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THE ROLE OF NKX PROTEINS IN NEURONAL AND GLIAL SPECIFICATION

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”När målet är vunnet är mödan glömd”

Visdomsord från min fars mor, 1946

ABSTRACT

During development, different classes of neurons and glia are generated from proliferative progenitor cells lining the ventricles of the brain and the lumen of the spinal cord. A central issue in developmental neuroscience is to understand the mechanisms by which these cells are generated in space and over time. In the ventral spinal cord, the expression profile of homeodomain (HD) proteins defines five progenitor domains that each will give rise to a distinct type of neuron. Two closely related HD transcriptional repressors, Nkx6.1 and Nkx6.2 (Nkx6), are expressed by progenitors of the ventral spinal cord. We provide evidence that different levels of Nkx6 repressor activity in progenitor cells are a critical determinant of ventral neuronal fate, assigning both redundant and specific roles for these proteins in neuronal specification. A reduction in Nkx6 activity further permits V0 interneurons to be generated from progenitors that lack HD proteins normally required for their generation, providing direct evidence for a model where HD proteins direct specific cell fates by actively repressing the expression of transcription factors that direct alternative fates.

In the ventral spinal cord, sMNs and oligodendrocyte precursors (OLPs) are sequentially generated from a domain defined by the expression of Olig2. We show that the generation of sMNs and OLPs in the ventral spinal cord is essentially missing in mice lacking Nkx6 function. In contrast, the same HD proteins instead act to suppress OLP specification in the ventral hindbrain. The divergent roles for Nkx6 proteins seem to reflect that OLPs in the spinal cord and hindbrain are produced by distinct ventral progenitor domains. While a ventral specification of OLPs is well established, it has remained unclear whether also more dorsal progenitor cells give rise to oligodendrocytes. We provide *in vivo* and *in vitro* evidence that oligodendrocytes are produced also by dorsal progenitors in the spinal cord and hindbrain and that the specification of these cells may result from the progressive evasion of dorsal BMP signalling over time. Together, our data suggest that oligodendrocytes are generated from multiple dorsoventral origins in the spinal cord and hindbrain, and indicate that the activation of Olig2 at different positions is controlled by distinct genetic programs.

The observation that the loss of sMNs in the spinal cord of *Nkx6* mutant mice correlates with an extinguished expression of the sMN determinant Olig2 has led to a model where Nkx6 proteins act strictly upstream of Olig2. However, in the hindbrain of *Nkx6* mutant mice the initial expression

of Olig2 is intact and despite this all sMNs are missing, indicating a parallel requirement for Nkx6 and Olig2 proteins in the generation of sMNs. Visceral motor neurons (vMNs) are generated immediately ventral to sMNs. The transcription factor Phox2b has been found to be an important determinant of these cells, but other factors involved have not been identified. We show that the HD protein Nkx2.2 is sufficient to mediate the expression of Phox2b. Furthermore, while the activities of Nkx6.1 and Nkx6.2 are dispensable for the initial generation of vMNs, they are required to prevent a parallel program of more dorsal interneuronal differentiation and to ensure a proper migration and axonal projection formation of vMNs. Thus, Nkx2 and Nkx6 proteins appear to have complementary roles in the establishment of vMN identity in the hindbrain. Taken together, our data suggest that both visceral and somatic motor neuron differentiation rely on the combined activity of cell intrinsic determinants, rather than on a singe key determinant of neuronal cell fate.

Neuronal diversity is established by mechanisms that operate in space and over time. Advances have been made in regard to the mechanisms that restrict and direct neuronal generation in space, but less is known about the mechanisms that underlie how neural progenitors produce distinct types of neurons in a specific temporal order. We addressed this issue by studying a population of progenitor cells in the ventral hindbrain that gives rise to vMNs and serotonergic (S) neurons. Each hindbrain segment, or rhombomere (r), initially generates vMNs, but all the rhombomeres except for r4 switch to producing S neurons at a defined time point. We found that the temporal and spatial generation of vMNs and S neurons critically relies on the integrated activity of Nkx- and Hox-class HD proteins. A primary function of these proteins is to coordinate the activation of Phox2b in space and time. Phox2b, in turn, functions as a binary switch in deciding whether progenitors differentiate into vMNs or serotonergic neurons. Taken together, these data indicate that determinants that control spatial patterning may be associated also with temporal patterning and require that expression patterns are dynamic and modulated over time.

LIST OF PUBLICATIONS

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

I **Vallstedt, A.,** Muhr, J., Pattyn, A., Pierani, A., Mendelsohn, M., Sander, M., Jessell, T. M., and Ericson, J. (2001). Different levels of repressor activity assign redundant and specific roles to Nkx6 genes in motor neuron and interneuron specification. Neuron 31, 743-755.

II. Pattyn, A*., **Vallstedt, A*.,** Dias, J. M., Sander, M., and Ericson, J. (2003). Complementary roles for Nkx6 and Nkx2 class proteins in the establishment of motoneuron identity in the hindbrain. Development 130, 4149-4159.

III. Pattyn, A., **Vallstedt, A.,** Dias, J. M., Samad, O. A., Krumlauf, R., Rijli, F. M., Brunet, J. F., and Ericson, J. (2003). Coordinated temporal and spatial control of motor neuron and serotonergic neuron generation from a common pool of CNS progenitors. Genes Dev 17, 729-737.

IV. **Vallstedt**, **A*.,** Klos, J*., Ericson, J. (2004). Multiple Dorsoventral Origins of Oligdendrocyte Generation in the Spinal Cord and Hindbrain. Submitted.

* These authors contributed equally to this work.

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ABBREVIATIONS

AP	Anteroposterior
bHLH	basic Helix Loop Helix
BMP	Bone Morphogenic Proteins
CNS	Central Nervous System
DV	Dorsoventral
FGF	Fibroblast Growth Factor
HD	Homeodomain
HDAC	Histone Deacytelase Complex
OLP	Oligodendrocyte precursor
pMNs	somatic motor neuron progenitor domain
pMNv	visceral motor neuron progenitor domain
S	Serotonergic
Shh	Sonic Hedgehog
sMN	somatic motor neuron
TGF- β	Transforming Growth Factor β
vMN	visceral motor neuron
Wnt	Wingless-Int

INTRODUCTION

The diverse functions of the vertebrate central nervous system (CNS) range from sensory perception and motor coordination to behaviour and memory. These highly complex tasks ultimately depend on precise connections formed between distinct nerve cells, of which there is an immense array of functionally different types. The two other principal cell types of the CNS are astrocytes and oligodendrocytes, collectively called glial cells, and they are likewise required for proper functioning of the CNS. Amongst their important properties are to insulate axons, provide structural support, regulate water and ion balance, and maintain the blood-brain barrier.

A central issue in developmental neuroscience, and a field that has made substantial progress during recent years, is to understand the mechanisms by which the cells that constitute the mature CNS are generated in space and over time in the developing embryo. A fundamental early step is the allocation of a group of ectodermal cells as precursors of the entire CNS. These cells form an epithelial sheet called the neural plate that, through multiple stages of growth and movement, will form a hollow structure termed the neural tube. All cells of the CNS will ultimately differentiate from proliferative neuroepithelial progenitor cells, which throughout the course of development line the lumen of the neural tube.

Numerous studies have addressed the question of how an essentially uniform population of progenitor cells can give rise to the large variety of cell types that populate the CNS. From these studies, some fundamental principles have emerged; cells in the neural tube acquire a regional identity by virtue of the position they occupy along two neural axes, dorsoventral (DV) and anteroposterior (AP). The exposure to regionally restricted signalling factors that operate over these two axes is thought to define the positional identity of a cell. Signalling along the anteroposterior axis of the neural tube establishes the main subdivisions of the CNS; the forebrain, midbrain, hindbrain and spinal cord (Lumsden and Krumlauf, 1996) and function in concert with the dorsoventral signalling system that has a more pronounced role in establishing cell type diversity within each of these anteroposterior subdivisions (Campbell, 2003; Jessell, 2000; Rallu et al., 2002). With these advances, neural cell groups with distinct anatomical features, such as the floor plate, roof plate and isthmus, have come to be recognized as key sources of secreted factors that establish

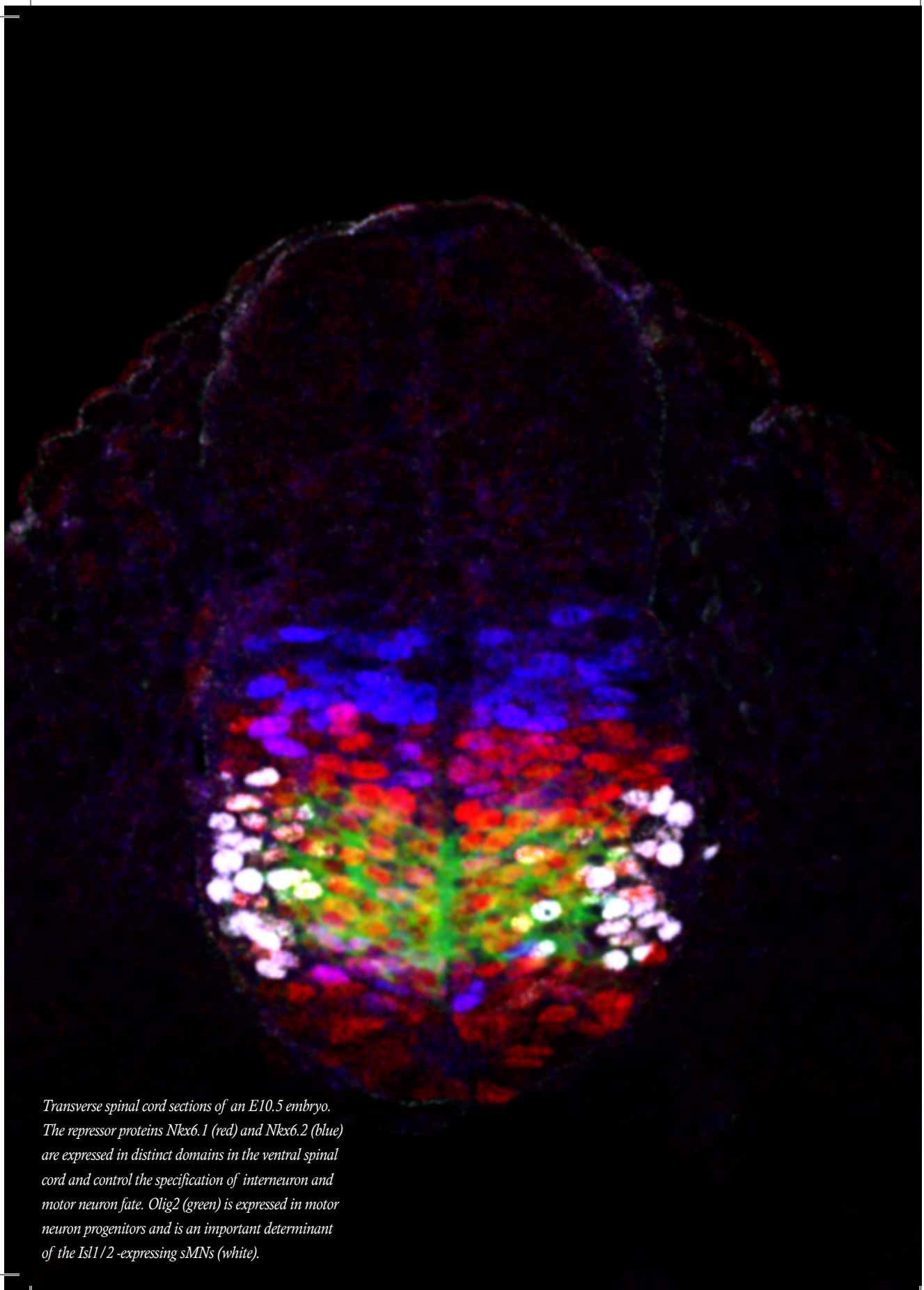
regional patterns and differences along the axes. Additionally, it has become evident that the actions of a relatively small group of signalling factor families, such as the hedgehogs, TGF-βs, Wnts, retinoids and FGFs, may underlie many features of regional specialisation within the neural tube.

How are these signalling molecules interpreted to create positional identity at the level of the progenitor cell? Increasing evidence suggests that different categories of transcription factors, many containing homeodomain DNA binding motifs, act as mediators of these signals and play essential roles at several steps in neural induction (e.g. Sox genes), regional patterning (e.g., Nkx, Dlx, and Pax genes) and cell fate determination (e.g. LIM and Ets genes). A common theme in regional, or spatial, patterning of the neural tube is the establishment of domains of progenitor cells that express different sets of transcriptions factors. The delineation of these domains is achieved through cross-repressive interactions between pairs of transcription factors. The transcription factors, in turn, are thought to regulate intrinsic cellular programs which cause progenitors to adopt specific cell fates (Briscoe and Ericson, 2001; Campbell, 2003). Programs of spatial patterning are further integrated with pan-neuronal pathways that control the selection of neural progenitors and their commitment to differentiation, involving Notch signalling, bHLH and Sox proteins (Bertrand et al., 2002).

The description above presents a simplified overview of the generation of cell diversity within the CNS. In reality, the development of the CNS demonstrates numerous complexities. For example, while several studies have shown the involvement of the mechanisms described above in specification of neuronal subtypes, it is more uncertain as to what extent the generation of glial cell types is governed by similar mechanisms. Furthermore, the same progenitor domain can give rise to different cell types at different time points during development. The generation of cells in a specific temporal order is most prominent during neurogenesis in the retina and neocortex, although increasing evidence suggests it to be important also in other parts of the developing nervous system. Thus, in addition to spatial patterning of the CNS, there is also a temporal aspect to take into account when contemplating mechanisms underlying cell specification.

In my studies, I have addressed issues of cell specification in the context of the caudal nervous system; the developing spinal cord and hindbrain. I will discuss our findings in relation to the current literature and with a special emphasis on the role of Nkx proteins in the following processes:

- Spatial patterning and cell specification in the ventral spinal cord
- Establishment of neuronal identity in the ventral hindbrain
- Oligodendrocyte generation in the spinal cord and hindbrain



Transverse spinal cord sections of an E10.5 embryo. The repressor proteins Nkx6.1 (red) and Nkx6.2 (blue) are expressed in distinct domains in the ventral spinal cord and control the specification of interneuron and motor neuron fate. Olig2 (green) is expressed in motor neuron progenitors and is an important determinant of the Isl1/2 -expressing sMNs (white).

SPATIAL PATTERNING AND CELL SPECIFICATION IN THE VENTRAL SPINAL CORD

Progress has been made during recent years in terms of identifying the extracellular signals and cell-intrinsic programs that commit cells to specific neuronal fates. A considerable amount of the acquired information has come from studies in the spinal cord. The physiology and anatomy of spinal neurons are well defined and relatively simple, making it an experimentally suitable system for addressing basic questions of patterning and cell specification. The vertebrate spinal cord is responsible for the coordinated control of locomotor function and the relay of sensory information to higher order brain centers. These two functional systems are segregated anatomically. The neurons that participate in motor output and body movements reside in the ventral spinal cord, whereas neurons that process and relay sensory information from the body are located predominantly in the dorsal part of the spinal cord (Brown, 1981).

Signalling in the neural tube

In many developing tissues, the generation of distinct cell types at different positions is initiated by the action of secreted signalling molecules from local organising centers. In the spinal cord, signalling from the ventrally positioned notochord and floor plate triggers the differentiation of different ventral cell types (Placzek, 1995). Similarly, signals from the overlying ectoderm and the most dorsal neural tube structure, the roof plate, have a prominent role in specifying dorsal cell fates (Lee et al., 2000; Liem et al., 1995). In the dorsal spinal cord, Bone Morphogenic Proteins (BMPs) and Wnts can account for a major part of the inductive activities (Lee and Jessell, 1999; Muroyama et al., 2002), whereas the secreted protein Sonic Hedgehog (Shh) is the predominant signal of the ventral spinal cord (Jessell, 2000). Furthermore, data have indicated that these pathways intersect and that BMPs act to limit the ventralizing activity of Shh (Liem et al., 2000). In addition, studies have suggested a role for retinoic acid-mediated signalling derived from the paraxial mesoderm in the specification of certain ventral neurons (Novitch et al., 2003; Pierani et al., 1999).

Certain signals have the feature of directing distinct cell fates at different concentration thresholds, and thus function as morphogens (Vincent and Briscoe, 2001). Shh is believed to possess such an activity and to pattern the ventral neural tube by graded signalling in a long range fashion. Several observations support this idea. Shh is both necessary (Chiang et al., 1996; Ericson et al., 1996) and sufficient (Marti et al., 1995; Roelink et al., 1995) for the induction of most ventral neuronal cell types. Cell-autonomous activation (Hynes et al., 2000) or inhibition of the Shh pathway pathway (Briscoe et al., 2001) implicates that Shh act as a direct long range morphogen. Progressive two- to threefold changes in Shh concentration generate five distinct classes of ventral neurons, where neurons originating from the most ventral regions of the neural tube require the highest concentrations (Ericson et al., 1997a; Ericson et al., 1997b).

The establishment of ventral progenitor domains

The findings summarized above suggest that the graded activity of Shh provide positional information to progenitor cells, linking cell position to cell fate in the ventral spinal cord. However, they also pose the problem of how neural progenitor cells interpret the graded Shh signal. Studies have shown that Shh signalling establishes distinct ventral progenitor domains by

regulating the spatial pattern of expression of a set of transcription factors of the homeodomain (HD) containing Pax, Nkx, Irx and Dbx families (Briscoe et al., 2000; Ericson et al., 1997b; Goulding et al., 1993; Pierani et al., 1999; Qiu et al., 1998) as well as the basic helix-loop-helix (bHLH) protein Olig2 (Novitch et al., 2001). These proteins can be subdivided into class I or Class II proteins based on their regulation by Shh signalling. The Class I proteins (Pax6, Pax7, Dbx1, Dbx2 and Irx3) are expressed by neural progenitor cells in the absence of Shh signalling and their expression is repressed by Shh (Briscoe et al., 2000; Chiang et al., 1996; Ericson et al., 1997b; Pierani et al., 1999). Their expression has instead been found to be enhanced by retinoid signalling (Novitch et al., 2003; Pierani et al., 1999). In contrast, the expression of Class II proteins (Nkx6.1, Nkx6.2, Nkx2.2, Nkx2.9 and Olig2) in neural progenitors depend upon exposure to Shh, with different Class II proteins being induced at different Shh concentration thresholds (Briscoe et al., 2000; Briscoe et al., 1999; Novitch et al., 2001; Pabst et al., 2000; Qiu et al., 1998). Interestingly, the expression of Olig2 is dependant on both Shh and retinoid signalling (Novitch et al., 2003).

The combinatorial expression of Class I and Class II proteins defines five separate progenitor domains in the ventral spinal cord, each with a distinct profile of protein expression (Briscoe and Ericson, 1999). However, the differential regulation of these proteins by Shh only accounts for a part of the mechanism by which distinct progenitor domains are formed. Downstream of Shh signalling, cross-repressive interactions between pairs of Class I and Class II proteins define the spatial extent of individual progenitor domains and establish sharp boundaries between adjacent domains (Briscoe et al., 2000; Muhr et al., 2001). This mechanism ensures that cells within individual domains will express a distinct combination of progenitor proteins, thus creating a spatial patterning of transcription factors along the DV axis of the ventral spinal cord. The profile of Class I and Class II protein expression within individual progenitors also appears to direct what neuronal subtypes the cells will give rise to (Figure 1). In support for this, several of these patterning proteins have the ability to induce the ectopic generation of specific neuronal subtypes when misexpressed outside their normal domains (Briscoe et al., 2000; Pierani et al., 2001; Paper I).

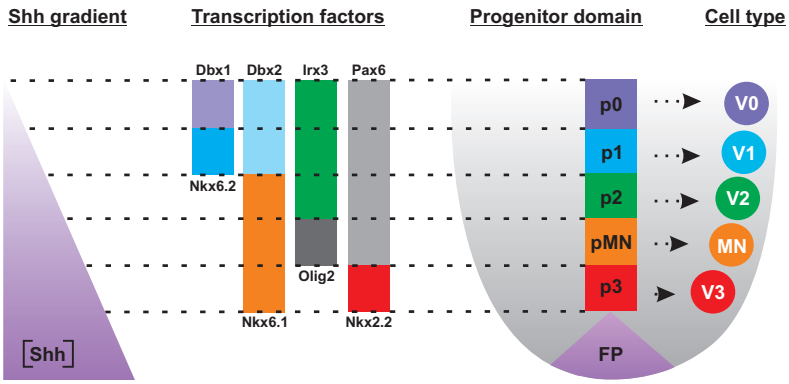


Figure 1. Model of Shh mediated neural patterning. A gradient of Shh regulate the expression of different HD proteins and the bHLH protein Olig2. Cross-repressive interactions between pairs of these proteins generate five distinct progenitor domains that each will give rise to a specific cell type.

In addition, the analysis of mouse mutants has provided evidence that the activities of individual Class I and Class II proteins are required to establish progenitor cell domains and to direct ventral neuronal fate (Briscoe et al., 1999; Ericson et al., 1997b; Mansouri, 1998; Pierani et al., 2001; Sander et al., 2000; Zhou and Anderson, 2002; Paper I).

The mechanisms by which Shh regulate Class I and Class II expression are still fairly elusive, partly due to the cross-repressive relationship between Class I and Class II proteins. One possibility would be that Shh directly induce expression of Class II proteins, presumably through a pathway that would involve the Gli proteins (Matise and Joyner 1999; Persson et al. 2002; Wijgerde et al. 2002 and Meyer and Roelink 2003). In this scenario, the repressive action of Shh on Class I proteins could be achieved directly or indirectly by the repression of Class I proteins by Class II proteins. Alternatively, the promotion of Class II proteins could be mediated by the direct repression of Class I proteins by Shh, thus allowing Class II proteins to be expressed close to the source of Shh. However, this appears unlikely since in *Nkx6.1/Nkx6.2* compound mutant mice the domain of expression of the Dbx Class I proteins expands ventrally all the way down to the Shh-secreting floor plate (Paper I). This observation would be more consistent with the idea that a Shh induced expression of Class II proteins is needed for ventral exclusion of Class I proteins.

Ventral progenitor domains and cell fate

The five progenitor domains that are defined by the patterned expression of Class I and Class II proteins are termed p3, pMN, p2, p1 and p0 in a ventral to dorsal manner (Briscoe et al., 2000). Examination of the relationship between progenitor cells and specific neuronal cell types using genetic markers has revealed that distinct classes of neurons arise from specific progenitor domains positioned along the DV axis. Based on these studies, V0-V3 interneurons develop from the p0 to p3 domains, respectively, and somatic motor neurons arise from the pMN domain (Figure 1). V0-V3 cells were first recognised as distinct classes of interneurons based on their specific expression profiles of homeodomain transcription factors. V0 cells are labelled by Evx1 (Moran-Rivard et al., 2001), V1 cells by En1 (Saueressig et al., 1999), V2 by Lim3 (Ericson et al., 1997b; Sharma et al., 1998; Tanabe et al., 1998) and Chx10 (Ericson et al., 1997b; Tanabe et al., 1998) and V3 by Sim1 (Ericson et al., 1997b). These ventral interneurons are thought to be involved in the modulation and coordination of locomotor control. For example, a recent study has identified an important role for V0 interneurons in the coordination of left-right locomotor activity necessary for walking movements (Lanuza et al., 2004). Somatic motor neurons project axons to the skeletal muscles of the body and are defined by the expression of Isl1 and HB9 (Arber et al., 1999; Tanabe et al., 1998; Tsuchida et al., 1994).

V3 interneurons are generated from the most ventral domain, the p3 domain, and are dependent on the selective expression of Nkx2.2 for their generation (Briscoe et al., 1999). In the pMN domain, the combined activities of Nkx6.1, Olig2 and Pax6 are required for the proper production of somatic motor neurons (sMNs) (Ericson et al., 1997b; Lu et al., 2002; Sander et al., 2000; Zhou and Anderson, 2002). The ectopic expression of Nkx6.1 induces sMNs and the loss of Nkx6.1 activity results in a decreased production of sMNs (Briscoe et al., 2000; Sander et al., 2000). Interestingly, Nkx6.1 is expressed in the three most ventral domains, the p3, pMN and p2 domains, but its sMN inducing activity is only evident in the pMN domain. In the p3 domain, this appears to be due to the expression of Nkx2.2, which overrides the activity of Nkx6.1 and promotes the generation of V3 interneurons. In the p2 domain, located immediately dorsal to the pMN domain, the combination of Nkx6.1 with Irx3 leads to the production of V2 interneurons instead of sMNs (Briscoe et al., 2000). The phenotype of *Nkx6.1* mutant mice further indicates that the activity of Nkx6.1 is required for the correct generation of both V2 interneurons and

MNs (Sander et al., 2000). The p1 domain generates V1 interneurons and is characterised by the selective expression of Nkx6.2, which also is required for the proper production of V1 neurons from this domain (Paper I). Most dorsal in the ventral neural tube, p0 progenitors that express Dbx1 generate V0 interneurons (Matise and Joyner, 1997). The expression of Dbx1 is essential for the generation of these cells (Pierani et al., 2001). Both the p1 and p0 domain express the Dbx1-related protein Dbx2 (Shoji et al., 1996), but the precise role of this protein remains to be determined.

The derepression model

What is the mechanism by which the progenitor domain proteins exert their functions? The antagonistic relationship between specific Class I and Class II proteins raise the possibility that many of these proteins are transcriptional repressors. In line with this, most of the Class I and Class II proteins has been found to work as transcriptional repressors through the recruitment of members of the Groucho/TLE corepressor complex (Muhr et al., 2001). Groucho/TLE proteins are a family of co-repressors that do not directly bind DNA, but are recruited to promoters via the interaction with DNA binding, sequence specific, transcription factors (Fisher and Caudy, 1998). Groucho/TLE proteins are thought to mediate transcriptional repression by modulating chromatin structure through the interaction with the Histone Deacytelase Complex (Chen et al., 1999; Choi et al., 1999a).

The finding that progenitor factors repress transcription helps to explain their cross-repressive interactions, but leads to questions of how they act in a combinatorial manner to promote specification of cell identity. Rather than conventional activator models of cell fate determination, the suggested model is one in which the progenitor factors inhibit many different cell fates, except the one appropriate for the domain in which they are expressed (Muhr et al., 2001). In this view, the way that progenitor factors direct certain cell fates is by repressing the factors that promote alternative fates. A basis of this model is that repression is exerted at two sequential steps; one step operates a the level of the progenitor proteins themselves and a second step of repression is applied on neuronal subtype determinant factors that have a downstream role in directing neuronal subtype fates (Muhr et al., 2001). The subtype determinants, in turn, would be predicted to control the identity of neurons by regulating properties such as cell migration, morphology, axon path finding and neurotransmitter phenotype. Thus, according to this model, spatial pattern of neurogenesis in

the ventral neural tube is achieved through the repression of repressors—a derepression strategy of neuronal fate determination.

Several observations provide support to this model. In *Pax6* mutant mice the cross-regulatory inhibition of Nkx2.2 is relieved, allowing it to expand into the more dorsal pMN domain and promote the generation of V3 interneurons at the expense of sMNs (Ericson et al., 1997b). Nkx2.2 has been found to repress the expression of Olig2, which is required for sMN generation from the pMN domain (Novitch et al., 2001). Thus, in the absence of the repressive actions of Pax6, Nkx2.2 is able to repress Olig2 or downstream determinants of sMN fate, or both. Moreover, the ability of Nkx2.2 or Nkx6.1 to induce V3 neurons or sMNs, respectively, when ectopically expressed in more dorsal positions of the spinal cord has been found to rely on them functioning as repressors (Muhr et al., 2001). In ventral progenitor cells, the inductive activities of Nkx6.1 and Nkx2.2 appear to depend, at least in part, on their ability to act as repressors of their complementary class I proteins, Dbx2 and Pax6 respectively (Ericson et al., 1997b; Sander et al., 2000). But in the dorsal neural tube, progenitor cells lack expression of many of the ventral class I repressor proteins. Consequently, dorsal neural progenitors must also express repressors of sMN and V3 neuronal differentiation—repressors that are themselves subject to repression by Nkx6.1 or Nkx2.2.

Additional support for a cell fate model based on repression is presented in Paper I. The function of Dbx1 is normally required for the generation of Evx1+ V0 neurons from the p0 domain, as there is a complete lack of these cells in *Dbx1* mutant mice (Pierani et al., 2001). In *Nkx6.2* mutant mice, the expression of Dbx1 expands ventrally into the p1 domain, leading to an ectopic generation of V0 neurons at the expense of V1 neurons. Interestingly, in *Nkx6.1* mutant/*Nkx6.2* heterozygous embryos Evx1+ cells are ectopically generated by ventral neural progenitors in the entire ventral spinal cord in the absence of Dbx1 expression. It is worth noting that the closely related protein Dbx2 is expressed in ventral progenitor cells in these mutants, raising the possibility that generation of ectopic Evx1+ cells is achieved through a possible redundancy between these two genes. However, progenitors in the pMN domain express very low levels of Dbx2 in *Nkx6.1* mutant/*Nkx6.2* heterozygous embryos, arguing against a function for Dbx2 in the generation of these ectopic V0 cells. Thus, in *Nkx6.1* mutant/*Nkx6.2* heterozygous embryos the net level of Nkx6.1 and Nkx6.2 (collectively referred to as Nkx6) repressor activity is enough to repress

Dbx1 but not Evx1. These data support the idea that Nkx6 proteins inhibit V0 neuronal fate by repressing the Class I protein Dbx1 and independently by repressing the V0 neuronal subtype determinant Evx1. They also substantiate a model where homeodomain proteins are required for a certain fate through their ability to repress other repressors of that fate, and not by directly activating downstream determinants.

Another interesting finding that stems from the analysis of *Nkx6* mutant mice is the importance of repressor activity levels for ventral neuronal fate (Paper I). Duplication of many homeodomain proteins during evolution has resulted in the overlapping expression of pairs of closely related homeodomain proteins; such as Dbx1/Dbx2, Pax3/Pax7, Nkx2.2/Nkx2.9 and Nkx6.1/Nkx6.2 (Mansouri, 1998; Mansouri and Gruss, 1998; Pabst et al., 2000; Pierani et al., 2001; Shoji et al., 1996). Both distinct and/or redundant roles in spinal cord patterning have been suggested for some of these protein pairs (Briscoe et al., 1999; Mansouri and Gruss, 1998; Pierani et al., 2001) (Paper I, II, III). Nkx6.2 expression is normally restricted to the p1 domain where it promotes the generation of En1+ V1 neurons. However, when misexpressed ectopically at high levels in the spinal cord, Nkx6.2 mimics the function of Nkx6.1 by inducing the generation of V2 and sMNs. In addition, in mice lacking Nkx6.1 function, the expression of Nkx6.2 is expanded to encompass the entire ventral part of the spinal cord. Under these circumstances, Nkx6.2 provides a "fail-safe" activity in the generation of the remaining sMNs observed in the Nkx6.1 mutant. In the absence of both Nkx6.1 and Nkx6.2, a close to complete lack of sMNs is observed and Evx1+ V0 neurons are generated in the entire ventral spinal cord at the expense of other cell fates. Both proteins also repress the expression of Dbx2, although Nkx6.1 appears to be a more efficient repressor of Dbx2 than Nkx6.2. as Nkx6.2 is co-expressed with Dbx2 in the p1 domain but keeps Dbx2 expression at a low level. The basis of these divergent functions for Nkx6 proteins appears to reside in different repressor activity levels of these proteins in different progenitor domains (Figure 2). In the p1 domain, Nkx6.2 negatively regulates its own expression to ensure a low level of expression. This low level of expression permits Dbx2 to be expressed, thereby ensuring the repression of Nkx6.1 in the p1 domain. Thus, differences in the level of repressor activity of Nkx6.1 and Nkx6.2 in different domains appear to be crucial for the correct generation of ventral neuronal cell types, providing a mechanism by which an enhanced diversification of neuronal cell types can be achieved.

A model of cell fate determination based on derepression must involve the existence of a pathway responsible for activating the expression of downstream determinants, since the key upstream transcription factors work as repressors. At one extreme, target gene activation could be achieved through uniformly expressed transcriptional activators whose constitutive functions would be regulated by the repressive functions of homeodomain proteins in each domain. Alternatively, the relevant transcriptional activators could themselves be regulated by extrinsic signalling pathways, and different activators may be dedicated to the generation of specific cell types. Recent studies have implicated retinoid signalling and the activator functions of retinoid receptors to be required at several phases of motor neuron specification in the spinal cord (Novitch et al., 2003; Sockanathan et al., 2003). Previous studies have also suggested that retinoid signalling is involved in the generation of V0 and V1 neurons (Pierani et al., 1999). Thus, retinoid-mediated transcriptional activation might have a widespread role in the specification of ventral neuronal subtypes, favouring a model of more commonly expressed activators in the neural tube.

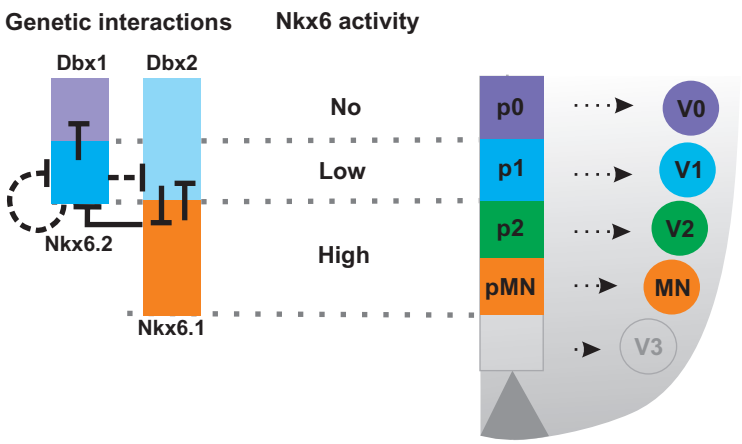


Figure 2. Regulatory interactions between Nkx and Dbx proteins in the ventral spinal cord. These interactions result in different levels of Nkx6 protein activity in different ventral domains, which promote the generation of distinct neuronal subtypes.

Additionally, through the characterization of a conserved HB9 enhancer, another study have found that non-specific general-activator proteins such as E2F and Sp1 are capable of driving widespread low level transcription of HB9, a protein specifically expressed in somatic motor neurons, in many cell types throughout the neural tube (Lee et al., 2004). The function of these general activators appears to be constrained by multiple repressor proteins, including Nkx2.2 and Irx3 in non-motor neuron cells, providing further support for the derepression model of gene regulation in the neural tube.

Neurogenesis and cell fate

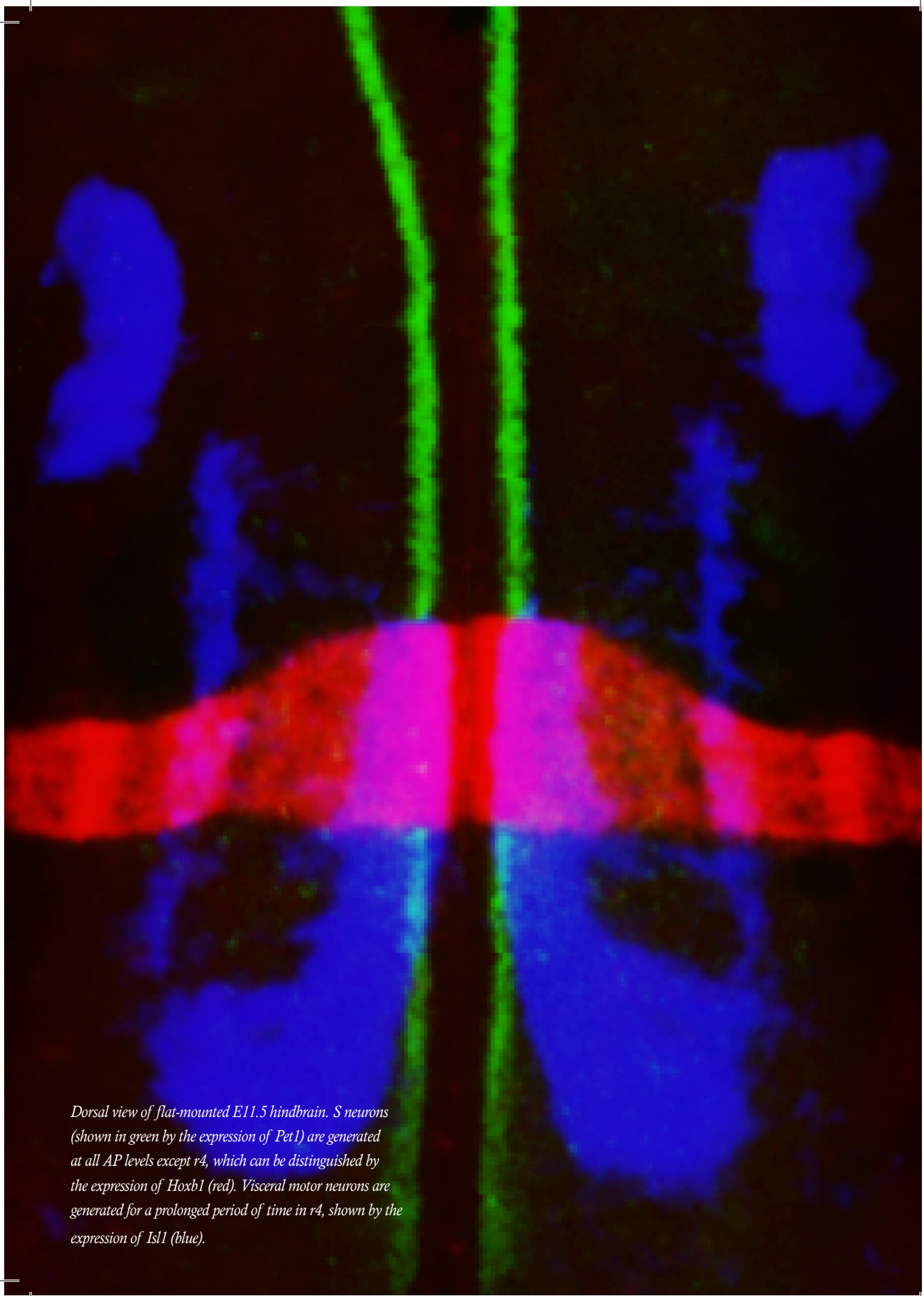
The specification of neural progenitors involves the parallel activation of several genetic programs – a program of neurogenesis that controls the selection of neural progenitors and their commitment to differentiation, and a program specifying the identity of progenitors and their subsequent neuronal subtype. The combinatorial activities of homeodomain proteins in neural progenitors play a major role in specifying the subtype identity of neurons. A number of basic helix-loop-helix (bHLH) transcription factors related to the drosophila genes achaete scute and atonal are expressed in neural progenitors and have well-established roles in more general aspects of neurogenesis; they promote cell cycle withdrawal, promote the generation of neurons at the expense of glia and induce genes common for many types of neurons, so called pan-neuronal genes (Bertrand et al., 2002). The proneural bHLH proteins are controlled by the Notch signalling pathway, which through local interactions between cells control the timing and number of cells that commit to differentiation (Appel and Eisen, 1998; Chitnis et al., 1995; Kageyama and Ohtsuka, 1999).

While bHLH proteins promote the formation of neurons from progenitor cells, the actions of Sox1-3 proteins keep progenitors in an undifferentiated state. The capacity of bHLH proteins to promote differentiation has been found to critically rely on their ability to suppress Sox1-3 expression in neural progenitor cells (Bylund et al., 2003). In addition to their more general roles in neurogenesis, increasing evidence has pointed towards a more specific role for some bHLH proteins in the establishment of unique cell fates (Bertrand et al., 2002; Parras et al., 2002; Pattyn et al., 2004). For example, similar to the homeodomain progenitor proteins in the ventral spinal cord, cross-inhibitory regulation between bHLH proteins Neurogenin 1 (Ngn1), Math1 and Mash1 has been shown to determine discrete progenitor domains and neuronal fate in the dorsal spinal cord (Gowan et al., 2001).

To ensure the proper generation of neurons, it appears likely that programs of neurogenesis must be coordinated with pathways conferring neuronal subtype identity. In support for this, studies have provided evidence for regulatory interactions between proneural bHLH proteins and homeodomain transcription factors specifying neuronal identity (Dubreuil et al., 2002; Lee and Pfaff, 2003; Mizuguchi et al., 2001; Novitch et al., 2001; Scardigli et al., 2001). For example, in the pMN domain, the Nkx6 homeodomain proteins inhibit the expression of repressors of sMN fate, while allowing the bHLH protein Olig2 to be expressed (Novitch et al., 2001; Paper I; Paper IV). Olig2, in turn, has been shown to promote the expression of downstream sMN determinants and to be required for the expression of the proneural bHLH protein Ngn2 (Mizuguchi et al., 2001; Novitch et al., 2001; Zhou et al., 2001). Also, Olig2 participates in the maintenance of the pMN domain through its repressive interactions with the p2 progenitor domain protein Irx3 (Lu et al., 2002; Novitch et al., 2001; Zhou and Anderson, 2002). Thus, these studies show that Olig2 has a dual role in integrating motor neuron subtype specification and generic neuronal differentiation and cell cycle exit.

Downstream of progenitor proteins

A number of transcription factors, many of which contain homeodomains, have been identified that are expressed in spinal cord neurons as they become postmitotic. Analysis of *Evx1* mutants has revealed an essential role for Evx1 in establishing V0 and repressing V1 neuronal identity in postmitotic cells (Moran-Rivard et al., 2001). Even though it is unclear whether Evx1 function as a repressor, these results indicate that repressive interactions not only in progenitor cells, but also in postmitotic neurons, might be important for the consolidation of neuronal identity. In support for this, the homeodomain protein HB9 has been found to be required to suppress the expression of V2 interneuronal subtype genes in sMNs, which are generated in *HB9* mutants but display cell migration and axon pathfinding defects (Arber et al., 1999). Loss of the En1 gene does not cause a dramatic change in cell identity, although En1 seems to be required for the proper axonal pathfinding of V1 interneurons. In contrast, the Isl1 gene is essential for the generation of sMNs (Pfaff et al., 1996). Thus, while some of the identified downstream factors are essential for the generation of certain neurons, others seem to contribute to the regulation of more specific functions, such as axon pathfinding.



Dorsal view of flat-mounted E11.5 hindbrain. S neurons (shown in green by the expression of Pet1) are generated at all AP levels except r4, which can be distinguished by the expression of Hoxb1 (red). Visceral motor neurons are generated for a prolonged period of time in r4, shown by the expression of Isl1 (blue).

ESTABLISHMENT OF NEURONAL IDENTITY IN THE VENTRAL HINDBRAIN

The hindbrain, or brainstem, show structural characteristics that are similar to the spinal cord. In a manner analogous to the spinal nerves, which relay sensory and motor functions to and from the trunk and extremities, the cranial nerves of the hindbrain serve specific functions for the head, neck and internal viscera. Basic behaviours necessary for survival, such as feeding, sleep and emergency responses are organised by the hindbrain and consist of relatively simple, stereotypic motor responses. Although the hindbrain is much more complex than the spinal cord and the cranial nerves mediate more intricate regulatory systems, the organisation follows the basic pattern of the spinal cord, with sensory functions localized dorsally and motor functions ventrally. In addition, like the interneurons in the spinal cord, the hindbrain contains ensembles of interneurons that coordinate reflexes and motor patterns mediated by the cranial nerves.

Patterning in the hindbrain

The strategies that are used to establish dorsoventral patterning in the spinal cord appear also to control cell identity and patterning along the dorsoventral axis of more anterior structures. For example, Shh is required for the specification of cell types in the ventral hindbrain (Chiang et al., 1996), and homeodomain proteins are expressed in specific progenitor domains in a similar fashion (Ericson et al., 1997b). However, the hindbrain differs from the spinal cord in that it during development passes through a segmented phase with a series of seven swellings, termed rhombomeres (r1-r7), forming along the anteroposterior axis of the neural tube (Lumsden and Krumlauf, 1996). Besides from being anatomically distinct, the rhombomeres are distinguished from one another by limited cell mixing (Cooke and Moens, 2002), specific expression of Hox genes and characteristic phenotypes of the constituent neurons (Cordes, 2001; Lumsden and Krumlauf, 1996). The identity of neurons in rhombomeres appears to be influenced by a spatial pattern of transcription factor expression along the anteroposterior axis that is made up by the Hox genes, together with other rhombomere-specific genes like Krox20 and Kreisler (Melton et al., 2004). In this way, while the dorsoventral positional values of a progenitor cell determine which general subtype of neuron it will become, anteroposterior position determine which cranial nerve a motor neuron belongs to. In line with this, Hox genes are involved in specifying the subtype identity of motor neurons, both at spinal cord and hindbrain levels (Cordes, 2001; Dasen et al., 2003; Melton et al., 2004). Hox genes are expressed in a distinct rhombomeres in a nested, or overlapping, fashion and are regulated by retinoic acid and FGFs, in addition to cross-regulatory interactions and regulation by other transcription factors such as Krox20 and Kreisler (Bel-Vialar et al., 2002; Gavalas and Krumlauf, 2000; Melton et al., 2004).

Cranial motor neurons

Cranial motor neurons can be classified into three types based on the dorsoventral position, axon pathway and synaptic target: somatic motor neurons (sMNs), general visceral motor neurons and special visceral motor neurons (also called branchial). sMNs innervate somite-derived muscles that control eye and tongue movements. The general visceral motor neurons project to parasympathetic neurons that innervate glands, smooth muscles, cardiovascular and gastrointestinal systems. The special visceral motor neurons, in turn, innervate branchial arch derived muscles that control jaw movements, facial expression, larynx and pharynx. During development, the

axons of motor neurons exit the hindbrain from even numbered rhombomeres through specific cranial nerves (nV-nXII). While axons of sMNs exit ventrally from the neural tube, axons of general and special visceral (collectively referred to as vMN) exit dorsally. Although vMNs and sMNs are both generated from ventral progenitors, they originate from separate progenitor domains along the DV axis (Ericson et al., 1997b). vMNs are produced from the most ventral domain termed the pMNv domain, which is topologically equivalent to the p3 domain in the spinal cord, that expresses Nkx2.2, Nkx2.9, Nkx6.1 and Nkx6.2 (Briscoe et al., 1999; Sander et al., 2000; Paper III). sMNs, in turn, are generated from the pMNs domain which is located immediately dorsal to the pMNv domain. This domain lacks the expression of Nkx2.2 and Nkx2.9, but expresses Nkx6.1, Nkx6.2, Pax6 and Olig2 (Ericson et al., 1997b; Novitch et al., 2001; Sander et al., 2000; Zhou and Anderson, 2002; Paper II). Note that in contrast to the spinal cord, Nkx6.1 and Nkx6.2 are co-expressed in ventral progenitors at this level (Paper III).

The generation of vMNs and sMNs differ also along the AP axis of the hindbrain. vMNs are produced along almost the entire AP extent of the hindbrain (r2-r7) (Pattyn et al., 2000), whereas the generation of sMNs is confined to more caudal levels, r5-r7 in chick or r5 and r7 in mouse (Arber et al., 1999). Similar to the spinal cord, sMNs are generated from Olig2+ progenitors in the pMNs domain, which is located immediately ventral to the p2 domain that expresses Irx3 and generates V2 interneurons (Briscoe et al., 2000; Novitch et al., 2001; Zhou and Anderson, 2002). Ventrally, the pMNs domain is abutted by the pMNv domain that is recognised by the expression of Nkx2.2. However, in the anterior hindbrain the expression of Olig2 is absent at stages of neurogenesis and Irx3 forms a boundary with Nkx2.2 (Paper II), indicating that the pMNs domain is absent at this level. In support for this, V2 interneurons appear to be generated immediately dorsal to vMN neurons at this level, showing that the dorsoventral patterning and position of neurons differ at anterior and caudal levels of the hindbrain (Paper II).

Specification of somatic motor neurons

As in the spinal cord, Pax6, Olig2 and Nkx6 proteins are required for the generation of sMNs in the hindbrain. Both Olig2 and Nkx6 proteins function as repressors and one role for these proteins is to repress the expression of other repressor that function to suppress a sMN fate (Muhr et al., 2001; Novitch et al., 2001). In line with this, Nkx6 proteins are necessary to prevent the repressors

Dbx1, Dbx2 and Nkx2.2 from being expressed in pMNs progenitors (Sander et al., 2000; Paper I; Paper II; Paper IV), while Olig2 suppresses the p2 domain protein Irx3 (Lu et al., 2002; Novitch et al., 2001; Zhou and Anderson, 2002). In addition, as discussed previously, Olig2 is important in sMN differentiation through its ability to promote cell-cycle exit and neuronal differentiation by promoting the expression of the proneural gene Ngn2 (Mizuguchi et al., 2001). Since Nkx6 proteins are both sufficient and required for the expression of Olig2 in the spinal cord (Novitch et al., 2001), a model in which Nkx6 proteins act upstream of Olig2 in sMN generation has emerged (Novitch et al., 2001; Zhou and Anderson, 2002). In this model, the primary requirement for Nkx6 proteins in the sMN pathway would be to ensure the expression of Olig2. However, despite that sMNs are missing in the hindbrain, the expression of Olig2 is left intact at stages of neurogenesis in *Nkx6* mutant mice (Paper II). Thus, at least at hindbrain levels, it appears that Olig2 is not sufficient to specify a sMN fate in the absence of Nkx6 proteins, indicating a parallel requirement for these proteins in sMN generation. As Nkx6 and Olig2 proteins act as repressors, it appears likely that they function to suppress different sets of repressor proteins from the pMN domain. These findings reinforce the idea (Briscoe et al., 2000) the specification of ventral cell types rely on the combined repressor activities of Class I and Class II proteins, rather than on a single key determinant of neuronal fate.

Specification of visceral motor neurons

Pax6 is required for the specification of sMNs in the hindbrain (Ericson et al., 1997b). In *Pax6* mutants, the expression domain of Nkx2.2 is expanded dorsally and the loss of sMNs is accompanied by a corresponding increase of vMNs (Ericson et al., 1997b). Phox2b is an important determinant of vMN fate and is expressed in pMNv progenitors during stages of neurogenesis and in postmitotic vMNs. As such, it is both necessary (Pattyn et al., 2000) and sufficient (Dubreuil et al., 2000) for the generation of the vMN phenotype. In line with this, the expression of Phox2b also expands dorsally in Pax6 mutant mice (Mizuguchi et al., 2001). Olig2 coordinately promotes pan-neuronal properties and cell-type specificity in the generation of sMNs (Mizuguchi et al., 2001; Novitch et al., 2001). In a similar manner, Phox2b promotes cell cycle exit and neuronal differentiation through the upregulation of proneural bHLH proteins Ngn2 and Mash1 and simultaneous repression of negative regulators of neurogenesis (Dubreuil et al., 2002). In addition, misexpression experiments have demonstrated a role for Phox2b in downregulating Pax6 and

Olig2. (Dubreuil et al., 2002). Thus, the similar function of Phox2b and Olig2 in coupling general aspects of neuronal differentiation with cell type specificity might represent a common rationale to modulate the number of neurons produced as a function of their type. An important difference between Olig2 and Phox2b, however, is that Phox2b appears to function as an activator, while Olig2 is a repressor (Dubreuil et al., 2002; Novitch et al., 2001).

Given that Phox2b is such an important determinant of vMN generation, an important issue to resolve is the mechanisms by which the expression of Phox2b is activated in the hindbrain. Nkx2.2 and Phox2b are co-expressed in hindbrain vMN progenitors. The observation that both Nkx2.2 and Phox2b are dorsally expanded in *Pax6* mutants, leading to the ectopic generation of vMNs, raises the question of whether there are any regulatory interactions between these proteins. In support for this, misexpression of Nkx2.2 in hindbrain progenitor cells is sufficient to mediate the ectopic activation of Phox2b (Paper II). Several studies have also indicated the importance of Hox genes for the expression of Phox2b (Davenne et al., 1999; Gaufo et al., 2000; Paper III). Through the identification of a conserved Phox2b proximal enhancer that mediates rhombomere restricted expression, Phox2b has recently been found to be a direct target of Hoxb1 and Hoxb2 (Samad et al., 2004). In this study, the actions of Nkx2.2 were found to enhance the Hox mediated transcriptional activation of Phox2b via its function as a repressor. Moreover, the ectopic induction of Phox2b-expressing vMNs in the hindbrain required the combined activities of Hox and Nkx2.2 proteins (Samad et al., 2004), further establishing Nkx2.2 as an important player in vMN generation. Arguing against this is the observation that mice lacking Nkx2.2 protein show no defect in vMN generation (Briscoe et al., 1999). However, Nkx2.9 is expressed in a similar fashion to Nkx2.2 in the hindbrain and *Nkx2.9* mutant mice show only minor defects in vMN generation (Pabst et al., 2003), suggesting that these related proteins work in a redundant fashion during the specification of vMNs. In support for this, Nkx2.2 and Nkx2.9 mediate similar activities when expressed at ectopic locations in the neural tube (Briscoe et al., 1999). However, Nkx2.9 is unable to compensate for the loss of Nkx2.2 in the generation of hindbrain serotonergic neurons (discussed below), indicating differences in the intrinsic properties of these proteins (Briscoe and Ericson, 1999; Paper III). Thus, the analysis of mice mutant for both these proteins will be an important future experiment in order to resolve the precise role for Nkx2 proteins in vMN generation. Nevertheless, considering that Nkx2.2 is required to suppress sMN

generation in the p3 domain of the spinal cord (Briscoe and Ericson, 1999), a prediction from these experiments would be a loss of vMN generation in favour of sMN production.

Nkx6.1 and Nkx6.2 proteins are also expressed in vMN progenitors. While being essential for the generation of sMNs, they are dispensable for the initial establishment of vMN identity (Paper II). However, they are required to prevent differentiating vMNs from initiating a parallel program of V0 neuronal differentiation (Paper II). Furthermore, Nkx6.1 and Nkx6.2 are expressed in postmitotic vMNs and are necessary for the correct pathfinding and migration all vMNs regardless of anteroposterior origin, although different degrees of severity are observed along the AP axis (Sander et al., 2000; Paper II). Since Nkx6 proteins are expressed in both progenitors of vMNs and in the postmitotic neurons, it is difficult to definitely link the requirement for these proteins in migration and axon pathfinding to postmitotic neurons. An alternative explanation could involve a change in the environment encountered by differentiating neurons due to the loss of Nkx6 proteins. Further, it is difficult to establish if the early role of Nkx6 proteins to prevent a parallel induction of the V0 determinant Evx1 reflects a requirement in progenitors and/or postmitotic neurons. However, the migration and axonal pathfinding defects observed in *Nkx6* mutant mice are not likely to be a secondary effect of the activation of Evx1 in differentiating vMNs, since vMNs in *Nkx6.1* single mutant mice show similar but milder defect without the ectopic expression of Evx1 (Paper II). Further, the expression of netrin receptors in vMNs is altered in *Nkx6.1* mutant embryos (Muller et al., 2003), arguing for a cell-autonomous role for Nkx6 proteins in differentiating vMNs. Nkx6 proteins appear to function as repressors in progenitor cells (Muhr et al., 2001), but it is conceivable that in postmitotic neurons the activities of these proteins would in a context-dependant manner require the interaction with different sets of proteins than in progenitor cells and possibly reflect activator functions instead. In line with this, other Nkx proteins have been shown to possess activator and repressor functions in non-neural cells (Choi et al., 1999b; Watada et al., 2000).

In summary, it appears that different sets of homeodomain proteins influence complementary and distinct features of vMN differentiation: Hox proteins regulate vMN identity along the AP axis, Nkx2 proteins are likely to be important for the activation of Phox2b, Phox2b in turn is essential for the very generation of these cells and Nkx6 proteins prevent parallel programs

of interneuronal differentiation as well as being important for the correct migration and pathfinding of postmitotic vMNs.

From visceral motor neurons to serotonergic neurons – temporal mechanisms

While mechanisms of spatial patterning confer neural progenitors with a positional identity along the dorsoventral and anteroposterior axis that direct the fate of cells, distinct cell types are also generated in a specific temporal order, often from common pools of neural progenitor cells. Thus, cell diversity in the CNS is obtained through mechanisms that operate both in space and over time in the developing embryo. In this way, temporal identity can be viewed as another axis of information that a progenitor cell can use to create cell type diversity. Issues of temporal identity have been addressed in different systems; for example the drosophila CNS, the vertebrate retina, the vertebrate cortex as well as more caudal parts of the nervous system (Pearson and Doe, 2004). These studies have indicated that the sequential production of different cell types reflects temporal changes in progenitors, either through intrinsic mechanisms or in response to extrinsic factors. Despite of this, few temporal determinants have been identified.

An intriguing feature of the pMNv domain is that it subsequent to the generation of vMNs gives rise to another type of neuron; serotonergic (S) neurons. In the study presented in Paper III, the sequential generation of vMNs and S neurons from Nkx2.2+ pMNv progenitors was examined. Birthdating studies showed that each rhombomere initially generates vMNs, but around E10.5 in the mouse all the rhombomeres except for r4 switch to producing S neurons. Instead, r4 continues to generate vMNs for a prolonged time period, raising two important questions; how do progenitors change from vMN to S neuron generation and what prevents r4 from making this switch?

As discussed earlier, Phox2b is required for the generation of vMNs in the hindbrain. In r2–r3 and r5–r7, the switch from vMN to S neuron generation was found to coincide with a downregulation of Phox2b in the most ventral portion of the pMNv domain, leaving these progenitors expressing Nkx2.2 only. In r4, however, Phox2b expression is maintained in the entire pMNv domain up until late time points (E12.5). r4 is further distinguished from the other rhombomeres by its specific expression of Hoxb1, which has been found to be essential in the establishment of r4 identity (Gaufo et al., 2000; Studer

et al., 1996). Strikingly, r4 of *Hoxb1* mutant embryos was found to generate S neurons and lack the prolonged generation of vMNs. Moreover, Nkx6.1 and Nkx6.2 are required to maintain Hoxb1 expression in the ventral part of r4. In *Nkx6* mutant mice, r4 begins to generate S neurons around the time point that expression of Hoxb1 is lost. These findings suggest that Hoxb1 suppresses the switch to S neuron generation, possibly by directly activating Phox2b in progenitors at this level (Samad et al., 2004). In addition, in *Hoxb2* mutant embryos the expression of Hoxb1 is downregulated at a later time point than in mice lacking Nkx6 function, leading to a similar but milder phenotype than in *Nkx6* mutants.

In conclusion, Hoxb1 appears to suppress S neuron production by prolonging the expression of Phox2b in the ventral part of r4, a notion that is supported by the observation that *Phox2b* mutant mice prematurely generate S neurons at all hindbrain levels (Paper III). Further, mice lacking Nkx2.2 function fail to downregulate the expression of Phox2b in the most ventral portion of the pMN domain and these mice lack S neuron generation (see below). Thus, the integrated activities of Nkx- and Hox class homeodomain proteins coordinate the expression of Phox2b in progenitor cells. In turn, the downregulation of Phox2b in ventral hindbrain progenitors seems to be a key event in the switch from vMN to S neuronal generation (Figure 3). DV and AP patterning have generally been analyzed independently, leaving open the issue as to what degree these patterning mechanisms are integrated. The late role of Nkx6 proteins in maintaining the expression of Hoxb1 in r4 progenitors reveals a close regulatory link between factors conferring DV and AP identity at time points after the initial establishment of patterning along both axes.

To a certain degree, the role of Phox2b in promoting early-born vMNs and suppressing late-born S neurons show similarity to the temporal determinant Hunchback in the drosophila CNS (Isshiki et al., 2001). Hunchback is required for the temporal identity of first-born cells in neuroblast lineages and when neuroblasts are forced to continually express Hunchback, late-born cells are suppressed (Isshiki et al., 2001). Depending on the spatial identity of the neuroblast, Hunchback expressing cells can give rise to different cell types and Hunchback is therefore correlated with a temporal identity and not a cell type identity. Phox2b, however, is more correlated to a specific cell type, as misexpression experiments show that Phox2b induces cells with a vMN phenotype (Dubreuil et al., 2000). Whereas Hunchback and Phox2b promotes

early-born cells, a temporal factor in the vertebrate cortex that suppresses early-born fates and promotes late-born cells has recently been identified; Foxg1 (Hanashima et al., 2004). The developmental potential of cortical progenitor cells has previously been found to become restricted with time, as late cortical progenitors transplanted to the cerebral cortex of a younger host are limited to the generation of later born fates (Frantz and McConnell, 1996; McConnell and Kaznowski, 1991). Interestingly, the study on Foxg1 function suggests that the competence of late cortical progenitors to generate the earliest-born cell fate is maintained, but actively suppressed, and that Foxg1 is required in later progenitors to suppress this earliest-born cell fate (Hanashima et al., 2004).

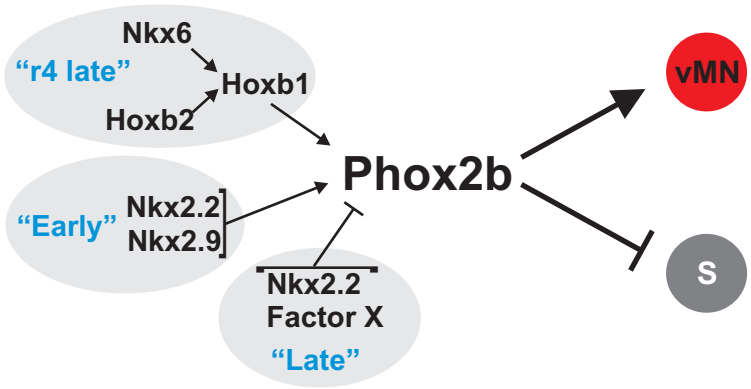


Figure 3. Model of vMN and S neuron generation in the ventral hindbrain. Nkx and Hox HD proteins coordinate the spatial and temporal expression of Phox2b in neural progenitors. In turn, Phox2b acts as a molecular switch that determines whether progenitors select a vMN or S neuron fate.

How the switch from vMN to S neuron generation in the hindbrain is initiated is unclear, but it appears to require the temporal downregulation of Phox2b in progenitors (Paper III). Interestingly, Nkx2.2 has been implicated in the induction of Phox2b and is furthermore required for the temporal downregulation of the same factor. How these opposing roles of Nkx2.2 are achieved is not known, but might involve a signal from the floor plate that could induce or activate a co-factor (Factor X) that is necessary for Nkx2.2

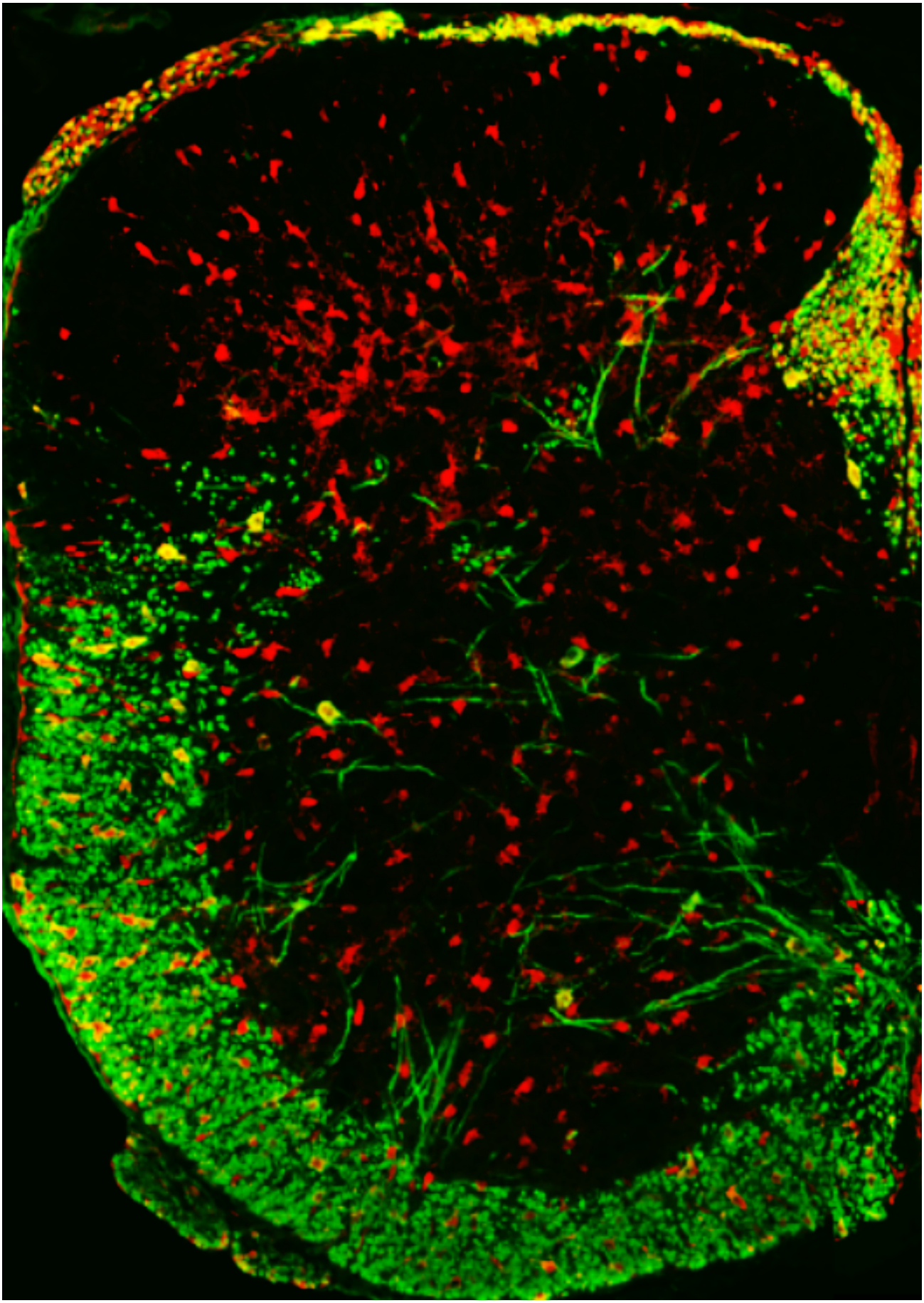
to repress Phox2b (Figure 3). In the spinal cord, somatic motor neurons and oligodendrocytes are sequentially generated in the pMN domain. Studies in the chick have revealed that the switch from producing motor neurons to start producing oligodendrocytes may be triggered by an expansion of Nkx2.2 expression into the Olig2+ domain (Zhou et al., 2001). Thus, a common theme in temporal specification of the spinal cord and hindbrain appears to be a change in gene expression over time of determinants that are also involved in the control of spatial patterning. In this view, the temporal control of cell fate may be mechanistically similar to spatial patterning, but merely require that expression patterns are dynamic and modulate over time. Studies from the vertebrate retina and the drosophila CNS have suggested that intrinsic changes in the progenitor play a large role in determining temporal identity in these systems. In the vertebrate cortex, spinal cord and hindbrain, it is less clear whether intrinsic or extrinsic changes play the primary role. In the future, it will be interesting to investigate the mechanisms, intrinsic or extrinsic, that lie behind the temporal profiles of gene expression patterns in these systems.

Development of serotonergic neurons

In contrast to the local-circuit interneurons that coordinate reflexes and motor patterns mediated by the cranial nerves, serotonergic neurons are long projecting interneurons that through widely dispersed connections regulate a number of complex functions of the CNS. They are located in two groups of nuclei called the caudal and rostral Raphe nuclei and data suggest that these groups are located anterior and posterior to r4, respectively (Paper III). While descending projections from the caudal Raphe nuclei to the spinal cord influence muscle tone in the motor system and pain perception, ascending projections from the rostal nuclei to the forebrain regulate such diverse function as wake-sleep cycles, appetite control, thermoregulation, anxiety and behaviour (Rubenstein, 1998).

A number of proteins have been implicated in the development of serotonergic neurons. One of the first to be identified was the homeodomain proteins Nkx2.2. Mice mutant for Nkx2.2 display a close to complete lack in S neurons at all hindbrain levels (Briscoe et al., 1999), with the exception of the most anterior nuclei (called the dorsal raphe nuclei) originating from r1 (Briscoe et al., 1999; Ding et al., 2003). The bHLH protein Mash1 has recently been found to be important for the specification of S neurons, as there is a complete lack of these neurons at all levels of *Mash1* mutant embryos (Pattyn et al 2004).

Mash1 is coexpressed with Nkx2.2 in pMNv progenitors and is important as a proneural gene for the production of postmitotic neurons and as a determinant of the serotonergic phenotype in the activation of more downstream determinants (Pattyn et al., 2004). While, Nkx2.2 and Mash1 appear to have upstream roles in the activation of downstream determinants of S neuron fate, the subsequent differentiation of serotonergic neurons has been shown to involve the homeobox protein Lmx1b (Ding et al. 2003), the Ets-domain protein Pet1 (Hendricks et al., 2003) and the zinc-finger proteins Gata3 (van Doorninck et al., 1999) and Gata2 (Craven et al., 2004). Together, these studies have indicated that the transcriptional program of S neuron differentiation is to some extent regulated differently in the rostral versus caudal Raphe nuclei. The precise genetic programs controlling serotonergic differentiation is unclear, although it seems to involve either a parallel activation of Gata3, Lmx1b and Pet1 (Pattyn et al., 2004) or a more upstream role of Lmx1b (Ding et al., 2003) in a genetic cascade of these proteins.



OLIGODENDROCYTE GENERATION IN THE SPINAL CORD AND HINDBRAIN

While neurons in the nervous system are primarily responsible for transfer of information, they are dependant on, interact with and are surrounded by glial cells. Glial cells constitute more than 90% of the cells in the mature vertebrate CNS and only 10-20% in drosophila, implying that glial function is crucial for the increased neurological complexity that has emerged during evolution. Traditional views of glial function have proclaimed passive and mere supportive roles for glia in nervous system function. However, increasing observations of more specific functions for astrocytes and oligodendrocytes are beginning to shatter this view and points towards interesting and vital roles for these cell types in the operation of the CNS. Astrocytes have general roles in providing structural support, regulating water and ion balance, and maintaining the blood-brain barrier (Rowitch, 2004). Recent data has also shown that astrocytes participate in cell-cell signalling through, for example, modulation of synaptic transmission (Newman, 2003). Oligodendrocytes form myelin sheaths that insulate axons, providing an essential enhancement in signal transduction of nerve impulses (Barres and Barde, 2000). Additionally, oligodendrocyte precursor cells form synapses with GABA-releasing interneurons in the hippocampus, suggesting the existence of complex interactions between neurons and glia (Lin and Bergles, 2004).

Neurons versus glia

A fundamental question in developmental neurobiology is how a relatively simple and undifferentiated neuroepithelium can give rise to the remarkable cellular diversity of the CNS. Neurons, astrocytes and oligodendrocytes are all specified from proliferating progenitor cells in the ventricular zone of the spinal cord and brain and studies in the spinal cord have shown that neurons are generated prior to glia (Rowitch, 2004). When differentiating neurons leave the proliferative ventricular zone, they concomitantly exit the cell cycle and begin to express neuronal specific markers (Jessell, 2000). In contrast, during gliogenesis cells leave the ventricular zone as proliferative precursors that migrate to eventually become widely dispersed in the CNS (Miller, 2002). Thus, with respect to glial cells, the main phase of proliferation might occur long after the initial commitment of a relatively small proliferative precursor cell population, making the specification and differentiation of glia somewhat more difficult to study than neurons. In the case of oligodendrocytes, the terminal differentiation occurs largely postnatally and not all cells differentiate but some remain as slowly dividing oligodendrocyte precursors in the adult CNS (Miller, 2002).

Substantial progress has been made towards understanding the mechanisms that underlie the development of neurons, as numerous studies have demonstrated the importance of extrinsic signalling molecules and patterning of transcription factors in this process (Jessell, 2000). Less is known about the mechanisms behind the specification of different glial cells. However, studies have begun to indicate that glia might be specified through analogous mechanisms to neurons. Initial indications of this stemmed from the observation that oligodendrocyte precursor cells (OLPs) emerge from a discrete region of the ventral neural tube (Pringle and Richardson, 1993; Timsit et al., 1995; Warf et al., 1991), rather than from diffuse locations along the dorsoventral axis as their scattered distribution in the adult CNS would imply.

Specification of spinal cord oligodendrocytes

OLPs are generated from a restricted domain in the ventral spinal cord that also generates sMNs, termed the pMNs domain (Hall et al., 1996; Pringle and Richardson, 1993). These OLPs can be defined by the selective expression of PDGFRα (Hall et al., 1996; Pringle and Richardson, 1993) and Sox10 (Kuhlbrodt et al., 1998) and are first detected at around E12.5 in the mouse. Thus, sMNs are generated between E9 and E12 (Jessell, 2000) and followed

closely by the generation of OLPs, which has lead to the suggestion of a close developmental relationship between these two cell types (Richardson et al., 2000). Additionally, Shh is both sufficient and necessary for oligodendrocyte induction in the ventral spinal cord, indicating further parallels with the development of neurons (Orentas et al., 1999; Poncet et al., 1996; Pringle et al., 1996).

The bHLH proteins Olig1 and Olig2 are specifically expressed in the pMNs domain of the spinal cord (Lu et al., 2000; Takebayashi et al., 2000; Zhou et al., 2000). Additionally, both proteins are expressed up to late stages in the oligodendrocyte precursors. Functional analysis in mice has show that Olig1 and Olig2 are required for the establishment of the pMNs domain and a null mutation of Olig2 alone results in a failure of development of all cells originating from the pMNs domain; sMNs and OLPs (Lu et al., 2002; Zhou and Anderson, 2002). In *Olig2* and *Olig1/2* mutant mice, the expression of the p2 domain protein Irx3 expands ventrally, leading to an ectopic generation of V2 interneurons at the expense of sMNs (Lu et al., 2002; Zhou and Anderson, 2002). Interestingly, the subsequent loss of oligodendrocyte generation in these mutants is accompanied by an ectopic generation of astrocytes, suggesting that glial subtypes are normally generated from different domains in a manner similar to the generation of different neuronal subtypes from distinct domains.

Consistent with the early requirement for Nkx6 proteins in the expression of Olig2 and generation of sMNs, these proteins are also required for the generation of OLPs from the ventral spinal cord (Paper IV). In Nkx6 mutants, the loss of Olig1/2 is correlated with a dorsal expansion of Nkx2.2 into the pMNs domain. Since Nkx2.2 is an established repressor of Olig2 expression (Novitch et al., 2001), it is conceivable that the requirement for Nkx6 proteins for the expression of Olig2 reflects their role in suppressing Nkx2.2.

Neuron to glial switch

The requirement for Olig1/2 proteins in the generation of both sMNs and OLPs raises the question of how progenitors in a common domain switch from producing neurons to generating glial cells. In the forebrain, the downregulation of proneural activity appears to be an important step in the onset of gliogenesis, as loss of proneural genes Ngn2 and Mash1 leads to the premature generation of astrocytes (Nieto et al., 2001). In addition, biochemical studies in neural stem cell cultures have shown that the proneural gene Ngn1

inhibits transcription of astrocyte differentiation factors by two independent mechanisms (Sun et al., 2001). Studies in the chick spinal cord has shown that the onset of oligodendrocyte differentiation is preceded by the downregulation of Ngn1 and Ngn2, and the ectopic expression of Olig2 in the absence of Ngn1/2 induces OLPs (Zhou et al., 2001). However, in *Ngn1/2* mutant mice the generation of oligodendrocytes is not affected (Rowitch, 2004), indicating that the neuron to glial switch in the pMNs domain is more complex.

In the chick, the collaboration of Olig2 with Nkx2.2 has been implicated in the temporal switch from sMNs to OLPs, as the domains of Olig2 and Nkx2.2 expression switch from being mutually exclusive to overlapping just prior to the appearance of OLPs in the pMNs domain (Zhou et al., 2001). In addition, misexpression of Olig2 together with Nkx2.2 promotes the generation of ectopic OLPs. However, studies in mice have showed that at stages of oligodendrogenesis the expression of Olig2 and Nkx2.2 do not significantly overlap (Fu et al., 2002; Paper IV) and the loss of Nkx2.2 does not reduce the initial specification of OLPs from the pMNs domain (Qi et al., 2001). Thus, in mice, an Olig2-Nkx2.2 interaction is not likely to play a major role in the specification of OLPs from the pMNs domain. Instead, Nkx2.2 is probable to have a role in suppressing Olig2 expression, and oligodendrogenesis, as indicated by the analysis of Nkx6 mutant mice (See above; Paper IV).

Analysis of mutant zebrafish embryos has provided evidence that Notch signalling is required to maintain a subset of Olig2+ progenitors until the oligodendrogenic phase starts in the pMNs (Park and Appel, 2003). Interestingly, forced expression of Notch1 blocked neurogenesis and resulted in an increased generation of OLPs, but without changing the timing of OLP generation. These data indicate that Notch signalling is permissive rather than instructive and acts together with other temporal and spatial factors to specify oligodendrocytes. In a recent study, the HMG protein Sox9 was demonstrated to play an important role in the specification of both oligodendrocytes and astrocytes (Stolt et al., 2003). Sox9 is expressed in both glial progenitors and glial cells and loss of Sox9 leads to a decrease in the number of astrocytes and oligodendrocytes and a concomitant increase in sMNs and V2 interneurons, suggesting that Sox9 is a major component of the neuron-glial switch in the developing spinal cord. Thus, the switch from neuronal to glial production in the pMNs domain seems to involve the Notch pathway and the transcription factor Sox9, coupled with downregulation of proneural activity.

Oligodendrocyte origins

The ventral origin of oligodendrocytes in the spinal cord is well established and has been demonstrated in a broad range of species including xenopus, humans, chick, mouse and rat (Hajihosseini et al., 1996; Maier and Miller, 1995; Ono et al., 1995; Pringle and Richardson, 1993; Warf et al., 1991). Nonetheless, it has remained unclear whether also other progenitors give rise to oligodendrocytes and the possible contribution of dorsal neuroepithelium to oligodendrocyte generation has been under intensive investigation and considerable debate (Noble et al., 2004; Richardson et al., 2000; Spassky et al., 1998). Initial studies of PDGFRα expression and culture of ventral and dorsal spinal cord suggested that oligodendrocyte precursors were only present in ventral regions of the spinal cord (Ono et al., 1995; Warf et al., 1991). A dorsal source of oligodendrocyte precursors in spinal cord regions was suggested from initial chick-quail transplantation studies (Cameron-Curry and Le Douarin, 1995), while a subsequent analysis of similar chimeric spinal cords argued that dorsal neuroepithelium produces astrocytes but not oligodendrocytes (Pringle et al., 1998). Moreover, *in vitro* assays have suggested the existence of a glial-restricted precursor cells (GRP) that can be derived from both dorsal and ventral parts of the spinal cord and give rise to astrocytes or oligodendrocytes in culture (Gregori et al., 2002; Rao et al., 1998). While neural specification *in vivo* is tightly linked to the position of individual progenitor cells in the developing CNS (Jessell, 2000; Temple, 2001), the potency of single cells in culture has been found to increase by the use of commonly used culture conditions (Gabay et al., 2003), thus questioning whether the *in vitro* potential of progenitor cells reflects their endogenous capacity *in vivo*.

In the study presented in Paper IV, the positional specification of oligodendrocytes in the spinal cord and hindbrain was examined. Olig1 and Olig2 genes are required for the generation of all oligodendrocytes in the entire CNS (Lu et al., 2002; Zhou and Anderson, 2002). In addition to the ventral expression of Olig1 and Olig2, expression of these proteins in a dorsal domain of the hindbrain was observed in a recent analysis (Liu et al., 2003). A detailed examination of the dorsal spinal cord revealed co-expression of scattered Olig2+ cells with progenitor markers specific for dorsal neuroepithelium, Pax7 and Gsh1, suggesting that some Olig2+ cells are generated from the dorsal the spinal cord (Paper IV). In line with this, explants derived from both dorsal and ventral spinal cord and hindbrain tissue give rise to Olig2+ oligodendrocytes in culture. Importantly, the explants were cultured in the absence of FGF2, as

FGF2 has been found to ventralize dorsal tissue and induce the expression of Olig2 and oligodendrocyte differentiation (Chandran et al., 2003; Gabay et al., 2003). The expression of Olig2 in dorsal explants was linked to Pax7, showing that explants retain their dorsal identity *in vitro*.

Additional support for a generation of oligodendrocytes from dorsal progenitors stems from an analysis of *Nkx6* mutant mice (Paper IV). Under normal conditions, oligodendrocyte precursor can be detected by the expression of PDGFRα (Hall et al., 1996; Pringle and Richardson, 1993) and Sox10 (Kuhlbrodt et al., 1998) in the Olig1/2 expressing pMNs domain from around E12 in the mouse and at subsequent time points, migrating OLPs are distributed throughout the spinal cord. In mice lacking Nkx6 function Olig1/2, Sox10 and PDGFRα expression in the ventral spinal cord is virtually absent, showing that Nkx6 proteins are essential for the generation of pMN domain-derived oligodendrocytes. However, since these proteins are not expressed in the dorsal part of the spinal cord, they are predicted not to affect putative dorsally specified oligodendrocytes. In support for this, Olig1 and Olig2 expressing cells can be detected in the dorsal neuroepithelium at E15 of *Nkx6* mutant mice and these cells differentiate along the oligodendrocyte lineage *in vivo* and *in vitro*. Taken together, these data strongly suggest that oligodendrocytes in the spinal cord are generated from both ventral and dorsal progenitor cells.

An interesting observation is that the first Olig2+ cells located in the dorsal progenitor domain can be detected several days after ventral OLPs are starting to be generated from the pMNs domain (at E15). Considering that neurons in the dorsal spinal cord appear to be generated up to later embryonic stages than ventral neurons (Jessell 2000, Helms and Johnson 2002), this observation is consistent with the idea that gliogenesis in the spinal cord takes place subsequent to neurogenesis (Figure 4). Local BMP signalling from the roof plate has a central role in the initial establishment of dorsal progenitor identity and in the generation of dorsal neuronal subtypes (Lee and Jessell, 1999). BMPs also function to suppress more ventral fates that are dependent on Shh signalling, including sMNs and oligodendrocytes (Liem et al., 2000; Mekki-Dauriac et al., 2002). In explant assays, BMP7 suppresses oligodendrocyte differentiation from dorsal progenitors while BMP antagonists enhance generation of dorsal Olig2+ cells in dorsal explants isolated at early developmental stages (Paper IV). Thus, the timing of Olig1/2 induction in dorsal progenitors might involve a progressive evasion of BMP signalling from the roof plate, possibly due to the

increase in size that the spinal cord show at these late developmental stages.

Specification of oligodendrocytes at different axial levels

Oligodendrocytes in the forebrain have been proposed to arise from ventral regions and, like their spinal cord counterparts, require Shh signalling (Nery et al., 2001; Spassky et al., 2001; Tekki-Kessaris et al., 2001). However, studies have also indicated the generation of oligodendrocytes from other regions of the forebrain (Gorski et al., 2002; Ivanova et al., 2003; Levison and Goldman, 1993). Thus, it is possible that the generation of oligodendrocytes from multiple positions is a general feature of the CNS. Subtype identity and functional properties of neurons are tightly linked to the position of origin within the CNS. Increasing evidence suggests the existence of functional differences between subpopulations of oligodendrocytes, as some oligodendrocytes has been found to establish synapses with GABAergic interneurons in the hippocampus and others remain as undifferentiated precursors over extended periods of time (Lin and Bergles, 2004; Zerlin et al., 2004). However, it remains to be determined whether such differences are correlated with the generation of oligodendrocytes from distinct progenitor populations in the CNS. In addition, it is difficult to assess the relative contribution of oligodendrocyte precursors derived from different positions in the CNS to the mature population of oligodendrocytes, since it is formally possible that an initially very small population specified from certain region might proliferate rapidly and eventually give rise to a substantial number of cells and vice versa.

Although Olig1/2 proteins are required for the generation of all oligodendrocytes regardless of the developmental origin in the CNS (Lu et al., 2002; Zhou and Anderson, 2002), the requirement for Olig1 and/or Olig2 differs at different axial levels. Oligodendrocytes fail to develop in the spinal cord of mice lacking Olig2 function, showing that Olig1 is inadequate to compensate for the loss of Olig2 (Lu et al., 2002). In the brain however, oligodendrocytes develop normally in mice mutant for Olig2, indicating that at this level Olig1 is necessary and sufficient for oligodendrocyte development in the absence of Olig2 function (Lu et al., 2002). In support for this, oligodendrocytes in the brain are missing in *Olig1/2* compound mice (Zhou and Anderson, 2002).

The expression of Olig1 is still present in the pMNs domain of *Olig2* mutant mice, but the integrity of the pMNs domain is compromised as the expression of p2 progenitor marker Irx3 is expanded ventrally and V2 neurons are

ectopically generated (Lu et al., 2002). Although present, Olig1 is not sufficient to promote the generation of both cell types originating from this domain; sMNs and OLPs, raising the possibility that the requirement for Olig2 in ventral OLP specification reflects its role in patterning and in the establishment of the pMNs domain. In contrast to the homogeneous expression in the ventral pMNs domain, the expression of Olig1/2 can be detected in scattered cells in the lateral part of the progenitor domain in the dorsal hindbrain and spinal cord as well as in the ventral anterior hindbrain (see below, Paper IV), making it unlikely that Olig1/2 participate in patterning at these positions. An interesting observation is that in *Olig2* mutant mice, migrating Olig1+ cells can be detected in the dorsal part of the spinal cord, although no cells are generated from the ventral domain (Figure 6, Lu et al., 2002). Thus, it is possible that Olig1 function is sufficient for the initial specification of dorsally-derived OLPs, but that these cells fail to progress in their differentiation due to the lack of Olig2.

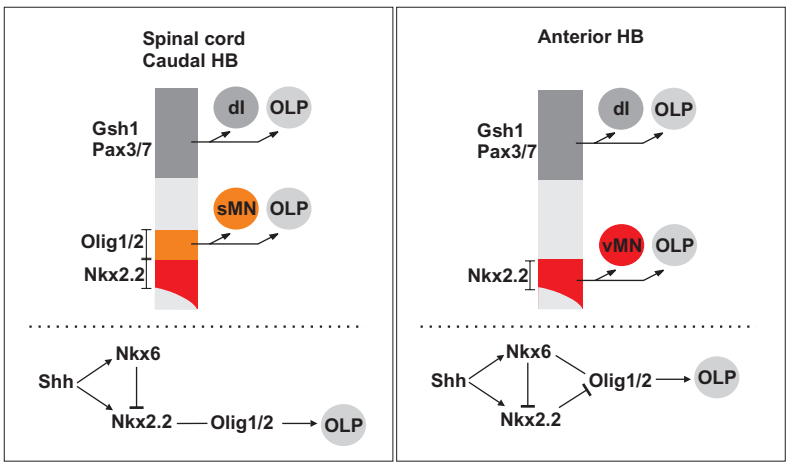


Figure 4. Model of oligodendrocyte generation in the spinal cord and hindbrain. Dorsal progenitors expressing Gsh1 and Pax3/7 generate OLPs at both levels. In the ventral spinal cord and caudal hindbrain, sMNs and OLPs are sequentially generated from the Olig2+ domain. In contrast, the anterior hindbrain generate OLPs from the Nkx2.2+ domain that also generates vMNs. OLPs are generated from different domains in the anterior vs caudal neural tube and are likely to be specified through distinct genetic programs,

Similar to the spinal cord, in the caudal hindbrain sMNs and oligodendrocytes are generated from the Olig1/2+ pMNs domain that is located dorsal to the Nkx2.2-expressing domain (Ericson et al., 1997b; Zhou and Anderson, 2002). In contrast, the anterior hindbrain lacks a pMNs domain and do not express Olig1/2 at stages of neurogenesis. Instead the Nkx2.2+ pMNv domain is broader and borders the p2 domain (Paper II). Taking this into account it is reasonable to consider that the induction of Olig1/2 expression and specification of OLPs will occur differently in the anterior hindbrain compared to more caudal levels. In line with this, expression of Olig1/2 can first be detected at E12.5 in scattered cells within the Nkx2.2-expressing pMNv domain in the anterior hindbrain (Paper II; Paper IV). Furthermore, while Nkx6 proteins are essential for oligodendrocyte specification in the ventral spinal cord caudal hindbrain (Novitch et al., 2001; Paper IV), the loss of these proteins in the anterior hindbrain instead lead to a premature induction of Olig1/2 expression and OLP differentiation (Paper II; Paper IV). A common phenotype in the brain and spinal cord of *Nkx6* mutant mice is the dorsal expansion of Nkx2.2 expression. In the anterior hindbrain, this dorsal expansion correlates well with the ectopic induction of oligodendrocyte precursor markers. Thus, while Nkx2.2 inhibits OLP differentiation in the ventral spinal cord and caudal hindbrain, the same protein appear to promote the generation of OLPs at anterior hindbrain levels (Figure 4). These data indicate that the activation of Olig1/2 and oligodendrocyte differentiation at different axial positions is controlled by distinct genetic programs.

CONCLUSIONS AND FUTURE PROSPECTS

During development, neurons and glia are generated from proliferative neuroepithelial progenitor cells lining the ventricles of the brain and the lumen of the spinal cord. The patterned expression of transcription factors of the homeodomain (HD) protein family provides progenitors with a positional identity that will influence the fate of cells. The work presented in this thesis has aimed towards providing insight into the role of some Nkx homeodomain proteins in the specification of cell fate in the caudal nervous system. Our findings indicate diverse functions of these proteins in the spatial and temporal generation of cell types in the developing spinal cord and hindbrain.

The two closely related HD proteins Nkx6.1 and Nkx6.2 (collectively referred to as Nkx6) are expressed in overlapping domains of the ventral part of the ventral spinal cord. In a series of gain- and loss of function experiments, we investigated the role of these proteins in the establishment of progenitor cell identity and neuronal cell differentiation of the spinal cord. Nkx6.1 and Nkx6.2 were found to display both redundant and distinct functions in the specification of ventral neuronal subtypes. These functions reflect differences in the level of repressor activity of Nkx6.1 and Nkx6.2, where the amount of repressor activity directs different classes of neurons with somatic motor neurons (sMNs) requiring the highest and V0 neurons the lowest repressor activity. Thus, in the ventral spinal cord, different levels of Nkx6 repressor activity provides a potential for enhanced diversification of ventral neuronal subtypes. A reduction in Nkx6 activity further permits V0 interneurons to be generated from progenitors that lack Dbx1, a HD protein normally required for their development (Pierani et al., 2001). These observations provide direct evidence for a previously suggested model of ventral neuronal specification (Muhr et al., 2001), where HD proteins direct specific cell fates by actively repressing the expression of transcription factors that direct alternative fates.

The loss of sMNs in the spinal cord of *Nkx6* mutant mice correlates with an extinguished expression of the bHLH protein Olig2 (**Paper I**; Novitch et al., 2001), which is likewise required for the generation of these cells (Lu et al., 2002, Zhou and Anderson, 2002). This observation has led to a model

where Nkx6 proteins are strictly upstream of Olig2 in the generation of sMNs. However, we found that the initial expression of Olig2 is intact in the hindbrain of *Nkx6* mutant mice and despite this all sMNs are missing. This observation indicates a parallel requirement for Nkx6 and Olig2 proteins in the generation of sMNs, reinforcing the idea that the combined activities of progenitor proteins are required in ventral cell specification.

In the hindbrain, visceral motor neurons (vMNs) are generated immediately ventral to sMNs. The transcription factor Phox2b has been found to be an important determinant of these cells (Pattyn et al., 2000), but other factors involved have not been identified. We show that the HD protein Nkx2.2 is sufficient to induce the expression of Phox2b. Furthermore, while the activities of Nkx6.1 and Nkx6.2 are dispensable for the initial generation of vMNs, they are required to prevent a parallel program of more dorsal interneuronal differentiation. These proteins are also necessary to ensure a proper migration and axonal projection formation of vMNs. Thus, Nkx2 and Nkx6 proteins appear to have complementary roles in the establishment of vMN identity in the hindbrain. Taken together, our data suggest that both visceral and somatic motor neuron differentiation rely on the combined activities of cell intrinsic determinants, rather than on a single key determinant of neuronal cell fate.

During development, neuronal diversity is established by mechanisms that operate in space and over time. Advances have been made in regard to the mechanisms that restrict and direct neuronal generation in space. The spatial pattern of expression of transcription factors along both the DV and AP axis enable neural progenitors at different positions to acquire distinct molecular identities, which direct the fate of neurons. Compared to spatial patterning, less is known about the mechanisms that underlie how neural progenitors produce distinct types of neurons in a specific temporal order.

We addressed this issue by studying a population of progenitor cells in the ventral hindbrain that gives rise to vMNs and serotonergic (S) neurons. Each hindbrain segment, or rhombomere (r), initially generates vMNs, but all the rhombomeres except for r4 switch to producing S neurons at a defined time point. By investigating this phenomenon, we found that the temporal and spatial generation of vMNs and S neurons critically relies on the integrated activity of Nkx- and Hox-class HD proteins, which are proteins that confer DV and AP identity respectively. A primary function of these proteins is to coordinate the

activation of Phox2b in space and time. Phox2b, in turn, functions as a binary switch in deciding whether progenitors differentiate into vMNs or serotonergic neurons. Taken together, our data indicate that determinants that control spatial patterning may be associated also with temporal patterning and require that expression patterns are dynamic and modulated over time.

In the developing CNS, gliogenesis is believed to occur subsequent to neurogenesis. During recent years, data have begun to indicate that the generation of glial cells depends on the patterned expression of transcription factors, much in the same way as for neurons. Oligodendrocytes, the myelinating glial cells of the CNS, are dependent on the expression of Olig1/2 for their generation and have been found to be produced from a restricted ventral domain, the pMN domain, that prior to oligodendrogenesis produce somatic motor neurons (Lu et al., 2002; Zhou and Anderson, 2002).

While a ventral specification of oligodendrocyte precursors from the pMN domain is well established, it has remained unclear whether also other progenitor cells in the spinal cord give rise to oligodendrocytes. We provide *in vivo* and *in vitro* evidence that oligodendrocytes are produced also by progenitors located in the dorsal spinal cord and hindbrain and that the specification of these cells may result from the progressive evasion of dorsal BMP signalling over time. Further, we found that Nkx6.1 and Nkx6.2 are required for the generation of ventrally derived oligodendrocytes in the spinal cord. Interestingly, in the ventral anterior hindbrain, the same HD proteins instead act to suppress oligodendrocyte specification. These divergent roles for Nkx6 proteins seem to reflect that oligodendrocytes in the spinal cord and hindbrain are produced by distinct ventral progenitor domains. While oligodendrocyte precursors are generated dorsal to Nkx2.2-expressing progenitors in the spinal cord, these cells are produced within the $Nkx2.2^{+}$ domain in the anterior hindbrain. Together, these data suggest that oligodendrocytes are generated from multiple dorsoventral origins in the spinal cord and hindbrain, and indicate that the activation of Olig2 at different positions is controlled by distinct genetic programs.

Future prospects

Significant insight has been obtained in the mechanisms that control the generation of cells in the developing CNS and numerous studies have demonstrated the importance of extrinsic signalling molecules and patterning

of transcription factors in this process. Despite of this, many questions remain to be resolved. For example, while increasing evidence suggests that HD proteins that pattern the ventral neural tube direct the fate of cells through repression of factors that promote alternative fates, the molecular mechanisms by which this is achieved are poorly understood. In theory, a possible model could be that the HD proteins themselves and the downstream determinants lack binding sites specifically for the proteins expressed in the domains in which they are expressed and contain binding sites for progenitor proteins present in other domain. However, this remains to be determined and evidence for the direct interaction of HD proteins with DNA of other progenitor factors and downstream genes is currently lacking.

A cell fate model based on repression also involves the existence of transcriptional activators, general or more cell-type specific, that activate the expression of downstream factors. How activators and repressors converge on a transcriptional level to regulate expression of genes in the process of cell fate determination will be an important area of future research. In addition, Nkx factors have been found to be bifunctional and act as both repressors and activators (Choi et al., 1999b). As both Nkx2.2 and Nkx6 HD proteins are present in postmitotic cells, it is possible that they have later functions that reflect activator functions instead and require the interaction with other sets of proteins than in progenitor cells.

Spatial patterning of transcription factors provides progenitor cells with a positional identity that influence the fate of the cells. While the importance for a temporal generation of cells is well established in the cortex and retina, increasing data suggest an importance for this in other parts of the nervous system. Initial studies have indicated that the temporal generation of cells in the spinal cord and hindbrain involves changes in expression of factors that also confer spatial cues. In the future, it will be interesting to investigate the mechanisms that lie behind the temporal profiles of gene expression patterns in these systems.

The available data suggests that oligodendrocytes are generated at multiple dorsoventral positions in the caudal nervous system and that their specification is controlled by distinct genetic programs. In addition, new functions for glia in the CNS are being discovered. An important question to address is whether different origins correlate with functional differences. In order to resolve this,

possible markers that distinguish cells originating from different positions need to be identified. In this, the use of Cre-recombinant mice where cells originating from the dorsal and ventral neuroepithelium can be labelled specifically might prove a valuable tool.

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