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SPATIAL AND TEMPORAL MECHANISMS OF CELL FATE DETERMINATION IN THE DEVELOPING CNS

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Cover picture depicts a composition based on serotonergic neurons, with Lmx1b in orange and Gata3 in white.

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For my parents,

José Dias & Arminda Cardoso

ABSTRACT

The generation of neural cell diversity in the developing central nervous system relies on mechanisms that provide spatial and temporal information to neural progenitor cells. The deployment of morphogen gradients is an important strategy to impart spatial information to the field of responding cells. In this process, cells translate different concentrations of signal into the expression of distinct sets of cell fate-determining transcription factors, which determine cell fate as progenitors leave the cell cycle and differentiate into neurons. However, the mechanisms by which time regulates cell fate determination are poorly understood. The aim of this thesis is to better understand the mechanisms of spatial and temporal patterning in the specification of neural cell types.

In the ventral half of the neural tube, the graded activity of Sonic hedgehog (Shh) has been proposed to specify the patterned generation of distinct neuronal subtypes. It remains unclear, however, whether non-graded mechanisms of Shh signaling also contribute to this process. We show that Shh-induced Nkx2 proteins intrinsically amplify Shh responses and that this activity is important to specify floor plate (FP) and V3 fates in the ventral spinal cord. Conversely, Pax6 antagonizes Shh signaling and constrains its inductive activity over time. Furthermore, our data suggest that the spatial patterning of FP and V3 cells reflects a switch of neuronal potential in neural progenitors and not a requirement for different concentrations of Shh. Together, this study indicates that the output of graded Shh signaling depends on dynamic and non-graded changes of competence in responding cells.

At the hindbrain level, the progenitor domain dorsally abutting the FP generates visceral motor neurons (vMN) at early stages of development. To better understand the genetic program of vMN specification, we studied the role of proteins expressed in vMN progenitors during this process. We show that Nkx2.2 is sufficient to activate the expression of Phox2b, an important determinant of vMN fate. Moreover, the redundant activities of Nkx6.1 and Nkx6.2 proteins are not required for the generation of vMNs, but are important to prevent the parallel activation of dorsal cell fate differentiation programs and to ensure proper migration and axonal projection of vMNs. Thus, our data establish complementary roles for Nkx2.2 and Nkx6 proteins in the establishment of vMN identity.

In contrast to spatial patterning, the mechanisms that regulate the sequential generation of distinct cell types from a common pool of progenitors remain poorly resolved. To better understand these mechanisms we analyzed the sequential generation of vMN and serotonergic neurons (5HTN) from a common pool of Nkx2.2^{+} progenitors in the ventral hindbrain, and found that the temporal specification of these cell types depends on the integrated activities of Nkx and Hox proteins to regulate the temporal expression of Phox2b. In turn, Phox2b functions as a cell fate selector promoting vMN and repressing 5HTN fate. To further understand the vMN-to-5HTN switch, we screened for factors that could regulate this process, and identified Tgfβ2 as a signal that executes the switch through a temporal cross-repressive interaction with Phox2b. Moreover, we show that prolonged Shh activity establishes the initial period of vMN fate and induces Tgfβ2 expression with a temporal delay. Together, our studies reveal that a Shh-Tgfβ signaling relay mechanism regulates the sequential generation of vMNs and 5HTNs in a dynamic process that can be modulated by determinants controlling spatial patterning.

LIST OF PUBLICATIONS

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

I Lek, M.*, **Dias, J.M**.*, Marklund, U., Uhde, C.W., Kurdija, S., Lei, Q., Sussel, L., Rubenstein, J.L., Matise, M.P., Arnold, H.H., Jessell TM and Ericson J. (2010). A homeodomain feedback circuit underlies step-function interpretation of a Shh morphogen gradient during ventral neural patterning. Development 137, 4051-4060.

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, 4149-59

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 **Dias, J.M**., Klos-Applequist, J.M., Alekseenko, Z., Ang, S-L. and Ericson, J. A temporal signal relay mechanism by Shh and Tgfβ underlies the sequential specification of motor neurons and serotonergic neurons in the developing CNS. Manuscript

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Other Publications

Holz,A., Kollmus, H., Ryge, J., Niederkofler, V., **Dias, J.**, Ericson, J., Stoeckli, E.T., Kiehn, O., Arnold, HH. (2010).The transcription factors Nkx2.2 and Nkx2.9 play a novel role in floor plate development and commissural axon guidance. Development 137, 4249-60.

Zheng, X.*, Linke, S.*, **Dias, J.M.*,** Zheng, X., Gradin, K., Wallis, T.P., Hamilton, B.R., Gustafsson, M., Ruas, J.L., Wilkins, S., Bilton, R.L., Brismar, K., Whitelaw, M.L., Pereira, T., Gorman, J.J., Ericson, J., Peet, D.J., Lendahl, U., Poellinger, L. (2008). Interaction with factor inhibiting HIF-1 defines an additional mode of cross-coupling between the Notch and hypoxia signaling pathways. Proc Natl Acad Sci U. S. A. 105, 3368-73.

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LIST OF ABBREVIATIONS

5HTN	Serotonergic neuron
AP	Anteroposterior
BDNF	Brain derived neurotrophic factor
bHLH	basic Helix-Loop-Helix
BMP	Bone morphogenic protein
DV	Dorsoventral
FGF	Fibroblast growth factor
FP	Floor plate
GDNF	Glial cell line-derived neurotrophic factor
GFP	Green fluorescent protein
HD	Homeodomain
HH	Hamburger Hamilton
IGF-1	Insulin growth factor 1
RA	Retinoic acid
RP	Roof plate
Tgfβ	Transforming growth factor beta
vMN	Visceral motor neuron
Wnt	Wingless-related MMTV integration site

INTRODUCTION

The vertebrate central nervous system (CNS) contains hundreds of functionally distinct neuronal subtypes that establish specific synaptic connections with other neurons. These connections are the basis of the complex neural circuits that allow the brain to perform its many tasks, from sensory perception and motor coordination to behavior and memory. In addition to neurons, the adult brain contains two other major cell types, astrocytes and oligodendrocytes, collectively termed macroglial cells. Astrocytes provide structural support, maintain the blood-brain barrier and participate in cell-cell signaling, neuropeptide production and modulation of synaptic transmission. Oligodendrocytes form myelin sheaths that insulate axons thereby allowing fast conduction of electrical impulses. Oligodendrocytes also provide trophic support for neurons by producing neurotrophic factors such as BDNF, GDNF and IGF-1 (Rowitch and Kriegstein, 2010).

The generation of the mature nervous system, therefore, critically depends on the specification of a vast number of distinct neuronal and glial cell types from a population of neural progenitor cells during embryonic and early postnatal development. The generation of cellular diversity depends on mechanisms that operate both in space and over time. In the early neural tube, the activity of local inductive signals delineates two orthogonal axes of spatial information. The intersection of the information provided by these axes endows neural progenitors with unique positional information for the determination of cellular identity. Time also plays an important role in establishing neural diversity as it has been observed that defined populations of neural progenitors sequentially produce different cell types at different developmental time points. Moreover, at later developmental stages, neural progenitors cease to generate neurons and begin to generate glial cells (Jacob et al., 2008; Jessell, 2000; Lumsden and Krumlauf, 1996; Pearson and Doe, 2004).

The work presented in this thesis aims to understand the mechanisms that underlie the generation of cellular diversity within the CNS during embryonic development. In particular, we are interested in understanding the interplay between local inductive signals and cell intrinsic molecular networks in the control of spatial and temporal specification of neural cell types.

ESTABLISHMENT OF THE NEURAL TUBE

The vertebrate nervous system develops from the neural plate, a neuroepithelial sheet of multipotent proliferating progenitor cells. This structure generates all the neurons and glia that constitute the adult nervous system. The neural plate is induced in the dorsomedial region of the embryonic ectoderm, in a process that depends on the spatial and temporal regulation of the activity of several signaling pathways before and during gastrulation, including BMPs, FGFs and Wnts (reviewed in Stern, 2006). As

development proceeds, the medial region of the neural plate forms a hinge and the edges of the neural plate thicken causing the neural plate to fold up. When the lateral edges of the neural plate meet, the neuroepithelial structure closes and separates from the overlaying epidermis, forming the neural tube (Gilbert et al., 2006). During this process, locally secreted signals, mainly belonging to the hedgehog, Wnt, FGF, Tgfβ and RA families, define two orthogonal axes of spatial information, the anterior-to-posterior (AP) and the dorso-to-ventral (DV) axes, which provide unique positional coordinates within the neural tube. This positional information is translated in neural progenitors in the activation of specific transcriptional programs. In turn, these programs define the functional properties of cells as progenitors exit the cell cycle and differentiate into neurons. In addition, pan-neuronal pathways including Notch signaling and proneural genes (such as the bHLH transcription factors) have been shown to integrate with spatial information in order to regulate the acquisition of subtype identity (Bertrand et al., 2002). At the postmitotic stage, newly generated neurons are still plastic in their identity. Local extrinsic signals and/or electric activity generated in the early born neurons, integrate with the transcriptional programs initiated at the progenitor stage to further diversify neuronal identity (Dasen et al., 2003; Dasen et al., 2005; De Marco Garcia et al., 2011). In addition to these spatial mechanisms, time also plays an important role in establishing neural diversity but the molecular mechanisms underlying this process remain largely unresolved. However, studies in the *Drosophila* nerve cord and vertebrate CNS indicate that changes in extrinsic signals and/or in intrinsic cellular properties of neural progenitors overtime provide a basis for this process (Jacob et al., 2008; Jessell, 2000; Lumsden and Krumlauf, 1996; Pearson and Doe, 2004).

SPATIAL PATTERNING OF THE NEURAL TUBE

Anterior-Posterior patterning

Patterning along the AP axis is initiated around the time of neural induction, and results in the initial division of the neural tube into four regionally distinct domains along the rostrocaudal axis: forebrain, midbrain, hindbrain and spinal cord (Figure 1). In the early gastrula stage, neural plate cells express markers characteristic of an anterior (forebrain-like) identity. The acquisition of more posterior identities depends on the activity of caudalizing signals, derived from the paraxial mesoderm (Wnts) and primitive streak (FGFs), in presumptive posterior neural plate cells. In the presence of FGFs, the graded activity of Wnt signaling induces midbrain, hindbrain and spinal cord identities, with an increasing requirement of longer or higher levels of Wnt signaling for progressively more caudal identities (Itasaki et al., 1996; Muhr et al., 1999; Nordstrom et al., 2006). As development progresses, the initial crude regionalization of the neural tube is further refined. At hindbrain and rostral spinal cord levels, RA secreted by the somites induces the expression of a set of Hox genes in neural progenitors. The activity of these genes determines caudal hindbrain and rostral spinal cord identity, repressing the generation of cells with a more rostral character. By

contrast, the opposing activities of FGFs derived from the regressing primitive streak induce the expression of more caudal Hox genes in neural progenitors in a concentration-dependent manner. The opposing activities of RA and FGF signals therefore constitute an important mechanism to refine positional identity at this level of the neural tube. However, RA and FGF are not sufficient to induce caudal identities on neural cells in vitro, with these activities being dependent on previous exposure of neural progenitors to Wnt signaling (Bel-Vialar et al., 2002; Liu et al., 2001; Nordstrom et al., 2006). Thus, the combinatorial activities of Wnt, FGF and RA signaling induce molecularly distinct domains along the AP axis of the neural tube (Nordstrom et al., 2002; Nordstrom et al., 2006).

The acquisition of AP identity by neural progenitors is accompanied by the establishment of several signaling centers within the developing neural tube. These signaling centers, also designated as "secondary organizers", function as a source of secreted factors that further refine the local neural identity (Echevarria et al., 2003). In the neural tube, three main local signaling centers are specified: the anterior neural ridge (ANR), located in the anterior end on the neural tube; the zona limitans intrathalamica (ZLI) in the middle of the diencephalon; and the isthmic organizer (IsO) located at the midbrain-hindbrain boundary. The ANR secretes FGF8, which has an important role in the specification of the anterior areas of the forebrain and, together with Shh and Wnt signals, regulates regional patterning (Aboitiz and Montiel, 2007). The caudal region of the forebrain forms the diencephalon and is divided into three domains, rostral-to-caudal, designated prosomeres 1-3 (p1-p3). The ZLI is located between p2 and p3 and its activity is important for the histogenesis of the diencephalon and, at later stages, for the patterning of the thalamus. The ZLI expresses Shh which mediates the morphogenetic properties of this organizer (Lim and Golden, 2007). At a more caudal position, the IsO plays an important role in the development of the midbrain and rostral hindbrain. The IsO secretes FGF8 which is both required and sufficient for the development of midbrain and rostral hindbrain structures (Chi et al., 2003; Crossley et al., 1996; Martinez et al., 1999). The secreted factor Wnt1 is also expressed near the isthmus and is required for the maintenance of FGF8 expression such that in Wnt1 mutants the IsO is not properly induced and most of the midbrain and rostral hindbrain structures are not established (McMahon et al., 1992).

Dorso-Ventral patterning

The neural tube is also patterned along the DV axis, resulting in the generation of distinct cell types at defined positions along this axis. Two main signaling centers are established in the neural tube: the floor plate (FP) ventrally and the roof plate (RP) dorsally (Figure 1). FP cells are induced in the medial region of the neural plate by the activity of a group of axial mesodermal cells that form the notochord (Gilbert et al., 2006). The notochord and at later stages also the FP provide inductive signals that pattern the ventral half of the neural tube. These structures secrete the morphogen Sonic hedgehog (Shh) whose activity is sufficient and required to mediate ventral patterning. Ectopic expression of Shh induces the differentiation of FP cells and ventral cell types,

while elimination of Shh activity results in the loss of ventral cell identities (Chiang et al., 1996; Ericson et al., 1996; Marti et al., 1995; Roelink et al., 1995). On the other hand, BMP signals derived from the epidermis flanking the neural plate initiate the specification of the RP and when the neural tube closes dorsally, these cells begin to differentiate (Gilbert et al., 2006).
The RP expresses several proteins of the BMP/GDF and Wnt family, which provide dorsal patterning information. Exposure of neural tissue to BMPs or misexpression of constitutively active forms of BMP receptors in neural progenitors is sufficient to induce dorsal cell fates at the expense of more ventral ones (Liem et al., 1997; Panchision et al., 2001; Timmer et al., 2002). Conversely, reduction of BMP signaling results in a loss of the most dorsal cell fates (Chesnutt et al., 2004). Similarly, gain- and loss-of-function experiments have shown that proteins of the Wnt family, namely Wnt1 and Wnt3a, can also induce dorsal cell fates at the expense of ventral neural identities (reviewed in Ulloa and Marti, 2010).

Together, these studies indicate that patterning along the DV axis of the neural tube is established by two opposing signaling activities: one, originating ventrally from the notochord and FP cells, is mediated by a ventral-to-dorsal gradient of Shh signaling that induces ventral cell types and represses dorsal fates; the other, provided dorsally by the RP, is mediated by BMPs and Wnts and represses ventral identities while promoting dorsal cell fates. At intermediate regions of the neural tube, retinoid signaling emanating from the somites adjacent to the neural tube induces the generation of interneuron subtypes at this level (Pierani et al., 1999).

Patterning of the ventral neural tube
As previously mentioned, patterning of the ventral half of the neural tube is mediated by the activity of Shh. Shh is produced as a precursor protein that contains an N-terminal signaling domain (Shh-N) and a C-terminal catalytic domain (Shh-C). The catalytic domain promotes an autocatalytic cleavage of the precursor protein, releasing the signaling domain. The Shh-N fragment is then modified with a cholesterol group at the C-terminus and a palmitate group at the N-terminus. This final bilipidated Shh-N molecule constitutes the biologically active form of Shh (Chen et al., 2004; Porter et al., 1996). Active Shh is secreted from the notochord and FP cells via Dispatched (Etheridge et al., 2010; Kawakami et al., 2002) as a large multimer complex that spreads from ventral to dorsal regions of the neural tube, resulting in a high ventral to low dorsal concentration gradient. Analysis of a green fluorescent protein (GFP) tagged version of Shh (GFP-Shh) has allowed a detailed analysis of the Shh gradient over time (Chamberlain et al., 2008). Punctae of GFP-Shh protein accumulate at the ventricular, apical pole of neural progenitors (the region facing the lumen of the neural tube). Over time, cells closer to the ventral midline are exposed to increasingly higher concentrations of Shh, and at the same time the Shh gradient expands dorsally. Additionally, GFP-Shh protein is localized to the basal region of primary cilia on neural progenitor cells, supporting previous studies showing the importance of this structure for intracellular transduction of Shh signaling (Huangfu and Anderson, 2006). In vitro

studies have shown that Shh can induce the generation of distinct ventral neuronal subtypes at different concentration thresholds. Moreover, the concentration of Shh that is required to induce distinct neuronal cell types correlates with the position of their generation in the neural tube, such that more ventrally generated cells require higher concentrations of Shh (Ericson et al., 1997). Together these data indicate that Shh acts as a long range morphogen that directs the differentiation of neural progenitors to specific neuronal subtypes by providing progenitor cells with positional information. In turn, neural progenitors are able to sense differences in Shh activity and translate these differences into specific neural cell fates.

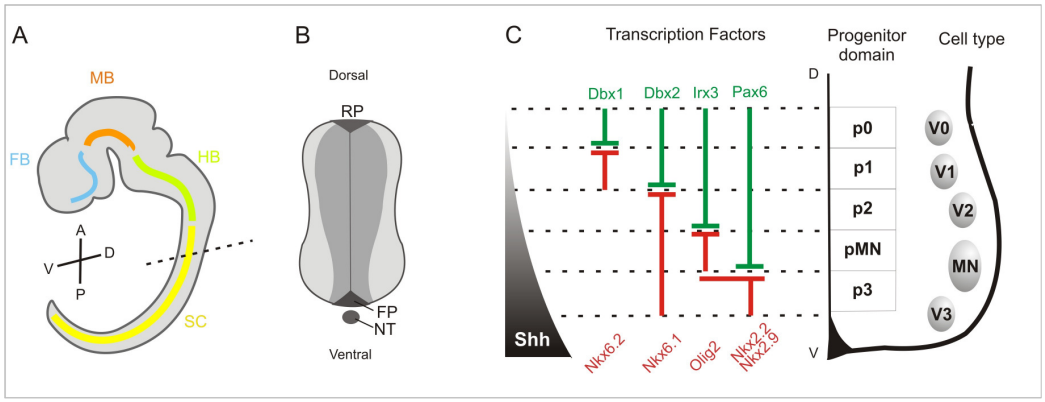

Figure 1. Patterning of the developing vertebrate CNS. (A) Schematic illustration of a developing embryo indicating the major subdivisions of the CNS along the AP axis: forebrain (FB), midbrain (MB), hindbrain (HB) and spinal cord (SC). Anteroposterior (AP) and dorsoventral (DV) axes are indicated. **(B)** The neural tube is patterned along the DV axis trough the activity of signaling factors provided by the roof plate (BMPs, Wnts), somites (RA) and notochord/floor plate (Shh). **(C)** Patterning of the ventral neural tube by Shh signaling. Shh, secreted by the notochord (NT) and floor plate (FP), regulates in a concentration dependent manner the expression of a set of HD- and bHLH-containing transcription factors (Class I and II proteins) and cross-repressive interaction between pairs of Class I and II proteins refine and stabilize the expression domains. The combinatorial expression of Class I and II proteins establishes five ventral progenitor domains (p0-p3, pMN) and their combined activity directs the fate of the differentiating neurons. Class I proteins are represented in green and Class II proteins in red. The same patterning mechanism is conserved at more anterior levels of the neural tube.

At the molecular level, Shh regulates the neural expression of a group of transcription factors that are characterized by the presence of homeodomain (HD) DNA binding motifs or basic Helix-Loop-Helix (bHLH) sequences (e.g. Olig2). Depending on their regulation by Shh, these transcription factors are grouped in two classes: Class I and Class II proteins. While Shh signaling induces the expression of Class II transcription factors at different concentration thresholds, Class I proteins are repressed by Shh. Some members of the Class II proteins have been shown to be directly regulated by the intracellular effectors of Shh signal (**Paper I,** Lei et al., 2006). By contrast, the repression of Class I proteins by Shh is indirect and mediated by Class II proteins (Pachikara et al., 2007). As a result, the graded activity of Shh in neural progenitors establishes a patterned expression of transcription factors with distinct dorsal (Class II) and ventral (Class I) borders of expression, thereby defining different progenitor domains (Figure 1). The majority of the transcription factors regulated by Shh are repressors (Muhr et al., 2001), and selective pairs of Class I and Class II proteins,

exhibiting complementary patterns of expression, are able to repress one another's expression. This mechanism of cross-repression allows not only maintenance of the expression domains of these proteins, but also the establishment of a sharp boundary between adjacent progenitor domains. Ultimately, the combined activity of Shh and selective transcriptional cross-repressive interactions results in the emergence of distinct progenitor domains characterized by a unique combinatorial expression profile of distinct transcription factors. In the ventral spinal cord, such processes establish five major progenitor domains (p0-p3; pMN) (Figure 1). The establishment of the different progenitor domains is a dynamic process. For instance, the two most ventrally expressed genes in the neural tube, Foxa2 (which demarcates FP cells) and Nkx2.2 (which is expressed immediately dorsal to FP cells) are not expressed at early stages of neural tube patterning. Instead, the ventral midline expresses Olig2, which at late stages of neural patterning is expressed dorsal to Nkx2.2 (Figure 1). With time, Nkx2.2 expression is activated at the ventral midline of the neural tube, concomitant with a dorsal expansion and ventral downregulation of Olig2. Thus, the establishment of the different progenitor domains occurs through a process of progressive ventralization (**Paper I,** Dessaud et al., 2010; Dessaud et al., 2007; Jeong and McMahon, 2005). The activation of successively more ventrally-expressed transcription factors correlates with an increased requirement for higher levels of Shh signaling for their induction (Briscoe et al., 2000; Dessaud et al., 2007; Ericson et al., 1997). Interestingly, increases in the time of exposure of neural progenitors to a defined concentration of Shh also has a ventralizing effect, indicating that neural progenitors are able to integrate both intensity and duration of Shh signaling (Dessaud et al., 2007).

As previously discussed, Shh signaling and cross-repressive interactions result in a patterned expression of HD and bHLH transcription factors, establishing distinct progenitor domains. While these Class I and II transcription factors act in a combinatorial manner to specify neuronal fate, because the majority of them work as transcriptional repressors in cell fate specification, a model of neural cell fate determination based on repression of alternative fates has been proposed. Such regulatory logic would ensure that a defined progenitor domain will activate only one program regulating the specification of a given neuronal subtype. The activation of defined programs of subtype determination subsequently controls the identity of the neuronal cells generated by regulating processes such as cell body migration and settlement, axon pathfinding and neurotransmitter identity (Muhr et al., 2001).

The Shh pathway

So far I have described the biological outcomes of Shh signaling in the patterning of the ventral neural tube, but an important question remains: how is Shh signaling translated at the cellular level into a transcriptional response?

The Shh receptor complex consists of two transmembrane proteins, including Patched (Ptc), to which Shh binds, and Smoothened (Smo), which initiates the intracellular Shh signaling cascade. The activity of Smo is regulated by Ptc, such that in the absence of Shh, Ptc inhibits Smo activity, while the binding of Shh to Ptc relieves Smo from this

repression (Figure 2). In turn, the activity of Smo seems to be required and sufficient to transduce graded Shh signaling: in *Smo* mutant embryos, ventral cell fates are not generated (Wijgerde et al., 2002; Zhang et al., 2001), whereas constitutively active forms of Smo can induce several ventral cell fates along the dorsoventral axis (Hynes et al., 2000). Moreover, specific activation or inhibition of Smo by small molecules recapitulates the graded responses to different concentrations of Shh (Dessaud et al., 2007). In addition to Ptc, Shh also binds other cell surface proteins that regulate Shh signaling, such as Hhip1, Cdo and Boc. Binding of Shh to Hhip1 inhibits Shh signaling by sequestering the ligand, whereas binding to Cdo or Boc positively regulates the transduction of Shh signaling in a mechanism that synergizes with Ptc1 (Tenzen et al., 2006; Yao et al., 2006). Furthermore, the expression of these Shh-binding proteins is regulated by Shh signaling, such that Ptc1 and Hhip1 are upregulated and Cdo and Boc are downregulated in response to Shh signaling (Jeong and McMahon, 2005; Tenzen et al., 2006). These feedback regulatory mechanisms are important for the interpretation of Shh signaling by responding cells (discussed below).

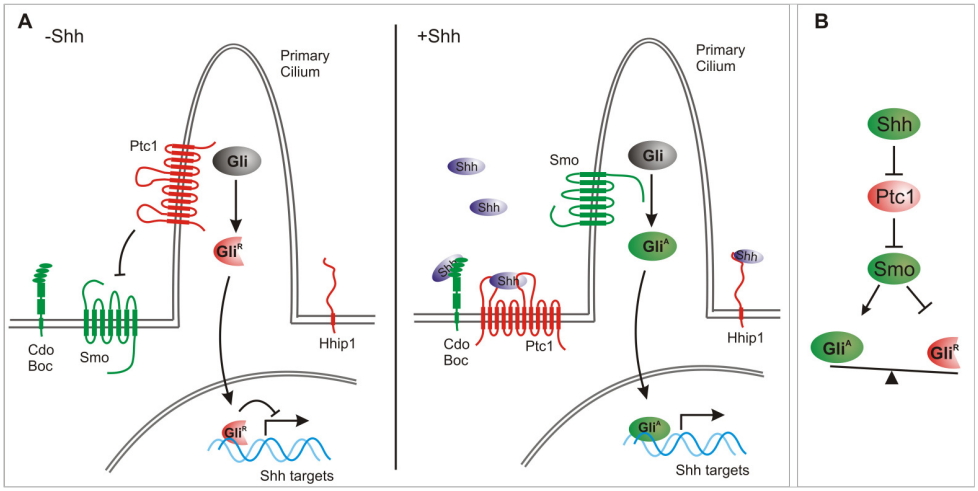

Figure 2. Schematic diagram of vertebrate Shh pathway (A) In the absence of Shh, Ptc1 localizes to the primary cilium and represses the activity of Smo and accumulation of Smo in the cilium. Under these conditions, Gli proteins are completely degraded or partially processed by the proteosome and the resulting truncated forms of Gli proteins (GliR) translocate to the nucleus where they repress the transcription of target genes. Binding of Shh to Ptc1 releases the repression on Smo. Ptc1 is removed from the cilium with concomitant ciliary accumulation of Smo. The activation of Smo inhibits proteolitic processing of Gli proteins resulting in the accumulation of activator form of Gli proteins (GliA), which translocate to the nucleus where they activate target genes. Other cell surface molecules present at the cell surface also bind Shh: Hhip1 blocks the activation of the pathway and Cdo/Boc proteins enhance the activation of the pathway possibly by increasing the presentation of Shh to Ptc1. **(B)** Simplified overview of functional interactions in the Shh pathway.

In vertebrates, primary cilia have an important role in the transduction of Shh signaling. Cilia are extensions of the cell membrane that contain a core microtubule structure and exhibit intra-flagelar transport (IFT). Mutations that affect ciliogenesis or IFT display Shh-related phenotypes (Ashique et al., 2009; Huangfu and Anderson, 2005; Huangfu et al., 2003). Furthermore, most components of the Shh pathway localize to this structure and display dynamic patterns of localization depending on the status of Shh signaling (Figure 2). In the absence of Shh, Ptc localizes to the cilium and prevents the

accumulation of Smo in this structure. Binding of Shh to Ptc results in the removal of Ptc from the cilium and concomitant accumulation of Smo (Corbit et al., 2005; Haycraft et al., 2005; Rohatgi et al., 2007). Ultimately, Smo regulates the activity of a group of transcription factors of the Gli family. In vertebrates this family consists of three members, Gli1-3. All three proteins are expressed in the neural tube and their transcriptional properties are regulated by Shh signaling. In the absence of Shh, Gli2 and Gli3 proteins are processed to generate a transcriptional repressor (GliR), whereas in the presence of Shh, both proteins are stabilized in their full-length activator form (GliA). Thus, Shh regulates the cellular ratio of activator vs. repressor activity of Gli (GliA vs. GliR) (Figure 2). Gain-of-function experiments indicate that different levels of GliA can recapitulate the graded patterning activity of Shh (Lei et al., 2004; Stamataki et al., 2005), suggesting that the total net of Gli activity in a cell determines the transcriptional output of Shh signaling. In the neural tube, Gli2 is argued to be the main contributor to GliA activity and in *Gli2* mutant mice the most ventral cell types (FP and V3 interneurons), that require higher levels of Shh signaling, are not generated (Ding et al., 1998; Matise et al., 1998). On the other hand, mutants for *Gli3*, which has been proposed to be the major mediator of GliR activity in the neural tube, display a dorsal expansion of intermediate subtype identities (Persson et al., 2002), indicating that Shh signaling expands dorsally in these mutants. In *Shh* or *Smo* mutants, the ventral cell types are not generated (Chiang et al., 1996; Wijgerde et al., 2002). Additional removal of Gli3R function (*Shh;Gli3* or *Smo;Gli3* compound mutants) rescues the generation of the intermediate cell fates (except FP or p3 fates) (Litingtung and Chiang, 2000; Wijgerde et al., 2002), suggesting that the regulation of GliR activity by Shh is important in the patterning of the intermediate region of the ventral half of the neural tube. The generation of the most ventral cell fates is, however, dependent on the levels of activator (GliA) (**Paper I,** Litingtung and Chiang, 2000; Wijgerde et al., 2002).

These data have led to the proposal of a model in which the ventral-high to dorsal-low concentration gradient of Shh is translated to a gradient of activator-to-repressor Gli activity in the neural tube (Dessaud et al., 2008). However, these data also support a model in which the induction of the most ventral progenitor domains is dependent on a threshold of GliA activity, while the induction of intermediate progenitor identity is more dependent on the graded regulation of GliR levels by Shh signaling. In addition to the concentration of Shh ligand, the duration of Shh signaling also regulates the patterning activity of Shh, with longer periods of exposure to Shh inducing more ventral cell fates (Dessaud et al., 2007; Ericson et al., 1997; Roelink et al., 1995). A proposed model of temporal adaptation that explains this phenomenon, argues that the sensitivity of cells to ongoing Shh signaling decreases with time of exposure to Shh due to the induction of negative feedback inhibitors (e.g. Ptc1). In this process, the concentration of Shh is converted into a period of intracellular Gli activity, and the maintenance of a given level of Gli activity is dependent on the exposure of cells to higher concentrations of Shh over time. Thus, the maintenance of periods of high Gli activity is correlated with the progressive establishment of more ventral progenitor identities (Dessaud et al., 2007).

NEUROGENESIS

Once neural progenitors have acquired a unique molecular identity that will define the identity of the cell types to be generated, they activate a program of neurogenesis that allows for the generation of mature, fully differentiated neurons. The bHLH family of transcription factors plays an important role in activating the differentiation program in neural progenitor cells. These proneural proteins, which in the mouse CNS include Ngn1-3, Ascl1/Mash1 and Math1, bind DNA as heterodimers with E-proteins. In turn, these protein complexes activate a set of target genes that regulate several aspects of the neurogenic process including cell cycle exit, downregulation of progenitor characteristics, migration from the progenitor zone and activation of pan-neuronal genes (Bertrand et al., 2002; Guillemot, 2007). Downstream of proneural genes, the Sox4 and Sox11 proteins activate pan-neuronal genes independently of cell cycle exit (Bergsland et al., 2006). In addition to activating generic neuronal gene programs, proneural genes also regulate the acquisition of neuronal subtype characteristics in a region-specific manner. An example is the activity of Ngn2 in MN progenitors in which it is required for the activation of Hb9, a transcription factor important for the acquisition of somatic MN identity (Lee and Pfaff, 2003). Importantly, the activity of Ngn2 cannot be replaced by other proneural genes, such as Ascl1/Mash1 (Parras et al., 2002). Thus, proneural genes integrate the activation of both generic and subtype-specific neuronal programs.

The rate of neurogenesis must be tightly regulated in order to prevent the premature depletion of the progenitor pool with time. To this end, Notch signaling and Sox1-3 proteins counteract proneural activity by maintaining cells in a proliferative undifferentiated state. Activation of the Notch signaling pathway is dependent on the interaction of the extracellular domain of the Notch receptor with its ligand, which is expressed by neighboring cells. This interaction results in a γ-secretase-mediated cleavage of the Notch intracellular domain (NICD), which in turn translocates to the nucleus where it interacts with the DNA binding protein CSL, converting it from a repressor to an activator. One of the targets of the CSL-NICD activator complexes are the Hes1 and Hes5 transcription factors. These proteins are able to block neurogenesis by repressing the expression of proneural genes or by interacting with E-proteins, preventing the formation of the E-protein:proneural protein complexes (Bray, 2006). In this way, Notch signaling maintains cells in a proliferative and undifferentiated state by reducing the levels of expression and activity of proneural genes. Sox1-3 proteins are expressed in most progenitor cells and also play a role in maintaining cells in an undifferentiated state. In contrast to Notch signaling, Sox1-3 proteins counteract neurogenesis, not by regulating the expression of proneural genes but by blocking the neurogenic activity of the proneural protein complexes (Bylund et al., 2003; Graham et al., 2003; Holmberg et al., 2008). The downregulation of Sox1-3 proteins by proneural genes is, therefore, an important step in the progression of the neurogenic program (Bylund et al., 2003). Interestingly, proneural proteins also activate the expression of Sox21 in progenitor cells, which promotes neurogenesis. This establishes a mechanism in which the balance between the activities of Sox1-3 and Sox21 proteins regulates the

maintenance of cells in a progenitor state or the initiation of differentiation (Sandberg et al., 2005). Overall, these studies reveal a tight balance between promoting (proneural genes, Sox4/11) and counteracting (Notch, Sox1-3) activities in the initiation of the differentiation process.

TEMPORAL CELL FATE SPECIFICATION

Early studies of the developing vertebrate cerebral cortex provided evidence that many neuronal cell types are generated in a defined temporal order from a common pool of progenitors (Berry, 1994). It is now well established that the developing CNS contains multipotent neural progenitor cells that generate distinct neuronal cell types in a temporally defined sequence. In vertebrates, the generation of neurons and glial cells also follows a temporal order. Importantly, these temporal aspects of cell fate determination are conserved across different species. Together, these observations have established the importance of time, in addition to space, in cell fate identity determination. Since each cell type reflects the identity of the progenitor cells from which they originated, multipotent progenitors change their identity with time in order to generate distinct cell populations. This raises the question of how progenitor cells transit from one temporal identity to the next. In an extreme scenario, the transitions between consecutive progenitor temporal identities could result from changes intrinsic to the progenitor cell. In this situation, neural progenitor cells would initially be responsive to extrinsic spatial information, defining their initial identity. Subsequently, however, progenitor cells would become refractory to extrinsic signals and intrinsic molecular mechanisms would initiate a sequence of stereotypic changes resulting in the generation of distinct cell identities. At the other extreme, the transitions between different progenitor identities could result from changes in the environmental signals to which progenitor cells are exposed during development. If changes in environmental cues occur in a stereotypical manner, this would result in the generation of distinct cell fates in a defined temporal order (reviewed in Pearson and Doe, 2004).

The process of temporal specification has been studied in several regions of the developing CNS and in different model organisms, including *Drosophila* nerve cord and the vertebrate cerebral cortex, retina, spinal cord and hindbrain. Our studies have focused in the ventral region of the developing hindbrain in vertebrates, but I will briefly describe different model systems and the main principles regulating temporal aspects of cell fate specification.

The *Drosophila* CNS

The embryonic *Drosophila* CNS develops from multipotent progenitors, the neuroblasts (NBs), which divide in an asymmetric manner to self-renew and generate a smaller daughter cell called the ganglionic mother cell (GMC). The GMC then divides, usually once, to give rise to two post-mitotic neurons or glia (reviewed in Doe, 2008). Most NBs go through several rounds of asymmetric divisions, thereby establishing a

NB lineage that generates specific neural cell types in a defined temporal sequence. Several studies have established that within a lineage, NBs express five different transcription factors in a defined sequential order: Hunchback (Hb)→Kruppel (Kr)→ Pdm→ Castor (Cas)→ Grainyhead (Grh). This defines five consecutive molecularly distinct periods that correlate with the production of different cell types (Figure 3). Gain- and loss-of-function experiments with several of these transcription factors have shown that they are required and sufficient to specify the birth order (temporal identity) of neurons in several NB lineages (Grosskortenhaus et al., 2005; Grosskortenhaus et al., 2006; Isshiki et al., 2001). For example, the loss of Hb in the CNS results in the loss of early-born neurons characteristic of the Hb^{+} temporal window. Conversely, its continuous expression in neuroblasts results in the prolongation of the production of early-born neurons at the expense of later-born neurons (Figure3) (Isshiki et al., 2001). Importantly, the ability of these temporal genes to confer temporal identity is restricted to the NB. Different NB lineages, which produce distinct cell types, express the same sequence of temporal genes. This indicates that temporal identity genes do not specify cell fate *per se*, but rather that their activity is integrated with other cues (for example spatial cues) to activate downstream programs of cell fate specification.

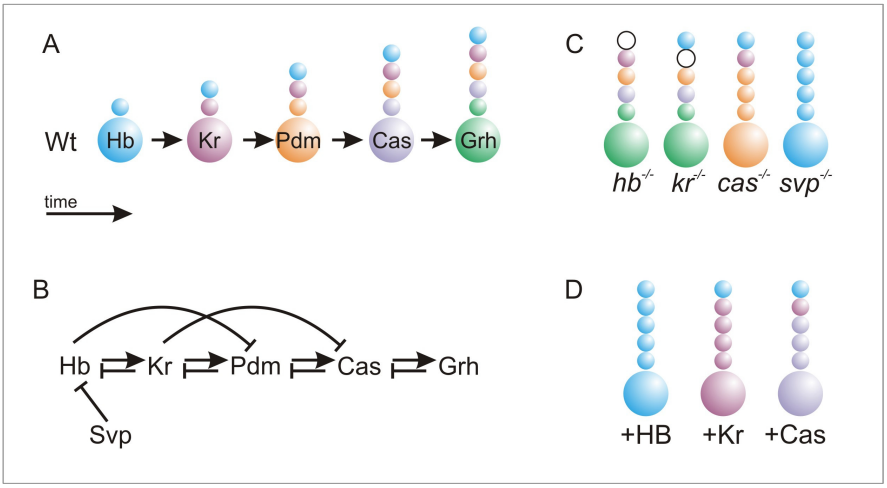

Figure 3. Temporal identity genes in *Drosophila* (A) *Drosophila* neuroblasts (large circles) express five distinct transcription factors (TF) in a defined temporal sequence during embryogenesis. The expression of each TF is associated with a post-mitotic progeny (small circle) of a different temporal identity. (B) Summary of the known regulatory interactions between temporal identity genes and switching factor Svp. Cas is both a temporal identity genes and a switching factor. (C, D) Effects of loss-of-function (C) and gain-of-function (D) of temporal genes in cell fate determination.

These studies raise the question of the nature of the mechanisms that drive the successive expression of the different temporal genes. Gain- and loss-of-function experiments indicate that the temporal genes establish genetic cross-regulatory interactions, in which a given gene activates the next gene in the cascade and represses the previous gene and the next one plus one (Figure3). With the exception of Cas, which is required for the expression of Grh, the temporal genes are not required for the expression of those proceeding them, but rather, regulate the timing of their expression (Isshiki et al., 2001; Maurange et al., 2008). Cell cycle progression is important for the

first temporal transition (Hb→Kr). Blocking the G2-M transition or cytokenesis prevents the downregulation of Hb, thereby maintaining the NBs at an early identity state (Grosskortenhaus et al., 2005). In addition to cell cycle progression, the Hb→Kr transition also requires the activity of the orphan nuclear receptor Seven up (Svp). Svp represses the expression of Hb and its expression in NBs correlates with the timing of Hb downregulation. Moreover, loss of Svp function results in a prolongation of Hb expression and generation of neurons of the corresponding temporal identity at the expense of later-born neurons; whereas early activation of Svp induces the generation of neurons with an identity characteristic of the Kr temporal window (Figure 3) (Kanai et al., 2005; Mettler et al., 2006). Hence, cell cycle progression and Svp activity are important regulators of the Hb→Kr transition, indicating that they work in this cascade as temporal switch factors. Cas also shows properties of a switch factor, as indicated by the prolongation of Pdm expression and the corresponding temporal identity in *Cas* mutants (Grosskortenhaus et al., 2005; Tran et al., 2010). In this case, Cas seems to operate as a temporal switch factor and a temporal identity determinant.

In summary, a temporal gene cascade operates in a NBs lineage to specify, in a cell autonomous manner, neural temporal identities. In addition, these temporal genes establish cross-regulatory interactions that, together with other intrinsic factors (such as cell cycle progression and Svp), modulate the progression of the temporal gene cascade in the NBs. Furthermore, the observations that NBs do not progress through their lineages in a synchronized manner in the developing CNS, and that when cultured in vitro NBs undergo the temporal Hb→Kr→Pdm→Cas→Grh gene cascade, have been used to exclude a role for extrinsic factors in the regulation of temporal identity progression (Brody and Odenwald, 2000; Grosskortenhaus et al., 2005). However, these culture experiments do not exclude the presence of feedback signaling from GMCs or post-mitotic cells to the NBs. Nevertheless, collectively these observations have been used to argue for a model of intrinsic regulation of temporal identity in the *Drosophila* CNS. In the future it will be important to unravel the mechanisms that regulate the activation of temporal switch factors, such as Svp and Cas, to better understand the contribution of intrinsic and extrinsic mechanisms in temporal fate specification in the *Drosophila* CNS.

The vertebrate Cerebral Cortex

The mammalian cerebral cortex is characterized by its stratified organization into six morphologically distinct layers. The first cortical neurons produced by cortical progenitors form the preplate and later born neurons migrate towards the preplate and split it into the marginal zone (upper layer) and the subplate. At later stages of development, the cortical progenitors cease to generate neurons and begin to produce glial cells (McConnell, 1991). Birthdating studies of early progenitor cells demonstrated that cortical neurons are generated in a defined temporal sequence and in an inside-out order, with early born neurons occupying deep layers (layers 5 and 6) and late born neurons settling in more superficial layers (layers 4, 3, 2) of the cortex. Additionally, lineage tracing experiments showed that cortical progenitors are

multipotent and able to generate neurons of different layers (Walsh and Cepko, 1988, 1993). At the onset of neurogenesis, the progenitor cells are located in the ventricular zone (VZ) and the majority of these cells divide asymmetrically to generate a daughter cell that remains in the VZ and another that differentiates and migrates away. At mid/late stages of neurogenesis, daughter cells leaving the VZ move into the subventricular zone (SVZ) where they divide symmetrically before differentiating, thus establishing a secondary progenitor pool. The progenitors located in the VZ generate early-born neurons (located in deep layers) while those located in the SVZ produce late-born neurons (located in upper layers) (Noctor et al., 2004; Noctor et al., 2008).

In contrast to *Drosophila*, very few factors have been shown to specify temporal identities in cortical progenitors. One example is the zinc finger transcription factor *Fezf2*. *Fezf2* is expressed in early cortical progenitors. In *Fezf2* mutant mice there is a loss of early-born neurons (layers 5, 6) and increased production of late-born neurons. Moreover, expression of *Fezf2* in late progenitors induces the generation of early fates at the expense of late fates (Chen et al., 2005; Molyneaux et al., 2005). Brn1/2 transcription factors are expressed at high levels in SVZ progenitors and Brn1/2 compound mutants show a loss of late-born neurons, indicating that these factors could play a role in specifying late fates (Sugitani et al., 2002). The mechanisms that regulate the temporal expression of these cell fate determinants, however, remain unknown.

The different laminar fates are generated in a synchronous temporal order, suggesting the existence of extrinsic cues that regulate the birth order of the different cell types. Transplantation experiments have also shown that young cortical progenitors can generate neurons with characteristics of late born neurons when transplanted into old host brains. Interestingly, this process requires progenitor cells to progress through cell cycle division, suggesting that cell fate is locked after the last cell division. However, when old progenitors are transplanted into young hosts, they still generate neurons with characteristics of late born neurons. These results suggest that young progenitors are multipotent and respond to extrinsic temporal cues that determine the temporal identity of the neurons generated, while late progenitors are restricted in their competence to respond to early extrinsic cues and generate young cell fates. Therefore, temporal cell fate specification in the cerebral cortex results from the interplay of extrinsic temporal cues and intrinsic states of competence of progenitor cells (Desai and McConnell, 2000; Frantz and McConnell, 1996; McConnell and Kaznowski, 1991). Surprisingly, both isolated single cortical progenitors and mouse embryonic stem cells can, when cultured in vitro, generate the different neuronal laminar fates and glial cells in a temporal sequence that recapitulates the in vivo process. While these findings do not rule out the role of extrinsic factors in the control of the transition between successive temporal fates, they indicate that the signals required are encoded within a neural lineage (Gaspard et al., 2008; Shen et al., 2006).

Following the generation of cortical neurons, cortical progenitors generate glial cells. Cytokines of the IL-6 family can, in vitro, promote cortical gliogenesis by activating the JAK-STAT signaling pathway in progenitor cells. In vivo, differentiated cortical

neurons express Cardiotrophin-1 (CT-1), a member of the IL-6 family, and the activity of this ligand is important to promote gliogenesis. Thus, the neurogenic-to-gliogenic switch in cortical progenitors is regulated by a feedback loop that involves signaling between progenitors and their progeny (Barnabe-Heider et al., 2005). However, CT-1 is released by differentiated neurons during early stages of neurogenesis without promoting a gliogenic switch, indicating that early neurogenic progenitors are refractory to gliogenic signals. In neurogenic progenitors, many astrocyte-specific genes are maintained in a repressed state via DNA methylation, implying that epigenetic silencing is important to establish competence states. Consistently, deletion of DNA methyltransferase 1 in cortical progenitor cells results in premature production of astrocytes and reduction of the neurogenic period (Fan et al., 2005). Thus, the regulation of the epigenetic status of glial genes is also an important step in the acquisition of gliogenic competence. In this regard, COUP-TF I/II transcription factors seem to play an important role in the gliogenic switch. These proteins are expressed in early neurogenic progenitors and downregulated before the onset of gliogenesis and their knockdown results in the maintenance of the epigenetic silencing of glial genes and failure to initiate gliogenesis (Naka et al., 2008). Interestingly, COUP-TF proteins are the vertebrate homologues of the *Drosophila* Svp and in both *COUP-TF* and *Svp* mutants the generation of early fates is prolonged at the expense of later ones. The neurogenic bHLH proteins Ngn1/2 and Ascl1/Mash also have a role in regulating the intrinsic potential of progenitor cells, biasing them towards a neurogenic fate and inhibiting gliogenesis (Cai et al., 2000; Nieto et al., 2001). bHLH proteins inhibit gliogenesis by interfering with the JAK-STAT pathway; Ngn1 sequesters the co-activator complex CBP/p300 preventing the formation of the active STAT3 complex and downstream activation of glial genes. This mechanism prevents the activation of the glial program even if progenitors are exposed to gliogenic cues (Sun et al., 2001).

The vertebrate Retina

The vertebrate retina is composed of six major neuronal types (ganglion, horizontal, bipolar and amacrine cells; cone and rod photoreceptors) and one glial cell type (Müller cells) that are organized in space in three layers: the outer nuclear layer, containing cone and rod photoreceptors; the inner nuclear layer composed of horizontal, bipolar, amacrine and Müller cells; and the inner most layer with retinal ganglion cells. Retinal progenitor cells (RPCs) generate all retinal cell types. Lineage tracing studies of single RPCs during early development of the retina have shown that many of these cells generate clones containing all major retinal cell types (Holt et al., 1988; Price et al., 1987). In addition, birthdating studies have demonstrated that retinal cells are generated in a conserved temporal sequence but in overlapping intervals. Retinal ganglion cells, horizontal cells and cone photoreceptors are generated first, followed by amacrine cells. Rod photoreceptors, bipolar cells and Müller glia are produced last (Cepko et al., 1996; Livesey and Cepko, 2001). These observations have, thereby, established that the retina contains multipotent progenitor cells that generate different cell types in a defined temporal order during development. Several observations support the notion that the regulation of temporal identity progression in the retina is largely dependent on cell-

intrinsic mechanisms. Experiments of progenitor cell co-culture in which early/late progenitor cells were placed in a late/early progenitor environment respectively, indicated that although the environment can affect the relative numbers of cell types, it has no effect in the temporal identity of the cells generated by early/late progenitor cells. Additionally, when isolated RPCs are cultured in vitro, they generate clones with a neural composition similar that generated in vivo by single RPC labeling, suggesting that the signals required to control the production of the different cell fates are encoded within the neural lineage itself (Cayouette et al., 2003).These observations argue that RPCs go through different states of competence, during which they are able to generate specific cell types in a process that is largely defined intrinsically.

In the embryonic *Drosophila* CNS, the sequential expression of a cascade of genes initiated by Hb defines successive states of progenitor competence, during which different neural fates are specified (discussed in *The Drosophila CNS*). Analysis of the function of Ikaros, a vertebrate orthologue of the *Drosophila* Hb, during retina development indicates that mechanisms similar to those regulating temporal progenitor competence in *Drosophila* neuroblasts may also operate in the retina (Elliott et al., 2008). Ikaros is expressed by early RPCs that generate retinal ganglion cells, horizontal cells and amacrine cells, but downregulated in late RPCs. In addition, *Ikaros* mouse mutants exhibit a reduction in the generation of early-born cell types without affecting the generation of late-born cell types, and expression of Ikaros in late RPCs is sufficient to induce the generation of early cell fates at the expense of late-born cell types. Similarly to Hb, Ikaros does not seem to operate as a cell fate determinant. However, Ikaros may regulate the expression of early-born cell type determinants such as Prox1 in RPCs thereby instructing progenitor cells to generate early-born cell types. Together, these findings support a role for Ikaros in conferring competence to RPCs to generate early-born cell types during development of the retina. The mechanisms promoting downregulation of Ikaros over time, and hence controling the transition between an early progenitor competence to a late progenitor competence state, are unknown. However, and in contrast to the to the Hb-to-Kr transition in *Drosophila*, this transition does not seem to require progression through the cell cycle.

The vertebrate Spinal Cord

In the ventral spinal cord, the graded activity of Shh signaling regulates the spatial expression of a set of transcription factors along the DV axis. The combined activities of these proteins establish distinct progenitor domains that generate specific neuronal cell types. As a result, five distinct progenitor domains are established: p3, pMN, p2-p0 (Figure1). Following the period of neurogenesis, progenitor cells switch to the production of glial cells. Lineage tracing experiments of neural progenitors in the pMN domain have shown that these progenitor cells first generate motoneurons (MN) then oligodendrocytes (Leber et al., 1990), establishing that the pMN domain contains multipotent progenitors that go through a neuronal-to-glial switch in competence during development. The neighboring progenitor domains, p2 and p3, have also been shown to generate interneurons followed by astrocytes (Rowitch et al., 2002). Several factors

have been implicated in regulating the switch in the pMN. For example, Shh, which is responsible for the establishment of the pMN domain, is also required for the generation of both MN and oligodendrocytes at later stages (Orentas et al., 1999), indicating that the specification of these cell types is dependent on extrinsic signals. Moreover, transplantation assays of both early and late pMN progenitors into young hosts indicate that these progenitors, like cortical progenitors, become restricted in their neural potential over time. Early progenitors generate MNs and oligodendrocytes, while old pMN progenitors only produce oligodendrocytes (Mukouyama et al., 2006).

The pMN domain is characterized by the expression of Olig2. Interestingly, Olig2 is expressed by progenitors during the time of MN and oligodendrocyte specification, and gain- and loss-of-function studies support a role for Olig2 in the generation of MNs and oligodendrocytes (Mizuguchi et al., 2001; Novitch et al., 2001; Park et al., 2002; Sugimori et al., 2007; Zhou and Anderson, 2002).Thus, Olig2 behaves as a bi-functional transcription factor that promotes both neuronal and glial cell fates. During the period of MN generation, $Olig2^{+}$ progenitor cells express the proneural factor Ngn2, which has been suggested to cooperate with Olig2 in promoting the specification of MN fate. The transition to a gliogenic phase is accompanied by the downregulation of Ngn2, and this event has been proposed to be necessary to allow pMN progenitors to switch to a gliogenic period (Mizuguchi et al., 2001; Novitch et al., 2001; Zhou et al., 2001). Additionally, it has been observed that the transition to gliogenesis is accompanied by the dephosphorylation of Olig2. Functional studies have provided evidence that this post-translational modification regulates the activity of Olig2; in its phosphorylated form Olig2 promotes MN specification, whereas dephosphorylation promotes oligodendrogenesis. This change of Olig2 activity seems to reflect changes in preferred binding partners, from Olig2 homodimers to Olig2:Ngn2 heterodimers, such that whereas Olig2 homodimers repress oligodendrocyte fate and create a permissive environment for MN specification, the dephosphorylation of Olig2 increases its affinity for Ngn2, reducing the amount of Ngn2 available to activate MN-specific genes and thereby promoting glial fate (Li et al., 2011). These results indicate that Olig2 is a cell fate determinant for both MN and oligodendrocyte fates and that the switch between these two competence states is mediated by post-translation modifications. The identity of the phosphatases/kinases that regulate this process remain unknown, but their identification and the mechanisms regulating their activity will be an important step in understanding the signals that control the neuronal-to-glial switch.

An additional factor that has been shown to affect the neuronal-to-glial switch is the transcription factor Sox9, a member of the Sox family of proteins that contains an HMG box DNA-binding domain, and together with Sox8 and Sox10 forms the SoxE subgroup of proteins. SoxE proteins have been shown to play an important role in the specification, lineage progression, survival and terminal differentiation of oligodendrocytes (reviewed in Stolt and Wegner, 2010). Conditional deletion of *Sox9* in the developing spinal cord revealed that Sox9 is important for the correct temporal specification of oligodendrocytes. In these mutants, the pMN progenitors fail to generate oligodendrocytes and this is accompanied by a prolongation of MN

generation. However, the expression of Sox9 in pMN progenitors is initiated during the period of active MN production, raising the question of whether Sox9 is working as a switch signal or simply conferring progenitor cells the competence to respond to the real switch signal. Members of the SoxD subgroup, Sox5 and Sox6, are co-expressed with Sox9 in pMN progenitors, and SoxD mutant mice (*Sox5* and *Sox6* compound mutants) display premature generation of oligodendrocytes (Stolt et al., 2006). In contrast to SoxE proteins, SoxD proteins are transcriptional repressors, enabling them to interfere with the transcriptional activation mediated by SoxE proteins. This suggests that a balance between SoxD (Sox5, 6) mediated repression and SoxE (Sox9) mediated activation may regulate the timing of oligodendrogenesis. The bHLH protein Hes5 has been shown to interact with one member of the SoxE group (Sox10), preventing DNA binding and activation of target genes (Liu et al., 2006). At present, it is not known whether similar mechanisms are involved in regulating the activity of Sox9 in pMN progenitor cells to affect the balance between the activities of SoxE and SoxD proteins. Another intriguing question is how the activities of SoxD/E proteins and the mechanisms regulating Olig2 de-phosphorylation are integrated in the regulation of the progression from neurogenesis-to-gliogenesis.

The vertebrate Hindbrain

In the ventral hindbrain, high levels of Shh signaling establish a progenitor domain, located dorsal to the FP, which expresses the HD transcription factor Nkx2.2. During development, this progenitor domain generates visceral motor neurons (vMNs) followed by a period of serotonergic neuron (5HTN) production. During the period of vMN neurogenesis these progenitor cells express the paired-like homeobox transcription factor Phox2b. Gain- and loss-of-function experiments have established an important role for this transcription factor in the specification of vMN fate. Broad expression of Phox2b in neural progenitors of the developing neural tube is sufficient to induce vMN fate, while ablation of its function in mice results in the failure to generate this cell type (Dubreuil et al., 2002; Dubreuil et al., 2000; Hirsch et al., 2007; Pattyn et al., 2000). Analogous to the temporal genes defined in *Drosophila* neuroblasts, Phox2b can be viewed as a temporal gene that confers progenitor cells the competence to generate early-born cell types. Interestingly, loss of Phox2b also results in the premature generation of 5HTNs (discussed in **Paper III**). At later stages, $Nkx2.2^{+}$ progenitor cells cease to generate vMNs and begin to produce 5HTNs. This is accompanied by the downregulation of Phox2b and the expression of high levels of the forkhead transcription factor Foxa2 (**Paper III,** Jacob et al., 2007). Loss of Foxa2 activity results in the prolongation of the generation of vMNs and the blocking of 5HTN fate; while expression of high levels of Foxa2 in early progenitors represses early-born neurons and promotes premature generation of late-born 5HTNs. These results have therefore suggested that Foxa2 is important not only to instruct the serotonergic fate but also to regulate the transition between early and late progenitor competence states (Jacob et al., 2007). In similarity to Cas in *Drosophila* neuroblasts, Foxa2 has been argued to operate in progenitor cells as a temporal fate determinant and as a switch factor. This study raises the question of how the expression of Foxa2 is

activated in progenitor cells at the appropriate time. Although the mechanism is unknown, the fact that Phox2b and Foxa2 establish cross-repressive interactions suggests that the activation of feed-back loops may play an important role in this process. In wild-type mice, at one level of the hindbrain (rhombomere 4), the vMN-to-5HTN switch does not occur, resulting in the prolongation of vMN generation at the expense of 5HTN fate. This is the result of the concerted actions of Nkx6.1/6.2 and Hoxb1 transcription factors which operate as a switch brake, thus maintaining progenitors in an early competence state (**Paper III**). The role of Foxa2 and other factors in the vMN-to-5HTN switch are further explored in the discussion of Papers III and IV.

CELL FATE SPECIFICATION IN THE VENTRAL HINDBRAIN

The hindbrain plays an important role in regulating basic functions of an organism such as breathing, heart rate, blood pressure and motor activity coordination. During development, this region of the neural tube becomes divided into eight segments or rhombomeres (r) (r1-r8) along the AP axis. This results in progenitor cells acquiring different molecular properties with time (for example adhesion properties), restricting their ability to intermingle with neighboring cells (Tumpel et al., 2009). The segmentation process of the hindbrain is tightly coupled to the expression of transcription factors of the Hox family. These genes are expressed along the AP axis of the hindbrain in a nested or overlapping pattern, establishing distinct domains defined by a code of Hox gene expression. This code of Hox activity is important in specifying the identity of each rhombomeric segment. In turn, the induction and maintenance of *Hox* gene expression is regulated by several signaling pathways (FGFs and retinoids) and transcription factors (e.g. Krox20 and Kreisler) (Cordes, 2001; Lumsden and Krumlauf, 1996). During the early stages of hindbrain development, the expression of each *Hox* gene is uniform within a segment. Then, as neurogenesis proceeds, it becomes restricted to specific domains along the DV axis where it influences the specification of the neuronal cell types (**Paper III,** Davenne et al., 1999). The unique positional identity along the AP axis provided to progenitors cells is also integrated with DV positional information. In this way, DV information establishes the general cell fate identity, i.e. motoneuron vs. interneuron; while AP information defines a specific neural subtype, e.g. the cranial identity of the motor neurons.

Cranial motor neurons

Motor neurons (MN) are unique among neurons generated in the CNS as they extend their axons outside the neural tube to innervate muscles directly or indirectly. MN generated in the developing hindbrain (designated cranial motor neurons) control muscles involved in eye, head and neck movement, feeding, speech and facial expression. Based on their targets, cranial MNs are classified into somatic motor neurons (sMN), general visceral motor neurons and special visceral motor neurons. sMNs innervate skeletal muscles directly. General visceral motor neurons project to

parasympathetic neurons that innervate cardiac muscles and smooth muscles of the viscera and special visceral motor neurons innervate branchial arch-derived muscles directly. General and special visceral motor neurons are collectively referred to as vMNs.

sMNs and vMNs also diverge in the trajectories that their axons use to leave the neural tube. The axons of sMNs exit the neural tube through ventral exit points, whereas those of vMNs choose dorsal exit points. Both sMNs and vMNs are generated in the ventral hindbrain. However, these two classes of MNs derive from different progenitor domains and are specified by different transcriptional programs. vMNs are derived from a progenitor domain (pvMN) located directly dorsal to the FP that expresses the transcription factors Nkx2.2/2.9, Nkx6.1/6.2 and Phox2b (Briscoe et al., 1999; Pattyn et al., 2000). In turn, sMNs derive from the progenitor domain (pMN), located immediately dorsal to the pvMN, that expresses the transcription factors Olig2, Nkx6.1/6.2 and Pax6 (Ericson et al., 1997; Zhou and Anderson, 2002). The generation of vMNs and sMNs differs along the AP axis of the hindbrain. vMNs are generated along the entire AP axis of the hindbrain, except in r1 level. By contrast, sMNs are only generated at caudal levels of the hindbrain (r5 and r7 in the mouse) and at spinal cord levels (Arber et al., 1999; Lumsden and Krumlauf, 1996; Pattyn et al., 2000).

Specification of somatic motor neurons

Most studies addressing the specification of sMNs have been focused on the spinal cord level. In this region of the neural tube, the progenitor domain that generates sMNs, pMN, is flanked ventrally by p3 progenitors that generate v3 interneurons and dorsally by p2 progenitors that give rise to V2 interneurons. The pMN progenitors express the HD transcription factors Pax6, Nkx6.1/6.2 and the pMN domain specific bHLH transcription factor Olig2. Both Olig2 and Nkx6 (including Nkx6.1 and Nkx6.2) have important roles in the generation of sMNs. They are sufficient to induce ectopic MNs in the neural tube and in *Olig2* or *Nkx6.1/6.2* compound mutant mice sMN are not generated (Lu et al., 2002; Novitch et al., 2001; Vallstedt et al., 2001; Zhou and Anderson, 2002). Additionally, these proteins function as transcriptional repressors, indicating that their role is to prevent the expression of repressors of the MN fate in pMN progenitors (Muhr et al., 2001). In line with this, Nkx6 and Olig2 proteins repress the expression of the proteins Dbx1/2 and Irx3, respectively, which have been implicated in blocking MN induction (Briscoe et al., 2000; Novitch et al., 2001; Sander et al., 2000; Vallstedt et al., 2001). Furthermore, Olig2 has also been shown to promote the differentiation of sMNs by regulating the expression of the bHLH pro-neural gene Ngn2 (Novitch et al., 2001; Zhou and Anderson, 2002). Nkx6 proteins are required for the expression of Olig2 in the spinal cord indicating that, at this level, Nkx6 proteins operate upstream of Olig2 in the sMN specification pathway (Novitch et al., 2001).

Specification of visceral motor neurons

All vMNs (including general and special visceral motor neurons) are generated from the pvMN domain that expresses the transcription factors Nkx2.2/2.9 and Nkx6.1/6.2

(**PaperII,** Briscoe et al., 1999; Ericson et al., 1997; Muller et al., 2003).The period of vMN generation correlates with the expression of the paired-like homeobox gene Phox2b in vMN progenitors, and gain- and loss-of-function experiments have demonstrated the important role of Phox2b in the specification of vMNs. Loss of Phox2b function in mice results in a complete loss of vMN production, while ectopic expression of Phox2b in chick neural progenitors is sufficient to induce transcriptional programs and axonal projections characteristic of vMNs (Dubreuil et al., 2000; Hirsch et al., 2007; Pattyn et al., 2000). In addition to its role in instructing vMN fate, Phox2b promotes neural progenitor cell cycle exit and the initiation of generic neuronal differentiation programs. This results from the ability of Phox2b, in combination with Nkx2.2, to activate the expression of the pro-neural gene *Ascl1/Mash1*, which in turn promotes neuronal differentiation. Moreover, Phox2b also promotes downregulation of the expression of the inhibitors of neurogenesis, *Hes5* and *Id2*. Accordingly, the expression of Ascl1/Mash11 in Nkx2.2^{+} pvMN progenitors is downregulated and there is a reduction of the numbers of progenitor cells that exit the cell cycle in *Phox2b* mutants. Phox2b also represses the expression of Olig2 and Pax6, known repressors of the vMN fate. Interestingly, and in contrast to most patterning genes, Phox2b seems to operate as a transcriptional activator (Dubreuil et al., 2002). These studies have therefore established an important role for Phox2b in coupling subtype-specific and generic neuronal differentiation programs involved in the generation of vMNs. The pivotal role of Phox2b in specifying vMN fate raises the question of the factors that regulate its expression in progenitors. In the pvMN domain, Phox2b is co-expressed with Nkx2.2 and in *Pax6* mutants, the expression of Nkx2.2 and Phox2b expands dorsally with a corresponding ectopic production of vMNs (Ericson et al., 1997), raising the possibility of the existence of regulatory interactions between these two proteins. While the activity of Phox2b does not affect Nkx2.2 expression (Dubreuil et al., 2002; Pattyn et al., 2000), ectopic expression of Nkx2.2 in hindbrain neural progenitors is sufficient to induce Phox2b (**PaperII,** Samad et al., 2004). However, in *Nkx2.2* mutant mice the expression of Phox2b and the generation of vMN are not affected. pvMN progenitors also express Nkx2.9 but *Nkx2.9* mutants show only minor defects in vMN generation (Pabst et al., 2003). Functional studies indicate that Nkx2.2 and Nkx2.9 mediate partially redundant activities when ectopically expressed in the neural tube and that they have redundant activities in the specification of FP cells (**PaperI**, Briscoe et al., 1999). It is therefore possible, that Nkx2.2 and Nkx2.9 proteins may also have redundant activities in the specification of vMNs, thereby explaining the normal generation of this cell type in the *Nkx2.2* and *Nkx2.9* single mutants. I will further discuss the role of Nkx2.2/2.9 proteins in vMN specification during the discussion of PaperII, in which I will talk about some preliminary results from the analysis of *Nkx2.2/2.9* compound mutants at the hindbrain level. Hox genes have also been shown to regulate the expression of Phox2b (**PaperIII,** Davenne et al., 1999; Gaufo et al., 2000; Gaufo et al., 2003). The isolation and functional characterization of a conserved non-coding regulatory sequence that recapitulates the expression of Phox2b in the ventral hindbrain at r4 level, provided evidence that *Hox* genes (in this study Hoxb1 and/or Hoxb2) can regulate directly the activation of Phox2b in pvMN progenitors (Samad et al., 2004). Furthermore, *Hox* genes cooperate with the repressor

activity of Nkx2.2 to induce robust expression of Phox2b in the hindbrain (Samad et al., 2004). These studies, therefore, indicate that the regulation of Phox2b involves the integrated activities of Nkx2, Nkx6 (discussed in **Paper III**) and Hox genes.

Serotonergic neurons

During embryonic development the ventral region of the hindbrain generates serotonergic neurons (5HTNs) that produce the neurotransmitter serotonin. Here, 5HTNs are generated in two clusters and are later organized in the raphe nuclei. Cells in the rostral cluster are born in r1-r3 and contribute to the raphe nuclei B6-B9, while the cells of the caudal cluster are born caudal to r4 and contribute to the B1-B5 raphe nuclei. r4 does not generate 5HTN, thereby creating a gap between the two clusters (**Paper III,** Bartonicek et al., 1964; Jensen et al., 2008). The rostral group largely projects anteriorly to several regions of the brain including the cortex, the limbic region and the midbrain, and modulates circuits involved in emotional responses, circadian rhythm and energy balance. By contrast, cells of the caudal group mainly send descending projections to the caudal hindbrain and spinal cord, and modulate many physiological processes such as cardiorespiratory homeostasis, thermoregulation, and nociception. As a result, the serotonergic system regulates the activity of a vast number of neural circuits in the CNS, involved in many physiological processes and behaviors. Consequently, the dysfunction of this system has been associated with several neurological and psychiatric disorders such as anxiety, depression, aggression and schizophrenia, among others (Gaspar et al., 2003; Hensler, 2006; Lucki, 1998; Sodhi and Sanders-Bush, 2004).

5HTNs are born from a ventral progenitor domain that expresses Nkx2.2 and, with the exception of r1, the generation of 5HTNs is preceded by a period of vMN production. The serotonergic progenitor cells express several transcription factors that have been shown to be important for specification of the 5HTN fate: Nkx2.2, Foxa2 and the proneural gene Ascl1/Mash1. In *Nkx2.2* mutants, all 5HTNs are lost with the exception of cells generated at r1 that localize to the dorsal raphe nuclei. The persistence of this rostral group of 5HTNs has been attributed to the redundant activity of Nkx2.9, which is also expressed in serotonergic progenitors (Briscoe et al., 1999). The primary function of Nkx2.2 in promoting 5HTN specification is to repress the expression of Phox2b, a determinant of vMN and repressor of 5HTN fate (see above and discussion of **Paper III**). The forkhead transcription factor Foxa2 is also expressed in serotonergic progenitor cells and its activity is required for the activation of serotonergic fate determinants (Jacob et al., 2007). In addition, Foxa2 has also been proposed to regulate the transition between vMN and 5HTN generation. Ascl1/Mash1 is expressed both in vMN and 5HTN progenitors. However, its activity is only required for the production of 5HTNs. In *Ascl1/Mash1* mutant embryos, the generation of vMNs is not affected, but after this period, progenitor cells fail to exit the cell cycle and the production of 5HTN is blocked. Introduction of Ngn2 coding sequence into the Ascl1/Mash1 locus is sufficient to rescue neurogenesis but does not rescue the generation of 5HTNs. Therefore, Ascl1/Mash1 seems to be important to provide proneural activity and to

activate the serotonergic program during the period of serotogenesis (Pattyn et al., 2004). However, this study cannot rule out that the failure of Ngn2 to rescue the specification of 5HTN fate results from the activation of neuronal-subtype specification programs that can repress the serotonergic program. Together, these studies have revealed critical roles of Nkx2.2, Ascl1/Mash1 and Foxa2 function in activating downstream transcriptional networks that determine 5HTN fate.

In post-mitotic cells, at least three factors are activated in parallel: Lmx1b, Gata3 and Pet1, and their activities have been shown to be important for the maturation and survival of 5HTNs. Analysis of *Lmx1b* mouse mutants showed that this gene is not essential for the generation of 5HTN precursors but, rather, is important for the maintenance of the serotonergic phenotype. In these mutants the initial expression of Pet1 and Gata3 is unaffected, but eventually lost. By contrast, Lmx1b activity is required for the expression of genes necessary for the synthesis (*Tph1/2*), vesicular transport (*Vmat2*) and re-uptake after synaptic release (*Sert*) of serotonin (Cheng et al., 2003; Zhao et al., 2006). Analysis of *Gata3*$^{-/-}$/Wt chimeras and *Gata3* mutants rescued with noradrenergic agonists, indicates a differential requirement for Gata3 in the generation of 5HTN along the rostro-caudal axis, with Gata3 playing a more important role in the generation of caudal 5HTNs than of the rostral group (Pattyn et al., 2004; van Doorninck et al., 1999). One reason for this may be the partial redundancy with the related factor Gata2, which is required for the generation of 5HTN in r1 and that is sufficient to induce several serotonergic markers at this level, including Gata3, Lmx1b and Pet1 (Craven et al., 2004). Interestingly, misexpression of Mash1 throughout the spinal cord is sufficient to activate the expression of Gata3, indicating that Gata3 may operate downstream of Ascl1/Mash1 (Tiveron et al., 2003). Importantly, the specification of 5HTN precursors is not affected in Gata3 mutants, as the expression of Lmx1b and Pet1 is not affected. Expression of Pet1 in the CNS is restricted to the 5HTN lineage and proceeds from that of Lmx1b and Gata3. Ablation of Pet1 function in mice results in a decrease of ~70% in the numbers of 5HTN, with the remaining cells exhibiting normal migration patterns and clustering into raphe nuclei. However, genes involved in the production and transport of serotonin (*Tph* and *Sert*) are expressed at lower levels in the remaining 5HTNs (Hendricks et al., 2003). The identification of Pet1 binding sites in close proximity to these genes has suggested a direct regulation by Pet1. The observation that these genes are also downregulated in *Lmx1b* mutants, before the downregulation of Pet1, suggests that these two factors cooperate in the regulation of late serotonergic genes (Hendricks et al., 1999; Hendricks et al., 2003; Zhao et al., 2006). At later stages Pet1 also seems to play an important role in the proper maturation of the serotonergic system (Liu et al., 2010). The mechanisms by which some 5HTNs are still generated in *Pet1* mutants is unknown, but it may be due to heterogeneity within the serotonergic population. Indeed, whole-genome profiling of 5HTNs has revealed a great number of genes differentially expressed in different groups of 5HTNs, supporting the existence of distinct subpopulations within the serotonergic system (Wylie et al., 2010).

AIMS

The overall focus of the work presented in this thesis was to better understand the mechanisms regulating neural cell fate specification during embryonic development of the vertebrate CNS and the interplay between local inductive signals and intrinsic molecular programs in this process.

Specific aims:

- To investigate the role of Shh-induced homeodomain proteins in the interpretation of Shh activity during ventral cell fate specification.
- To analyze the role of Nkx2 and Nkx6 proteins in the specification of motor neurons in the hindbrain.
- To study the role of Nkx and Hox proteins in the sequential generation of motor neurons and serotonergic neurons from a common pool of neural progenitor cells.
- To identify and characterize the factor(s) that control the MN-to-5HTN temporal fate switch.

RESULTS AND DISCUSSION

A HOMEODOMAIN FEEDBACK CIRCUIT UNDERLIES STEP-FUNCTION INTERPRETATION OF A SHH MORPHOGEN GRADIENT DURING VENTRAL NEURAL PATTERNING (PAPER I)

In the ventral neural tube Shh plays an important role in the spatial patterning of cell fate identity. The current model that explains the activity of Shh poses that the exposure of a target field of cells to increasing concentrations of Shh induces more ventral cell identities. However, mice lacking *Ptc* function generate several ventral cell types, despite the inability of progenitor cells to detect differences in the ambient concentration of Shh. In addition, ventral patterning is partially restored in *Shh* or *Smo* mutants after additional removal of *Gli3* function, indicating that some aspects of ventral patterning can occur in the absence of graded Shh signaling activity. Furthermore, genetic analysis of several mouse mutants of Gli proteins and Gli-mutant combinations show similar phenotypes, in which FP cells and V3 neurons are generally missing while more dorsal cell fates are generated. These observations raise the possibility that some aspects of spatial patterning of ventral cell types do not require graded Shh signaling activity.

Shh signaling regulates the expression of transcription factors in progenitor cells. Interestingly, the spatial expression of some of these transcription factors has been shown to be dynamic over the period during which ventral patterning is established. Once progenitor domains are established, Nkx2.2 is expressed in p3 progenitors located dorsal to FP cells. Strikingly, we observed that the expression of Nkx2.2 in the ventral neural tube exhibited a dynamic spatial expression pattern. At early stages, Nkx2.2 is expressed in the ventral midline, together with the FP determinant Foxa2. With time its expression expands dorsally with a concomitant downregulation in differentiating Foxa2/Shh^{+} FP cells in the ventral midline. This observation prompted us to examine the role of Nkx2.2 in the establishment of FP and V3 cell fates.

Analysis of compound mouse mutants for *Nkx2.2* and the closely related gene *Nkx2.9* (*Nkx2* mutants), revealed an important requirement for Nkx2 proteins in the generation of FP cells and V3 neurons. In order to understand whether Nkx2 proteins were sufficient to induce these cells types we missexpressed Nkx2.2 in the chick neural tube. Previous studies had shown that Nkx2.2 can induce the expression of markers of the V3 fate in the neural tube (Briscoe et al., 2000). However, those studies did not reveal a role for Nkx2.2 in the induction of FP fate. The expression vectors used in these studies are known to require some time to initiate gene expression (Briscoe et al., 2000). Since the expression of Nkx2.2 in the ventral midline is restricted to early stages, we tested whether expression of Nkx2.2 at early stages could induce FP. For this, we expressed Nkx2.2 or Nkx2.9 under the control of a promoter that drives rapid gene expression in neural progenitors (Bylund et al., 2003). Under these conditions, Nkx2 proteins induced FP cells. Together, these results revealed a role for Nkx2 proteins in the induction of FP

and V3 fates. Importantly, the inductive activities of Nkx2 proteins depend on the stage of neural development; at early stages Nkx2 proteins induce FP fate, while at later developmental stages they induce V3 neuron fate. These observations raised two important questions: 1) what are the mechanisms by which Nkx2 proteins induce FP cells; 2) what is the nature of the signal that controls the choice between FP cells or V3 neurons induction? Foxa2 is a determinant of FP identity and its expression is regulated directly by Gli-mediated transcriptional activation (Sasaki and Hogan, 1994; Sasaki et al., 1997; Weinstein et al., 1994). The early expression of Nkx2.2 in the neural tube resulted in the induction of Foxa2 before the onset of the expression of Shh, indicating that Nkx2.2 is able to activate the expression of Foxa2, which in turn activates the FP program. However, our results also indicate that the initial ectopic expression of Foxa2 is mediated by the repressor activity of Nkx2.2, implying an indirect mechanism for the activation of Foxa2 by Nkx2.2. Since Foxa2 is regulated directly by Gli proteins, we tested weather Nkx2.2 could induce the expression of Foxa2 by influencing the intracellular strength of Shh. For this purpose, we isolated an enhancer element that recapitulated the expression of Nkx2.2 in the chick neural tube which contained one Gli binding site (GBS) essential for the activity of the element. During our studies, a similar element was published by the Matise lab, which was shown to recapitulate the expression of Nkx2.2 in the mouse neural tube. The activity of this element was also shown to be dependent on the GBS (Lei et al., 2006). This tool allowed us to monitor the overall Gli-transcriptional activity in neural progenitors in the neural tube, and we observed that expression of Nkx2.2 resulted in an increase of Gli transcriptional activity. In addition, the increase of Gli activity was accompanied by the downregulation of *Gli3* expression, a major suppressor of Shh target genes. To test if the reduction of Gli repressor activity was important for the induction of FP by Nkx2.2, we co-expressed Nkx2.2 with a truncated form of Gli3 (Gli3R), which acts a dominant inhibitor of Gli activity (Persson et al., 2002). Maintenance of Gli3R activity abrogated the FP inducing activity of Nkx2.2. Together these data indicate that Nkx2.2 activity enhances cell intrinsically the responses of neural progenitor to Shh signal in a process, at least in part, mediated by the downregulation of *Gli3*.

Next, we addressed whether other HD transcription factors could influence the response of neural progenitors to Shh signaling. Pax6 expression is negatively regulated by Shh and its activity represses the expression of Nkx2.2. In addition, removal of Pax6 function from *Shh* mutants partially rescues ventral patterning defects in the developing telecenphalon (Fuccillo et al., 2006). These observations prompted us to examine if Pax6 could influence the response of neural progenitors to Shh signaling. Using gain-of-function experiments in the chick neural tube we were able to show that the activator activity of Pax6 repressed the induction of FP cells and up-regulated the expression of *Gli3*. Importantly, co-expression experiments of Nkx2.2 with Pax6 indicated that Pax6 activity counteracted the Nkx2.2 mediated regulation of Foxa2 and *Gli3* expression, in a process independent of Nkx2.2/Pax6 cross-repressive interactions. Analysis of *Pax6* mutants showed that Pax6 was not absolutely required for the expression of *Gli3*. However, its activity was important for the maintenance of the ventral expression of

Gli3. Moreover, removal of *Pax6* function in *Gli2* mutants rescued the induction of the Nkx2.2 (Lei et al., 2003) and FP domains.

Collectively, these data reveal the establishment of a feedback circuit in which transcriptional targets of Shh modulate, in a cell intrinsic manner, the cellular responsiveness to Shh signaling. This mechanism is important for the correct patterning of the neural tube. In the ventral spinal cord, Nkx2 proteins are required for the induction of FP cells and they mediate this activity by amplifying Shh responses in a cell intrinsic mechanism by repressing *Gli3* and Pax6. Conversely, Pax6 has an opposing function, to antagonize Shh signaling by up-regulating the level of GliR. Currently, we do not know if the regulation of *Gli3* by these HD proteins is direct. Preliminary analysis of the *Gli3* locus reveals the presence of conserved binding sites for Pax6 and Nkx2.2. However, further studies will be required to verify the functionality of these binding sites. Moreover, we cannot rule out the possibility that these HD proteins might influence the translation of the *Gli3* transcript or the processing and/or nuclear localization of the Gli3 protein. Our studies also raise the question of whether other members of the Class I/II transcription factors can modulate the cellular responses to Shh. Our preliminary data indicates that Irx3 can repress the expression of Nkx2.2 and the induction of FP cells. A more detailed analysis will be necessary to understand if Irx genes can modulate the levels of GliR as in the case of Pax6.

Another important observation from our results is the role of time in regulating the induction of FP and V3 fates by Nkx2.2. Since both cell types arise from Nkx2.2$^+$ progenitors, the ability of Nkx2.2 to sensitize cells to Shh signaling does not explain the sequential generation of these cell types. Graded Shh signaling is also unlikely to explain this observation. As development proceeds, the amplitude of the Shh gradient increases (Chamberlain et al., 2008), which is reflected in the increase and dorsal expansion of the expression of the Shh target gene *Ptc1* (Marigo and Tabin, 1996). Our observations indicated that FP is induced before V3 neurons, at a time when the ambient concentration of Shh is lower. If the generation of FP and V3 cells would follow the gradient model of Shh activity, FP cells would require higher levels of Shh signaling and, therefore, be generated after V3 neurons. FP cells express glial-like traits and the initiation of gliogenesis after the neurogenic period is accompanied by the reduction of the expression of pro-neural genes. Because the induction of FP cells occurs before the expression of pro-neural genes in the neural tube, we tested if a switch from non-neuronal to neuronal progenitor potential could mediate the selection between the two cell fates. The p3 domain expresses the pro-neural genes Ngn3 and Ascl1/Mash1. When we co-expressed Nkx2.2 with Ngn3 or Ascl1/Mash1, the ability of Nkx2.2 to induce FP was blocked and instead V3 neurons were generated. In addition, scattered expression of Ngn3 or Ascl1/Mash1 in the FP domain was sufficient induce the expression of the V3 marker *Sim1*, without significantly affecting the expression levels of *Shh*. These results support that a switch in the neuronal potential of progenitor cells, rather than graded Shh signaling, accounts for the selection of cell fate choice from Nkx2.2$^+$ progenitors.

Consistent with our data, previous studies demonstrated that the induction of FP or V3 cell fate by Shh depends on the developmental stage. In these studies, ectopic expression of an active form of Shh in the neural tube of HH10-12 chick embryos induced V3 identity, while expression of Shh at neural plate stage (HH8) induced FP identity (Patten and Placzek, 2002; Ribes et al., 2010). Similar results were also observed when intermediate regions of the neural tube from different developmental stages were exposed to Shh. This argues that the change in competence of neural cells to respond to Shh is intrinsic, and reinforces our model in which cell fate choice is gated by the acquisition of neurogenic potential. Additionally, this model allows us to explain the restriction of the induction of FP in response to Shh signaling with time. Because differentiated FP cells express Shh, their induction would result in the maintenance of high level of Shh and in a continuous induction of FP cells throughout the neural tube. The change in competence of neural progenitors in response to neurogenic cues allows a spatial restriction in the induction of FP cells, even in the presence of high levels of Shh. Using explants of naïve neural plate cells, Ribes et al. observed that the induction of FP cells requires a higher concentration of Shh than the one required to induce p3 progenitors. This observation was used to support a model of graded Shh in the induction of these fates. In addition, neural cells in this system are only competent to generate FP cells during the first 12h of culture. Interestingly, q-PCR analysis of gene expression showed that only with a concentration of Shh that induces FP there is a robust induction of Nkx2.2 by 12h of culture. This indicates that the generation of FP and V3 fates, in response to different concentrations of Shh, does not reflect different requirements of Shh signaling in cell fate specification, but rather a difference in the temporal kinetics to reach the threshold level to induce robust expression of Nkx2.2, which is required for the generation of both cell types.

In the neural tube, time and neurogenic potential of neural progenitors influences the selection between FP and V3 fate in response to Nkx2 proteins activity. This process is therefore a good model to understand the influence of cellular competence in the activity of defined factors. In this way, it would be interesting to identify the direct target genes of Nkx2 proteins by chip-sequencing methods and the influence of time or the activation of neurogenic programs in the selection of targets genes by Nkx2 proteins. Combining such experiments with global RNA sequencing of progenitor cells would further provide important insights to the transcriptional activity of Nkx2 proteins during this process.

COMPLEMENTARY ROLES FOR NKX6 AND NKX2 CLASS PROTEINS IN THE ESTABLISHMENT OF MOTONEURON IDENTITY IN THE HINDBRAIN (PAPER II)

In the hindbrain, Nkx2.2$^+$ progenitors generate visceral motor neurons (vMNs) during early stages of development. In addition, this progenitor domain also expresses the HD transcription factors Nkx2.9, Nkx6.1 and Nkx6.2. In order to better understand the mechanisms regulating cell fate specification, and in particular the genetic program that underlies the generation of vMN in the developing hindbrain, we analyzed the role of Nkx2.2 and Nkx6 (Nkx6.1 and Nkx6.2) proteins in this process.

Using gain-of-function experiments in the chick neural tube, we were able to show that the expression of Nkx2.2 in progenitor cells of the hindbrain is sufficient to mediate the activation of Phox2b. In turn, Phox2b is sufficient to induce vMN fate without inducing the expression of Nkx2.2 (Dubreuil et al., 2002). A later study from the Rijli group provided evidence that the repressor activity of Nkx2.2 cooperates with Hox proteins in the activation of Phox2b expression (Samad et al., 2004). These results, therefore, indicate that Nkx2.2 acts upstream of Phox2b in the induction of vMNs. The mechanism by which the repressor activity of Nkx2.2 promotes the expression of Phox2b remains unknown. Based on our studies in the spinal cord (**Paper I**), a possible mechanism would be that Nkx2.2 mediates this process by regulating the levels of Gli activity in neural progenitors. However, analysis of an enhancer element that recapitulates the expression of Phox2b at r4 level (Samad et al., 2004) does not seem to contain a Gli-binding site consensus sequence. In addition, Nkx2.2 can still induce the activation of Phox2b expression in conditions of blocked Shh signaling (José Dias, unpublished observations), arguing that Shh signaling is not required for the activity of Nkx2.2 in this process. Analysis of *Nkx2.2* mutant mice has shown that the expression of Phox2b and the generation of vMNs are not affected (Briscoe et al., 1999), questioning the role of Nkx2.2 in the generation of vMNs. However, it has been proposed that the normal generation of vMNs in *Nkx2.2* mice could reflect a functional redundancy with closely related gene *Nkx2.9* (Briscoe et al., 1999). We have recently been able to test this hypothesis by analyzing the generation of vMNs in *Nkx2.2* and *Nkx2.9* (*Nkx2*) compound mutants. In these mutant mice, the expression of Phox2b is not activated in ventral progenitors at the caudal hindbrain and vMN are not generated. These results support that Nkx2.2 and Nkx2.9 provide redundant activities in the specification of vMNs. Previous studies have shown that Nkx2.2 represses the generation of sMNs in the spinal cord, most likely by repressing the expression of the sMN determinant Olig2 (Briscoe et al., 2000; Novitch et al., 2001). In *Nkx2* mutants the expression domain of Olig2 expanded ventrally and this was accompanied by ectopic ventral generation of sMNs. In addition, expression of Olig2 in Nkx2.2$^+$ progenitors using an Nkx2.2 enhancer element (described in **Paper I**) was sufficient to repress the expression of Phox2b, even in the presence of Nkx2.2 activity (José Dias, unpublished data). These data argue that an important role for Nkx2 proteins is to repress the expression of repressors of the vMN fate.

In contrast, we observed that vMNs are generated in normal numbers in *Nkx6.1* and *Nkx6.2* compound mutants (*Nkx6* mutants). The activities of Nkx6.1 and Nkx6.2 proteins however, seem to be important to repress parallel programs of differentiation in the vMN lineage, in particular the expression of *Dbx* genes and the V0 determinant Evx1. The de-repression of the V0 program in vMN progenitors, in contrast to the de-repression of the sMN program, is not sufficient to affect the generation of vMNs.

The expression of Nkx6 proteins is maintained in most differentiated vMN suggesting that these proteins may have a role in later aspects of vMN differentiation. Indeed, analysis of *Nkx6* mutant mice at late developmental stages revealed that both cell body migration and axonal projection of vMns were affected. Most vMNs migrate dorsally settling close to the exit point where their axons leave the neural tube. In *Nkx6* mutants this dorsal migration was slower and eventually, the vMNs settled in a more ventral position. The facial branchial motor neurons (fbMN) generated in r4 exhibit a more complex migration pattern. These cells are generated at r4 level and subsequently migrate caudally along the midline through r5 until they reach r6, where they migrate dorsally to form the facial nucleus. In *Nkx6* mutants, we observed that the fbMN failed to initiate their caudal migration and instead initiated a dorsal migration within r4 level. Since the *Nkx6.2* mutant mice contain a *tau-LacZ* expression cassette under the control of the *Nkx6.2* locus, we were able to visualize the axonal projections of vMNs. The analysis of the expression of *tau-LacZ* revealed that loss of Nkx6 function affected axon pathfinding, with the more caudal groups of vMns showing more severe defects. At this level, the majority of the axons after reaching dorsal positions in the neural tube failed to recognize the exit points and instead projected caudally or rostrally within the CNS. Together our results reveal a role for Nkx6.1 and Nkx6.2 proteins in regulating migration and axon pathfinding of vMNs. This function seems to be evolutionarily conserved, as a similar role for Nkx6.1 has been described in *Drosophila*, zebrafish and *Xeponus* (Broihier et al., 2004; Dichmann and Harland, 2011; Hutchinson et al., 2007). The activity of Nkx6 proteins in vMN progenitors is important for the correct specification of vMN. This raises the question of whether the role of Nkx6 proteins in neuronal migration and axon guidance reflects a function of these proteins in post-mitotic neurons. In *Nkx6.1* mutants, the r4-fbMN do not activate the expression of Evx1, however, their caudal migration is affected. This argues that the early role for Nkx6 proteins, in repressing the expression of Evx1, is not responsible for the migration defects observed in these mutants. In addition, in *Nkx6.1* mutants, fbMN show changes in the expression of netrin guidance receptors (*Unc5h3* and *neogenin*) (Muller et al., 2003), arguing for a cell autonomous activity of Nkx6.1. More recently, studies in the spinal cord have shown that the expression of Nkx6.1 in Nkx6.1$^-$ sMN results in the acquisition of axonal projections characteristic of Nkx6.1$^+$ sMNs (De Marco Garcia and Jessell, 2008). These data provide additional support for a cell-autonomous function of Nkx6 proteins in the correct differentiation of vMNs. However, we cannot rule out the possibility that loss of Nkx6.1 activity results in changes in the progenitor environment that can affect some aspects of vMN differentiation. Ultimately, the generation of mouse lines that allow the conditional

deletion of Nkx6.1 function in post-mitotic vMNs will be required to determine the role of these proteins in post-mitotic MNs during the differentiation process.

In contrast to reports in the spinal cord where Nkx6 protein function is required for the maintenance of Olig2 (a determinant of sMN), we found that the initial expression of Olig2 is left intact in the caudal hindbrain of *Nkx6* mutants but that all sMNs are missing. These results promote a model for a parallel requirement for Nkx6 and Olig2 proteins in the progression of sMN fate determination in the hindbrain level.

COORDINATED TEMPORAL AND SPATIAL CONTROL OF MOTOR NEURON AND SEROTONERGIC NEURON GENERATION FROM A COMMON POOL OF CNS PROGENITORS (PAPER III)

In the hindbrain, the generation of vMNs from Nkx2.2$^+$ progenitors is followed by the production of serotonergic neurons (5HTN) (Briscoe et al., 1999). However, 5HTNs are initially detected as two morphologically distinct groups of cells (a rostral and a caudal group) which results from an interruption in their generation along the AP axis of the hindbrain. We mapped this gap to r4 and, using BrDU birthdating experiments, we were able to show that at this level the generation of vMN is prolonged. This indicates that Nkx2.2 progenitors, located in r1-r7, sequentially generate vMN and 5HTN, except those located in r4 that maintain the production of vMN. Due to the simpler nature of this system and the well defined order in the generation of vMN and 5HTN, we used it to study the mechanisms that control the sequential generation of distinct cell types from a common pool of progenitors.

Since cell fate specification is initiated at the progenitor stage, we analyzed the temporal expression of several transcription factors in Nkx2.2$^+$ progenitors at levels where the vMN-5HTN fate switch occurs. During the period of vMN production most progenitors co-expressed Nkx6.1, Nkx6.2 and the vMN determinant Phox2b. The transition to the serotonergic phase correlated with the downregulation of Phox2b expression. Importantly, the prolongation of vMN production in r4 correlated with the maintenance of the expression of Phox2b. Furthermore, loss of Phox2b activity resulted in the lack of vMN production and premature generation of 5HTNs at all axial levels of the hindbrain, including r4. Altogether, our observations indicate that the activity of Phox2b plays a key role in selecting the cell type generated by Nkx2.2$^+$ progenitors. Moreover, this activity is restricted to progenitor cells as deletion of Phox2b in post-mitotic vMNs does not result in the activation of the 5HTN program (Coppola et al., 2010).

Previous studies have shown that the function of Nkx2.2 is required for the generation of 5HTNs in most axial levels of the hindbrain (Briscoe et al., 1999). This prompted us to examine the changes of gene expression in progenitor cells in the absence of Nkx2.2 activity. In *Nkx2.2*, mutants we observed that at the time when cell fate switch occurs the expression of Phox2b and the generation of vMNs were maintained, revealing an

important role for Nkx2.2 in regulating the switch process. In addition, progenitor cells located in r1 are still able to generate 5HTNs in the absence of Nkx2.2 function, indicating that Nkx2.2 does not have an essential role in the activation of the serotonergic program. The molecular mechanism through which Nkx2.2 regulates the switch process remains unknown. Taking into account the progressive, ventral-to-dorsal repression of Phox2b expression in the progenitor domain, we suggested that a signal produced by the FP induced or activated an unknown factor in Nkx2.2 progenitors which would be necessary for Nkx2.2 to repress Phox2b expression. In Paper IV we provide some experimental evidence that implicates the ability of Nkx2.2 to modulate the cellular response to Shh signaling in the regulation of the vMN-5HTN switch.

Our analysis of *Nkx2.2* mutants indicated that HD transcription factors involved in DV patterning can also have a role in regulating the sequential generation of cell fates. We therefore examined if other DV patterning genes expressed in Nkx2.2 progenitors could also influence temporal cell fate specification. Nkx6.1 and Nkx6.2 are expressed in these progenitors during the period of vMN and 5HTN generation. While the initial generation of vMNs is not affected in *Nkx6.1;Nkx6.2* compound mutants (*Nkx6* mutants, **Paper II**) we observed a premature termination of vMN production in r4 accompanied by ectopic generation of 5HTN. These observations revealed a specific requirement for Nkx6 proteins in maintaining the generation of early-born vMN fate. Since Nkx6 proteins are expressed by all Nkx2.2^{+} progenitors in the hindbrain, we argued that their selective activity in r4 had to be indirect. In the hindbrain, the combined activity of different Hox genes defines the identity of each rhombomere. Hoxb1 is selectively expressed in r4 and its activity is important to establish r4 identity. We observed that loss of Nkx6 function resulted in a reduction of the ventral expression of Hoxb1 from E10.5 which correlated with the downregulation of Phox2b expression. In addition, analysis of *Hoxb1* mutants revealed a premature termination of MN production and ectopic generation of serotonergic cells, without changes in Nkx6 proteins expression. Interestingly, we observed that in *Hoxb2* mutants the expression of *Hoxb1* was downregulated at a later stage (from E11.5) and consistently we observed a milder phenotype in the changes of vMN and 5HTN production, as compared to *Nkx6* mutants. These observations suggest that Nkx6 proteins operate upstream of Hoxb1 in maintaining the expression of Phox2b and prolonging the period of vMN production. Later studies provided evidence that Hoxb1 can directly regulate the expression of Phox2b (Samad et al., 2004), suggesting the existence of an intrinsic molecular mechanism important to maintain progenitors in an early temporal identity. The expression of Hoxb1 in r4 is regulated by a complex network of auto- and cross-regulation between different *Hox* genes (e.g. Hoxb1, Hoxa1, Hoxb3) and other transcriptional regulators (e.g Krox20, PIASxβ, RA signaling) (Garcia-Dominguez et al., 2006; Gavalas et al., 2003; Wong et al., 2011). How the activity of Nkx6 proteins feeds into this regulatory network is, at the moment, unknown. Future analysis of the changes in gene expression of r4-Nkx2.2^{+} progenitors in *Nkx6* mutants may provide important insights into this regulatory mechanism.

In this study we have begun to address the molecular mechanisms that regulate the sequential generation of vMNs and 5HTNs from a common pool of Nkx2.2$^+$ progenitors. From early stages, Nkx2.2$^+$ progenitors are competent to generate both vMNs and 5HTNs and the selection between these two cell fates relies on the activity of Phox2b, which promotes early-born vMN fate and represses late-born 5HTN fate. In this aspect, the role of Phox2b resembles that of Hunchback in *Drosophila* neuroblasts in determining early temporal identity. However, and in contrast to Hunchback, missexpression studies have shown that the activity of Phox2b in neural progenitors correlates with the acquisition of a specific cell fate, the vMN fate (Dubreuil et al., 2000).

Our studies established that the regulation of Phox2b expression is a key step in the control of the vMN-5HTN fate switch. Strikingly, we found that the integrated activities of Nkx and Hox proteins can maintain the expression of Phox2b, possibly through direct activation by Hoxb1 (Samad et al., 2004). This reveals the presence of cross-talk between DV and AP patterning mechanisms, which have traditionally been studied separately, and their importance in establishing diversity in the neural tube. Yet, the signal(s) that control the termination of Phox2b expression and how they cooperate with Nkx2.2 in this process remains unknown. Identifying such signal(s) is a key issue, as it will allow us to begin to understand the mechanisms used by progenitor cells to measure time and coordinate the generation of different cell types during vertebrate CNS development.

A TEMPORAL SIGNAL RELAY MECHANISM BY SHH AND TGFB UNDERLIES THE SEQUENTIAL SPECIFICATION OF MOTOR NEURONS AND SEROTONERGIC NEURONS IN THE DEVELOPING CNS (PAPER IV)

In our previous study (**Paper III**), we defined a key role for Phox2b in regulating the vMN-5HTN cell fate switch in ventral hindbrain Nkx2.2$^+$ progenitors. Analysis of the dynamics of Phox2b expression at caudal levels of the hindbrain revealed a progressive repression in a ventral to dorsal wave. Thus, a signal originating from the FP region and that progresses dorsally has been proposed to explain the switch process (**Paper III**, (Pattyn et al., 2004)). The nature of such signal, however, and the mechanisms regulating its temporal expression were unknown.

In this study we set out to identify factors that could terminate the production of vMN and induce premature generation of 5HTNs. For this, we used chick electroporation together with an *Nkx2.2* gene regulatory element (**Paper I**) to selectively activate several signaling pathways during the period of vMN generation in Nkx2.2$^+$ progenitors. Of the pathways tested - Shh, Notch, Wnt (canonical pathway), Tgfβ superfamily (Tgfβ and BMP pathways) and nuclear receptors (RxR) – only the activation of the Tgfβ pathway resulted in premature induction of 5HTNs.

The transforming growth factor-β (Tgfβ) proteins have been shown to have an important role in cell proliferation, differentiation and apoptosis. In a simplified overview of the Tgfβ signaling pathway, Tgfβ ligands (Tgf-β1, -β2,- β3) bind to two distinct transmembrane serine/threonine receptors, the Tgf-β type I (Tgfbr1) and Tgf-β type II (Tgfbr2) receptors. This results in the stabilization of a heterotetrameric complex (composed by two type I and two type II receptors) that leads to the phosphorylation and activation of the type I receptors by the constitutively active kinase of the type II receptors. When activated, the kinase of receptor type I phosphorylates Smad2/ 3 transcription factors at C-terminal serines. This event facilitates the interaction of Smad2/3 proteins with Smad4 and subsequent translocation to the nucleus. Here, the trimeric Smad complex binds to DNA to regulate gene expression. In addition to the Smad-mediated transcriptional signaling, the activated receptor type I can also activate other signaling pathways such as mitogen-activated protein kinases (MAPKs) (e.g. p38, Jnk and ERK), Rho-like GTPases and PI3 kinases.

From our screen, we observed that the expression of an activated form of the type I receptor ($Tgfbr1^{CA}$) in $Nkx2.2^{+}$ progenitors during the period of vMN generation resulted in reduced generation of vMNs and premature production of 5HTNs. Interestingly, broad expression $Tgfbr1^{CA}$ in the neural tube only induced the generation of 5HTNs from the Nkx2.2 progenitor domain. In addition, conditional deletion of *Tgfbr1* in the ventral neural tube resulted in a prolonged production of vMN production and delayed initiation of serotogenesis. These results show that Tgfβ signaling in $Nkx2.2^{+}$ progenitors controls the selection between vMN and 5HTN fate.

Tgfβ proteins signal through Tgfbr1-Tgfbr2 complexes and expression analysis of Tgfβ ligands indicated that *Tgfβ2* was expressed in the ventral hindbrain. Moreover, its expression in Nkx2.2 progenitors correlated temporally with the termination of vMN production and induction of 5HTN. This suggests that signaling initiated by Tgfβ2 can mediate the vMN-5HTN cell fate switch. Supporting this hypothesis, exposure of ventral neural progenitors from the rostral hindbrain to Tgfβ2 protein resulted in premature induction of 5HTN cells. Moreover, the induction of Tgfβ2 expression is delayed in mice lacking Nkx2.2 function, which have prolonged production of vMN and lack the generation of serotonergic cells (**Paper III**). Taken together, these results strongly suggest that activation of Tgfβ signaling by Tgfβ2 ligand regulates the vMN-5HTN cell fate switch in $Nkx2.2^{+}$ progenitors.

The activity of Phox2b in Nkx2.2 progenitors promotes early-born vMN fate and represses late-born 5HTN fate (**Paper III**). Using gain- and loss-of-function experiments we showed that Tgfβ signaling represses the expression of Phox2b. Since maintenance of Phox2b expression under conditions of activated Tgfβ signaling abolished the premature induction of 5HTN, a key role for Tgfβ signaling in the vMN-5HTN switch is to repress Phox2b expression. In turn, analysis of *Phox2b* mutants revealed an early activation of Tgfβ2 expression. Our data, therefore, indicate that Phox2b and Tgfβ2 establish genetic cross-repressive interactions. At early stages, the activity of Phox2b delays the activation of the expression of Tgfβ2 in Nkx2.2

progenitors ensuring a period of vMN generation. With time, the expression of Tgfβ2 is activated resulting in the repression of Phox2b and transition to 5HTN generation. The mechanism underlying this cross-repressive interaction is unknown. Phox2b has been shown to operate as a transcriptional activator in the induction of vMN fate (Dubreuil et al., 2002). To date, no transcriptional repressor activity has been associated with this factor, suggesting that the repression of Tgfβ2 is indirect. Tgfβ signaling activates the intracellular Smad2/3 pathway (usually referred to as canonical pathway) and we observed that Smad3 is expressed in Nkx2.2 progenitors during vMN and 5HTN neurogenesis. However, expression of a constitutively active form of Smad3, either alone or in combination with Smad4, did not result in premature induction of 5HTNs. In addition to the canonical Smad pathway, Tgfβ signaling also activates other signaling pathways (e.g. MAPK, PI3K). Therefore, it is possible that Tgfβ2 mediates the repression of Phox2b through the activation of non-canonical pathways or by a combination of canonical and non-canonical signaling pathways. The identification of the molecular mechanisms that repress Phox2b expression would allow us to determine if this process reflects a direct repressive role of the Tgfβ pathway or if it involves the induction of intermediary factors.

A previous study has proposed that the expression of the forkhead transcription factor Foxa2 in Nkx2.2 progenitors mediates the vMN-5HTN switch by promoting the downregulation of the expression of Phox2b (Jacob et al., 2007). However, our study does not support such role for Foxa2. First, we observed robust co-expression of Foxa2 and Phox2b during the period of vMN neurogenesis, arguing against cross-repressive interactions between these two transcription factors in vivo. Secondly, the repression of Phox2b by Foxa2 in overexpression experiments in the chick neural tube reflects the induction of FP fate, as observed by the activation of several FP markers and the downregulation of Nkx2.2 and of pan-neuronal progenitor markers. These observations are supported by previous studies showing a role for Foxa2 in the activation of Shh expression and specification of FP fate. In addition, we were not able to detect significant changes in the expression of Foxa2 in conditions where the switch process is affected, namely after activation of Tgfβ signaling or in *Tgfbr1*, *Nkx2.2* and *Phox2b* mutants. However, we cannot rule out a regulatory activity for Foxa2 in this process, as we observed that its activity is important for robust expression of Tgfβ2, at least at r1 level. To date, genetic studies of the function of Foxa2 in the vMN-5HTN temporal switch has been restricted to a region of the hindbrain where this process does not occur (r1) (Jacob et al., 2007). In order to determine the role of Foxa2 in the switch process such analysis needs to be extended to regions where the process occurs.

The identification of Tgfβ2 as a temporal switch signal raises the question of as to what mechanisms trigger the activation of Tgfβ2 in the Nkx2.2^{+} lineage. We observed that expression of Shh in the neural tube was sufficient to induce the sequential generation of vMN and 5HTN. Importantly, Shh also induced the expression of Tgfβ2 with a temporal delay. These observations show that Shh signaling is sufficient to activate molecular programs that provide neural progenitors with spatial and temporal information. The activation of Shh signaling resulted in the expression of Phox2b,

Nkx2.2 and Foxa2 before the onset of Tgfβ2 expression. While Phox2b activity has a negative effect on Tgfβ2 expression, analysis of *Nkx2.2* and *Foxa2* mutants revealed a role for these factors in the correct temporal activation of Tgfβ2. Together, these data suggest that genes induced by Shh at early stages of development influence the timing of Tgfβ2 expression.

In paper I, we showed that Nkx2.2 can amplify Shh responses in a cell intrinsic manner. Accordingly, exposure of ventral neural tissue from *Nkx2.2* mutants to high concentrations of Shh could rescue the generation of 5HTNs, suggesting that the strength of Shh signaling is important for the switch process. Supporting this idea, exposure of ventral hindbrain neural tissue to a high concentration of Shh resulted in premature generation of 5HTNs. These results link Shh signal strength with the switch process and raise the question of whether Shh could directly regulate the expression of Tgfβ2. The identification of conserved elements in the *Tgfβ2* locus that are sufficient to recapitulate the ventral expression of Tgfβ2 will be important in dissecting the molecular mechanisms that regulate the temporal expression of Tgfβ2.

In this study we examined the sequential production of vMNs and 5HTNs from a common pool of Nkx2.2^{+} progenitors in the ventral hindbrain, and identified Tgfβ2 as a new regulator of this temporal switch process. Our data indicate that Tgfβ signaling mediates this process by repressing the expression of the vMN determinant Phox2b, allowing the determinants of 5HTN fate, such as Foxa2, to operate. Moreover, the expression of Tgfβ2 is regulated by Shh and repressed by Phox2b, which allows a temporal delay of Tgfβ2 expression in the Nkx2.2 lineage. Our studies reveal the importance of cross-repressive mechanisms in regulating sequential specification of neuronal fate. Such mechanism resembles the cross-regulatory interactions established during spatial cell fate specification. However, in temporal patterning, and in contrast to spatial patterning, determinants of different cell fates can be co-expressed (e.g. Phox2b and Foxa2). This suggests a model of cell fate program dominance in which the subordinate cell fate program only becomes activated after the repression of the determinants of the dominant cell fate.

CONCLUSIONS AND FUTURE PERSPECTIVES

The general aim of the work presented in this thesis was to further our understanding of the mechanisms that regulate the establishment of cellular diversity during CNS development. To this end, we have focused our studies in the ventral region of the neural tube, allowing us to study the mechanisms of spatial and temporal cell fate patterning.

By studying the generation of FP cells and V3 interneurons we have shown that Nkx2 proteins, classically viewed as operating downstream of Shh signaling, establish a cell-intrinsic amplification feedback loop that strengthens cellular responses to Shh signaling. In addition, the specification of FP and V3 progenitors relies on changes in the neurogenic potential of progenitors over time, rather than on a gradient of Shh signaling. This process reveals a novel mechanism to diversify Shh responses independent of the extrinsic concentration of Shh. Our studies on the temporal specification of vMNs and 5HTNs in the ventral hindbrain have revealed an important function of Shh signaling in temporal cell fate specification in addition to its role in spatial patterning. Shh signaling specifies the primary vMN fate by inducing Phox2b at early developmental stages and, at later stages, triggers the expression of Tgfβ2 which executes the vMN-to-5HTN fate switch. Similarly to spatial patterning, cross-repressive interactions are important in this process. However, while in spatial patterning distinct cell fate determinants are expressed in mutually exclusive domains, in temporal patterning these determinants can be co-expressed but, the establishment of a hierarchical relationship between cell fate determinants only allows one cell fate program to be active at a time. Furthermore, our studies have demonstrated that transcription factors involved in different aspects of spatial cell fate specification are also integrated with the mechanisms that regulate temporal patterning.

During development, the competence of neural progenitors to respond to inductive signals changes over time. For example, the ability of Shh to induce FP fate is restricted to a developmental time window during early stages of development (Le Douarin and Halpern, 2000; Ribes et al., 2010; Strahle et al., 2004). We found that a non-neuronal-to-neuronal switch in progenitor potential defines an initial window of competence for the induction of FP fate. However, how the switch in neurogenic potential is triggered remains unresolved. Our data indicate that the specification of FP and V3 fates by Nkx2 proteins is gated by the acquisition of neurogenic potential by progenitors. In the future, it would be interesting to investigate how proneural programs affect the specification of cell fates by Nkx2 proteins.

Our studies have also revealed a new role for Nkx2 proteins in providing non-graded regulation of Shh signaling, which is important for the specification of cell fates (FP and V3 neurons) and for the regulation of temporal switch mechanisms (vMN-to-5HTN switch). This process involves, at least in part, the regulation of *Gli3* expression and therefore the balance of GliA and GliR within the cell. A genome-wide

characterization of the target genes of Nkx2 proteins, for instance by chip-sequencing, should provide a better understanding of the pathways regulated by Nkx2 proteins that are important to modulate the levels of Gli activity in neural progenitors.

The identification of the Tgfβ pathway as the signal that executes the vMN-to-5HTN switch represents an important step in the understanding of the mechanisms that regulate temporal cell fate specification. Although Shh signaling plays the lead role in the temporal activation of the switch signal, the molecular details underlying this process remain sketchy. The identification of conserved regulatory sequences controling the spatial and temporal expression of Tgfβ2 in the ventral hindbrain would be instrumental in dissecting the molecular mechanisms that regulate the activation of the switch process. Furthermore, analysis of the transcriptional changes occurring in the Nkx2.2^{+} lineage over the period of the switch would provide a greater understanding of its regulatory mechanisms.

5HTNs innervate virtually the entire CNS and regulate numerous physiological and behavioral processes, including mood, appetite, locomotion and cognition. Thus, abnormal serotonergic function is implicated in several neurological and psychiatric diseases including autism, depression and movement disorders. With the advent of technologies that allow the generation of patient-specific induced pluripotent stem (iPS) cells, the identification of the signals that control the generation of 5HTNs can be applied to direct patient-specific iPS cells to the serotonergic lineage and in this way, provide an important experimental platform to study the aetiology of diseases associated with the serotonergic system.

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