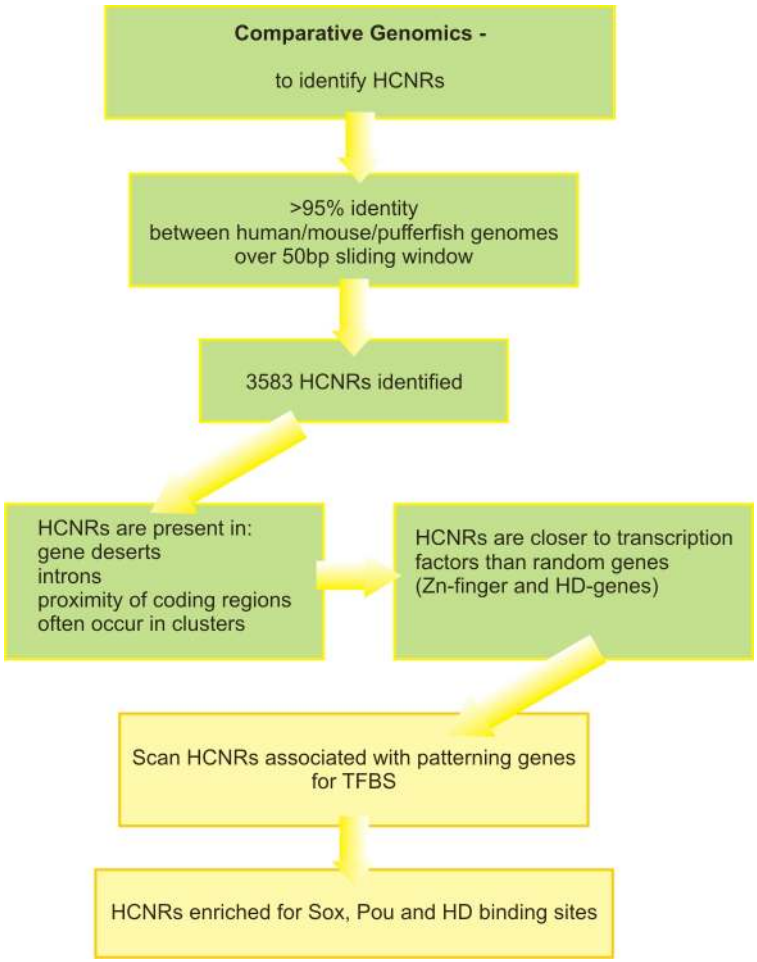

*Figure 3. Chart summarizing the findings in (Sandelin et al., 2004) and first section of **Paper I**.*
*Sandelin and co-workers identified non-coding regions showing over 95% identity between the human, mouse and pufferfish genomes, here termed Hyper Conserved Non-coding Regions (HCNRs), and found that these regions are statistically closer to transcription factors genes than to random genes. In **Paper I**, we extend the concept of HCNRs and evaluate HCNRs associated with HD transcriptions factors involved in dorsoventral patterning for transcription factor binding sites (TFBS). We show that such HCNRs are enriched for binding sites for S, P and HD proteins. Green indicates findings from (Sandelin et al., 2004), while yellow depicts findings from **Paper I.***

SPHD$^+$ HCNRs ASSOCIATED WITH CNS GENES.

Dach locus.

Our finding of S, P and HD binding sites being overrepresented in HCNRs in the proximity of patterning genes, prompted us to investigate if other CNS expressed genes were associated with SPHD$^+$ HCNRs. It has been shown that HCNRs associated with the *Dach* locus can act as CNS specific enhancers driving expression of a reporter gene construct *in vivo* and acting over a near Mb distance (Nobrega et al., 2003). Nobrega and coworkers took advantage of a ß-galactosidase (ß-gal) reporter assay in transgenic mice to show that 7/9 putative enhancer regions with extreme conservation located in a gene desert surrounding the *Dach* locus, could reproduce Dach expression in a distinct set of tissues in the mouse. However, the factors mediating this function remained unknown. We examined the Dach locus and found that the previously defined enhancers were highly enriched for SPHD$^+$ TFBS (**Paper I**).

Other genes.

We next asked if the SPDH$^+$ HCNRs could be part of a more general transcriptional program governing the expression of genes in the CNS. We evaluated if the presence of SPHD binding sites within HCNRs could be predictive of CNS expression. Out of the 3583 previously characterized HCNRs (Sandelin et al., 2004), we identified a subset of 675 HCNRs, corresponding to 19% of the total HCNRs, that were enriched for SPHD (p-value < 0.05). Based on these regions we identified 430 closest neighbor genes. This number is lower than the number of SPHD$^+$ HCNRs, since HCNRs often occur in clusters.

Next we retrieved 172 cDNAs out of the 430 identified closest neighbor genes, from the RIKEN FANTOM2 full-length cDNA library. In addition, we retrieved 69 randomly selected control genes from the same library. We analyzed the expression pattern of these genes in the CNS at two developmental stages E10.5 and E12.5. We found that 70% of the genes linked to SPHD$^+$ HCNRs were expressed during the analyzed developmental stages in the CNS, compared to only 26% in the control group. When we analyzed the available literature, we found that an additional 13% of the closest neighbor genes had been reported to be expressed in the CNS. The corresponding number of for the control group was 10%.

It is also important to mention, that the number of genes expressed in the CNS may in fact be higher than is shown in our screen, as problems with *in situ* probe preparation and labeling may have resulted in genes scoring as false negatives (**Paper I**).

In summary, at least 83% of the closest neighbor genes were found to be expressed in the developing CNS at the stages analyzed, suggesting that the association to SPHD$^+$ HCNRs is a strong predictor of CNS expression (**Paper I**).

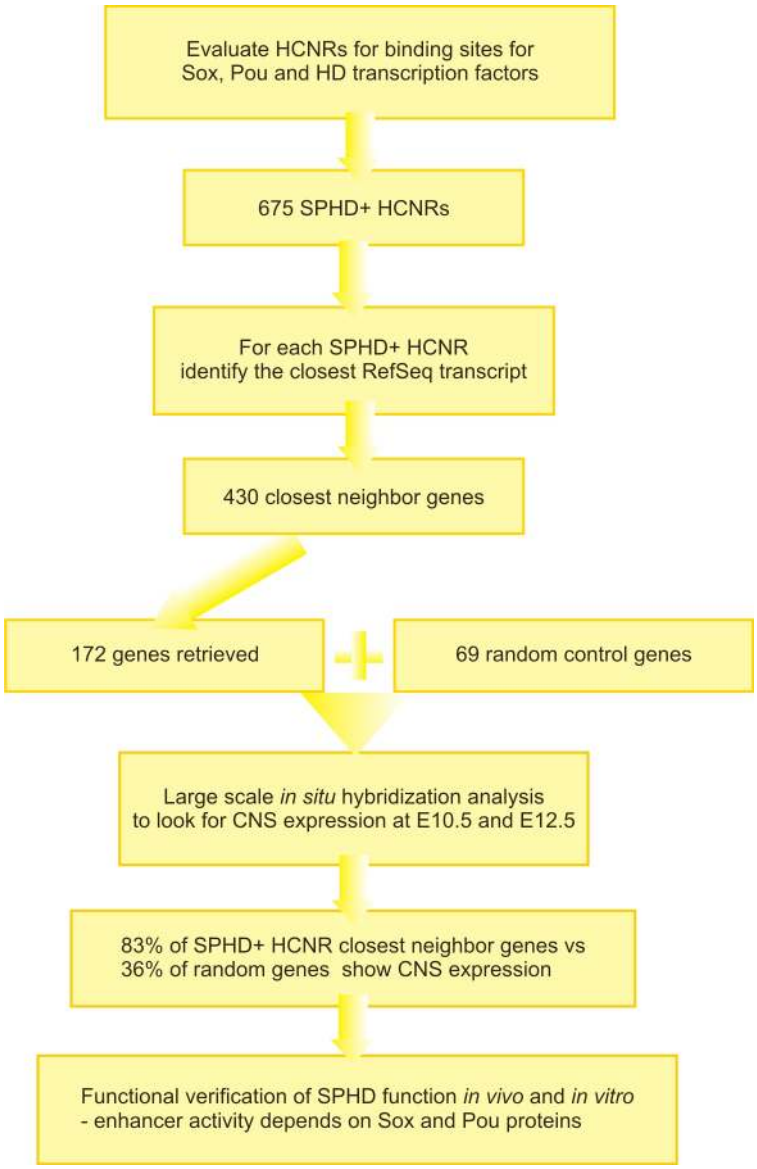

Figure 4. Chart summarizing findings from second section of ***Paper I****.*
Out of approximately 3600 HCNRs, 675 HCNRs showed enrichment for Sox, Pou and HD binding sites. For each HCNR the closest neighbor gene was located and collected. 172 closest neighbor and 69 random control genes were analyzed for expression in the developing CNS at E10.5 and 12.5 by in situ histochemistry. 83 % of closest neighbor genes were found to be expressed in the developing CNS compared to 36 % of the random control genes. Further, the putative enhancer function of SPHD$^+$ HCNRs was confirmed, both in vivo and in vitro.

Genomic Hotspots.

It was shown by Spitz and co-workers that genomic regions with extreme conservations in the *HoxD/Lunapark* locus can acts a global control regions influencing the expression of several structurally unrelated genes located within the same region of DNA (Spitz et al., 2003). Also the *Irx* cluster has been shown to be associated with shared/global regulatory sequences (Sandelin et al., 2004; Woolfe et al., 2005). The existence of regions with co-expression domains of unrelated genes

points to global regulatory mechanism acting over large chromosomal distances (Hurst et al., 2004).

Upon analysis of the HCNRs in the proximity of the *HoxD/Lunapark* locus, we observed that these regions were also enriched for S, P and HD binding sites (**Paper I**). Therefore we asked, if $SPHD^{+}$ HCNRs could be involved in global control of gene regulation.

We examined 43 regions associated with $SPHD^{+}$ HCNR (p-value ≤ 0.01) where at least five genes were present within 2 Mb distance up- or downstream of the HCNR and at least one of those genes was known to be expressed in the CNS. We evaluated the expression of the identified genes by *in situ* hybridization and additionally performed literature searches. We found that in 40 out of 43 loci, several of the examined genes (in addition to the previously known CNS expressed gene) where expressed in the CNS (**Paper I**).This suggests that $SPHD^{+}$ HCNRs appear to define genomic hotspots for CNS-expressed genes.

In summary, we are able to show that $SPHD^{+}$ HCNRs are strong predictors of CNS expression, as at least 80% of the genes associated with such regions where expressed in the developing CNS. Additionally, we show data indicating, that $SPHD^{+}$ HCNRs may be associated with genomic hotspots for CNS expression. It is noteworthy, that close physical localization of genes along the chromosome, is not always predictive of similar expression patterns. For example the homeobox genes *Six2* and *Six3*, although located next to each other in a region containing a large number of highly conserved non-coding sequences, are expressed in non-overlapping complex patterns. It has been suggested that the presence of a strong insulator sequence (described below) between *Six2* and *Six3* accounts for their distinct expression patterns (Barski et al., 2007).

FUNCTIONAL VERIFICATION OF SOX AND POU REQUIREMENT.

Our results described above, indicate that a large number of genes may be controlled by a transcriptional program involving Sox, Pou and HD proteins. To verify the functional requirement of Sox and Pou proteins, we isolated a putative enhancer region ($SPHD^{+}$ HCNR p-value ≤ 0.0111) of the bHLH E-box protein TCF12, which is broadly expressed in the neural progenitors in the developing CNS and conserved between mouse and chick (Neuman et al., 1993; Uittenbogaard and Chiaramello, 2002). This region contained four consensus binding sites for Sox and Pou transcription factors (**Paper I**). Homotypic clustering of transcription factor binding sites (TFBS) within enhancers has been reported for over 66% of known developmental enhancers (Gotea et al., 2010).

We inserted the putative TCF12 enhancer region, referred to as TCF12-HCNR, into a reporter construct upstream of the minimal ß-globin promoter and a ß-gal reporter. To evaluate if the putative TCF12-HCNR enhancer could drive expression in the neural tube, we electroporated this construct into Hamburger Hamilton (HH) stage 10-12 chick embryos and scored for ß-gal expression after 24 hours. We observed extensive expression of ß-gal in the electroporated side of the neural tube, indicating that the TCF12-HCNR has enhancer activity in neural progenitors *in vivo*. In contrast, electroporation of the minimal ß-globin promoter alone did not lead to any ß-gal expression (**Paper I**).

As it has been shown that mutations in S and P TFBS abolish the ability of Sox and Pou proteins to bind to the DNA (Ambrosetti et al., 2000; Josephson et al., 1998; Tanaka et al., 2004), we mutated the S and P binding sites within the TCF12-

HCNR. We found that ß-gal expression was lost or significantly diminished by mutations in all but one of the sites, which supports that the enhancer activity of TCF12-HCNR is dependent on Sox and Pou proteins and suggests cooperative mode of action (**Paper I**). Additionally, we were able to show in a cell transfection assay, that the TCF12-HCNR enhancer was activated synergistically by Sox3 and the Pou protein Brn4 (**Paper I**).

Sox protein dependent gene regulation is thought to function in a partner protein dependent manner. Binding of Sox proteins alone to a regulatory region, has been shown to be insufficient to activate gene transcription, but cooperation with a partner protein, such as a member of the Pou family, which binds to a nearby site, creates an active complex capable of transcriptional activation or repression (Kondoh and Kamachi, 2010). This is in line with that the enhancer activity of the TCF12-HCNR is dependent on both Sox and Pou proteins. The availability of partner protein and the specific sequence flanking the STFBS determines what factor partners up with the Sox protein (Kondoh and Kamachi, 2010).

PROPOSED MODEL.

Based on the data described above, we suggest a model where Sox and Pou proteins may act as generic activators of expression in the CNS, while the spatial restriction of expression is dependent on the action of HD proteins.
Most CNS expressed proteins, including the genes involved in patterning along the DV axis, exhibit distinct spatially restricted expression patterns, suggesting that there must be mechanisms that control generic activation of gene expression in a spatial manner. It has been shown that HD proteins act as repressors and that cross-repressive interactions between pairs of HD proteins are involved in establishing progenitor domains in the developing neural tube (Briscoe and Ericson, 2001; Muhr et al., 2001). We propose a common transcriptional logic for SPHD^{+} HCNR-linked genes, where Sox and Pou proteins act as generic activators of CNS expression, while the HD proteins act to restrict the spatial extent of expression (Figure 5).

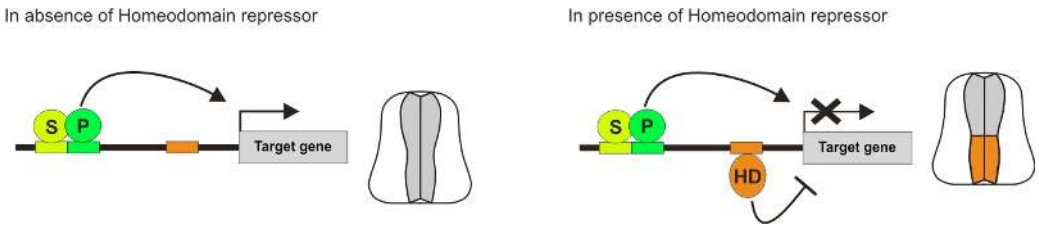

Figure 5. Schematic drawing illustrating the SPHD model. Sox and Pou proteins act as common activators of gene expression in the CNS, while HD transcription factors work to spatially restrict gene expression to specific positions within the developing CNS. Target gene expression in the SC is indicated in grey and HD expression in orange. Importantly, while SPHD^{+} HCNRs are depicted as a single element is this picture, these elements often show high clustering tendencies.

Another member of the Sox family of proteins, Sox21, has been shown to have an opposing role to Sox1-3 (Sandberg et al., 2005). Sox21 is, in contrast to Sox1-3, expressed in the intermediate zone in the neural tube. Since Sox21 has been shown to share the same consensus TFBS as Sox1-3, it may be that Sox21 competes with Sox1-3 for binding sites when neural progenitors leave the cell cycle (Uchikawa et al., 1999). Sox21 could therefore be involved in coordinating the on/off expression of a large number of $SPHD^{+}$ HCNR associated genes as the neural progenitors differentiate into neurons.

Interestingly, Sox and Pou proteins are widely expressed in embryonic stem cells (ESCs), and have been shown to co-occupy putative enhancer regions of genes in ESCs (Boyer et al., 2005). However, many CNS expressed genes (including the HD patterning genes Nkx2.2 and Pax6) are suppressed in ESCs. It may be that the $SPHD^{+}$ HCNR genomic hotspots may be involved in silencing, in addition to activating, genes located within a given genomic territory.

Our study focused on genes expressed in the CNS. However, as the development of other organs has been shown to involve Sox, Pou and HD proteins, it may be that the shared expression profile of many key genes between these organs reflect a underlying common transcriptional logic (Henseleit et al., 2005; Kume, 2005; Lioubinski et al., 2003; Rosenfeld et al., 1996; Thor et al., 1991).

OTHER CIS-REGULATORY FUNCTIONS.

An interesting aspect of non-coding regions showing extreme levels of conservation is if they, in addition to functioning as cis-regulatory enhancers, could act as gene silencers.

Enhancers can act as nucleation sites for transcriptional activators. It is believed that enhancer-promoter interactions are brought about by looping of intervening DNA facilitated by protein-protein interactions. It is possible that silencers act through blocking the binding of activators to enhancer regions by competition for TFBS or by recruiting chromatin remodeling factors and closing the chromatin structure (Sakabe and Nobrega, 2010). Although highly probable in theory, exploring a putative silencer function might be harder due to difficulties finding an appropriate read-out system. One possible approach is to use reporter constructs with a strong promoter sequence to evaluate putative silencer function. Additionally, such function may be dependent to a large degree on genomic context, which will be lost upon isolation of putative silencer regions. Also, in the case of enhancer regions, isolation from genomic context may bear effects on the ability to drive expression and therefore isolated enhancers may be reported to positively regulate gene expression in tissues were they endogenously are kept silent. Therefore, it is important to apply complementary evaluation methods to gain a better understanding of the functions of these non-coding regions.

It may also be that HCNR regions are involved in RNA secondary structure formation (Sakabe and Nobrega, 2010), however this aspect is beyond the scope of this thesis and will not be discussed here.

Recently, sequences that act as insulators of enhancer activity have been identified. Such sequences are located between enhancers and promoters and act to block the spreading of enhancer or silencer activity, either by preventing interactions between enhancers/silencers and promoters or by preventing spreading of

heterochromatin (Sakabe and Nobrega, 2010; Wallace and Felsenfeld, 2007). Genome wide mapping of insulators point to that they delineate genomic hotspot regions (Kim et al., 2007). Also, regions that do not have enhancer activity *per se*, but are able to boost enhancer activity have been identified in the mammalian genome (Smith et al., 2008).

DISTRIBUTION AND FUNCTION.

HCNRs or other similar regions are found in various locations in the genome, including intronic, 3' upstream, and 5' downstream of the regulated gene (**Paper I** (Woolfe and Elgar, 2008)). A small fraction of HCNRs or similar regions have been reported to be found in 3' and 5' UTRs (Woolfe and Elgar, 2008). Taken together, this suggests that HCNRs or similar regions can function irrespective of position, in contrast to promoter regions. Only a minority of HCNRs show any evidence of transcription, based on available ESTs (Woolfe and Elgar, 2008). It may be that these represent unannotated coding sequences. Alternatively, some of these regions may carry cis-regulatory function, as regions aligning with known ESTs have been shown to drive reporter gene expression in a spatially and temporally specific manner (de la Calle-Mustienes et al., 2005). Yet another possibility is that they have post transcriptional regulatory function. It has also been suggested that HCNRs within 3' or 5' UTRs are remnants of gene duplication events which occurred during evolution, as such regions have been reported within the Hox and Irx clusters (Woolfe and Elgar, 2008). Additionally, since many HCNRs have different orientations in different species, it appears as HCNRs have undergone inversion events during evolution without affecting their function, implying that their function is orientation independent (Woolfe and Elgar, 2008). On the other hand, transposition events appear to be rare underlining the importance of gene specific association of HCNRs (Woolfe and Elgar, 2008).

Our HCNR definition of > 95% identity allows for only a small number of insertions/deletions/mutations, however other definitions allow for more variation in the sequence of HCNRs, as they do not code for proteins and therefore should not be sensitive to missence of frameshift mutations. For example, insertion of two linkers into the enhancer regions of the *Dach* gene had no effect on ability to drive expression *in vivo* (Poulin et al., 2005). In contrast, we show that mutations in the core TFBS have affect on the activity of the TCF12-HCNR (**Paper I**). It may be that while the TFBS are sensitive to mutations, regions between them are not.

The functionality of conserved non-coding regions is still being evaluated. It is noteworthy that when Nobrega and coworkers deleted two large conserved non-coding regions spanning approximately 1 Mb on mouse chromosomes 3 and 19, no significant effect was seen on viability or phenotype of the mouse mutants (Nobrega et al., 2004). Additionally, PCR analysis showed that the expression levels of genes flanking the deleted regions were unaffected in 98% of tissues analyzed. This raises the question of what role highly conserved region have *in vivo*. However, in Nobregas analysis, only 1 of 15 of the most conserved regions within those 1 Mb deletions were able to drive reporter gene expression in a tissue specific manner, suggesting that the regions deleted were not enriched for enhancer function. Also, in the study of Ahituv and co-workers, the deletion of ultra-conserved elements did not lead to any phenotype (Ahituv et al., 2007). Although, as the regulatory landscape of the given genes is not fully understood, it may be that redundant regulatory

sequences are able to compensate for the deletion or that minor phenotypes where overlooked by the assays used. A study by King et al. from the ENCODE[2] pilot project reported the lack of function for 40% of constrained regions, implying that there is not as strong a relationship between conservation and function as previously thought (King et al., 2007). Further, the analysis pointed to that approximately 50% of functional elements in the genome, did not show conservation between species. It may be so that a substantial fraction of regulatory sequences are responsible for human specific features, and therefore would not be conserved in evolutionary distant species. These studies highlight the fact, that regions conserved across large evolutionary distances may not have critical functional roles. In contrast, studies, which take into account conservation between more distant species than human, and mouse, e.g. pufferfish, have shown a significantly positive relationship between conservation and functionality (de la Calle-Mustienes et al., 2005; Pennacchio et al., 2006).

A study by Werner and coworkers points to the existence of five enhancer regions within 100 kb of Sox10 and show by a reporter assay in transgenic mice that these regions are able to drive expression in distinct, but overlapping, spatial and temporal specific manner at different developmental time points (Werner et al., 2007). This underlines the importance of evaluating enhancer function at several developmental stages in order to get at more comprehensive understanding of enhancer functions as domains of expression change over time and may be overlapping. Interestingly, the enhancer regions identified by Werner et al. do not show conservation in amniotes, although Sox10 functions as a key regulator of neural crest development in all vertebrates, indicating that the relationship between conservations of gene regulation and gene function may in some cases be complex (Werner et al., 2007). Further, the overlapping activity of some Sox10 enhancers may provide a level of fail-safe mechanism of gene regulation. It may be that this type of mechanisms, can explain the apparent lack of phenotypes upon deletions of regulatory regions.

Importantly, these studies bring forward the importance of *in vivo* assays to evaluate the functionality of non-coding conserved region and point to that the relationship between conservation and functionality may be more complex than originally estimated.

IMPLICATIONS OF EXTREME CONSERVATION.

In our study we applied a less stringent criteria of HCNRs than used by Bejerano and co-workers, where they define a Ultra Conserved Element (UCE) as having 100% conservations over at least 200 bp (Bejerano et al., 2004). In contrast, our HCNR criteria allow for 5% variation in sequence between species. Interestingly, Bejerano et al report that the UCE exhibit approximately 20 times

[2] The National Human Genome Research Institute (NHGRI) launched a public research consortium named ENCODE, the **Enc**yclopedia **O**f **D**NA **E**lements, in September 2003, to carry out a project to identify all functional elements in the human genome sequence. The project started with two components - a pilot phase and a technology development phase.

lower chance of mutations, compared to the rest of the genome, implying a level of active evolutionary mechanism, e.g. negative selection or reduced mutation rate, acting on these regions. Selection to maintain protein function, protein-nucleic acid interactions or RNA-RNA interactions does not result in near to total conservation over long stretches of DNA (Bejerano et al., 2004). Similar to our findings, Bejerano et al. found that the 250 UCEs located in introns and between genes were located close to transcription factors and genes expressed early in development (**Paper I** (Bejerano et al., 2004)). Also Woolfe and co-workers show that non-coding regions exhibiting high levels of conservation, (74-98% identity between human and pufferfish genomes over at least 100 bp), can act as tissue specific enhancers as shown in zebrafish embryos (Woolfe et al., 2005). Importantly, most of these regions were located in and around developmental regulators (Woolfe et al., 2005). This suggests that these regions have also been subject to stringent evolutionary mechanisms and consistent with key gene function.

Bejerano were unable to trace UCE to evolutionary distant species such as *C.elegans* or *D.melanogaster*, suggesting that the bulk of UCE evolved fast in chordates and then became frozen in birds and mammals (Bejerano et al., 2004). Woolfe and colleagues were also unable to identify HCNRs in invertebrates (Woolfe et al., 2005). This is line with the common developmental features and processes of chordates, such as the presence of a notochord and a hollow dorsal nerve cord. Further, given the extreme conservation of regulatory regions it may be that evolutionary variation has occurred by tweaking regulatory regions, rather than the developmental genes themselves (Sakabe and Nobrega, 2010). It is noteworthy that non-coding regulatory sequences, parallel to the ones found in the human genome, have been identified around similar genes in nematodes (Vavouri et al., 2007).

Interestingly, in a large scale transgenic mouse study of close to all non-exonic ultraconserved regions identified in the human genome, Visel and co-workers found that in addition to the ultraconserved non-coding regions that act as enhancers of developmental genes. In addition, they show that other non-coding conserved regions, which are under similar evolutionary constraint but exhibit a lower level of conservation between mouse and human, are equally enriched for enhancers (Visel et al., 2009a).

OTHER TECHNIQUES.

In our study, we have taken advantage of combining comparative genomics and TFBS prediction with a large scale expression analysis. This methodology, although laborious and time consuming, has the advantage of revealing additional information about spatial and temporal expression not made available by conventional large-scale screening methods (e.g. microarrays, ChIP on Chip). For example, Lmx1a was revealed to be selectively expressed in the ventral midbrain at the time of specification of dopaminergic neurons in this screen, and later shown to play a critical role in the development of dopaminergic neurons (Andersson et al., 2006).

Chromatin immunoprecipitation (ChIP) can be used to pull down transcription factor binding DNA regions from living cells, based on the antibodies binding to the transcription factor bound to these regions. These DNA sequences can then be purified and analyzed with the help of microarrays (ChIP on Chip). However, traditional microarrays are limited as far as the presence of sequences with repetitive features and cross hybridizations which prompted the development of

new tools for analysis such as ChIP-SAGE (based on DNA sequences bound to transcription factors), ChIP-PET (where more DNA is covered compared to regular ChIP on Chip) and high throughput sequencing ChIP-Seq (reviewed in (Sakabe and Nobrega, 2010)). In an elegant study, Visel et al, take advantage of the developmental near-ubiquitous co-activator p300 in a ChIP-Seq analysis to predict tissue specific enhancers, which they validate *in vivo* for 87% of the regions tested (Visel et al., 2009a). ChIP-Seq has also allowed for the identification of 55.000 putative enhancers in the human genome (Heintzman et al., 2009).

As transcription factors are prevented from binding to densely packed chromatin, the actual functionality of enhancer regions is dependent on the chromatin structure. Therefore, experimental methods are necessary to determine which regulatory regions are active in a cell type specific manner (Sakabe and Nobrega, 2010). Based on DNA methylation patterns of transcriptionally active and inactive promoters and enhancers as well as various histone acetylations, a code for transcriptional activity has been proposed (Bernstein et al., 2006). Analysis has indicated differences between promoters and enhancers in their methylation code and certain histone acetylations have been shown to be associated with enhancers, in line with local opening of chromatin structure and facilitation of transcription. However, this may not be as straightforward, since recently reports of the presence of similar methylation patterns in active and silent DNA have been published (reviewed in (Sakabe and Nobrega, 2010)).

Recent studies have reported differences in core promoter structure between tissue specific genes and housekeeping genes. The majority of genes expressed in a tissue specific manner are associated with TATA-box containing promoters in contrast to housekeeping genes, whose promoters often are TATA-less and overlap with a CpG island (Gomez-Skarmeta et al., 2006). Interestingly, key developmental genes appear to be an exception to this rule, as most transcription factors described in (Sandelin et al., 2004) have TATA-less promoters (Gomez-Skarmeta et al., 2006). Studies have shown that choice of promoter used in reporter constructs, has influence over the enhancers that can be detected, underlining the importance of increasing our understanding of interactions between cis-regulatory regions and promoters (Gomez-Skarmeta et al., 2006). Additionally, mutating several components of the transcription initiation complex has been shown to have only limited effect on gene expression. Therefore the previously thought generic components of the transcription initiation complex, may govern expression of specific classes of genes, adding another level of complexity to the regulation or transcription (Holstege et al., 1998).

The advances during the recent years emphasize that computational analysis is a powerful tool used to identify important factors in biological processes, however in order to increase the power of these methodologies a deep understanding of the underlying biological processes is required. By combining traditional biology with refined computational methodologies it should be possible to direct searches towards more specific biological questions.

FUTURE DIRECTIONS.

As many of the computational and high throughput techniques mentioned previously, do not differentiate between functional, non-functional and type of cis-

regulatory region, functional verification of HCNRs is critical for understanding their importance in governing and regulating gene expression. Cell culture based assays evaluating putative regulatory regions based on ability to induce reporter gene expression, offer an inexpensive and high throughput alternative, however such methods may be affected by artificial high level expression or cell line artifacts. Studying HCNRs in a whole organism system is advantageous as it provides important information about spatial and temporal expression. As transgenic mouse models are expensive and time consuming, other experimental systems, such as zebrafish, *Xenopus* or chick, may offer greater opportunity to evaluate the function of large numbers of HCNRs.

Recently, cis-regulatory modules that mediate expression of genes in pre-synaptic neurons (Liu et al., 2009) as well as enhancers driving expression in the heart have been characterized (Narlikar et al., 2010). The identification of tissue specific enhancers offers an opportunity to access gene function in a tissue specific manner. For example, targeted deletion of a tissue specific enhancer in mouse, leading to abolished expression in the tissue in question, would allow for studying gene function of otherwise lethal mutations. Additionally, enhancers could be used to drive expression of Cre-recombinase in a tissue and/or time specific manner. A new approach that may be promising in cis-regulatory region predictions is to make use of know interaction of pairs of transcription factors as tissue specific functional units (Hu and Gallo, 2010).

However, the more we unravel the more complex the cis-regulatory machinery appears to be. In a recent study by Smith and coworkers, demonstrated the modulation of enhancer activity, as they showed that the activity of a enhancer region of the *TAL1* gene, involved in development of the haematopotic system, can be boosted by a non-enhancer adjacent region (Smith et al., 2008). Further, it has recently been shown some cis-regulatory regions preside within exons of neighboring genes, implying that the search space used when searching for cis-regulator sequences, may need to be changed to encompass these regions. It is also important to keep in mind, that cis-regulatory regions only in part determine genes expression, as epigenetic factors such as nucleasome positioning, DNA methylation and histone acetylation also are involved in regulation of gene expression.

Lastly, to unravel the regulatory networks governing gene expression over time and space, the combined knowledge from computational, *in vitro* and transgenic assays will be needed.

Motoneurons (MNs) play an important role in the control and regulation of bodily functions including movement, metabolism and nutritional state by acting on muscles, visceral ganglia and peripheral organs. A MN is classically defined as a neuron with its cell body located within the CNS and that projects its axon to directly or indirectly innervate muscles. Based on their targets, MNs are classified into somatic MNs (sMN), which directly innervate skeletal muscles, and visceral MNs (vMN). vMNs are further divided into special vMN, which directly innervate brachial muscles and general vMN, which indirectly innervate cardiac muscles and the smooth muscles of the viscera. General vMN synapse to ganglionic neurons that make synaptic connections with the muscles.

During development instructive positional information from signaling centers in and adjacent to the neural tube provide developmental cues governing cell fate. All MNs are born from ventrally located progenitor cells in the neural tube in response to high levels of the morphogen Shh secreted by the notochord and FP (Briscoe and Ericson, 2001). Visceral and somatic MNs are generated from adjacent but distinct ventral progenitor domains (Ericson et al., 1997b). In the developing hindbrain (HB) vMNs are born from Nkx2.2 expressing progenitors directly dorsal to the FP while the more dorsal sMN progenitor domain expresses the bHLH protein Olig2 (Briscoe et al., 1999; Ericson et al., 1997b; Novitch et al., 2001). Therefore, the initial diversification of MN subtypes is dependent on patterning along the DV axis. Nkx2.2 and Olig2 not only serve as progenitor domain markers but are also involved in governing the developmental programs of MN, e.g. Nkx2.2 is involved in initiating the cell type specific differentiation program of vMN by up regulating the expression of generic vMN transcription factors such as Phox2b, Tbx20 and Nkx6 (Pattyn et al., 2003a; Pattyn et al., 2003b; Song et al., 2006).

Additional diversity is generated by patterning mechanisms acting along the AP axis of the neural tube and involves the nested but overlapping expression of the Hox family of transcription factors (Cordes, 2001; Jacob et al., 2001; Tumpel et al., 2009) resulting in positional distinction between seven or eight metameric compartment called rhombomeres (r). This level of diversification underlies the generation of different cranial nerves in the hindbrain (Bell et al., 1999; Jacob and Guthrie, 2000; Jungbluth et al., 1999; Warrilow and Guthrie, 1999) Additionally, the rhombomeric origin may contribute to the diversity within one cranial nerve, as trigeminal MNs generated from r2 and r3 project into different peripheral branches (Warrilow and Guthrie, 1999).

The high complexity level of the cranial nerves cannot be explained by such limited number of parameters; therefore yet another diversification mechanism must exist. It is known that at other levels of the CNS, for example the cortex and the retina, time is an important factor in the process of cell and subtype specification from a given progenitor domain (Pearson and Doe, 2004). However, it is unclear how vMNs subtypes are established in the developing HB. In order to address the question of diversity generation over time, we focused our attention to the vagal nerve (**Paper II**)

THE VAGAL NERVE.

The vagal nerve (the 10th cranial nerve) is the longest of the cranial nerves and was before referred to as the "wandering nerve". The vagal nerve exits the brainstem via the jugular foramen and its different sub branches innervate various targets such as larynx, pharynx, heart, lungs, intestines and gut to perform distinct functions at these targets (Ruffoli et al., 2010). In addition to motor (efferent) output to various organs, the vagal nerve carries sensory (afferent) information back to the CNS, in fact only 10-20% of the vagal nerve fibers comprise of motor fibers (Ruffoli et al., 2010). The vagal nerve represents the major parasympathetic component of the autonomic nervous system and many vital bodily functions have been shown to be regulated by the vagal nerve, ranging from sweating and thermoregulation, heart rate, gastrointestinal peristalsis to regulation of blood sugar levels and pancreatic insulin response (Szekely, 2000; Teff, 2008). Additionally, decreased vagal functions have been implicated as a risk factor for cardiovascular disease (Thayer and Lane, 2007). Further, vagal nerve stimulation therapy is used to treat a vast variety of neuro-psychological disorders, ranging from epilepsy and depression to obesity (Gillig and Sanders, 2010; Hatton et al., 2006). Interestingly, vagal activity has also been associated with infant growth and socio-emotional development by stimulating gastric motility, which mediates weight gain in infants as well as in mother-child interactions promoting the social behaviors of attentiveness, facial expressions and vocalizations (Field and Diego, 2008). Taken together, the many distinct roles for the vagal nerve at different targets imply that a large number of subtypes of mature vagal neurons must exist. However, little is known about how initial vagal vMN diversity is established during embryonic development.

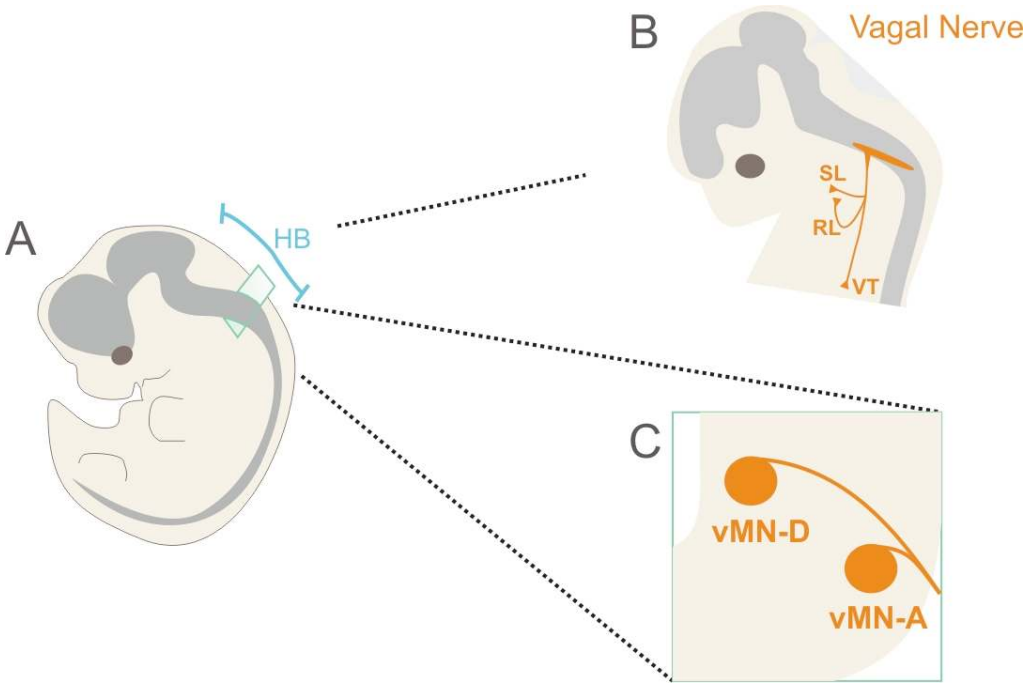

Figure 6. Schematic illustration of the vagal nerve. Drawing of an E12.5 mouse embryo with hindbrain (HB) indicated in blue. Close up of embryo head with the vagal nerve drawn in orange and the three main vagal branches are indicated: superior laryngeal branch (SL), recurrent laryngeal branch (RL) and the vagal

trunk branch (VT). Cross section of the caudal HB with the vagal nuclei motor neuron nucleus of the 10th cranial nerve (vMN-D) and nucleus ambiguus (vMN-A) indicated.

The cell bodies of the MN component of vagal nerve are organized into two separate nuclei in the caudal HB, the dorso-medially located dorsal motor nucleus X, referred to here as vMN-D, and the more lateral nucleus ambiguus, the vMN-A. These vagal cell body aggregations give rise to longitudinal columns that stretch along the length of r7 and r8, and receive sensory input from the closely positioned tractus solitarius. A small fraction of vagal MNs are found in an intermediate zone between the vMN-A and vMN-D, and are closely associated with the intermedullary fascicles of the vagus nerve (Hopkins et al., 1996), and will not be considered as a separate entity in this thesis.

In contrast to axons of the somatic hypoglossal nerve, which exits in the ventral neural tube, vagal efferent axons exit the CNS at more dorsal positions to innervate a wide range of targets. The brachial motor component of the vagus nerve originates in the vMN-A, and has three branches; the pharyngeal branch innervates the muscles of the soft palate and pharynx, the superior laryngeal branch innervates the cricothyroid muscles and the recurrent laryngeal branch innervates the larynx. The vMN axons originating in the vMN-D project into the vagal nerve to innervate ganglionic neurons, which are located adjacent to each target organ. They innervate the glands of the larynx and pharynx, the thorax –including lungs, esophagus and heart where they regulate bronchoconstriction, peristalsis and heart rate, respectively, and to the abdomen including the stomach, the pancreas and the intestine where they regulate secretion and constriction of smooth muscles (Gillig and Sanders, 2010). Additionally it has been shown that the mature vMN-D is organized into longitudinal columns related to the innervation pattern by its subdiagphramic branches, as cells located medially innervate the stomach (corpus/antrum), pancreas and liver, lateral cells innervate the forestomach (funcus) and most laterally located cells project to the cecum and perhaps the heart (Hopkins et al., 1996). Also the vMN-A is organized in two major divisions; the first contains special vMN organized into three columns along the rostro-caudal axis dependent on innervation pattern – divided into the esophageal MNs, pharyngeal constrictor MNs and laryngeal MNs in a rostral to caudal manner. The second division of the vMN-A contains general visceral preganglionic neurons innervating the heart, lungs and other supradiagphragmic regions (reviewed in (Hopkins et al., 1996)). Note, the origins of the cardiac innervation of the vagal nerve have been less clear and have been attributed to both the vMN-D and vMN-A, however the majority of studies now point toward that the vMN-A provides the major contribution to heart innervation (Hopkins et al., 1996).

In humans, neurogenesis of the vagal nerve commences at about five weeks of gestation and cell migration occurs in the sixth week (Cheng et al., 2008). Most studies of the vagal nerve have concentrated on more mature aspects of vagal biology (Cheng et al., 2008). Several studies have focused on fetal development and shown that by gestational week 13 the vMN-D, is comprised of at least two sub nuclei, while two weeks later there are three sub nuclei and by week 21 all eight (or nine) known adult sub nuclei are distinguishable (Cheng et al., 2004; Cheng et al., 2008; Huang et al., 1993; Nara et al., 1991). Studies of the vMN-A have shown that vMN-A cells initiate their dorsal migration at around 6.5 weeks of gestation and the adult organization of the vMN-A is evident at around 10 weeks, while mature

characteristics appear at about 12.5 weeks of gestation (Brown, 1990). Additionally, observations indicate that the human vMN-A develops along a rostrocaudal temporospatial gradient (Brown, 1990).

Behavioral studies have shown that the neural circuits involved in vagal reflexes appear to be established during the early part of fetal life (Humphrey, 1968; Muller et al., 1981). Sporadic gastric peristalsis has been reported already at 14 weeks of gestation in human embryo (Sase et al., 1999) and swallowing has been observed at 12 weeks of gestation (Brown, 1990). Evidence points to that the structural development of the vagal nerve occurs in parallel with the functional maturation of target organs, such as the gut and heart (Allan et al., 1980; Sase et al., 2005; Sase et al., 1999).

Additionally, the vagal nerve appears to possess some regenerative potential, as it has been reported that upon injury Tuj1 expressing cells within the vMN-A up regulate expression of the intermediate filament Nestin, which is expressed in stem cells, and incorporate bromodeoxyuridine (BrdU) indicating that the cells have become mitotically active (Takaoka et al., 2009).

MOLECULAR IDENTITIES AND PROJECTION PATTERNS.

Prior to our study, little was known about early development of vagal vMN subtypes. Shortly after post mitotic vagal vMNs enter the mantle layer, they initiate a dorsal migration towards their adult settling positions close to the sulcus limitans delineating the alar-basal boundary in the neural tube. In order to study the specification of different vagal vMN subtypes we were interested in identifying subtype specific markers in the vagal nerve (**Paper II**).

We were able to discriminate between three groups of vagal vMNs based on subtype specific expression of transcription factors. The vMN-As comprises of one subtype, which is characterized by the expression of Isl1/2, Brn3a, Oct-6, Lhx4 and Cst. The vMN-D comprises of two subpopulations of neurons, one that expresses Nurr1 and Klf6 and another one that expresses Isl1/2. Also, we observed that the subtype specific characteristics appear before the neurons reach their final destinations, implying that subtype specific properties are established early during development (**Paper II**). The identification of subtype specific markers already at E9.5-12.5 allowed us to discriminate between different vagal subpopulation at early developmental stages, allowing for a detailed study of the early development of the vagal nerve.

In neuroscience, two techniques have been widely used to investigate the relationship and connections between the neuronal cell body and the axonal terminus. Anterograde tracing is used to trace neural connections from the cell body to their point of termination, while retrograde tracing traces from the axonal termination point back to the cell body. Both techniques allow for visualization of axonal transport by using either genetic or molecular tracers. Previous retrograde labeling experiments involving the vagal nerve have predominantly focused on fetal or mature/adult stages and therefore did not address the initial development and establishment of vagal projections (Hopkins et al., 1996).

By retrograde labeling of axons from the vagal nerve branches, using fluorescent conjugated dextrans, we show that MN subtype correlates with projection pattern already at E12.5 (**Paper II**). When we trace the nerve at the exit point from the CNS we label both the vMN-A and the vMN-D. Instead tracing the

nerve in the vagal trunk (VT) labeled the Nurr1^{+} population of the vMN-D and tracing the recurrent laryngeal nerve, which innervates the smooth muscles around the epiglottis, we labeled the vMN-A population. The Isl1/2^{+} population of the vMN-D likely corresponds to the branch of the vagal nerve that innervates the soft palate, although due to technical difficulties we did not succeed with exclusively labeling the vMN-DL cells.

We show that the formation of the vMN-A precedes that of vMN-D and that the subtype characteristic expression profile is a premigratory feature of both early and late born subtypes (**Paper II**). Retrograde labeling of axons at the vagal nerve exit point (EP) showed that Isl1/2^{+} vMNs had initiated their dorsal migration at E10.5 and settled in a position characteristic to the vMN-A by E12.5. At this time point, no vMN-D cells were labeled. First at later developmental stages did retrograde tracing label migratory Nurr1^{+} cells, in addition to the Isl1/2 population. At E12.5 both subtypes were located in their final positions and both subtypes were labeled by tracing from the EP.

The sequential appearance of vagal vMN subtypes, suggested to us that the Isl1/2^{+} vMN-A and the Nurr1^{+} vMN-D are born at different time points during development or that the vMN-D could be generated by a later conversion from the vMN-A subtype. By labeling mitotically active progenitors with the thymidine analogue BrdU and analyzing the cells labeled with subtype specific markers, we examined the relative birth date of vMN-A and vMN-D neurons. BrdU is incorporated into the DNA during the S phase of the cell cycle, which allows for discriminating between mitotically active and inactive cells. Our data show that the cells of the vMN-A are generated prior to the vMN-D cells (**Paper II**). Additionally, we observed that the Isl1/2^{+} vMN-DL population is generated after the Nurr1^{+} vMN-D cells (**Paper II**).

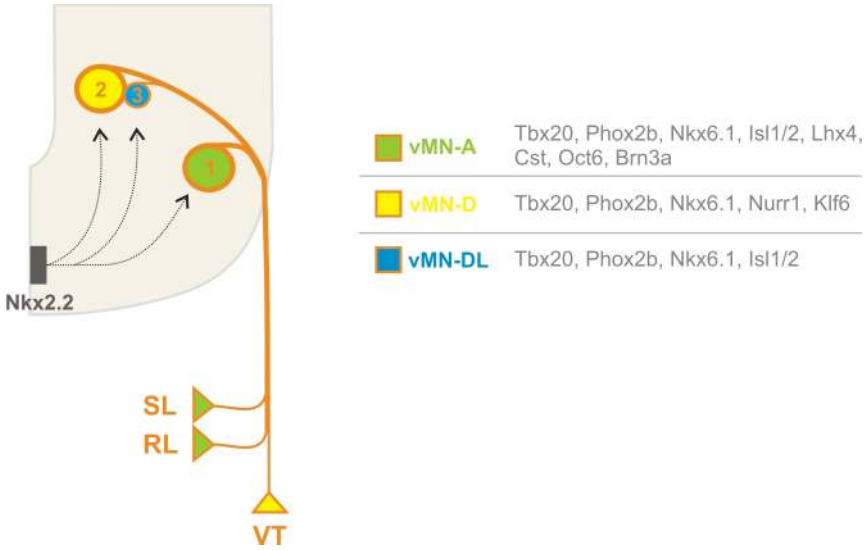

*Figure 7. Summary of findings from **Paper II**. Three subtypes of vagal visceral motor neurons, the vMN-A (green), the vMN-D (yellow) and the vMN-DL (blue) are distinguishable already at E12.5 and correspond to distinct projections in the periphery superior and recurrent laryngeal (SL and RL) and the vagal trunk nerve (VT). Additionally, these subtypes are generated sequentially independent of contact with peripheral targets or cell-cell contact.*

In summary, we show that the birth order of vMN is predictive of their subtype fate (**Paper II**). However, our data does in no way exclude further refinement of expression profile and innervation pattern at later developmental stages. It is highly probable that such refinement occurs, providing the vagal vMN with the necessary information and properties to refine the neuronal connections to various target tissue. An interesting observation has been made by Sato and colleagues where, by using fast voltage-sensitive dyes they monitor electrical impulses of vagal neurons, and show that the vMN-D acquires ability to generate action potential at day E13 in rat, while the vMN-A is still silent by E15-16 (Sato et al., 1998) suggesting that the relationship between temporal generation and functionality onset might not be straightforward.

GENERATION OF DIVERSITY.

The observation that different subtypes of vMN were generated sequentially over time, prompted us to investigate what mechanism underlies the diversification of vMN of the vagal nerve. A key question is what kind of mechanism is involved in the specification of the different subtypes. There are five conceptually different ways this can occur, namely:
1) Different subtypes of neurons may arise due to the signals they receive from their peripheral targets.
2) An instructive extrinsic signal may affect the cell type generated. In this case the progenitor population does not change over time, but the signal acts on post mitotic neurons giving them their identity.
3) An intrinsic cue may influence the progenitors, such that the progenitors change over time and therefore the neurons generated from them.
4) Signaling from earlier born populations may influence the fate of later born cells.
5) Combinations of the above.

We first investigated if subtype identity specification is dependent on signaling from peripheral targets. By isolating and *in vitro* culturing the $Nkx2.2^{+}$ domain from E9.5 embryos for 24, 48 and 72 hours we were able to recapitulate the generation of the three vagal vMN subtypes in a sequential manner. This indicated that subtype identity is independent of contact with peripheral targets (**Paper II**).

We next wanted to investigate if the decision to become a specific subtype is made at the level of progenitor or at early post mitotic level. We developed a single cell approach where we isolated the $Nkx2.2^{+}$ progenitors from caudal HB and dissociated the progenitors into single cells for culture. 45 minutes prior to sacrificing we inject pregnant females with BrdU to label the mitotic progenitors. Under our culture conditions, the $Nkx2.2^{+}$ progenitors differentiated into neurons based on Tuj1 immunoreactivity. We observed that a majority of post mitotic $Tuj1^{+}$ neurons co-expressed the vMN marker Phox2b in addition to Isl1/2, thus most likely represented vMN. In fact, we were able to observe single cells expressing vagal subtype specific markers Cst and Klf6 (**Paper II**).

Next, we labeled mitotic progenitors with BrdU and asked if different subtypes are labeled depending on the developmental time point of BrdU injection. We observed that when we isolate $Nkx2.2^{+}$ progenitors at 15-18 somite stage a majority of the $BrdU^{+}$ cells co-expressed Cst. In contrast, when we isolated the same progenitors from older 22-25 somite embryos, the majority of $Tuj1^{+}$ cells expressed

Klf6. These data suggest that post mitotic signals are not required for the expression of subtype markers (**Paper II**).

Forcing the cells to leave the cell cycle has been shown to be insufficient to alter cell fate decisions (Lobjois et al., 2008), therefore the potential effects on the cell cycle exit related to the experimental setup should have no effect on the subtypes generated in our experiments (**Paper II**)

MECHANISMS OF DIVERSITY GENERATION IN OTHER VERTEBRATE SYSTEMS.

Diversity within the neuronal lineages, at other CNS levels, is also established by the generation of different subtypes over time from a given progenitor domain. For example, in the retina and the cerebral cortex, different neuronal subtypes are produced in a sequential manner (Pearson and Doe, 2004).

In the retina, progenitors divide to give rise to seven major cell types organized into three layers present in the eye; outer nuclear layer (rods and cones), inner nuclear layer (horizontal, bipolar, amacrine and Muller cells) and ganglion cell layer. Birth dating with BrdU and lineage tracing using retrovirus have shown that the generation of each cell types occurs in a fixed but overlapping order, many progenitors are multipotent, sibling cells often have different fates and that progenitor clones vary in size and cell type composition (reviewed in (Pearson and Doe, 2004)). In summary, it appears that retinal progenitor cells (RPCs) have a bias toward producing some cells early and some late. Heterochronic experiments have shown that RPCs retain donor properties when transplanted into older cell environment, implying a strong contribution of cell intrinsic temporal identity cues (Belliveau and Cepko, 1999; Morrow et al., 1998; Rapaport et al., 2001). It is noteworthy that in prolonged cultures of old RPCs cells, the old progenitors start to give rise to early born fates (James et al., 2003). There seem to be differences in the developmental potential of young vs. old RPCs as the cells change their responsiveness to instructive signals suggesting that they go through competence stages (Cayouette et al., 2003). Additionally, late fates appear more sensitive to extrinsic signals, since the number of cells they give rise to can be modified (Ezzeddine et al., 1997). Cayouette and colleagues suggest that the cell intrinsic mechanisms play a major role in the retinal progenitors cell fate decision, as culturing RPCs in serum-free or serum containing media (where the environment is complex and changes with time) did not lead to any differences as to what cell types were generated (Cayouette et al., 2003). The diversification of the retina seems to involve both cell intrinsic and extrinsic cues. A purely extrinsic cue cannot account for the overlap in birthdates of cells, while exclusively intrinsic cues do not explain switch of old progenitors to produce early fates. Additionally, cell culture experiments suggest that extrinsic signals may be important in phenotype maturation, as cells in serum free cultures failed to turn on late and/or mature markers (Cayouette et al., 2003).

In the cerebral cortex it has been shown that early born cells make up the deepest layers while late born cells make up the outer layers of the cortex only the very first born neurons do not follow this rule and make up the outermost layer (Berry and Rogers, 1965; Campbell, 2005; McConnell, 1988; Shoemaker and Arlotta, 2010). Retroviral lineage tracing has shown that progenitors are multipotent (Walsh and Reid, 1995). Interestingly, data from heterochronic transplants indicate that cells that have not undergone their terminal division are competent to respond to

cues from the older host, in contrast to already post mitotic cells (McConnell and Kaznowski, 1991). However, older progenitors transplanted into an early environment, were unaffected (Frantz and McConnell, 1996). This implies that extrinsic cues play an important role in the cell fate specification of cortical progenitors and that the older progenitors undergo a progressive restriction of their developmental potential (Campbell, 2005).

Interestingly, we observed that as the Nkx2.2 domain expands over time in the *Pax6* mutants (Ericson et al., 1997b; Hill et al., 1991), the newly recruited Nkx2.2^{+} population of progenitor cells (referred to as Nkx2.2') gives rise to the late born subpopulation of vagal vMN similar to cells born from the endogenous Nkx2.2 domain at the same developmental time point (**Paper II**). The newly recruited Nkx2.2' cells do not generate the early born subtype vMN-A and instead begin directly to generate vMN-D cells, skipping the early subtype completely. It is tempting to speculate if this is a reflection of progressive restriction of developmental potential inherent to the progenitor cells or if it is a reflection of the overall developmental stage of the embryo.

It has been shown that in the pancreas, progenitors go though competence stages each allowing for the generations of specific subtypes of endocrine cells in a partially overlapping order (Johansson et al., 2007). This is reminiscent of the progressive change in the competence of progenitors is in the cortex and retina – an indication that the mechanisms of gradual restriction of progenitor competence may be extended outside the CNS.

In the developing SC, the combinatorial code of LIM HD proteins have been shown to play important roles in the development of distinct spinal motor columns by triggering sMN differentiation, specification of subtype identity and axonal path finding, by regulating guidance molecules (Jessell, 2000; Misra et al., 2009). Additionally, expression of other transcription factors e.g. Pea3, Er81, Runx1, Scip and Nkx6.1 have been shown to distinguish motor subtypes at the level of motor pools within the columns (Dasen et al., 2005; De Marco Garcia and Jessell, 2008; Lin et al., 1998; Livet et al., 2002). A number of studies have shown that the expression of Hox proteins in the SC governs the diversification of MN in the SC (Guthrie, 2007; Kanning et al., 2010; Misra et al., 2009). Additionally, it has been shown that early born sMNs, innervating the limb, influence the differentiation of late born MNs by the secretion of retinoic acid (Misra et al., 2009; Vermot et al., 2005). Thus it appears as if there are also temporal mechanisms influencing the generation of subtype diversity at several levels of the CNS.

Interestingly, in the ventral SC the p2 progenitor domain gives rise to two subtypes of V2 IN, the V2a and the V2b, involved in the network of locomotion (Kiehn, 2006). The transcription factor Foxn4 has been shown to be expressed in all p2 progenitors, but only to be required for the V2b fate (Del Barrio et al., 2007; Li et al., 2005). Foxn4 has also been shown to activate the Notch Delta signaling pathway to introduce asymmetry by lateral inhibition in the p2 domain, allowing for the generation of both V2a and V2b subtypes from an initially homogenous progenitor domain (Del Barrio et al., 2007). Del Bario and colleagues report that while electroporation of Delta4 at HH stage 14-16 blocked the generation of V2a neurons without increasing the V2b population, electroporation at stage 11-12 lead to a reduction of the number of both subtypes. This result points to differences that are time dependent and raises the question if the temporal component is involved in V2 IN subtype specification. However, there is no direct evidence of that V2a INs are generated before V2b INs, instead studies point to that the two V2 populations are generated simultaneously (Karunaratne et al., 2002; Liu et al., 1994).

DROSOPHILA NEUROBLASTS.

During the embryonic development the neurons of the CNS of *D. melanogaster* are generated from progenitor cells that are termed neuroblasts. Each neuroblast forms at a specific time and position by delamination from the neuroectoderm and is characterized by a unique expression profile of molecular markers. Neuroblasts, in turn, undergo asymmetric divisions to give rise to ganglion mother cells (GMCs), which divide one more time and give rise to either two neurons, two glia or a neuron and a glial cells (reviewed in (Pearson and Doe, 2004). It has been shown that every neuroblast is multipotent and gives rise to a unique clone of progeny in a reproducible order (Bossing et al., 1996; Schmid et al., 1999; Schmid and Tautz, 1997).

Several studies have shown that intrinsic temporal determinants govern the sequential delamination from CNS neuroblasts (Doe, 2008; Sousa-Nunes et al., 2010; Zhong, 2003). The transitions in cell type generation appear to be regulated by a series of transcription factors that regulate competence windows of the neuroblast, allowing the production of different cell types over time (Sousa-Nunes et al., 2010). The expression of the transcription factors Hunchback (Hb), Pdm, Castor (Cas) and Krüppel (Kr) has been shown to correlate with birth order of neurons but is also required for their subtypes (Brody and Odenwald, 2000; Cui and Doe, 1992; Isshiki et al., 2001; Kambadur et al., 1998). As the neuroblast divides, its progeny, the GMC, inherits its temporal expression profile and in turn confers the developmental fate to its progeny, the post mitotic neurons or glia (Doe, 2008). Several studies have defined the time and sequence of expression of these transcription factors; Hb, Kr, Pdm, Grainyhead and Cas, and showed that their expression correlated with temporal identity (Grosskortenhaus et al., 2005; Isshiki et al., 2001; Kambadur et al., 1998; Pearson and Doe, 2004). The expression of these temporal determinants did not correlate with, or limit, the generation of specific cell types, but instead appear to specify temporal identity in multiple lineages (Pearson and Doe, 2004). For example, Isshiki and colleagues showed that the loss of Hb expression resulted in a cell fate in the early lineage, but did not affect later born fates (Isshiki et al., 2001). Forced expression of Kr early in the lineage, on the other hand, directed cells toward later generated fates (Isshiki et al., 2001). The unique spatial and temporal identity of neuroblasts appears to be integrated to provide each cell with its unique developmental potential and fate.

Interestingly, we identified a number of homologs to the identified Drosophila temporal determinants Hb, Pdm1, Cas and Grainyhead to be expressed in the different vagal vMN subtypes. The vagal vMNs express for example Cst (vertebrate Castor), Krüppel like factor 6 (Klf6) and SCIP/Oct6 (**Paper II**). However, the order of expression is not easily transferable from the Drosophila model as well as the co-expression of Oct6 and Cst in the vMN-A adds to the level of complexity. In addition we did not identify any vagal phenotype in mice mutant for *Cst* (Joanna M Klos-Applequist and Mattias Karlén, unpublished data). Further, no identified phenotype was observed in the *Oct6* mutants (Mattias Karlén, unpublished data). The lack of phenotypes in these mutants could be explained by the co-expression of compensatory factors i.e. Lhx4, Isl-1 and Oct6/Cst, respectively, since multiple temporal homologs are co-expressed within the same subtype population (**Paper II**, Joanna M Klos-Applequist and Mattias Karlén, unpublished data). Interestingly, *Ikaros*, a mouse ortholog of the Drosophila *Hunchback* gene, is expressed in all early embryonic RPCs, and has been shown to

be both necessary and sufficient to confer early temporal competence of RPCs. The *Ikaros* protein is expressed in early RPCs, while late RPCs do not express Ikaros. Forced over expression of Ikaros in late RPCs leads to generation of early born fates, while in *Ikaros* deficient mice the number of cells of early fate is reduced. This suggests commonalities between the mechanism governing cell type diversification in the mammalian retina and that of the *Drosophila* neuroblast lineage and suggests that the loss of competence to generate early fates in late RPCs is coupled to the loss of Ikaros expression (Elliott et al., 2008). However, it is noteworthy that, the late fates are not affected in *Ikaros* mutants (Elliott et al., 2008). Preliminary analysis of embryos mutant for *Ikaros*, did not reveal any defects in the organization of and/or expression pattern in the vagal vMN subtypes (Joanna M Klos-Applequist preliminary results). Whether the temporal component is intrinsically common to all progenitors, inherited or induced, it is evident that it is an integral part in the decision to generate a certain neuronal subtype lineage.

SYMMETRIC AND ASYMMETRIC DIVISIONS.

By live fluorescent labeling in the zebrafish it has been shown that over 80% of neurogenic divisions generate pairs of neurons and only 11% of divisions are asymmetric and give rise to a progenitor and a neuron sibling in the hindbrain, indicating that unlike Drosophila (Lu et al., 2000), at least in this vertebrate system, asymmetric divisions do not play a large role in controlling if a cell will become a neuron or not (Lyons et al., 2003). After a BrdU pulsing, we isolated the ventral midline cells encompassing mainly the FP and the Nkx2.2 domain from E9.5 embryos, dissociated the cells and plated for *in vitro* culture. We scored to what extent $BrdU^+$ cell pairs expressed the same or different identities – based on Cst or Klf6 immunoreactivity. We observed that over 85% of cell pairs generated expressed the same identity, indicating that the large majority of divisions were symmetric in character (Joanna M Klos-Applequist, unpublished data). Clonal analysis in the chick has provided evidence that progenitors often give rise to clones of neurons of one single subclass, implying that a large portion of cell divisions in the avian CNS are symmetrical (Clarke et al., 1998; Lyons et al., 2003). However in the cortex, there is some evidence of asymmetric divisions, since isolated clones after expansion often generate two neurons with different characteristics (Qian et al., 1998). As data from different systems and/or developmental time points and locations, point to differences in the mechanisms for diversity generation, there appears to be a high level of complexity warranting further investigations.

SUBTYPE SPECIFIC DETERMINANTS.

Our study also reveals selective roles for Nkx6.1 and Nurr1 for the differentiation of vMN-A and vMN-D cells.

The role of Nkx6.1 in vMN-A specification.

The HD transcription factor Nkx6.1, and the closely related Nkx6.2, are both expressed in the ventrally located vMN progenitors in the hindbrain and both have been shown to have a partial overlapping requirement in the specification of MN fate at the neural progenitor stage (Briscoe and Ericson, 2001; Sander et al., 2000; Vallstedt et al., 2001). Nkx6.1 has been shown to have a role in MN generation,

however MNs are still generated at caudal SC levels in *Nkx6.1* mutant mice (Sander et al., 2000; Vallstedt et al., 2001). This can be explained by the derepression of Nkx6.2 expression in MN progenitors in *Nkx6.1* mutant mice, leading to a compensation for Nkx6.1 activity by Nkx6.2 (Vallstedt et al., 2001). In addition, the expression of Nkx6.1 and Nkx6.2 is also maintained in post-mitotic visceral MNs up to late developmental stages (Pattyn et al., 2003a).

Nkx6.1 is expressed in the both the early born vMN-A and late born vMN-D cells, however only the vMN-A population is effected in the mutants (**Paper II**). The *Nkx6.1* deficient embryos exhibit a significantly, albeit not complete, reduction of migrating presumptive Isl1/2^{+} vMN-A cells as compared to controls at E10.5. Additionally, *Nkx6.1* mutants lack the expression of early subtype markers e.g. Cst and Lhx4, supporting that the vMN-A population is missing in these mice. Whole mount analysis of vagal nuclei and axonal trajectories show that the vMN-A cell bodies are missing at caudal hindbrain levels and that there is a loss of SL and RL projections in the periphery (**Paper II**). In contrast, the late born Nurr1^{+} vMN-D populations as well as the VT projections appear unaffected in *Nkx6.1* mutant mice (**Paper II**). It is noteworthy, that the size of the vMN-D in *Nkx6.1* mutant mice appears to be increased. This can be explained by that some of the vMN-A cells may have taken on a cell fate characteristic of the vMN-D, leading to a change in their spatial location in the caudal hindbrain as scored by ß-Gal expression in the vMN-D (**Paper II**). Alternatively, the derepression of Nkx6.2 in the absence of Nkx6.1 may also, at least in part, explain increase in intensity of the ß-Gal staining in *Nkx6.1*$^{-/-}$ *Nkx6.2* $^{lacZ/+}$ embryos. A detailed analysis of the phenotype in *Nkx6.1* deficient mice will be needed to answer this question, however it is clear that Nkx6.1 is required in the specification of early born vagal vMN cells.

Nurr1 is required for manifestation of the vMN-D subtype.

We show that the orphan nuclear receptor Nurr1, a key factor in the specification and maintenance of midbrain dopaminergic neurons (Kadkhodaei et al., 2009; Wallen et al., 1999; Zetterstrom et al., 1997), is also involved in manifesting the vagal late born vMN-D subtype (**Paper II**).

It was previously shown that vagal efferent projection mutants exhibit subtle defects in the proximal axonal pattern in *Nurr1* mice (Castro et al., 2001; Nsegbe et al., 2004; Wallen et al., 2001; Zetterstrom et al., 1997). Further, a loss of RET expression has been described in the vMN-D nucleus in *Nurr1* mutants (Wallen et al., 2001). However, no detailed analysis of Nurr1 function in the caudal hindbrain has been available to date.

Since we identified Nurr1 as a subtype specific marker for the vMN-D, we were interested in its functional role during vagal vMN development. Interestingly, vMN-D subtype in the *Nurr1* mutants acquires certain properties characteristic of early born vMN-A neurons. In particular, the presumptive vMN-D cells acquire the expression of the vMN-A marker Isl1/2 in addition to the vMN-D marker Klf6, implying a mixed subtype phenotype (referred to as vMN-D'). However, these cells continue to migrate to their normal dorso-medial settling position in Nurr1 mutants, indicating that part of their original subtype identity is maintained in the *Nurr1* mutants, and therefore independent of Nurr1 function (**Paper II**). Additionally, our findings suggest that Nurr1 may be involved in direct downregulation of the expression of Isl1/2 in the late born vMN-D cells.

A significant loss of vMN-D neurons projecting into the VT branch of the vagal nerve was observed in *Nurr1* mutants. A significant number of RL projecting

cells were present among the vMN-D' cells, however we did not see any significant difference in the number of RL projecting cell in the vMN-A nor the expression pattern of vMN-A cells, indicating a vMN-D specific phenotype in *Nurr1* mutants (**Paper II**).

Since the vMN-D' cells continue to express KLF6 and migrate to their normal dorso-medial settling position in *Nurr1* deficient mice, Nurr1 alone cannot govern the specification of the vMN-D subtype cells and additional factors must contribute to the specification of these cells. Additionally, Nurr1 expression is initiated at early post-mitotic stages and therefore is expressed too late to explain the initial specification of late born vMN-D cells from Nkx2.2^{+} progenitors. It is still unknown what factor is involved in the initial step of vMN-D specification at progenitor level.

To summarize, we show that Nurr1 is specifically required to manifest functional properties of late born vMNs and to suppress early born traits, however Nurr1 must act together with other factors in the specification of the vMN-D subtype.

Nurr1 mutants die shortly after birth due respiratory defects coupled to the hindbrain (Wallen et al., 2001). As *Nurr1* mutants loose the majority of the VT projecting vMN-D cells, and gain number of vMNs projecting to the esophageal region, it may be that these erroneous innervations may be involved causing parts of the respiratory defects observed in *Nurr1* mutant animals.

SPECIAL VISCERAL PROPERTIES OF THE vMN-A.

Neurons of the adult vMN-A are defined as special vMNs, meaning that they share common traits with sMNs, in addition to their inherent visceral properties. The special vMNs innervate striated muscle fibers in the esophagus directly, similar to the direct innervation of skeletal muscles by sMNs. In contrast, the general vMNs, including the vMN-D, innervate smooth muscle targets via secondary postganglionic MNs. The esophageal striated muscle fibers, innervated by the vMN-A, have a neural crest origin in contrast to somite derived striated muscles innervated by sMNs from the SC (Patapoutian et al., 1995). Interestingly, in addition to visceral identity factors the vMN-A subtype expresses the LIM HD transcription factor Lhx4, which has been shown to be involved in the specification of sMNs at the time of cell cycle exit (Sharma et al., 1998). The special vMNs of the vMN-A initiate the expression of Lhx4 at the time they have become post mitotic. It would be interesting to find out if the late expression of Lhx4 in special vMN, is involved in the establishing the sMN-like properties of special vMNs. The late post mitotic expression of Lhx4 in vMNs may allow the vMN-A to maintain the generic vMN identity while exhibiting properties characteristic to sMN. In line with this, the miss-expression of Lhx3 in early differentiating vagal vMNs has been shown to convert them into sMNs of the hypoglossal nerve (Sharma et al., 1998).

SEROTONERGIC NEURONS.

At later stages of development, the Nkx2.2 progenitors give rise to yet another neuronal subtype, the serotonergic (S) INs. This generation is initiated at E10.5, after the generation of vMNs has ceased, at all rhombomeric levels except r4 in the hindbrain (Pattyn et al., 2003a). r4 instead continues a prolonged generation of vMN. The change from generation of vMN to S neurons appears to require the

temporal downregulation of the vMN determinant Phox2b in progenitors (Pattyn et al., 2003a). Nkx2.2 has also been implicated in S neuron development, as *Nkx2.2* deficient mice display a close to complete loss of S neurons (Briscoe et al., 1999). However, the regulation of S development appears more complex, as S neurons can be induced by high levels of Shh in cultures of *Nkx2.2* mutant tissue (Joanna M Klos-Applequist, unpublished data), indicating the Nkx2.2 is not required for S neuron generation. As Nkx2 proteins have recently been shown to be involved in strengthening Shh responses in a feed-forward amplification loop at SC levels, it may be that Nkx2.2 has a similar role in promoting S neuron development (Lek et al., 2010).

The molecular events that convert the generation of vMNs to S neurons from the Nkx2.2 progenitor domain, may display similarities to the temporal events in the production of vMN subtypes. However, it may be that the mechanisms governing the switch between vMN and S neuron generation, also reflects the larger molecular differences between vMNs and S neurons.

FUTURE DIRECTIONS.

In this study we give a comprehensive description of early vagal vMN development (E9 - E12.5) in the mouse. We show that at early developmental stages we are able to distinguish at least three subtypes of vagal vMN based on the expression of subtype specific markers and that subtype identity correlates with topography within the CNS and peripheral innervation pattern.

In our study of the generation of vMN subtypes, we have not been able to identify a true temporal determinant – although we have identified genes (Nkx6.1 and Nurr1) involved in manifesting subtype properties of vagal vMN. From a theoretical point of view a true permissive temporal determinant should fulfill the following criteria; 1) expression at appropriate time 2) loss of function mutants should exhibit absence of a temporal identity 3) misexpression should lead to generation of cells with a particular temporal identity (or the converse for a temporal determinant that acts via repression). In our search we have found candidates for determinants in homologs to the Drosophila temporal determinants (Hb, Pdm, Cst and Kr) (Sousa-Nunes et al.) and correlated their expression to specific vagal vMN subtypes. However, single mouse mutant for these genes do not exhibit a temporal phenotype in the vagal nerve. This might be due to redundancy, as several factors are expressed within the same vagal subtype, therefore it would be interesting to analyze compound mutants for these genes. The identification of a true temporal determinant would be a key discovery for understanding the mechanism governing the temporal specification of vMN subtypes.

Our *in vivo* and *in vitro* data show that vagal vMN subtypes are generated sequentially and suggests that the decision to become a specific subtype of vagal vMN is confined to the ventral midline and is independent of contact with peripheral targets. Further our data indicate that the subtype decision is made at the level of progenitor, is independent of cell-cell interaction and that the competence of $Nkx2.2^{+}$ progenitors to generated different temporal identities changes over time. However, the detailed mechanism underlying this process is unknown and further investigation is warranted. As subtype generation appear to be uncoupled from the time of initiation of Nkx2.2 expression in the progenitor domain, it is interesting to speculate how an intrinsic clock – if indeed such exists – would operate.

Additionally, it would be interesting to investigate if other cranial nerve subtypes are generated in a similar fashion in the hindbrain.

Interestingly, it is estimated that about 30% of animal genes are regulated by microRNAs (miRNA) (Farh et al., 2005; Lewis et al., 2005; Miranda et al., 2006). Although the precise mechanism of miRNA mediated silencing is unknown, studies indicate that miRNA may promote the destabilization of mRNA transcripts by de-adenylation, which leads to clearance or translational repression of the target mRNA (Bartel, 2004; Kapsimali et al., 2007; Zhang et al., 2007). Many miRNAs show spatially and temporally restricted expression patterns in the CNS and for example miRNA-218a are expressed in the vagal nerve nuclei in the zebra fish embryo (Kapsimali et al., 2007). As miRNAs have been shown to be involved in the temporal control of development in *C.elegans* by regulating the orthologs of the genes involved in temporal specification of *Drosophila* neuroblasts (Abrahante et al., 2003; Grosskortenhaus et al., 2005; Lin et al., 2003), it would be interesting to see if miRNA expression is restricted to a specific vagal subtype and if such restriction reflects any subtype specific features.

The vagal nerve is one of the major cranial nerves and many important bodily functions are dependent on correct vagal innervations and activity. Understanding the development of the vagal nerve may increase the understanding of vagal neuropathies and injuries and provide insight into potential therapeutic strategies. In a broader sense, the understanding neuronal diversification will provide insight into the mechanisms for guiding cell fates towards not only spatial but also temporal identities – allowing for more tools for *in vitro* manipulation of stem cells for therapeutic use.

The CNS, contrary to what its name implies, consists of around 90 % of non-neuronal cells collectively termed glia. It was originally thought that glial cells mainly maintained a passive role in the CNS, providing structural support and holding the neurons together (Rowitch and Kriegstein, 2010). An impressive body of data has since showed that glial cells play essential roles in the functioning CNS and that glial cell pathologies are detrimental to CNS function (Emery, 2010; Rowitch and Kriegstein, 2010). Glial cells are subdivided into microglia and macroglia, dependent on their functional roles and origin.

Microglia are the resident macrophages of the brain and spinal cord (SC) and are derived from hematopoietic precursors (Graeber and Streit, 2010). Since the blood-brain barrier prevents antibodies from entering the CNS, microglia act as the main form of active immune defense and presents antigens to activate T-lymphocytes in the CNS and can also develop into macrophages (Deng and Sriram, 2005; Graeber, 2010).

Macroglial cells are generated subsequent to neurogenesis (Rowitch, 2004) and are grouped into astrocytes and oligodendrocytes. Astrocytes are involved in providing structural and trophic support for neurons, maintaining the blood-brain barrier and have also been shown to regulate synaptogenesis and synaptic transmission (Freeman, 2010; Rowitch, 2004; Rowitch and Kriegstein, 2010).

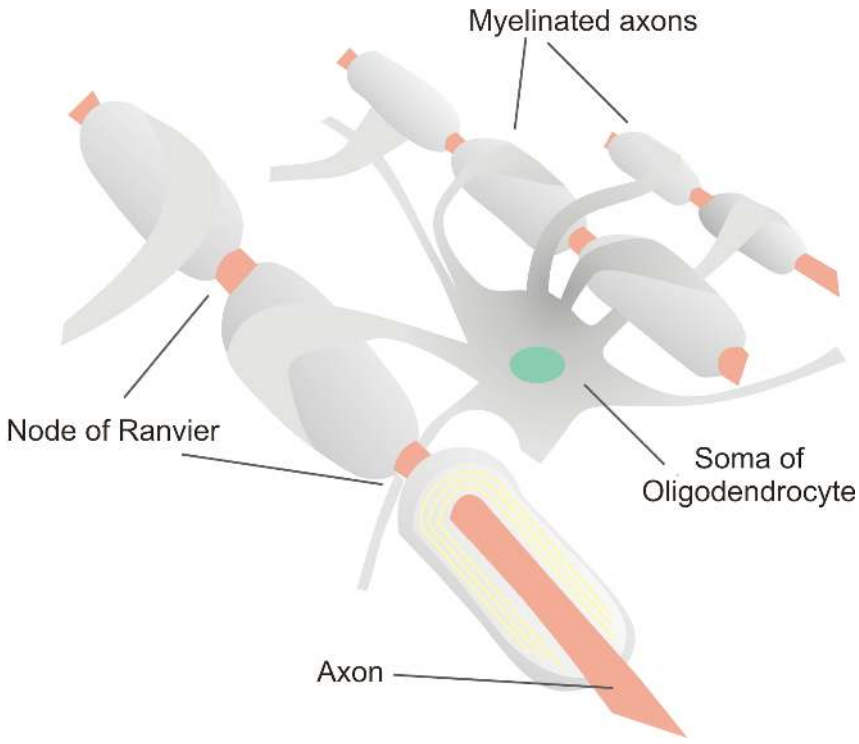

Figure 8. Illustration of an oligodendrocyte myelinating axons of central nervous system (CNS) neurons.

Oligodendrocytes are the myelinating cells of the CNS. They insulate axons by ensheating them with plasma membrane layers and promote the rapid and efficient conduction of electrical impulses by inducing clustering of sodium channels at the nodes of Ranvier (Kalsi et al., 2004; Kaplan et al., 1997). Oligodendrocytes can also provide trophic support for neurons by producing

neurotrophic factors such as BDNF, GDNF and IGF-1 (Bradl and Lassmann, 2010; Dougherty et al., 2000; Du and Dreyfus, 2002; Wilkins et al., 2003).

Additionally, it has been observed that improperly myelinated axons exhibit changes in microtubule stability and number, axonal transport rates as well as swelling and degeneration (Bradl and Lassmann, 2010; Dupree et al., 1999; Edgar and Garbern, 2004; Griffiths et al., 1998; Kassmann and Nave, 2008; Kirkpatrick et al., 2001) implying a role for oligodendrocytes in upholding axonal structure and function.

DEVELOPMENTAL ORIGINS.

Oligodendrocytes are like neurons and astrocytes, born in the ventricular zone of the CNS but once specified they migrate as proliferative precursors and undergo an extensive program of proliferation, migration, differentiation and myelination. Since mature oligodendrocytes occupy all regions of the CNS, oligodendrocyte precursor cells (OLPs) were originally thought to be generated evenly at all levels (Emery, 2010; Rowitch and Kriegstein, 2010). Additionally, radial glia, which are widespread throughout the CNS, were thought to trans-differentiate into OLPs, supporting the view of a widespread origin of OLPs (Choi and Kim, 1985; Hirano and Goldman, 1988).

Subsequently, studies in the SC suggested that OLPs are generated by a small group of progenitors close to the FP, the pMN progenitors, which give rise to sMNs at earlier stages of development ((Hall et al., 1996; Lu et al., 2002; Pringle and Richardson, 1993; Richardson et al., 2006; Rowitch, 2004; Warf et al., 1991; Zhou and Anderson, 2002). We and others have shown that the OLPs generated from this domain contribute to approximately 80-90% of the final oligodendrocyte population in the spinal cord (**Paper III**, (Cai et al., 2005; Fogarty et al., 2005).

The controversy of OLP origin in the Spinal Cord.

In the 90s two conflicting studies were published, concerning if progenitors outside the pMN domain produce oligodendrocytes *in vivo*. Although both studies applied a similar experimental approach they came to the opposite conclusion. From a series of quail-to-chick grafting experiments Cameron-Curry and Le Douarin argued that dorsal progenitors can generate oligodendrocytes (Cameron-Curry and Le Douarin, 1995) On the other hand, quail-chick grafting experiments performed by Pringle and co-workers provided data suggesting that dorsal progenitors can only produce astrocytes (Pringle et al., 1998). It is interesting to speculate how and why an, in theory simple, experimental approach led to such conflicting results. The idea behind the experiments; to remove either the dorsal or ventral part of the SC from one animal and replace with the corresponding part of the other species and then fate map the developing OLPs, is in its essence straightforward. The difficulties that arise are technical and the definition of a dorsal graft is most probably the culprit, especially since the dorsoventral origin of the graft had to be derived retrospectively upon analysis. Cameron-Curry and Le Douarin defined a dorsal graft as one with the presence of graft derived ependymal cells, assuming ependymal cells were derived equally from all DV levels throughout the SC. It was recently shown that the ependymal cells, that replace the neuroepithelial cells lining the embryonic lumen of the SC as it matures, are exclusively derived from the ventral half of the SC (Fogarty et al., 2005; Fu et al., 2003).This means that the grafts that Cameron-Curry and Le Douarin defined as dorsal, in fact had a ventral contribution, rendering their grafting

experiments inconclusive in retrospect. The study by Pringle et al. excluded grafts with ependymal cells from their analysis and concluded that only the ventral SC contributes to the OLP lineage.

The existence of a glial restricted progenitor cell that can be derived from both ventral and dorsal parts of the SC and give rise to both astrocytes and oligodendrocytes in culture has also been suggested by others (Rao et al., 1998). Additionally, several studies have reported that tripotent stem cells can be isolated from all levels of the SC (Gage, 2000; Seaberg and van der Kooy, 2003), implying that the OLPs and astrocytes progenitors (ASTs) are derived from a common progenitor pool. Further, explants isolated from dorsal SC gave rise to OLPs in culture, and OLPs also developed from neurospheres derived from animals lacking Shh signaling (Chandran et al., 2003). However, the existence of a tripotent stem cell/progenitor *in vivo* has been questioned ((Gabay et al., 2003) see later discussion) suggesting that deregulation of positional identity due to *in vitro* culturing conditions (including addition of Fibroblast Growth Factor (FGF) into culture media) is the reason for the observation of tripotent neural stem cells in culture. Therefore this observation may not reflect the endogenous potential of these cells *in vivo*. Indeed, we show that the addition of FGF2 to explant cultures *in vitro* induces oligodendrocyte differentiation in cells not fated to generated OLPs *in vivo* (**Paper III**). A study by Sussman and co-workers showed that explants isolated from mouse dorsal SC at E11.5 and cultured for a short time period did not generate OLPs, while OLPs developed in the corresponding ventral explants. However, in a subset of long term cultures of dorsal SC, Sussman and colleagues observed $O4^+$ OLPs, suggesting that the dorsal SC had the potential to generate OLP under appropriate conditions. Another study took advantage of a transgenic mouse expressing ß-gal under the PLP/DM20 promoter and also suggested a possible dorsal source of OLPs in the SC (Spassky et al., 2000). However, no direct evidence for the existence of dorsally derived OLPs *in vivo* had been presented.

We and Cai et al. observed the presence of $Olig2^+$ cells in the dorsal progenitor zone in the SC of wild type mice at E15.5 (**Paper III**, (Cai et al., 2005)). These cells showed co-expression of dorsal progenitor markers Pax7 and Gsh1/2. Formally, two explanations for this observation were proposed. First, oligodendrocytes were generated at dorsal levels of the SC. Second, $Olig2^+$ cells generated ventrally had invaded the dorsal progenitor space and turned on the expression of markers characteristic to dorsally derived cells.

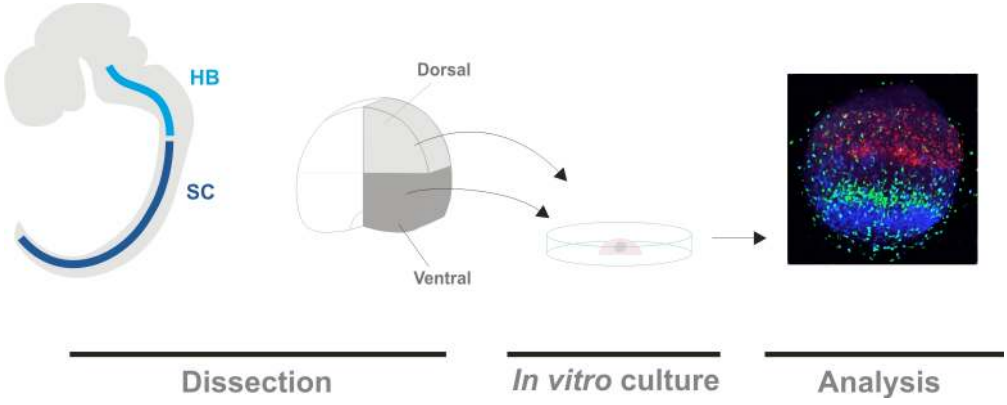

Figure 9. Illustration of the experimental approach for explants. Upon isolation of SC was divided into dorsal and ventral parts, which were cultured and scored for the presence of oligodendrocyte markers by immunohistochemistry.

By isolating ventral and dorsal tissue from the developing SC prior to any dorsal migration of ventrally derived OLPs, we were able to show that dorsal SC indeed had oligodendrogenic potential (Fig 9) (**Paper III**). We identified expression of OLP markers, Olig2, PDGFRα, O4 and the mature marker myelin-basic-protein (MBP) in dorsal SC cultures that had retained their DV identity. This implies that OLPs observed in the dorsal SC actually were generated there. The presence of dorsal progenitor marker expression in the cultures argues against the possibility that OLPs observed in dorsal explants were generated due to an *in vitro* induced ventralization. Further, our analysis of *Nkx6.1 Nkx6.2* (collectively referrred to as *Nkx6*) compound mutant mice, which lack the ventral pMN domain, showed that the dorsal SC generates oligodendrocytes *in vivo* (**Paper III**, (Cai et al., 2005)). Nkx6 proteins are only expressed ventrally and not dorsally, suggesting that the lack of them would not affect a putative dorsal OLP population. In control embryos $Olig1^+$ OLPs cells were distributed all over the SC, while $Olig1^+$ cells were in majority located in the dorsal half of the SC in *Nkx6* mutant mice. $Olig2^+$ cells in the mutants were also found to co-express characteristic markers of dorsal progenitors supporting their dorsal origin (**Paper III**, (Cai et al., 2005)). Importantly, $Olig2^+$ cells co-expressing Pax7 were also found in control embryos at E15, indicating that these cells were not an artifact only present in *Nkx6* mutants, but a naturally occurring population. These precursors had been overlooked in earlier studies, probably due to that they are generated later than the ventrally derived cells and at a time point when the vOLPs have already migrated into the dorsal parts of the SC, making it hard to distinguish a dorsally derived population. Since *Nkx6* mutants die at birth due to breathing failure, further *in vivo* analysis of differentiation and maturation of dOLPs was impossible (Sander et al., 2000; Vallstedt et al., 2001). Instead, we isolated dorsal SC tissue from embryos prior to onset of oligodendrogenesis and after culture we were able to observe $Olig^+$ cells expressing more mature oligodendrocyte markers O4 and MBP, indicating that these cells have the capacity to differentiate into oligodendrocytes (**Paper III**, (Cai et al., 2005))

The emergence of the first dorsally derived OLPs was found to be at E15, approximately 2 days after their ventral counterparts were observed (**Paper III**, (Cai et al., 2005)) and we estimated their contribution to the final oligodendrocyte population in the SC to be approximately 20-30% (**Paper III**). In contrast, Cai et al. estimate the contribution of $Olig2^+$ dOLPs to be around 8% at E14.5 based on co-expression of Pax7. This may be an underestimate of the real number, since progenitor markers are rapidly down regulated as cells leave the ventricular zone.

Further evidence for the existence of dorsally derived OLPs comes from lineage tracing studies by Fogarty and co-workers (Fogarty et al., 2005). By taking advantage of Cre-mediated recombination in transgenic mice, they were able to follow the neuronal and glial fates of cells that expressed the progenitor cell marker Dbx1 in the SC. Dbx1 (and Dbx2) is expressed in the p1, p0, dP6 and dP5 domains in the developing SC. Fogarty et al. conclude that the Dbx domain gave rise to a small number (3%) of OLPs in addition to radial glia, astrocytes and neurons. They find that the OLPs derived from the Dbx domain, were not evenly spread in the white matter, but mainly located at the level of their origin. This is in contrast to our data since, at least in *Nkx6* mutants; we were able to observe the dorsally derived $Olig1^+$ cells evenly distributed in the SC (**Paper III**). It may be that the lack of vOLPs in *Nkx6* deficient mice allows for more extensive migration of dOLPs as

compared to the wild type case. Additionally Fogarty et al. observed that some OLPs retained expression of the radial glia marker RC2 as well as radial processes, suggesting that they were derived from radial glia. Radial glia have classically been described as a transient cell type that continues to differentiate into astrocytes, however, in the last decade studies have suggested that radial glia may be multipotent stem cells (Alvarez-Buylla et al., 2000; Parnavelas and Nadarajah, 2001).

Comparing the dorsal source of oligodendrocytes studied in the *Nkx6* mutants (**Paper III**, (Cai et al., 2005)) and the dorsal Dbx1 derived oligodendrocytes, there is only a small overlapping domain between these two studies, the dP5. We found the contribution of dorsally derived OLPs to be 20-30% of the total OLP population at E18.5, based on the number of Olig1 expressing cells in *Nkx6* mutants, which is significantly higher contribution than the study by Fogarty et al. reported (**Paper III**). A further fate mapping study by Fogarty et al. using the Msx3-Cre line expressed in pD5-pD1 (i.e. the dorsal SC) reported that about 10-15% of OLPS at SC levels are derived from dorsally located progenitor cells (Richardson et al., 2006). In contrast to our study, where we did not observe any significant difference, Cai et al. reported an approximately 3-fold increase in the number of $Olig2^{+}/Pax7^{+}$ cells in the *Nkx6* mutant compare to wild type (**Paper III,** (Cai et al., 2005)). This implies that there may be an effect of the loss of Nkx6 on the generation of dorsal OLPs. The number of dOLPs in the *Nkx6* mutants might not truly reflect the wild type condition, although the differences in numbers between our study and the study of Cai et al. may be due to differences in *Nkx6* mutant background. In wild type embryos the number of dOLPs could be lower since dorsally specified OLPs in *Nkx6* mutants might propagate more efficiently due to the lack of ventrally derived OLPs which are likely to compete for the essential growth factor PDGFα (Calver et al., 1998). In summary, taking all three studies into account, a probable estimate of the contribution of dorsal OLPs is about 10-15% in the SC.

The failure of Pringle et al. to identify dorsally generated OLPs in their quail chick grafting experiments might have two explanations. The first, and perhaps most obvious, are potential species differences between avian and rodent/mammalian OLP origins in the SC. This may be analogous to the forebrain (FB), where fate mapping experiments in mouse have shown that both ventral and dorsal FB contribute to OLPs during development (Kessaris et al., 2006). In contrast, there seems not to be a dorsal source of OLPs in the chick FB (Olivier et al., 2001). However, recently the existence of dorsally derived OLPs has been reported in 10 day old chick embryos (Ulrika Marklund, unpublished data). A second explanation is, the markers used by Pringle et al to study the OLPs. As both we and Cai and co-workers show, the dorsally derived OLPs are born with at least a two day delay compared to their ventral counterparts, and exhibit a partially different expression profile (**Paper III**, (Cai et al., 2005). It might be that the use of MBP and Schwann cell myelin protein (SMP) by Pringle and colleagues, both late markers in the OLPs developmental program, as identifying markers for OLPs taken together with the lower numbers of dorsally derived OLPs (**Paper III**, (Cai et al., 2005) led to overlooking the potential dorsally derived population. Indeed, we were able to identify MBP expressing cells in explant cultures of dorsal SC first after 10 days of culture, corresponding to the fourth postnatal day.

Origins in the Hindbrain.

In the developing hindbrain OLPs are born from a ventral Olig1/2^{+} domain and their generation is dependent on Shh signaling (Alberta et al., 2001; Davies and Miller, 2001; Lu et al., 2002; Zhou and Anderson, 2002). In addition a more dorsal expression domain of Olig1/2 has been reported (**Paper III**, (Liu et al., 2003)). We mapped this domain to fall into the Pax3^{+}/Pax7^{+}/Gsh1/2^{+} co-expression progenitor domain, all defining markers for truly dorsally derived cells (Goulding et al., 1991; Hill et al., 1991; Sander et al., 2000; Valerius et al., 1995). By explant cultures we show that the dorsal Olig1/2^{+} cells *in vitro* have the capacity to generate OLPs based on Olig1/2, PDGFRα, NG2 and O4 expression. Importantly we show that control tissue from an Olig1/2^{-} domain does not possess the oligodendrogenic capacity, implying that the potential of the dorsal domain to generate OLPs *in vitro* is a true reflection of the *in vivo* potential and not a deregulated, or ventralized, artifact of the culture conditions.

Collectively, these data point to a gradual progression of OLP generation from restricted foci ventrally to dorsally in the SC – where OLPs are first observed generated from the pMN domain at E12.5 and 2 days later from the dorsal domain. Interestingly a similar ventral to dorsal theme has been observed in the FB, where the first OLPs originate from the medial ganglionic eminence (MGE) and anterior entopeduncular area (AEP) and come to migrate to populate the telencephalon and cerebral cortex. These are later joined by OLPs generated in the lateral ganglionic eminence (LGE) and finally OLPs arise in the postnatal cortex (Bradl and Lassmann, 2010; Gorski et al., 2002; Kessaris et al., 2008; Levison et al., 1993).

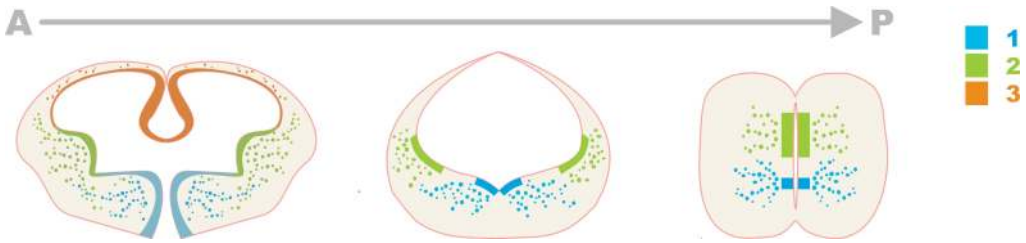

Figure 10. Schematic illustration the gradual progression of OLP generation . Progression from ventral to dorsal origins in the forebrain, hindbrain and SC. Arrow indicated anterior (A) to posterior (P) levels of the CNS. Numbers 1, 2 and 3 indicate the sequential order of generation of oligodendrocytes precursors from restricted foci.

SIGNALING PATHWAYS INVOLVED IN OLIGODENDROCYTE SPECIFICATION.

Shh signaling.

Shh signaling from the notochord and FP has been shown to be required both for the establishment of the ventrally located pMN domain and for the subsequent generation of MN and vOLPs (Alberta et al., 2001; Davies and Miller, 2001; Lu et al., 2002; Orentas et al., 1999; Pringle et al., 1996). Also, ventrally derived OLPs

generated at more anterior levels of the neural axis – i.e. in the telencephalon – have been shown to require hedgehog signaling (Tekki-Kessaris et al., 2001). Interestingly, it has been shown that ectopic expression of Olig1 can promote the generation of OLP independent of Shh signaling (Alberta et al., 2001), suggesting the existence of an alternative pathway for OLP generation. Since Shh signaling does not play a prominent role in cell fate specification in the dorsal SC, a Shh independent pathway may be utilized in the development of dOLPs.

However, cultures of dorsal SC explant by Sussman et al showed a partial requirement for Shh signaling in the development of $O4^+$ OLPs (Sussman et al., 2000). Additionally, in a series of chick explant experiments, Orentas and co-workers showed that dorsal SC explants treated with Shh can generate $O4^+$ OLP, however this ability was lost with time. Both studies did not in detail investigate contribution of Shh patterning effect on their cultures, which makes the interpretation of the result harder. It might be that the Shh treatment performed by Orentas et al. at earlier developmental stages, lead to a ventralization of the tissue and therefore a generation of OLPs, rather than reflected endogenous capacity (Orentas et al., 1999).

Cai et al. show that the addition of a neutralizing anti-Shh antibody to explant cultures had no effect on the generation of dorsal $Olig2^+$ OLPs, while the numbers of $Olig2^+$ cells in ventral explants was dramatically reduced. Further they show that OLPs can be derived from ESC cultures derived from Shh receptor *Smoothened (Smo)* deficient mice, suggesting a Shh independent pathway of OLP specification. Additionally, Cai et al. were able observe a small number of $Olig^+$ cells at E14.5 in *Shh* deficient SCs. At E18.5 the $Olig2^+$ cells had spread throughout the whole SC. Since $Shh^{-/-}$ mice die at birth the maturation and functionality of these Olig2 expressing OLPs cannot be followed *in vivo*. Instead Cai and co-workers observed MBP expressing cells in explant cultures of Shh deficient SCs indicating that OLPs in $Shh^{-/-}$ have the capacity to develop into mature oligodendrocytes (Cai et al., 2005).

In line with this, mice deficient for the Shh signal transducer Gli2 show a markedly reduced number of OLP at early developmental stage, but by late gestation, no significant difference is observed between wild type and mutant SCs (Qi et al., 2003). This might reflect the contribution and potential higher proliferation rate of dorsally derived OLPs, due to decreased competition for PDGFα (Calver et al., 1998), or due to rescue by other hedgehog signaling pathways.

BMP signaling.

During development Bone Morphogenic Protein (BMP) signaling from the RP and overlying ectoderm plays a central role in the establishment of dorsal progenitor cell identity (Helms and Johnson, 2003; Lee et al., 2000; Liem et al., 1997; Nguyen et al., 2000). BMP signals in the dorsal neural tube act to suppress more ventral fates, that in turn are Shh dependent, including the pMN derived OLPs (Hall and Miller, 2004; Mekki-Dauriac et al., 2002; Wada et al., 2000). Implanting BMP coated beads into the developing ventral SC of Xenopus embryos has been shown to locally inhibit OLP generation, while anti-BMP4 coated beads locally induced ectopic OLPs (Miller et al., 2004). Additionally, transgenic mice overexpressing BMP4 have decreased oligodendrogenesis and increased numbers of astrocytes (Gomes et al., 2003). We propose that a decrease in BMP signaling over time, due to the increase in the size of the neural tube, may influence the time of induction the dOLP in SC (**Paper III**). We exposed explants to either BMP7 or

BMP antagonists Noggin and Chordin (Piccolo et al., 1996; Zimmerman et al., 1996). Exposure to BMP7 completely blocked the dOLP generation, while the presence of BMP antagonists in cultures of explants isolated at E10.5 lead to a 3-4-fold increase in the number of $Olig2^+$ cells as well as premature induction of Olig2 expression as compared to controls. However, explants isolated at E12.5 where not responsive to treatment with BMP antagonists, in line with that the concentration of BMPs in the dorsal neural tube decreases over time (**Paper III**).

In line with this, a spatiotemporal modulation of BMP signaling has been shown to underlie the sequential generation of GABAergic neurons and OLPs from ventral progenitors in the developing forebrain (Yung et al., 2002).

Additionally, BMP signaling has been shown to have a role in the maturation of glial cells. Mice mutant for *BMP receptor 1a* and *1b* in the neural tube, exhibit a reduction in astrocyte number, while the number of OLPs at birth (P0) appears normal. However expression of mature markers MBP and PLP and the number of mature oligodendrocytes was reduced in the SCs deficient in BMP signaling (See et al., 2007), indicating a role for BMPs in the maturation process of OLPs. See and co-workers showed that the decrease in the number of MBP^+ cells was not due to altered precursor cell proliferation or apoptotic death of OLP cells.

Taken together, the data on BMP signaling in oligodendrogenesis, implies two temporally distinct roles for BMPs during OLP development. First, BMP signaling appears to have an inhibitory action on the induction/generation of oligodendrocyte precursor cells and second, BMP seems to be required for the maturation of oligodendrocytes into MBP expressing cells (Miller et al., 2004; See et al., 2007).

It is noteworthy that, upon injury in the adult CNS, BMP signaling is rapidly up regulated (Hall and Miller 2004) which would be consistent with a need for remyelination and therefore maturation of potential dormant OLPs in the adult. Also, BMPs have been suggested to have neuroprotective action (reviewed in (Hall and Miller, 2004).

FGF signaling.

Fibroblast Growth Factor (FGF) signaling has been shown to promote oligodendrogenesis in both cell and tissue culture ((Fogarty et al., 2005; Gabay et al., 2003) **Paper III**). For example, FGF2 is able to induce $Olig2^+$ $PDGFR\alpha^+$ cells in hindbrain (**Paper III**). Deciphering the real contribution of FGF signaling towards the oligodendrocyte lineage may be a difficult task. While it has been shown that progenitors in dorsal and ventral SC can generate neurons, astrocytes and oligodendrocytes after expansion *in vitro* (Chandran et al., 2003), Gabay et al. showed that culturing cells in the presence of FGF2 has a ventralizing effect. They demonstrate that neurosphere cultures derived from dorsal $Olig2^-$ and ventral $Olig2^+$ progenitors behaved as tripotent stem cells upon FGF2 treatment and could be differentiated along the neural, astrocytic and oligodendrocytic lineages. Upon analysis of progenitor marker expression in cultures treated with FGF2, Gabay et al. observed the upregulation of the ventral marker Nkx2.2 and downregulation of the dorsal Pax3, implying that the cultures had been ventralized. Additionally, they found that the process of ventralization involved endogenous Shh signaling (Gabay et al., 2003). Therefore, the tripotency of CNS stem cells appears to be an artifact of deregulation of dorsoventral identity due to the use of FGF2 for the expansion of cultures, rather the reflection of true *in vivo* potential.

Fogarty et al. provide data supporting the requirement for FGF signaling in the specification of dorsal OLPs. In cultures of dissociated SC they followed the fate

of cells derived from the Dbx1 domain and found that $Sox10^+$ OLPs developed in the cultures. Addition of the Shh antagonist cyclopamine, did not have an effect on the number of OLPs observed. Interestingly, blocking FGF signaling completely abolished the $Sox10^+$ cells in the cultures, implying a strict requirement for FGF in the development of dOLPs (Fogarty et al., 2005). On the other hand, Chandran and co-workers provide evidence that Shh and FGF2/MAPK signaling cooperate to induce $Olig2^+$ OLPs from neocortical precursors in culture (Chandran et al., 2003). Understanding the *in vivo* contribution of FGF signaling in the specification of dorsally derived OLPs would require targeted disruption of FGF signaling in the dorsal SC at the time of oligodendrogenesis. As it is currently unknown, which FGFs and FGF receptors may be involved, more studies are warranted before such an undertaking is feasible.

OLIG GENES.

The pMN progenitor domain which gives rise to both sMN and OLPs in the SC is defined by the expression of bHLH factors Olig1 and Olig2, collectively termed Olig1/2.

Functional studies have shown that Olig1/2 are required for oligodendroglial and sMN generation from the pMN domain during development. Olig1/2 and Olig2 mutant mice fail to establish the pMN domain and instead the presumptive pMN progenitors acquire the identity of $Irx3^+$ p2 progenitors. *Olig2* deficient mice fail to develop sMN and OLPs originating from the pMN domain. (Lu et al., 2002; Zhou and Anderson, 2002). The loss of the pMN domain in *Olig* mutants is accompanied by the ectopic generation of V2 INs and astrocytes, suggesting that these macroglial lineages are separated *in vivo* at early developmental stages. Additionally, the requirement for Olig1 and/or Olig2 appears to differ at different axial levels of the CNS. While *Olig1* mutants appear to normally develop OLPs in the brain, *Olig2* deficient mice fail to develop OLPs in the SC, indicating that Olig1 cannot compensate for the loss of Olig2 at caudal levels (Lu et al., 2002; Zhou and Anderson, 2002).

The role of Olig1 during development is less understood and while gain-of-function experiments suggest a role for Olig1 at the progenitor level (Lu et al., 2002; Park et al., 2002), *Olig1* deficient mice appear normal and exhibit only a mild delay in oligodendrocyte maturation at birth (Lu et al., 2002). However, recent evidence has shown that Olig1 plays a role in postnatal OLP maturation and in remyelination of brain lesions (Arnett et al., 2004; Ligon et al., 2006). *Olig1* deficient mice develop severe neurological deficits and die after 2-3 weeks, in line with that Olig1 has been shown to regulate the transcription of myelin-specific genes (MBP, Plp1 and Mag) and axonal integrity (Xin et al., 2005).

HOMEODOMAIN FACTORS IN OLIGODENDROCYTE SPECIFICATION.

Nkx2.2 is expressed in differentiating oligodendrocytes in both mouse and chick (Fu et al., 2002; Qi et al., 2001; Xu et al., 2000; Zhou et al., 2001). Nkx2.2 has been shown to be required for the maturation of oligodendrocytes and the expression of MBP and PLP is delayed and reduced in *Nkx2.2* mutants (Qi et al., 2001). However, the relationship between Nkx2.2 expression in progenitors and OLP origins is more complex when comparing species.

Distinct progenitor domains are characterized by distinct transcription factor expression profiles (see Introduction, Figure 2) and these transcription factors are involved in the specification of the cell type generated in each domain. It is therefore important to learn about the exact origin of OLPs in order to understand the specification of glial cells. A study by Sun et al. showed that PDGFRα^+ OLPs in the mouse SC are born from the pMN domain, which prior to oligodendrogenesis gives rise to MNs (Sun et al., 1998). In contrast to mouse, studies in chick showed that PDGFRα^+ OLPs in the avian SC come from the Nkx2.2$^+$ p3 progenitor domain (Xu et al., 2000). This challenged the notion of the special relationship of MNs and OLPs suggested from mouse studies (Richardson et al., 1997). During development, the expression domain of Nkx2.2 changes over time and extends progressively more dorsally to overlap with the Olig2$^+$ pMN domain. Mouse OLPs are born from the pMN domain before the expansion of Nkx2.2, while the OLPs in chick are generated from progenitors expressing Olig2 and Nkx2.2 after the Nkx2.2 expression has expanded dorsally. Interestingly, we observed a similar pattern in the mouse hindbrain, indicating that OLP generation and perhaps mechanism of specification varies along the AP axis in the mouse (**Paper III**). While Nkx2.2 inhibits OLP differentiation in the ventral SC and caudal hindbrain in mouse, in the anterior hindbrain Nkx2.2 appears to promote OLP generation. This indicates that, the activation of Olig1/2 and oligodendrocyte differentiation at different axial levels of the neural tube is governed by distinct genetic programs (**Paper III**). Whether these differences have any bearing on the developmental potentials and/or the properties of OLP remains to be investigated. Additionally, the expression profile of Nkx2.2 in OLPs generated dorsally and ventrally in the SC is also different – with only a small fraction of OLPs in *Nkx6* mutants (dOLP) expressing Nkx2.2 at E18.5 as compared to 30% in control animals (Paper III). Whether this prenatal molecular difference mirrors any functional property differences between dorsally and ventrally derived OLPs is unknown. Alternatively dOLPs may up regulate Nkx2.2 expression later in development as compared to their ventrally derived counterparts.

Nkx6.1 and Nkx6.2, collectively termed Nkx6, have been shown to be required for Olig2 expression in the SC and *Nkx6* deficient mice loose both sMN and vOLP expression in the ventral SC ((Cai et al., 2005; Novitch et al., 2001; Vallstedt et al., 2001) **Paper III**). Interestingly, Olig2 expression is maintained in the HB, and even ectopically expressed at anterior hindbrain levels, suggesting that Nkx6 proteins have different roles at different AP levels in the regulation of ventral oligodendrogenesis (**Paper III**).

Dorsally derived OLPs in the developing hindbrain seem not to be affected in the *Nkx6* mutant (**Paper III**).

An additional role for Nkx6.2 in the maturation of oligodendrocytes has been reported. Nkx6.2 appears to be involved in regulation of interactions between oligodendrocytes and axons at myelin paranodes (Southwood et al., 2004).

SOX GENES IN OLP DEVELOPMENT.

Members of the SoxE family of proteins, Sox8, 9 and 10, are expressed in the oligodendrocyte lineage. During early stages of development Sox9 is expressed in the progenitor zone in the CNS and later in glial cells and it has been shown to be required for the both astrocyte and oligodendrocyte development. Sox9 appears to

function in the neural to glial switch, since *Sox9* mutants exhibit prolonged neurogenesis (Stolt et al., 2003; Stolt and Wegner, 2010; Wegner, 2008).

OLP cells express Sox9 while still in the progenitor zone and *Sox9* deficient mice exhibit a large initial reduction in OLP numbers, implicating Sox9 in OLP specification (Stolt et al., 2003). Sox 10 is also expressed in early OLPs and persists throughout development into mature cells. It has been shown that Sox10 is essential for terminal differentiation (Stolt et al., 2002; Wegner, 2008), since terminal differentiation and myelin production are severely impaired in *Sox10* mutant mice. Sox 9 and 10 can functionally compensate for each other during early development (Stolt et al., 2002).

Sox8 is expressed after Sox9 but prior to Sox10 expression. OLPs in *Sox8* mutants appear to develop largely properly until birth, but display a delay in postnatal development and maturation (Stolt et al., 2004). Since the phenotype of *Sox8* deficient mice is less severe than that of *Sox9* and *Sox10* mutants, it has been suggested that Sox8 performs partially redundant roles with Sox9 and Sox10 during OLP development (Stolt et al., 2004).

Additionally, the SoxD proteins Sox5 and Sox6, have been reported to be involved in the temporal progression of OLP development, by regulating SoxE proteins to perform different functions at different time points during development (Stolt et al., 2006).

THE NEURON-TO-GLIA SWITCH.

At about E12, the pMN domain in the SC ceases to generate neurons and instead becomes gliogenic and gives rise to OLP (**Paper III**). Studies in the FB indicate that the neuron-to-glia switch involves the downregulation of proneural activity (Ngn2, Mash1) (Nieto et al., 2001). During MN generation, cells in the pMN domain express the proneural protein Ngn2 in addition to Olig2 and data from Zhou and co-workers show that Ngn2 is down regulated when OLP generation begins in chick (Zhou et al., 2001). Additionally, ectopic Olig2 expression in the absence of Ngn activity has been reported to induce OLP in chick SC (Zhou et al., 2001). However, *Ngn1/2* compound mutant mice do not exhibit premature OLP generation, indicating that, at least in mice, the neuron-to-glia switch is more complex(Rowitch, 2004).

Studies in zebrafish provide evidence that the Delta-Notch signaling pathway is involved in the neuron-to-glia switch as $Olig2^{+}$ cells only give rise to OLPs in Notch mutants. Forced expression of Notch1a resulted in increased generation of OLPs, with no effect on the timing of OLP production. Therefore, Notch signaling is necessary to maintain a portion of $Olig2^{+}$ cells into the gliogenic phase of development and Notch appears to have a permissive, rather than instructive, role in gliogenesis (Park and Appel, 2003).

Sox9 has also been implicated as a general component of the neuron-to-glia switch at SC levels, since *Sox9* deficient mice exhibit defects in both astro- and oligodendroglial specification accompanied by increased numbers of neurons (Stolt et al., 2003).

The expansion of Nkx2.2 into the Olig2 expressing pMN domain has been suggested to be involved in the neuron-to-glia switch in the chick SC. OLPs in chick are generated from progenitors expressing both Olig2 and Nkx2.2 and the expansion of Nkx2.2 expression coincides with the initiation of OLP production. In the mouse SC, however, Nkx2.2 and Olig2 are expressed in mutually exclusive progenitor domain (Fu et al., 2002) and *Nkx2.2*$^{-/-}$ do not exhibit any reduction in the initial

specification of OLPs from the pMN domain (Qi et al., 2001), making a similar process unlikely in the mouse. Instead, Nkx2.2 appears to repress the expression of Olig2 in mouse SC (**Paper III**)

OLIGODENDROGLIAL DIVERSITY.

Several studies (**Paper III**, (Cai et al., 2005; Fogarty et al., 2005; Rowitch and Kriegstein, 2010; Spassky et al., 2000; Sussman et al., 2000; Tekki-Kessaris et al., 2001)) have demonstrated the existence of several sources of oligodendrocytes in the developing SC and FB – it is now of importance to understand what this means. Do different sources of generation imply that cells become inherently different? From our current knowledge of developmental biology we assume that cells born at different positions in the embryo and that are under the influence of different positional cues differentiate into distinct cell types. Or can such cells converge into the same phenotypic endpoint? Several lines of evidence point to multiple pathways for oligodendrocytes development. Cai et al. showed that the dorsally generated OLP in the SC are Shh independent, in contrast to their ventral counterparts (Cai et al., 2005). Similarly, Shh is required for the specification of OLP in the ventral embryonic FB (Nicolay et al., 2007; Qi et al., 2002). A late wave of oligodendrocytes is generated from the embryonic cerebral cortex, which has no source of Shh, implying a different mechanism of OLP specification in line with the specification of dOLPs in the SC (Rowitch and Kriegstein, 2010). FGF signaling in culture has been shown to promote oligodendrocytes, although this may be due to ventralizing culture conditions (Chandran et al., 2003; Gabay et al., 2003; Kessaris et al., 2004). The evasion of BMP signaling over time has been proposed in the specification of dOLPs in the SC **(Paper III**).

Differences between properties of oligodendrocytes have been reported, but so far no connection has been made between embryonic origin and phenotypic and/or functional properties. Oligodendrocytes vary in morphology depending on the type of axon they myelinate and also the number of internodes they make (Bjartmar et al., 1994; Butt et al., 1994; Butt et al., 1997). Molecular differences in the expression of gap junction proteins between oligodendrocytes dependent on the size of the axon their myelinate have also been observed (Kleopa et al., 2004). Transplant studies of oligodendrocytes purified from rat optic nerve point to a high level of plasticity (Fanarraga et al., 1998), although it is unknown if all cells exhibit similar levels.

An argument against different properties of oligodendrocytes generated at different positions is that when dorsal and ventral oligodendrocyte populations were killed separately by targeted expression of Diphtheria toxin A in the FB, the different regional populations were able to spatially and functionally substitute each other without any behavioral consequence for the animals (Kessaris et al., 2006). Additionally, the early born ventral OLPs (vOLPs) in the FB have been shown to be eliminated and functionally replaced by oligodendrocytes born at more dorsal locations later during development (Kessaris et al., 2006). It is tempting to speculate about the causes of this; perhaps vOLP in the FB are a remnant of an evolutionary primitive source or maybe they have an embryological function and are no longer required in the adult.

Outside the SC, OLPs have been reported to exhibit two different expression profiles. One group expresses PDGFα (Hall et al., 1996; Pringle and Richardson, 1993),while the other expressed PLP/DM-20 (Perez Villegas et al., 1999; Spassky et al., 1998; Timsit et al., 1995) and both groups have been shown

to give rise to mature oligodendrocytes based on double-labeling experiment, suggesting the existence of two separate OLP populations in the brain. (Spassky et al., 1998). In the SC, PDGFRα expressing OLP are dominating, and PLP appears to label a later, more mature, developmental stage of these OLPs (Richardson et al., 2000; Spassky et al., 2000).

MYELINATION.

As the migrating OLPs reach their final positions they differentiate and mature into oligodendrocytes that associate with axons and ensheat them with membrane layers. The mechanisms of myelination and regulation thereof are beyond the scope of this thesis and will not be discussed here in detail. However, several lines of evidence point to a derepression model of differentiation, a concept familiar from the mechanisms of neural cell type generation in the neural tube. Myelination factors seem to be kept in an inactive state by protein sequestering and/or histone modification and chromatin compaction. Upon onset of myelination, activators – or repressor-of-repression – come into play allowing for the activation of the myelination process (reviewed in (Li et al., 2009))

This type of rigorous and strict regulatory network, involving extrinsic signals, transcription factors and epigenetic regulators allow for a tight regulation and maintenance of both progenitor cells and mature oligodendrocytes during development and also in the adult CNS.

OLIGODENDROCYTE PATHOLOGIES.

Myelination is essential for normal functioning of the CNS. Disruption of CNS myelin, due to injury, pathological degeneration (i.e. Multiple Sclerosis, Devic's Disease, Leukodystrophy) or other, leads to severe functional deficits and frequently to shortened lifespan. Oligodendrocytes are at high risk for damage under pathological conditions, due to the demands put on their metabolism. Proper myelination requires high metabolic rate and high ATP consumption, which results toxin formation and oxidative stress. Also, enzymes involved in synthesis of myelin require iron as a co-factor, and large intracellular storage of iron puts cells at risk for free radical formation. Oligodendrocytes have been reported to have low levels of anti-oxidative enzymes. Additionally, the endoplasmic reticulum in oligodendrocytes is under high throughput pressure, due to the large amount of protein productions required for myelination, and accumulation/retention of misfolded proteins is a often seen in oligodendrocyte pathologies (reviewed in(Bradl and Lassmann, 2010)).

REMYELINISATION.

An axon can upon demyelination either remain in its demyelinated state, and become vulnerable to axonal and neural death, or it can be remyelinated by the generation of oligodendrocytes that form short and thin internodes covering the area of lesion (Lassmann et al., 1997; Prineas et al., 1993; Zhao et al., 2005). This process is remarkably efficient and is dependent on the activation of a progenitor cell pool, characterized by their multipolar morphology and the expression of NG2 and PDGFRα. This population proliferates as it migrates to the lesion site where the

cells undergo terminal differentiation into mature oligodendrocytes (Zhao et al., 2005).

Studies have shown that oligodendrocytes can be generated along multiple developmental pathways and some differences in marker expression have been observed between distinct populations of OLPs (**Paper III**, (Cai et al., 2005; Liu and Rao, 2004; Mallon et al., 2002; Spassky et al., 2000). However, it is not known if this diversity has any bearing on myelination potential and/or myelination targets of oligodendrocytes. It may be that all OLPs in the adult are equivalent, or that different populations of OLPs – with different regenerative potential and differential responsiveness to growth factors - exist in the adult. From a therapeutic point of view, this is highly significant, since it may provide an opportunity for potential therapies of oligodendrocyte pathologies.

Interestingly, an area of the brain subjected to repeated demyelination, has the capacity to remyelinate from the pool of OLPs in that area if sufficient time is given for OLPs to repopulate the area between lesions. However, if exposed to continuous demyelination, an area of the brain does not recover and the numbers of OLPs present gradually diminish. Additionally, OLPs capacity to remyelinate lessens with increasing age (Zhao et al., 2005).

Although OLP are often found in non-repairing lesions in multiple sclerosis, mature oligodendrocytes are absent. It may be that oligodendrocyte differentiation is blocked by inhibitory signal; Notch ligand expression as well as TCF4/Tcf7L2 expression has been reported in these area suggesting that active WNT signaling may be involved (Fancy et al., 2009; John et al., 2002).

OLPs in the adult white matter express PDGFRα and Olig1 and upon demyelination up regulate Olig2 and Nkx2.2 (Fancy et al., 2004; Talbott et al., 2005; Watanabe et al., 2004), reminiscent of the developmental pathway for OLPs specification. Also, FGF2 has been shown to be up regulated in oligodendrocyte lesion areas (Hinks and Franklin, 1999), which may be involved in upregulation of Olig2 expression. Interestingly, BMP4, a factor associated with astrocyte differentiation, has also been shown to be up regulated in OLPs during remyelination (Fancy et al., 2004). The role of BMPs during remyelination is unknown, but may perhaps be opposite of their role during embryological development, or upregulation of BMP signaling may coincide with upregulation of BMP inhibitors thereby allowing for oligodendrocyte differentiation/specification. BMP signaling may instead play a role in a remyelination-associated process. In summary, the upregulation of Olig2, Nkx2.2, and BMP4 in dormant OLPs is associated with their reactivation and differentiation into mature oligodendrocytes. Further research into the molecular mechanisms governing remyelination will not only increase our understanding of this regenerative process but may identify new potential targets of therapeutic significance.

Another therapeutic aspect with some promise is transplanting glial progenitor cells (GPC), cultured *in vitro* from stem cell or isolated from tissue, into patients with congenital hypomyelination disorders. It appears as grafted cells distribute themselves along the neural axis and give rise to both astrocytes and oligodendrocytes and may hold promise for disorders of initial myelination and those reflecting congenital enzymatic deficiency. Interestingly, grafting experiments with GPCs derived from fetus vs adult has shown some bias in the properties of the oligodendrocytes dependent on origin. While adult derived GPC are predominantly biased toward rapid myelination, fetal GPC migrate further and differentiate into both astrocytes and oligodendrocytes. Therefore the source of cells has to be carefully considered for each disease (reviewed in (Goldman et al., 2008). A limitation that is important to consider is that ESCs based approaches carry the risk

of tumourgenesis if the graft is contaminated with ESCs, and therefore implantation of tissue-derived GPCs may be the clinically preferable choice.

Most studies are carried out in rodent model system and most, but not all, findings can be extrapolated to the human case. However, certain interspecies differences have to be considered – differences in the size and complexity of brain regions and the sheer numbers of oligodendrocytes, the timescale of myelination as well as regulatory differences of certain genes – and these may prove to be critical when establishing therapies in humans (Bradl and Lassmann, 2010).

FUTURE DIRECTIONS.

Why is developmental biology important to study? One answer is, that the pathways utilized during development to generate and specify different cell types are often reactivated upon lesions and or/pathologies in the adult, and may therefore carry a therapeutic potential. For example, understanding the early phases of oligodendrocyte development may have implications for therapies for demyelinating diseases. The recent emergence of several developmental pathways giving rise to mature myelinating oligodendrocytes, offer higher chances for finding therapies applicable in the adult. It is currently unknown, if oligodendrocytes from different developmental sources are inherently different or if they form a homogenous population of mature OLPs despite their diverse origins. It may be that although these cells perform the same function in the adult, their regenerative potential is different and thereby they would be different candidates for therapeutic manipulation. Further studies, involving lineage tracing into adulthood alone and combined with injury challenges and/or disease models may provide some insight into this matter. The use of Cre-recombinant mice where OLPs from different origins can be labeled specifically may prove to be a valuable tool. Additionally, lineage tracing will provide important information about the relative contribution of OLPs of different developmental origin to the general oligodendrocyte population.

It is not known if OLPs generated at different positions in the CNS myelinate different axons, although there is some data supporting such a division (Fogarty et al., 2005). Do dorsally derived oligodendrocytes myelinate sensory axons and ventrally derived oligodendrocytes myelinate axons of MNs? Does such a division, if it exists, reflects any functional differences or is it a stochastic division mostly based on location and proximity.

The identification of new markers for macroglia, through large-scale analysis such as microarray or other, will also provide important tools for further study of OLP and oligodendrocytes. Markers differentially expressed in different OLP and OL populations will provide a tool to identify cells not only during development, but also at the mature destination and provide means to correlate function to developmental origin.

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