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From Signaling to Form: The Coordination of Neural Tube Patterning

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Abstract

The development of the vertebrate spinal cord involves the formation of the neural tube and the generation of multiple distinct cell types. The process starts during gastrulation, combining axial elongation with specification of neural cells and the formation of the neuroepithelium. Tissue movements produce the neural tube which is then exposed to signals that provide patterning information to neural progenitors. The intracellular response to these signals, via a gene regulatory network, governs the spatial and temporal differentiation of progenitors into specific cell types, facilitating the assembly of functional neuronal circuits. The interplay between the gene regulatory network, cell movement, and tissue mechanics generates the conserved neural tube pattern observed across species. In this review we offer an overview of the molecular and cellular processes governing the formation and patterning of the neural tube, highlighting how the remarkable complexity and precision of vertebrate nervous system arises. We argue that a multidisciplinary & multiscale understanding of the neural tube development, paired with the study of species-specific strategies will be crucial to tackle the open questions.

Introduction

The formation of the vertebrate nervous system commences during gastrulation with the emergence of the neural tube (NT). In amniotes, the primordium of the NT appears as a thickened epithelium over the embryo's midline, the central region of which invaginates and the lateral edges rise and fuse to establish the dorsal midline of the NT (for a comprehensive introduction, refer to (Darnell and Gilbert, 2017; Gilbert and Barresi, 2017). As development proceeds, the spinal cord forms with the gradual addition of neural progenitors to the posterior end of the NT from uncommitted cells in the caudal region of the elongating embryo. The outcome is a bilaterally symmetrical pseudostratified epithelial tube, the basal surfaces of which form the lateral edges of the NT and the apical surfaces are oriented towards the central lumen that later becomes the central canal and ventricles of the nervous system. During the early phase of NT development, neural progenitors proliferate, and their cell bodies, containing their nuclei, undergo a stereotypic interkinetic nuclear movement (IKNM) coordinated with cell cycle progression (Lee and Norden, 2013). This results in a substantial increase in the number of neural progenitors and contributes to the remarkable enlargement of the NT (Kicheva et al., 2014). As neural progenitors differentiate into post-mitotic neurons, they detach from the apical surface of the neuroepithelium and migrate laterally, taking up residence basal to the progenitors.

Neural progenitors acquire distinct transcriptional identities that determine the specific cell type(s) that they will generate. These transcriptional programs depend on the location of the progenitor within the NT (Briscoe and Small, 2015; Dessaud et al., 2007; Jessell, 2000). For instance, in the ventral half of the developing spinal cord, the neuroepithelium is divided into six discrete domains arrayed along the dorsoventral (DV) axis. Each domain expresses a unique combination of homeodomain and bHLH transcription factors (Briscoe et al., 2000; Ericson et al., 1997). This transcriptional code governs the differentiation of progenitors into specific cell types, such as motor neurons and interneurons (reviewed in (Alaynick et al., 2011; Sagner and Briscoe, 2019)). Similar transcriptional codes are found in other regions of the NT and control the spatial pattern of neurogenesis (for reviews, see (Guillemot, 2007; Lai et al., 2016; Pearson and Placzek, 2013; Scholpp and Lumsden, 2010)). This principle, in which spatially restricted expression of transcriptional factors in neural progenitors leads to the spatially

segregated generation of distinct neuronal subtypes, represents the first step in the assembly of functional neuronal circuits. It facilitates the formation of correct synaptic connections between neighbouring cell types and ensures that newly generated neurons are positioned in locations where they are exposed to appropriate axon guidance signals. Thus, the subsequent function of the vertebrate nervous system is contingent upon these patterns of transcription factor gene expression in neural progenitors.

Here we will summarise current molecular and cellular understanding of how the posterior region of the nervous system, comprising the spinal cord, is formed and patterned. We will highlight how molecular and cellular processes spanning gene regulation, cell motility and tissue mechanics coordinate to generate the NT. The formation of the neural tube showcases how diverse strategies and routes are utilised throughout development to produce a highly conserved pattern across species.

1. Patterning the head-to-tail axis

The formation of the main body axis distinguishes head (rostral) from the future tail (caudal) early in development, concomitant with the formation of the primitive streak (Arnold and Robertson, 2009; Metzis et al., 2018). This process is critical for spinal cord formation. Despite shared similarities in gene expression and function, the spinal cord exhibits a closer lineage history to the mesodermal derived somites of the trunk than to neurons and glia in the brain (Brown and Storey, 2000; Davis and Kirschner, 2000; Tzouanacou et al., 2009). This provided evidence of a population of multipotent progenitors that generate spinal cord, somites and notochord during the process of axis elongation (Cambray and Wilson, 2007; Rito et al., 2023; Tzouanacou et al., 2009). These progenitors reside in the caudal lateral epiblast (CLE) at the posterior pole of vertebrate embryos and are termed neuromesodermal progenitors (NMPs) (**Figure 1A**) (Catala et al., 1996; Guillot et al., 2021; Wymeersch et al., 2016). NMPs form in a region of the embryo that expresses Wnt and FGF ligands and are characterised by the expression of the transcription factors *Cdx1,2,4*, *Sox2* and *Tbxt* (Henrique et al., 2015; Wymeersch et al., 2021). Whilst undergoing differentiation, NMPs must also self-renew to maintain a progenitor pool for complete axial elongation and understanding the specification and behaviour of NMPs has been the focus of studies in recent years (Wymeersch et al., 2021)

2.1 Signals specifying NMPs

2.1.1 Wnt and Fibroblast Growth Factor (FGF)

Wnt and FGF ligands are highly expressed during axis elongation and regulate the expression of Cdx1,2,4, *Tbxt* and *Sox2* in NMPs *in vivo* (Boulet and Capecchi, 2012; Goto et al., 2017; Martin and Kimelman, 2012; Rivera-Pérez and Magnuson, 2005; Tsakiridis et al., 2014; Yoshikawa et al., 1997) and *in vitro* (Beccari et al., 2018; Frith et al., 2018; Gouti et al., 2014; Lippmann et al., 2015; Moris et al., 2020; Olmsted and Paluh, 2021) (**Figure 1A**). Mutations in the Wnt pathway result in defects in axial elongation (Arnold et al., 2000; Cunningham et al., 2015; Erter et al., 2001; Garriock et al., 2015; Martin and Kimelman, 2012; Yamaguchi et al., 1999a). Initially, high levels of Wnt signalling induces *Tbxt* expression and marks the caudal region of the embryo (Liu et al., 1999; Metzis et al., 2018; Takada et al., 1994; Tsakiridis et al., 2014). Wnt expression is maintained caudally by a Tbxt-Wnt feedback loop, as shown by direct genomic interactions between Tbxt and Wnt effectors, which activate each others expression to sustain NMP self renewal (Amin et al., 2016; Arnold et al., 2000; Koch et al., 2017; Martin

and Kimelman, 2012; Yamaguchi et al., 1999b). Wnt signalling specifies paraxial mesoderm (Dunty et al., 2007; Koch et al., 2017; Martin and Kimelman, 2012), by upregulating T-box genes (Chalamalasetty et al., 2014; Chapman and Papaioannou, 1998; Yabe and Takada, 2012) and inhibiting the pro-neural gene *Sox2* (Takemoto et al., 2011).

FGF ligands are also expressed in caudal regions of the embryo (**Figure 1A**) and FGF signalling is required for *Tbxt*/*Sox2* expression (Boulet and Capecchi, 2012; Takemoto et al., 2005) and axial elongation (Deng et al., 1994; Olivera-Martinez et al., 2012). FGF signalling begins to attenuate as NMPs leave the progenitor zone and transition towards a neural identity (Corral et al., 2002; Mathis et al., 2001). As FGF signalling declines in these cells, Erk1/2 phosphorylation falls, triggering a remodelling of the chromatin landscape and removing repressive H3K27me3 marks resulting in expression of *Pax6* (Semprich et al., 2022). However, the downregulation of FGF signalling is not sufficient for the conversion of preneural cells to neural progenitors. Exposure to Retinoic Acid (RA) is also required (Corral et al., 2002; Patel et al., 2013; Sasai et al., 2014). Continued FGF signalling, by contrast, inhibits *Pax6* expression consistent with the antagonistic actions of RA and FGF in neural specification (Bertrand et al., 2000).

2.1.2 Retinoic Acid (RA)

As the spinal cord forms, there are significant changes in the tissue morphology and the surrounding signalling environment. Somites start expressing *Raldh2,* which synthesises RA. RA signalling contributes to the generation of neural progenitors during spinal cord formation (**Figure 1A**) (Cambray and Wilson, 2007; Corral and Storey, 2004; Gouti et al., 2017a; Henrique et al., 2015; Martin and Steventon, 2022; Wymeersch et al., 2021). *Raldh2* null mice generate *Sox1/2* positive pre-neural cells, however transition to *Pax6+* and *Olig2+* neural progenitors (NPCs) is RA dependent (Grandel et al., 2002; Molotkova et al., 2005). RA signalling negatively regulates FGF signalling, facilitating the progression of pre-neural cells to neural progenitors (Corral et al., 2003; Sirbu and Duester, 2006). Whilst RA is critical for specifying cells toward a spinal cord fate, excess RA signalling depletes the pool of NMPs and represses mesodermal differentiation (Gouti et al., 2017a). The RA degrading enzyme Cyp26a1 is expressed in the tailbud, repressing neural differentiation to maintain the balance of self-renewal and differentiation of NMPs under the control of Cdx2 (Gouti et al., 2017b; Rhinn and Dollé, 2012; Sakai et al., 2001; Savory et al., 2009; Young et al., 2009).

2.2 Gene expression during trunk formation

2.2.1 Homeobox genes: specifying axial identity in the spinal cord

Cells acquire distinct rostro-caudal identities before becoming neural progenitor cells (NPCs). This is driven by *Cdx* and *Hox* genes family members, which are also integral to driving the posterior growth of NMPs (**Figure 1A**) (Bel-Vialar et al., 2002; Metzis et al., 2018; Skromne et al., 2007). Wnt signalling induces expression of Cdx family transcription factors. Loss of *Cdx1/2/4* result in defects in axial elongation and reduced expression levels of Wnt and FGF ligands in the tailbud (Akker et al., 2002; Amin et al., 2016; Rooijen et al., 2012; Savory et al., 2009; Ven et al., 2011; Young et al., 2009). Cdx family members are required for *Hox* gene expression in NMPs and the resulting spinal cord (Bel-Vialar et al., 2002; Metzis et al., 2018; Skromne et al., 2007). Cdx2 removes repressive H3K27me3 marks (Mazzoni et al., 2013) and remodels 3D chromatin architecture (Rekaik et al., 2023) to allow *Hox* gene activation. Ectopic *Hox* gene expression rescues elongation defects in Cdx null mice (Young et al., 2009).

Hox genes impart axial identity (Denans et al., 2015; Hubert and Wellik, 2023; Mallo et al., 2010; Wacker et al., 2004). *Hox4-11* genes are expressed in the CLE and spinal cord (**Figure 1A**). Paralogs 4-8 are expressed in cervical/brachial NPCs, *Hox9* in thoracic regions and *Hox10-13* in lumbosacral regions (Philippidou and Dasen, 2013; Sagner and Briscoe, 2019). Pairs of *Hox* genes along the spinal cord exhibit cross-repressive interactions that prevent the generation of cells with mixed axial identities and HoxC members determine the specific subtype identity of spinal cord motor neurons (Dasen et al., 2005, 2003) Thus, *Cdx* and *Hox* gene expression patterns serve as a molecular map of axial identity in the spinal cord. However, how cells interpret the molecular map established by Hox and Cdx gene expression, is still unclear. Moreover, the cell type specific expression patterns and functions of Hox genes remains to be elucidated in cell types other than motor neurons.

2.2.2 Tbxt and Sox2 mediate mesodermal vs neural fate decisions

The Tbox factor *TbxT* and the SoxB genes *Sox2*/3 are expressed in NMPs in many species (**Figure 1B**) (Gouti et al., 2014; Henrique et al., 2015; Javali et al., 2017; Martin and Kimelman, 2012; Metzis et al., 2018; Wymeersch et al., 2016). The *TbxT* gene encodes the protein Brachyury, which was first identified by the birth of short-tailed mice (Chesley, 1935; Gluecksohn-Schoenheimer, 1938; Herrmann et al., 1990). During gastrulation, *TbxT* is expressed in the posterior epiblast and becomes restricted to the primitive streak and the axial & paraxial mesoderm. Subsequently, *Tbxt* expression is observed in the tailbud (Kispert et al., 1995; Rivera-Pérez and Magnuson, 2005; Wilkinson et al., 1990). This pattern is conserved across bilaterians (Knezevic et al., 1997; Schulte-Merker et al., 1994; Smith et al., 1991) highlighting the role of TbxT in the formation of the post cranial axis. TbxT remodels chromatin accessibility, opening and binding genomic sites of the Wnt signalling pathway, and promoting mesodermal differentiation (Amin et al., 2016; Gentsch et al., 2013; Gogolou et al., 2022). A higher ratio of *TbxT* expression over *Sox2* inititates greater cellular motility, the onset of EMT, and expression of *Tbox* genes. These all ensure NMP contribution to paraxial mesoderm specification at the expense of neural fate (Chapman and Papaioannou, 1998; Gentsch et al., 2013; Kinney et al., 2020; Romanos et al., 2021; Wilson et al., 1995)

For the correct proportions of spinal cord and paraxial trunk mesoderm, NMPs must self-renew and differentiate in the correct proportions. This is dependent on the balance of signalling pathways and gene expression. *Tbxt* and *Sox2* are both expressed at low levels in NMPs (Wymeersch et al., 2016) and individual *Tbxt+/Sox2+* cells can contribute to both neural and mesodermal tissues upon grafting (Gouti et al., 2014; Tsakiridis et al., 2014). Recent work has developed a model by which there are distinct routes for NMP cell fate decisions towards neural or mesoderm in response to changing signalling regimes (Meritxell Sáez et al., 2022). Rather than NMPs exhibiting a stable low level of *Tbxt* and *Sox2* expression, their expression is heterogenous (Toh et al., 2022). This is postulated to occur due to differences in signalling environment (Edri et al., 2019; Wymeersch et al., 2021) influencing *Sox2/Tbxt* expression through mutual co-repression at regulatory elements (Koch et al., 2017). However, recent data suggests NMP fate specification occurs independently of direct mutual antagonism between *Tbxt* and *Sox2* (Guibentif et al., 2021). Moreover, single-cell sequencing has revealed a role for RA in concert with Wnt/FGF signalling for NMP specification (Gouti et al., 2017a) indicating that further work is required to unravel the precise mechanisms of NMP cell fate decisions.

2.3 Transition from NMP to Neural Progenitor goes through a pre-neural intermediate

NMPs undergo a series of transitions that result in spinal cord formation (**Figure 1B**) (Koch et al., 2017; Romanos et al., 2021; Toh et al., 2022). Sox2 can act as a pioneer factor that remodels the chromatin landscape facilitating the expression of the gene regulatory network characteristic of NPCs (Iwafuchi-Doi and Zaret, 2016; Michael et al., 2020). The regulation of *Sox2* expression in forming neural progenitors occurs through Wnt/FGF dependent N1 Cis-regulatory element (CRE) (Takemoto et al., 2005; Uchikawa et al., 2003). Sox2 activity cross-regulates Wnt signalling by modulating the binding of the Wnt pathway transcription effector proteins TCF/LEF (Blassberg et al., 2022; Mukherjee et al., 2022) by directly binding to them (Zorn et al., 1999). This feedback loop ensures that Sox2 represses excess mesodermal differentiation by regulating Wnt signalling (Yoshida et al., 2014) and preventing delamination from the CLE (Kinney et al., 2020). In addition to activating *Sox2*, the spinal cord specific N1 CRE can be bound by Tbox genes which repress *Sox2* expression in cells committed to the mesoderm lineage (Koch et al., 2017; Takemoto et al., 2011). A delicate balance of signals and gene regulatory mechanisms control the proportions of self-renewal and differentiation in the CLE. However, once a commitment point is reached, *Sox2* initiates a transition from NMP to NPC through an intermediate pre-neural state (**Figure 1B**) downstream of a changing signalling environment and concurrent with the morphogenetic movements that will give rise to the NT.

The expression of *Nkx1*-2 in the caudal epiblast in a Sox2 and FGF-dependent manner is associated with the pre-neural state (**Figure 1B**) (Bae et al., 2004; Delfino-Machín et al., 2005; Gouti et al., 2017a; Rangini et al., 1989; Schubert et al., 1995; Simon and Lufkin, 2003; Spann et al., 1994). Lineage tracing experiments revealed the contribution of *Nkx1-2+* cells to the neural, mesodermal, notochord and neural crest derivatives, (Albors et al., 2018; Cooper et al., 2022; Corral et al., 2003; Sasai et al., 2014; Verrier et al., 2018). consistent with Nkx1-2 marking NMPs in addition to pro-neural cells. However, ectopic expression of *Nkx1*-2 in the pre-neural domain represses the induction of the neural progenitor markers *Irx3* and *Pax6* (Sasai et al., 2014) and maintains *Tbxt* expression in *vitro* (Tamashiro et al., 2012). *Nkx1*-2+ pre-neural cells form spinal cord progenitors downstream of RA signalling (Corral et al., 2002; Sasai et al., 2014) in concert with epigenetic remodelling (Patel et al., 2013), yet they retain the potential to give rise to mesoderm (Albors et al., 2018). These observations suggest that the pre-neural state is a checkpoint prior to neural differentiation and contributes to the balance of NMP cell fate dynamics (**Figure 1B**). However, the precise mechanism of how signals and differentiation trajectories of NMPs are integrated to drive cell fate specification is unclear. In particular, the requirement for NMPs to transition through a pre-neural state and how associated gene and epigenetic changes impact cell fate potential remains to be resolved.

2.4 Axial elongation drives formation of the body plan

Signalling and genetic mechanisms must be coordinated with changes in cell proliferation and movements for axial elongation to occur (Bocanegra-Moreno et al., 2023; Leber and Sanes, 1995). Several experiments revealed a pool of proliferative stem cells in the tailbud that drive axial elongation (Cambray and Wilson, 2002; Mathis and Nicolas, 2000; Nicolas et al., 1996). However, proliferation alone is not sufficient to elongate the body axis. Cell cycle disruption by genetic (Riley et al., 2010) and pharmacological means (Bénazéraf et al., 2010) is not deleterious for the formation of the spinal cord. The NT elongates through a process of convergence extension (CE) whereby cells from lateral regions of the body converge medially, fuelling elongation (**Figure 1C**) (Shih and Keller, 1992; Steventon et al.,

2016; Xiong et al., 2020). FGF signalling is required for CE (Bénazéraf et al., 2010; Ciruna and Rossant, 2001; Guillot et al., 2021; Steventon et al., 2016). Moreover, mutations in the non-canonical Wnt/planar cell polarity (PCP) pathway, such as the ligands *Wnt5a* and *Wnt11* (Andre et al., 2015; Heisenberg et al., 2000; Yamaguchi et al., 1999a) and intracellular effectors *Ptk7* and *Vangl2* (López-Escobar et al., 2018; Williams et al., 2014) also affect CE, resulting in a shorter rostro-caudal axis independently of cell signalling (Andre et al., 2015). These data highlight the importance of multiple mechanisms both at the tissue and the molecular level to generate the spinal cord pattern. Additional studies have also proposed rostral to caudal gradients of metabolism (Oginuma et al., 2017); extraembryonic tension (Kunz et al., 2023); and cell jamming (Mongera et al., 2018) upstream of cellular motility. Future studies will shed light on how extrinsic chemical and mechanical signals and intrinsic gene regulation properly pattern axial elongation and spinal cord formation.

2.5 Patterning mediolaterally: generating multiple cell types from the pre-neural tube

As NMPs differentiate towards spinal cord, they initially form a flat epithelial sheet called the neural plate. This gives rise to neural crest cells as well as NPCs (**Figure 1D**) (Albors et al., 2018; Brown and Storey, 2000; Frith et al., 2018; Lukoseviciute et al., 2021). Mesodermal cells underneath the neural plate secrete Bone Morphogenetic Protein (BMP) inhibitors (**Figure 1D**). These play an important role in the specification of NPC identity by protecting prospective NPCs from BMP ligands that are secreted laterally (Marchant et al., 1998; Wawersik et al., 2005). The opposing gradients of BMP ligands and inhibitors result in a low concentration of BMP activity at the border between pre-neural and non-neural epithelium where neural crest is induced (**Figure 1D**). This region of the embryo has a characteristic gene expression profile that includes *Pax7* and *Sox9* (Basch et al., 2006; Corral et al., 2003; García-Castro et al., 2002). The consequence is a mediolateral (ML) pattern of differentiation with distinct gene expression patterns. *Sox2* and *Pax7* are expressed in pre-neural cells (Martínez-Morales et al., 2011) and each gene gradually becomes segregated to neural or neural crest cells respectively (Roellig et al., 2017).

2. Neural tube closure is driven by cell movements and mechanical forces

Once the flat neural plate has acquired a 2D pattern across its mediolateral axis, the process of neurulation starts (**Figure 1C**). This serves as a good example of how morphogenesis and patterning require fine-tuned interplay of mechanisms acting across different scales for correct pattern formation. Mammals, birds and amphibians utilise two different mechanisms of NT formation along the rostro-caudal axis (Colas and Schoenwolf, 2001; Douarin et al., 1998). Along most of the amniote spinal cord, the flat neural plate forms folds that elevate the dorsal regions which bend and converge medially, subsequently fusing and forming a hollow tube (**Figure 1C**). By contrast, in the tailbud, the NT is shaped by a process known as “secondary neurulation”. From the 25 somite in chick, 30 in human and 31 in mouse embryos proceeding caudally (Catala, 2021; Copp et al., 1982; Müller and O’Rahilly, 1987; Schoenwolf, 1984), cells converge and condense into a medullary cord (**Figure 1Di**), that subsequently epithelializes (**Figure 1Dii**) and cavitates (**Figure 1Diii**) to create the lumen of the future NT (**Figure 1D**) (Douarin et al., 1998). Teleosts, on the other hand, have evolved a unique strategy for neurulation, which progresses through the formation of a solid rod (neural keel) that later opens a lumen (Araya et al., 2016). This has been compared to secondary neurulation but occurring throughout the rostro-

caudal axis. However, there is evidence to suggest that the folding of the neural keel folds is similar to the process of primary neurulation, prompting the need for further investigation to elucidate this process (Lowery and Sive, 2004; Werner et al., 2021).

In most of the spinal cord, primary neurulation begins with the neural plate bending at the middle hinge point (MHP), at the midline of the neural plate (**Figure 1Ci, ii**). The location of this hinge point is instructed by the secretion of Sonic hedgehog (Shh) and additional factors from the notochord (Patten and Placzek, 2002; Ybot-Gonzalez et al., 2002). Once the position of the MHP is established, neural plate bending is driven by intrinsic forces, that appear to be produced by cell shape changes. Bending is aided by apical actomyosin turnover under the control of the PCP pathway (Baldwin et al., 2022; Escuin et al., 2015; Nishimura et al., 2012; Nishimura and Takeichi, 2008; Ybot-Gonzalez and Copp, 1999) and acquisition of a wedge-like cell shape close to the midline (**Figure 1Cii**)(Schoenwolf, 1991, 1985; Smith and Schoenwolf, 1989). Changes in cell shape synchronize with mitosis and nuclear position (Ampartzidis et al., 2023; Sausedo et al., 1997; Smith and Schoenwolf, 1988), coupling cell cycle phase with morphogenesis, which has been proposed in zebrafish (Ciruna et al., 2006). Subsequently, the elevated neural folds bend at the dorsolateral hinge points (DLHP) (**Figure 1Cii**) instructed by Noggin (Ybot-Gonzalez et al., 2007) and converge to the dorsal midline, driven by extrinsic forces exerted by the lateral surface epithelium and its extracellular matrix (ECM) (Schoenwolf, 1991; Smith and Schoenwolf, 1991). However, intrinsic mechanisms, such as a dorsolateral increase in cell density (McShane et al., 2015) in concert with an apicobasal force exerted by apoptotic cells within the NT may facilitate bending at the DLHP (Roellig et al., 2022). It is important to note that neurulation differs along the rostrocaudal axis within the same embryos, moving from predominantly MHP-mediated anteriorly, to mostly driven by DLHP posteriorly (Shum and Copp, 1996; Ybot-Gonzalez and Copp, 1999).

During NT closure, an F-actin cable runs along the neural folds, mechanically coupling the entire folding neural plate (Galea et al., 2017; Nishimura et al., 2012). Surface ectoderm cell protrusions - regulated by Rho-GTPases- aid dorsal fusion during neural fold apposition (Bancroft and Bellairs, 1975; Hashimoto et al., 2015; Mak, 1978; Massarwa and Niswander, 2012; Ogura et al., 2011; Pyrgaki et al., 2010; Rolo et al., 2016; Schoenwolf, 1979; Waterman, 1976). Finally, NT fusion is facilitated by the focal anchorage of the folds to an integrin-β1 rich area (Molè et al., 2023, 2020).

Many TFs and signalling pathways have been shown to regulate cellular and mechanical events of NT closure. Zic2 controls the formation of cell protrusions necessary for NT closure (Rolo et al., 2016) and Grhl- family TFs are required for several adhesion and EMT processes downstream of Wnt/PCP (Kimura-Yoshida et al., 2015; Pyrgaki et al., 2011; Rifat et al., 2010; Senga et al., 2012; Werth et al., 2010). Wnt/PCP regulation of *Pax3*, *Cdx2* and *Zic2* as well as their cross-regulation play a role in neurulation (Ferras et al., 2012; Sanchez-Ferras et al., 2014; Savory et al., 2009; Zhao et al., 2013), although the precise mechanism is still unclear (Sanchez-Ferras et al., 2014).

While the influence of signalling on cellular events has been investigated, the influence of shape and mechanics on signalling has received less attention. Recent studies have suggested a regulation of the PCP pathway component Vangl2 by MyosinII (Matsuda and Sokol, 2021; Newman-Smith et al., 2015; Ossipova et al., 2015), whilst the Hippo pathway is also mechanically regulated during NT closure (Marshall et al., 2023). In addition, different aspects of cell metabolism as well as ECM composition

are crucial for correct neurulation (Castro et al., 2012, 2010; Copp and Greene, 2010; Dunlevy et al., 2007, 2006; Leung et al., 2017; Ybot-Gonzalez et al., 2005). Thus, NT closure offers a tractable system to study the interaction of tissue, cellular and molecular processes involved in the generation of tissue shape.

Once NT closure is complete, cell movements and rearrangements within the epithelium continue up to E9.5 in mouse and HH15 in chicken, before giving rise to the rostrocaudal, mediolateral and dorsoventral patterning that constitute the mature spinal cord (Bocanegra-Moreno et al., 2023; Leber and Sanes, 1995). Cellular movements first cease along the rostrocaudal axis (Leber and Sanes, 1995) then along the DV axis, as NPCs are specified into distinct domains (Erskine et al., 1998; Kicheva et al., 2014). The dispersion of cells is mostly isotropic across different NPC identities, however pMN progenitors persist with cellular rearrangements for longer (Bocanegra-Moreno et al., 2023). Movement along the ML axis is the last to stop, consistent with the radial movement of postmitotic neurons outside of the progenitor zone. From this moment onwards, the cellular rearrangements in the spinal cord are mostly due to dispersal and passive migration (Leber and Sanes, 1995).

In addition, cell body rearrangements such as IKNM maintain the epithelium in a fluid like state until E9.5 in mouse (Bocanegra-Moreno et al., 2023; Kicheva et al., 2014). After these rearrangements become more restricted as proliferation declines, the mechanical property of the epithelium transitions to a more glass-like behaviour (Bocanegra-Moreno et al., 2023).

3. Dorsoventral patterning

3.1 Opposing gradients

NPCs are exposed to antiparallel signals secreted from opposite poles of the NT that specify a dorsal to ventral spatial pattern (Alaynick et al., 2011). These signals act as morphogens – intercellular signalling molecules that exert their effects in a concentration dependent manner across developing tissues – to convey spatial information that organise and generate the diverse cellular subtypes of the NT (Kicheva and Briscoe, 2023). Sonic hedgehog (Shh) is produced ventrally by the notochord and later the floor plate (Echelard et al., 1993; Krauss et al., 1993; Yamada et al., 1993), while ligands of the BMP and Wnt families are secreted dorsally by the surface ectoderm and roof plate (Liem et al., 1997; Muroyama et al., 2002; Wine-Lee et al., 2004).

Numerous studies, taking advantage of gene deletions and *ex vivo* explants, have identified the requirement for these signals to generate the complement of NPCs found in the spinal cord (Andrews et al., 2017; Chiang et al., 1996; Lee et al., 2000; Liem et al., 1997; Litingtung and Chiang, 2000; Marti et al., 1995; Muroyama et al., 2002; Roelink et al., 1995; Wijgerde et al., 2002). In the NT, as in other growing tissues (Morishita and Iwasa, 2009), the graded signals are integrated along a single axis to provide precise spatial information and minimize patterning errors (Zagorski et al., 2017). However, *in silico* modelling has questioned whether two distinct gradients are necessary (Vetter and Iber, 2022), at least in some idealised cases, highlighting the need for further investigation.

The combination of antiparallel dorsal and ventral signalling gradients results in the generation of 11 dorsoventral domains of NPCs (**Figure 2A**). Each of these domains give rise to functionally distinct

neuronal subtypes which relies on a combination of signalling concentration (Briscoe and Ericson, 2001, 1999; Ericson et al., 1997, 1996; Stamataki et al., 2005; Yamada et al., 1993) and time duration (Dessaud et al., 2007, 2010). TFs, such as Nkx2-2, identifying progenitors closer to the source of Shh, require a higher concentration and duration of Shh exposure to be expressed than TFs such as Olig2, which are expressed in progenitors further from the ventral midline (**Figure 2B, C**). Mechanistically, *ex vivo* studies have shown that neural cells translate ligand concentration into proportional durations of Gli activity. This is achieved via a temporal adaptation system, that relies on the progressive desensitization of cells to Shh, mediated by Shh signalling inhibitor Ptch1 (Cohen et al., 2015; Dessaud et al., 2007) and aided by the movement of cells away from the source during NT growth (Kicheva et al., 2014). Thus, cells exposed to a lower concentration of Shh have a steeper and faster decline of downstream activity. This scenario poses the question of how cells convert time and dose dependent information from morphogens into spatially discrete domains of gene expression.

3.2 Intracellular morphogen signalling

3.2.1 Ventral neural tube

The molecular mechanism that converts the patterning signal inputs into distinct progenitor identities is best understood for Shh in the ventral NT. The intracellular response to Shh is mediated by Gli family TFs (Hui and Angers, 2011; Jiang and Hui, 2008), which have dual functions as transcriptional repressors (GliR) and activators (GliA) (Hui and Angers, 2011). A gradient of Gli activity is established by Shh signalling, resulting in expression of ventral and repression of dorsal TFs. The Gli gradient is decoded into gene expression through Gli-binding sites present in cis regulatory elements (CREs) of genes expressed in the ventral NT (**Figure 2D, E**) (Oosterveen et al., 2012; Peterson et al., 2012; Vokes et al., 2007).

A combination of molecular, genetic and modelling studies have uncovered a mechanism for decoding graded Shh signaling based on cross regulatory interactions between TFs expressed in neural progenitors (**Figure 2D**) (Briscoe et al., 2000; Novitch et al., 2001). Deletion of individual TFs lead to switches in NPC fate and expansion of TFs characteristic of the adjacent domain (Balaskas et al., 2012; Briscoe et al., 1999, 2000; Briscoe and Ericson, 2001; Ericson et al., 1997; Novitch et al., 2001; Vallstedt et al., 2001; Zhou et al., 2001a). In addition, forcing the expression of NPC TFs in chick embryos induced the corresponding NPC identity throughout the NT, suggesting that TFs also repress the gene expression programs of non-adjacent progenitor domains to impose their specific identity (Kutejova et al., 2016; Nishi et al., 2015). This has led to the idea that molecular distinct progenitor domains are established by a de-repression mechanism involving, in the ventral NT, 4 TFs: Olig2, Nkx2-2, Irx3, Pax6 downstream of Shh signalling that act by repressing alternative fates to allow the execution of a specific cell fate programme (**Figure 2B**). In this view the dynamics of the transcriptional network convert the graded signalling inputs into the discrete NPC identities and involves broadly acting activating inputs counteracted by spatially regulated transcriptional repressors (Cohen et al., 2014) (**Figure 2D, E**).

3.2.2 Dorsal neural tube

In the dorsal spinal cord, 6 distinct domains of NPC, dorsal progenitor (dp1) 1-dp6, are established (Lai et al., 2016) (**Figure 2A**). Whilst BMP and Wnt have been shown to induce dorsal populations, the GRN of the dorsal NT is less well characterized, partly due to the redundancy of the ligands. Overexpression

studies have shown that BMP induces *Pax6, Msx1, Msx2* expression in the dorsal part of the NT (Timmer et al., 2002) through the canonical Bmpr1-Smad1/5 pathway (Dréau et al., 2011; Hazen et al., 2012, 2011). Simultaneously, BMP represses intermediate proteins such as Dbx1-2, Cash1 and Atoh1 TFs (Hazen et al., 2011; Timmer et al., 2002). The duration of BMP signalling plays a role in setting expression boundaries (Tozer et al., 2013). However, different dose-response experiments suggested that concentration and duration of the signal only alter the number of cells converted to a specific fate and does not entirely prevent the emergence of any cellular state (Andrews et al., 2017). This discrepancy can be explained by the presence of several distinct BMP ligands that, rather than acting as a single morphogen, exhibit specific activities in regulating different dorsal progenitor identities (Andrews et al., 2018, 2017; Dréau and Martí, 2013).

A model has been proposed in which the dorsal NP domains are first subdivided into two classes comprising 2-3 domains each (Andrews et al., 2018). Subsequently, varying temporal exposure to BMPs may distinguish these progenitor identities (Tozer et al., 2013). Downstream of BMP signalling, different bHLH TFs are expressed in a domain-specific fashion and exhibit cross-repressive interactions. For instance, cross regulation between Atoh1 and Neurog1 specifies the dp1-2 boundary, and they restrict Ascl1 to dp3-5 (Gowan et al., 2001). In addition, Ptf1a induces *Pax2* in dp4 while suppressing TFs such as Tlx1/3 in dp5, via Prdm13 (Chang et al., 2013). Wnt signalling also promotes the sustained expression of dorsal TFs such as *Pax3*, which in turn enhances its own expression (Moore et al., 2013).

3.3 Morphogen responsive Cis-Regulatory Elements

CREs are the regions of non-coding genome that contain binding motifs for signalling pathway effectors and TFs that regulate gene expression (Davidson, 2010). Work in the ventral neural tube has revealed that CREs integrate three types of inputs to select neural progenitor specific gene expression: broad activators that promote gene expression in all neural progenitors; GliA and GliR input that introduces a spatial polarisation along the dorsal-ventral axis; and cell type specific repressors, responsible for the acquisition and commitment to a domain specific gene expression program (**Figure 2E**). The broad activators appear to include Sox2, which binds at CREs (Peterson et al., 2012), facilitating expression of NPC identity genes and resulting in a chromatin landscape that is broadly accessible across all NPCs (Delás et al., 2023). Additional steps are then responsible for determining the specific NPC gene expression programme that is activated in a progenitor domain. First, different levels of Shh signalling along the DV axis results in different levels of GliA and GliR proteins. These bind to motifs within CREs of NPC identity genes conferring a DV order to expression patterns (Oosterveen et al., 2012; Peterson et al., 2012). The expression of NPC identity genes, which in turn act as transcriptional repressors, bind to motifs within the accessible CREs to regulate expression of alternative fate genes (Kutejova et al., 2016; Nishi et al., 2015). As a consequence of the differential activation and binding of these repressive TFs at CREs, the specific NPC gene expression programme is selected (**Figure 2F**).

The exception to the differential binding mechanism is the ventral most p3 domain, which displays a distinct chromatin landscape. This differential accessibility is established by the pioneer TF FoxA2 (Delás et al., 2023) (**Figure 2F**). As shown by lineage tracing (Delás et al., 2023; Dessaud et al., 2007; Erskine et al., 1998; Kicheva et al., 2014) the process of p3 fate acquisition also highlights the importance of prior gene expression (such as FoxA2) on cell fate specification. Despite these findings identifying how the chromatin landscape is initiated in NPCs during identity acquisition, it remains

unclear how CREs regulate gene expression of NPC identity genes throughout neurogenesis and into gliogenesis in the spinal cord.

3.4 Precision of patterning

The boundaries between the 11 NPC domains are accurately positioned, with only modest intermixing of cell identities. The detailed mechanisms that explain boundary precision are still not fully elucidated, and different organisms appear to use distinct strategies. Zebrafish NPCs in the ventral NT exhibit a noisy response to Shh and NPCs, with different domain identities initially intermingled (Xiong et al., 2013) (**Figure 2G**). Cell sorting, based on a differential cadherin-mediated adhesion code ensures boundary sharpness (Tsai et al., 2020). In pMN cells, Olig2 represses cadherin *pcdh19*, lowering the heterotypic adhesion between NPCs of different identities and facilitating sorting (Tsai et al., 2020) (**Figure 2G**). This strategy based on cell sorting might have been employed in teleosts due to the higher cellular dispersal compared to amniotes seen during neurulation.

There is limited evidence that differences in adhesion are required in amniotes. Some intermixing has been observed in physiological conditions (Kicheva et al, 2014), which becomes more prominent when individual components of the GRN are deleted (**Figure 2H**) (Balaskas et al., 2012; Ericson et al., 1997; Exelby et al., 2021; Novitch et al., 2001) . Gene deletion studies have confirmed that a bistable switch between two nodes can explain the choice between mutually exclusive fates, but addition of a third node to the network, improves the sharpness of the boundary (Exelby et al., 2021; Perez-Carrasco et al., 2018). Therefore, boundary precision in amniotes appears to rely on intrinsic dynamics of the GRN. Lack of boundary precision might affect axonal trajectories and spinal sensory-motor circuit organization (Balaskas et al., 2019) but the degree of precision required for this remains to be determined.

4. Growth and cellular rearrangements

After the NPC specification phase is concluded, the “growth phase” of patterning begins (Kicheva and Briscoe, 2015) (**Figure 3A**) where it is critical that pattern is maintained and adjusts appropriately to changes in size. This poses several questions: What are the factors involved in tissue growth and how do they influence or are influenced by pattern formation? How do patterns scale with increase in spinal cord size within a single animal or across different organisms? An intriguing idea would be that tissue size and pattern scaling is dictated by the number of NMPs incorporated into the spinal cord. In fast developing species such as zebrafish, the NMP pool is composed of a finite number of cells (Attardi et al., 2018; Steventon et al., 2016). However in mammals the NMP pool persists for longer and is replenished by self-renewal (Cambray and Wilson, 2007; Tzouanacou et al., 2009). Altering the proportion of neural versus mesodermal NMP progenitors, as is the case in *Tbx6* and *Wnt3a* null mice, while reducing the formation of paraxial mesoderm, does not alter the final size of the spinal cord, but instead results in the generation of ectopic NT-like structures (Chapman and Papaioannou, 1998; Takada et al., 1994; Yoshikawa et al., 1997). This supports the view that number of NMPs alone is not sufficient to determine NT size (Takada et al., 1994; Takemoto et al., 2011; Yoshikawa et al., 1997). Even if number of NMPs does not dictate final size, their continuous addition to the forming NT, as well as the convergent extension of the neural plate (Steventon et al., 2016) contribute to the anisotropy of tissue growth in the rostro-caudal direction. After NT closure, the mediolateral and dorsoventral axes also increase in size and a transient inhibition of cell proliferation around E8.5 in mouse affects

the spinal cord mediolateral size, but has no longer an effect on its rostro-caudal length (Bocanegra-Moreno et al., 2023).

Proliferation rates in the spinal cord are similar across DV domains (Kicheva et al., 2014), however it is still unclear how uniform proliferation is regulated by signalling gradients. Irrespective of the mechanism, morphogens have been suggested to influence the cell cycle (reviewed in (Kuzmicz-Kowalska and Kicheva, 2021). At later stages (E11 in mouse), the major increase in size happens along the DV axis, following an anisotropic pattern across the DV domains (Kicheva et al., 2014; Kuzmicz-Kowalska and Kicheva, 2021) (**Figure 3A**). A proportion of NPCs divide asymmetrically, differentiating into postmitotic neurons (see next section), causing selective progenitor loss. Different domains exhibit different neuronal differentiation rates, that alter the size of the respective progenitor domain without affecting morphogen signalling. However, the different temporal dynamics of differentiation are still unclear. Local factors including gene regulation and Notch signalling (Henrique et al., 1995; Sagner et al., 2018) are crucial, as is morphogen control of the balance between proliferation and differentiation (Dréau et al., 2014; Gupta et al., 2022; Saade et al., 2017, 2013). Thus, the interplay between these levels of regulation is likely to be critical for distinct rates of NPC differentiation.

A consequence of the domain-specific rates of neurogenesis is the unequal growth of the different domains (**Figure 3A**). This means that patterns do not scale with size of the NT over time (Kicheva et al., 2014; Kicheva and Briscoe, 2015). Scaling can also be considered from an interspecies perspective, as the NT of different species display different growth speeds and dynamics (Kicheva et al., 2014), rates of differentiation (Delile et al., 2019a; Jang et al., 2022; Rayon et al., 2020, 2021), and final size (Steventon and Arias, 2017; Uygur et al., 2016). Despite these differences, patterning is conserved across most vertebrates (Kicheva et al., 2014; Uygur et al., 2016) and is robust to manipulations, as demonstrated by studies in mice, chicken, zebrafish and zebrafinch (Collins et al., 2018; Kicheva et al., 2014; Uygur et al., 2016). Understanding how morphogen gradients allow the scaling of size and patterning will improve as parameters such as gradient amplitude, decay length and downstream signalling cascade are integrated into computational models. Nonetheless, different hypotheses have been proposed, among which the existence of an expansion-repression mechanism that regulates morphogen gradient amplitude and the requirement for two opposing gradients (Ben-Zvi and Barkai, 2010; Collins et al., 2018; Kicheva and Briscoe, 2015; Shilo and Barkai, 2017; Zagorski et al., 2017).

In conclusion, patterns are mostly established early in embryogenesis, when the tissue is smaller in size. Therefore, the translation of the morphogen signal into gene expression is more efficient and precise at smaller scales, since the morphogen concentration difference is the highest the closest to its source. In addition, the early establishment of patterning followed by a growth phase provides opportunity for compensation in case of patterning errors (Kicheva and Briscoe, 2015).

5. Initiation of neuronal differentiation

The production of neurons from each of the progenitor domains depends on a cascade of signalling and morphogenetic events, resulting in the asymmetric division of NPCs, with daughter progenitor cells remaining in the medial ventricular zone (VZ), while the differentiated daughters delaminate, migrating laterally into the outer layer of the NT (**Figure 3B**) (Alaynick et al., 2011; Jessell, 2000; Leber and Sanes, 1995; Lee and Pfaff, 2001).

5.1 Interkinetic nuclear movements at the onset of differentiation

One of the first events of neurogenesis is the movement of cells out of the VZ. The onset of differentiation coincides with a decrease in the amplitude of Shh signalling (Saade et al., 2013) and is a striking example of an interplay between cell movement and signalling during cell fate decisions. During the early phase of NT development, divisions are predominantly symmetric and result in the expansion of the progenitor pool (Saade et al., 2013). NPCs display IKNM during the cell cycle resulting in mitosis at the apical ventricular zone and DNA synthesis basally (**Figure 3B**) (Bocanegra-Moreno et al., 2023; Kasioulis and Storey, 2018; Sauer, 1935). NPCs in the VZ are maintained in an epithelial sheet by adherens junctions that link progenitors at their apicolateral surfaces (Dady et al., 2012; Hatta et al., 1987; Hatta and Takeichi, 1986) and β1 integrin that attach cells to the basal surface (Long et al., 2016). N-cadherin at apical junctions connects neighbouring NPCs (Miyamoto et al., 2015) and is required for NPCs to remain in the VZ (Das and Storey, 2014a; Rousso et al., 2012). At the onset of differentiation, *FoxP2/4* and *Neurog2* expression promotes disassembly of cadherin complexes, resulting in differentiating cells leaving the progenitor zone (Das and Storey, 2014a; Rousso et al., 2012). Live imaging studies indicate that delamination happens through apical abscission (**Figure 3C**). In this process, N-cadherins at the apical surface are disassembled and actomyosin cables begin to constrict, cutting off the region containing the primary cilium, which is left behind on the apical side of the neuroepithelium as the differentiating cell leaves the VZ. The loss of the apical cilium renders the delaminated cell unable to respond to Shh signalling, contributing to differentiation (Das et al., 2012; Das and Storey, 2014b; Toro-Tapia and Das, 2020).

5.2 Breaking the pattern: Notch mediated lateral inhibition driving neurogenesis

Neuronal differentiation is asynchronous extending over several days in mouse and more than a week in human. The decision of a progenitor to differentiate relies on local communication between progenitors, mediated via the Delta-Notch pathway that balances differentiation and maintenance of a progenitor pool. Retention of progenitor identity occurs via activation of the transmembrane Notch receptor, promoting expression of Hairy/Enhancer of split (*Hes*) family TFs in NPCs, and repression of pro-neural genes such as *Neurog2* and *Ascl1* (Bertrand et al., 2002; Kobayashi and Kageyama, 2014; Ohtsuka et al., 1999; Shimojo et al., 2011). Cells committing to differentiate express the Delta ligand and down regulate expression of Hes TFs (Akai et al., 2005; Appel and Eisen, 1998; Chitnis et al., 1995; Henrique et al., 1995). Delta-like ligands are upregulated by pro-neural bHLH TFs *Neurog*2, Ascl1 (Henke et al., 2009) reinforcing neuronal commitment and Hes downregulation. Hes TFs exhibit negative autoregulation, resulting in an oscillatory and asynchronous pattern of their expression (Biga et al., 2021; Imayoshi et al., 2013; Manning et al., 2019). This property allows individual NPCs to respond differently to the same stimulus at any given time and is presumed to ensure an appropriate balance of self-renewal and differentiation. However, it remains unclear how the appropriate rate of neuronal differentiation and delamination of progenitors from the VZ is co-ordinated. Zebrafish studies suggest that upon reaching a threshold of local cell density, NPCs undergo a change in cell shape and are extruded from the VZ (Hiscock et al., 2018). Computational modelling also support the importance of mechanical constraints for this process (Guerrero et al., 2019). In addition, zebrafish studies suggest that the heterotypic adhesion code among different NP domain cells might facilitate their delamination (Rousso et al., 2012; Tsai et al., 2020). Future studies will identify the link between the local scale of Notch-Delta regulation of differentiation to a tissue level, which is present across the DV

axis of the spinal cord. Micro-domains of Notch-delta activity have been identified (Biga et al., 2021) and identifying how these relate to domain specific timing of differentiation across the spinal cord will be critical to tying these observations together.

5.3 bHLH patterns

For the correct specification of distinct subtypes of neurons in the spinal cord, the DV cellular identity must be communicated during differentiation to define the identity of the newly generated neuron. bHLH pro-neural genes, such as *Neurog1-3, Ascl1* and the *Atoh* family, are required for the first step of neuronal differentiation (**Figure 3D**) (Alaynick et al., 2011; Sagner and Briscoe, 2019). These pro-neural TFs drive neuronal differentiation by remodelling chromatin landscape either acting alone as pioneer factors (Aydin et al., 2019), or in concert with chromatin remodelling complexes (Păun et al., 2023). Pro-neural genes subsequently activate neuronal LIM-homeodomain genes specific to the various progenitor DV identities (**Figure 3D**) (Aydin et al., 2019; Borromeo et al., 2014). Gain and loss of function experiments reveal that as well as promoting differentiation of NPCs to neurons, pro-neural TFs also contribute to the generation of distinct subtypes of neurons (Borromeo et al., 2014; Chang et al., 2013; Gowan et al., 2001; Mizuguchi et al., 2006; Parras et al., 2002; Wildner et al., 2006). However, given the redundant expression of pro-neural genes throughout the spinal cord, as demonstrated by the requirement of Ascl1 for both dl3-dp5 neurons in the dorsal spinal cord and V2a/b in the ventral spinal cord (Alaynick et al., 2011; Sagner and Briscoe, 2019), additional mechanisms to establish neuronal diversity are required.

ChIP-seq experiments have identified that the same TFs that impose neural progenitor domain identity by repressing alternate NPC fates, also repress gene expression associated with alternative neuronal fates. This acts to ensure differentiation to the correct neuronal subtype (**Figure 3E**) (Kutejova et al., 2016; Nishi et al., 2015). This is exemplified in motor neuron differentiation, where expression of pMN marker *Olig2* peaks prior to neuronal differentiation, repressing Hes5 (Sagner et al., 2018) and alternate neuronal programs helping establish MN formation. Moreover, downstream of pro-neural TFs, a transcriptional code of LIM genes appears to transmit NPC identity to neurons (**Figure 3E**). For instance, putative V2a neurons express *Lhx3,* downstream of the pro-neural gene *Ascl1*, forming a tetrameric protein complex with LDB1 (a nuclear Lim interactor). Lhx3-LDB1 complexes activate *Chx10,* critical for the differentiation to V2a neurons. However, in the adjacent MNs, *Lhx3* and *Isl1,* downstream of the pro-neural factor Neurog2, form a hexameric complex with LDB1, which binds to distinct motifs, activating *Mnx1* specifying MN differentiation (**Figure 3E**) (Lee et al., 2008; Thaler et al., 2002). Despite this, the precise mechanisms by which heterogeneity in gene expression profiles can activate the next cell state whilst decommissioning the gene regulatory mechanisms that stabilised the previous cell state is still unclear. While Olig2 and Neurog2 both act to specify pMN and MN fate, they also display cross-repressive interactions (Lee et al., 2005). Thus, how Neurog2 drives differentiation by overcoming elevated levels of Olig2 is not yet clear. One putative mechanism is by post-translation modifications (PTMs) on Olig2 (Sun et al., 2011), Ascl1 (Ali et al., 2014) and Neurog2 (Ali et al., 2011) leading to their degradation by Cyclin dependant Kinase (CDK) mediated ubiquitination. However, it is not clear how PTMs on NPC and pro-neural genes are controlled in individual cells within a tissue to ensure the correct balance of differentiation and self-renewal in NPCs. Moreover, identifying how precise patterns of neural differentiation occur will require understanding multiple layers of gene regulation. For instance, how do differential CRE usage and accessibility, TF

binding, and changes in the 3D chromatin organisation come together to establish gene expression patterns that allow a small set of pro-neural genes to coordinate the diversity spinal cord neurons.

6. More than the spatial pattern: diversifying neural tube cell types over time

6.1 Neuronal temporal patterning

The spatial patterning of NPCs cannot account for the full diversity of neuronal subtype identity in the mature spinal cord. Numerous studies have provided evidence for temporally stratified neuronal production across the nervous system and the spinal cord (Benito-Gonzalez and Alvarez, 2012; Deska-Gauthier et al., 2019; Hayashi et al., 2018; Hollyday and Hamburger, 1977; Müller et al., 2002; Sagner et al., 2021; Stam et al., 2011). Temporal patterning appears to be domain intrinsic, adding complexity to the spatial organization and increasing the variety of neurons produced (**Figure 4Ai, ii**). A well-documented example of how temporal patterning contributes to establish a spatial and functional neuronal pattern is given by the generation of motor columns, containing molecularly distinct subtypes of MN that innervate different sets of body muscles (Francius and Clotman, 2014; Tsuchida et al., 1994) (**Figure 4B**). Lateral motor columns (LMC), that innervate limb musculature (Dasen et al., 2008, 2005; Dasen and Jessell, 2009), are divided into lateral LMC (LMCl) and medial LMC (LMCm) which innervate distinct targets and have different gene expression profiles. Birth dating studies identified that LMCl is born after LMCm and migrate past them to reach their lateral most position (Hollyday and Hamburger, 1977). The early born LMCm expresses *Isl1*, downstream of *Onecut* (Roy et al., 2012) and expresses *Raldh2*, triggering RA secretion (Sockanathan et al., 2003; Sockanathan and Jessell, 1998) (**Figure 4B**). RA signalling to differentiating MNs results in the expression of *Lhx1* in late born MNs and their specification as LMCl (Francius and Clotman, 2010; Kania and Jessell, 2003; Roy et al., 2012; Tsuchida et al., 1994). LMCl neurons also express miRNA9, which inhibits Onecut-mediated induction of *Isl1* (Luxenhofer et al., 2014) (**Figure 4B**). However, the precise regulatory events downstream of RA and upstream of the Isl1/Lhx1 cross-repressive events are still unclear, as is the full temporal sequence and the lineage relationships between the different motor columns *in vivo*. In addition, differential cadherin expression appears to drive the segregation of MNs into different motor columns in the chick (Fredette and Ranscht, 1994; Price et al., 2002), prompting questions about the interplay between temporal progression and the adhesion code.

Single cell RNA sequencing datasets and EdU birthdating studies *in vitro* and *in vivo* describe a shared temporal TF (tTF) code, which drives the sequential production of diverse neuronal subtypes, further partitioning the major neuronal classes (Delile et al., 2019b; Sagner et al., 2021) (**Figure 4Ai, ii**). Early, intermediate, and late-born progenitors and neurons express characteristic arrays of tTF that is common throughout the CNS and conserved across species. Onecut TFs are detected in the earliest-born neurons across all DV domains, intermediate neurons express *Pou2f2* and *Zfhx2–4*, while late-born neurons express *Nfia/b/x*, *NeuroD,* and *Tcf4* (Sagner et al., 2021) (**Figure 4Ai**). Notably, two V2a interneuron subtypes born at different times and characterised by *Zfhx3* or *Nfib* expression differentially control MNs connected to forelimbs and hindlimbs, (Hayashi et al., 2018), highlighting the role of the temporal code in specifying functional diversity.

Both NPCs and differentiated neurons possess a temporal patterning code, comprised of different tTFs (Sagner et al., 2021) ((**Figure 4Ai**). Further study is required to identify whether these tTFs regulate each other's expression and whether the progenitor code directly regulates the neuronal code. In Drosophila neuroblasts, which are equivalent to NPCs, sequentially expressed tTFs define identity windows to generate specific neuronal progeny (Doe, 2017; Maurange, 2020). In addition, further lineage tracing studies will be needed to elucidate the lineage relationship between sequentially born neurons and their progenitors.

6.2 Adding glia

6.2.1 Gliogenic switch

The early phase of neurogenesis in the CNS is followed by a period of glial cell production (Cochard et al., 1995; Kessaris et al., 2001; Miller and Gauthier, 2007; Qian et al., 2000) (**Figure 4C**). During the gliogenic phase, astrocytes and oligodendrocytes are produced (Cochard et al., 1995; Gao et al., 2014; Leber et al., 1990), which provide physical, functional and metabolic support to neurons. The transition in cell fate competency of NPCs from neural to glial is termed the 'gliogenic switch' (Kessaris et al., 2001; Miller and Gauthier, 2007) (**Figure 4Ci**), the timing of which is species and region-specific (Belmonte-Mateos and Pujades, 2022; Miller and Gauthier, 2007; Rowitch, 2004a; Rowitch and Kriegstein, 2010). In the mouse spinal cord, gliogenesis starts at E12-12.5 with the production of oligodendrocyte precursors (OPCs) in the pMN domain (Wu et al., 2006a; Zhou et al., 2001b; Zhou and Anderson, 2002a). In human embryos gliogenesis commences at Carnegie Stage (CS) 15 (Gestational Week -GW- 6) in ventral region and CS18 (GW7) in dorsal regions (Dady et al., 2022; Deneen et al., 2006; Rayon et al., 2021).

The gliogenic switch has been suggested to be a cell-intrinsic restriction in progenitor potential, as heterochronic transplantation of NPCs from gliogenic into neurogenic spinal cord failed to give rise to neurons (Mukouyama et al., 2006). The mechanisms driving this competence shift and the relationship to the temporal programme of neurogenesis are not fully understood, however increasing evidence highlights an interplay of intrinsic, extrinsic, and epigenetic signals. Cascades of TFs have been linked to this transition (Laug et al., 2018; Miller and Gauthier, 2007). At the end of the neurogenic phase, *Neurog1* and *Neurog2* expression in NPCs declines reducing neuronal production (Sun et al., 2001), while *Nfia/b* (Deneen et al., 2006)*, Sox9* (Stolt et al., 2003)*, GLAST* (Shibata et al., 1997)*, COUP-TFII* (Naka et al., 2008)*, Atf3* and *Runx2* (Tiwari et al., 2018) and other pro-gliogenic genes start being expressed (**Figure 4Ci**). Nfia and Sox9 are two well studied regulators of the gliogenic switch, activating several glial-specifc genes (Molofsky et al., 2013) (**Figure 4Cii**). Loss- and gain-of-function experiments demonstrated that *Nfia/b* and *Sox9* are necessary and sufficient to regulate glia production (*in vivo* and *in vitro*) and to terminate neurogenesis in a timely manner (Caiazzo et al., 2015; Deneen et al., 2006; Finzsch et al., 2008; Neves et al., 1999; Scott et al., 2010; Shu et al., 2003; Stolt et al., 2003; Tchieu et al., 2019). *Nfia* expression is regulated by Sox9 and Brn2 (Glasgow et al., 2017; Kang et al., 2012; Molofsky et al., 2013) (**Figure 4Cii**), however, it is unclear what determines activation of *Sox9* and further studies will be needed to elucidate the GRNs underlying this competence shift.

Different mechanisms regulate the production of different glial cells. Sox9 promotes astrocytic differentiation together with Nfia (Akdemir et al., 2020; Kang et al., 2012; Molofsky et al., 2013).

However, in OPCs, Sox9 promotes differentiation by cooperating with Olig2 and Sox10 (Küspert et al., 2011; Lopez-Anido et al., 2015; Stolt et al., 2002), which in turn inhibits *Nfia* (Glasgow et al., 2014) to prevent astrogenesis (Stolt et al., 2005) (**Figure 4Cii**). In OPCs, Sox9 expression decreases, while SOX10 remains expressed in mature oligodendrocytes (Finzsch et al., 2008; Stolt et al., 2003, 2002).

NOTCH signalling is a key extrinsic factor in glial production (Bansod et al., 2017; Taylor et al., 2007), putatively upstream of *Sox9/Nfia* expression (Martini et al., 2013; Taylor et al., 2007) (**Figure 4Cii**). However, studies in zebrafish and chick suggested that Notch might facilitate the gliogenic switch by maintaining the progenitor pool needed to produce glia (Deneen et al., 2006; Park and Appel, 2003). In addition, some studies have begun to uncover the role of epigenetic factors in glial fate control, such as timely DNA demethylation or histone acetylation of glial genes (Cheng et al., 2011; Fan et al., 2005; Koreman et al., 2018; Zhang et al., 2016), yet the detailed mechanisms remain poorly understood.

6.2.2 Oligodendrocytes

Oligodendrocytes are the myelinating cells of the spinal cord and are produced from a proliferative intermediate Oligodendrocyte Progenitor Cell (OPC). OPCs are one of the most migratory cell types in the spinal cord that rapidly disperse radially and then dorsoventrally from their site of origin (Rowitch, 2004b; Zhou et al., 2000). This results in their broad distribution in the mature spinal cord (Altman, 1966). Several mechanisms have been implicated in their migration (Xia and Fancy, 2021), from cell autonomous expression of *Sox9/Sox10* (Finzsch et al., 2008), regulation of cellular polarity (Miyamoto et al., 2008), extracellular cues (Tsai et al., 2002), chemoattraction (Tsai et al., 2003) and Wnt-mediated movement along blood vessels (Tsai et al., 2016).

The widespread distribution of OPCs fuelled an ongoing debate about the location of their origin (Richardson et al., 2006; Spassky et al., 2000) (**Figure 4D**). Their broad spatial location initially favoured the hypothesis that OPCs originated from all DV regions of the embryonic VZ (Altman, 1966). However, *in vivo* experiments suggested they exclusively originated from the ventral spinal cord (Noll and Miller, 1993; Pringle et al., 1998; Rowitch, 2004b), from the pMN (Sun et al., 2006) or p3 domain (Fu et al., 2002; Soula et al., 2001). However, other studies indicated multiple OPC sources (Cameron-Curry and Douarin, 1995; Spassky et al., 1998). Cre/Lox lineage tracing and *Nkx6* mutant mice studies revealed a distinct second wave of oligodendrocyte production from progenitors expressing *Pax7* and *Dbx1* located dorsally to the origin of first wave OPCs (Cai et al., 2005; Fogarty et al., 2005; Vallstedt et al., 2005) (**Figure 4D**). While the first wave of OPCs appears to be dependent on Shh signaling (Cai et al., 2005; Orentas et al., 1999; Poncet et al., 1996; Pringle et al., 1996; Richardson et al., 2000; Trousse et al., 1995; Wang and Almazan, 2016), the second wave appears to arise independently (Cai et al., 2005; Chandran et al., 2003; Kessaris et al., 2004), likely under the regulation of other signals (Chandran et al., 2003; Grinspan et al., 2000; Gross et al., 1996; Kessaris et al., 2004; Shimizu et al., 2005). The signalling histories of these cells as well as the regulation of *Olig2* in the dorsal spinal cord, far from its original ventral domain of expression, remain unclear. Similar uncertainty has emerged regarding OPC origins in the brain (Richardson et al., 2006), where different temporal and spatial waves of OPC production have been observed (Gorski et al., 2002; Kessaris et al., 2006). Notably, OPCs generated at early embryonic times appear to have been lost in adult animals, prompting questions about the significance and function of the two waves (Kessaris et al., 2006).

Why does the origin of OPCs matter? If mature OPCs, originating from NPCs exposed to different signaling histories (e.g., BMP vs Shh), exhibit indistinguishable functions and characteristics, it would imply unexpected plasticity in the downstream response to signals leading to fate convergence. Alternatively, the divergent lineage histories of these cells might lead to distinct properties and roles in the mature spinal cord that are currently unappreciated.

The debate over the spatial origin of OPCs parallels discussions about the lineage potential of their progenitors (Liu and Rao, 2003, 2004a; Noble et al., 2004) (**Figure 4E**). Studies *in vitro* have proposed the existence of a glial restricted progenitors (GRP) that produce OPCs and astrocytes, but not neurons (Rao et al., 1998; Wu et al., 2002). However, others identified a close lineage connection with somatic MNs, consistent with the location of primary OPC wave and shared *Olig2* expression requirement (Leber et al., 1990; Lu et al., 2002; Takebayashi et al., 2002; Zhou and Anderson, 2002b). Therefore, OPCs might arise from bipotent progenitor able to give rise to both MNs and OPCs (MNOP) (Leber et al., 1990; Lu et al., 2002; Takebayashi et al., 2002; Xing et al., 2022; Zhou and Anderson, 2002b). Alternatively, OPCs and MNs could emerge from distinct pools of pMN progenitors assigned early to one of the two lineages (Liu and Rao, 2003, 2004b; Rao and Mayer-Proschel, 1997; Scott et al., 2021). Another possibility is that OPCs originate from distinct NPCs that activate *Olig2* expression at later developmental times than pMN and arise from a domain neighbouring pMNs (Ravanelli and Appel, 2015; Wu et al., 2006b). Discrepancies between these studies stem from differences between *in vitro* potential and *in vivo* fate constraints, experimental methods, and analysis timing. New unbiased lineage tracing tools and a detailed timeline of embryogenesis will be beneficial in solving these long-standing questions.

6.2.3 Astrocytes

Astrocytes are the most abundant cells in the CNS and play a vital supporting role; promoting synaptogenesis, maintaining the blood brain barrier, producing trophic factors and contributing to homeostasis (Allen and Eroglu, 2017; Verkhratsky and Nedergaard, 2018). Despite their importance, the developmental principles behind astrocytic differentiation are still far from understood (Akdemir et al., 2020; Bayraktar et al., 2015) owing to the paucity of early markers for astrocyte precursor cells (APCs). In spinal cord, the earliest marker of APCs is GLAST, which is expressed in mouse as early as E11 (Shibata et al., 1997), while GFAP, a mature astrocyte marker, appears after E15.5 (Andræ et al., 2001; Eng, 1985).

There is evidence that astrocytes are produced in two waves, first from progenitors in the VZ, and later through an intermediate APC in the mantle of the spinal cord (Tien et al., 2012). The two pools of astrocytes are produced in a ventral-to-dorsal gradient (Tien et al., 2012; Tsai et al., 2012) that have temporally distinct programmes of gene expression (Chaboub et al., 2016; Molofsky et al., 2013).

Contrary to early suggestions postulating a uniform origin and function of astrocytes throughout the CNS, several lines of evidence suggest their functional, spatial and lineage heterogeneity (Bayraktar et al., 2015; Zhang and Barres, 2010). Several *in vivo* studies have shown that, like neuronal subtype identity, the molecular identity and spatial location of astrocytes depends on their origin (**Figure 4F**) (Hochstim et al., 2008; Sartoretti et al., 2022; Tsai et al., 2012; Vue et al., 2014). In the ventral spinal cord, the production of ventral astrocytes (vAs) from the p0-p3 domains (vA0-vA3) depends on a homeodomain transcriptional code, whose components are repurposed once neuronal identity is

established (**Figure 4F**). For example, the bHLH TF Tal1 is necessary and sufficient for vA2 astrocytes by suppressing *Olig2* (Muroyama et al., 2005), the combinatorial expression of Pax6 and Nkx6-1 regulate vA1-vA3 subtype identity (Hochstim et al., 2008; Zhao et al., 2014), and Dbx1 controls vA0/V0 interneuron balance (Sartoretti et al., 2022) (**Figure 4F**). Functionally, ablation of domain-specific astrocyte types results in incorrect synaptogenesis (Tsai et al., 2012) and the deletion of region-specific genes (e.g., *Sema3a* or *Kcnj10*) in ventral astrocytes caused death of specific classes of MNs and abnormal MN circuit organization and electrophysiological properties (Kelley et al., 2018; Molofsky et al., 2014). This evidence suggests lineage-dependent functional heterogeneity, which is just starting to be uncovered (Yoon et al., 2017).

Astrocyte lineage relationships are still to be determined (**Figure 4E**). Studies have proposed the existence of a GRP (Hirano and Goldman, 1988; Noble et al., 2004; Rao and Mayer-Proschel, 1997), in addition to a tripotent progenitor producing MNs, OPCs and astrocytes (Leber et al., 1990; Masahira et al., 2006; Rao et al., 1998). However, the evidence showing a common transcriptional programme for domain-specific interneurons and astrocytes production (Muroyama et al., 2005; Sartoretti et al., 2022) prompts further investigation into the neuronal and glial lineage relationships.

7. Conclusions & Future Perspectives

Numerous studies have provided insight into the formation and patterning of the spinal cord, highlighting how an interplay of gene regulation, cell movements and tissue mechanics is critical. The GRN underlying pattern formation has shown how the integration of cell intrinsic and extrinsic activating and repressive gene regulatory mechanisms organise cell fate decisions. In the future, advances in imaging and sequencing techniques that identify the 3D chromatin structure will help answer fundamental questions as to how layers of gene regulation are conveyed across the genome to produce precise changes in gene expression.

Recent studies have highlighted how the temporal dynamics of gene expression dictate cell commitment (Delás et al., 2023; Dessaud et al., 2007; Sagner et al., 2021). Future studies will need to account for time to explain morphogen response and lineage decisions in the spinal cord. Moreover, a high degree of convergence of multiple embryonic lineages into fewer transcriptionally distinct mature cell types has been observed in mouse and Drosophila scRNAseq datasets (Li et al., 2017; Russ et al., 2021). The combination of detailed molecular knowledge of cell type heterogeneity identified in scRNAseq datasets and new unbiased and high-throughput sequencing-based lineage tracing techniques will help resolve this complexity.

In addition to the molecular signature and histories of the differentiating neural cells, cell and tissue mechanics are crucial to explain the spatial patterning of the spinal cord. The feedback between tension, cytoskeleton and gene expression is just starting to be uncovered (Matsuda and Sokol, 2021; Newman-Smith et al., 2015; Nikolopoulou et al., 2017; Tsai et al., 2022) and new techniques are now available to further explore these forces in *in vivo* systems (Maniou et al., 2022). These multidimensional data will offer greater power to already existing quantitative models describing and predicting NT development (Bocanegra-Moreno et al., 2023; Maizels et al., 2023; Pezzotta and Briscoe, 2022; M. Sáez et al., 2022). In the future, the integration of these into *in silico* models will help make new predictions and test new hypotheses. Finally, while whilst the broad mechanisms governing spinal

cord patterning are conserved across different species, there are species-specific processes that are now starting to be uncovered (Jang et al., 2022; Rayon et al., 2021). Therefore, it will be important to elucidate the different strategies employed across the animal kingdom to achieve a final conserved pattern. Studying spinal cord patterning will help gain an understanding of fundamental processes through development and in particular the integration of molecular, cellular and tissue scale mechanisms will uncover the many ways that an organism has to make a pattern.

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Figure legends

Figure 1: Formation of the spinal cord is initiated during gastrulation

(A) Schematic of a HH10 chicken embryo (adapted from Rito et al. 2023). Hox paralogous groups (PG) are expressed in specific regions across the rostro-caudal axis in the developing spinal cord. Wnt/FGF ligands show a gradient of expression that is highest at the tail of the embryo, and Retinoic Acid (RA) is expressed in more rostral regions of the spinal cord. **Inset** indicates the spatial localisation of NMPs, pre-neural and neural progenitor cells. **(B)** Changes in cell state and gene expression signatures from NMP to neural following exposure to FGF, then RA signalling. **(C) (I)** Secretion of BMP ligands from the Lateral Plate mesoderm (LPM) and BMP inhibitors creates a gradient of BMP activity across the mediolateral axis of the overlying ectoderm. At the medial region of the ectoderm, BMP inhibition results in the formation of neural precursors. **(II)** Bending of the neural ectoderm starts at the Median Hinge Point (MHP) where wedge-like cells are observed. More dorsally, the NT bends at the Dorsolateral hinge points (DLHP). **(III)** The neural tube closes, resulting in the formation of the hollow neural tube. **(E)** Secondary neurulation is initiated as neural precursors converge and condense **(I)**. **(II)** Neural precursors in the tail subsequently epithelialize and form the medullary cord. **(III)** The medullary cord undergoes cavitation, which creates the lumen of the neural tube.

Figure 2: Patterning of the dorsoventral axis of the spinal cord

A) Schematic cross-section of an embryonic spinal cord with progenitor (left) and neuronal (right) DV domains. NT progenitors are patterned into 11 domains along the DV axis (dp1-dp6, p0-p2, pMN, p3), each of which gives rise to distinct neuronal subtypes (dI1-dI6, V0, V1, V2a, V2b, MNs, V3), characterized by the expression of specific TFs. FP, floor plate; RP, roof plate. **B**) Phase portrait depicting levels and duration of Shh signalling for the induction of *Pax6/Irx3, Olig2* and *Nkx2.2* in NPCs. *Nkx2.2* expression requires higher levels and duration of Shh. **C**) Sequential induction of NPCs markers in the ventral spinal cord. Increasing levels/duration of Shh signalling result in the sequential induction of first *Olig2* (orange) and, later, *Nkx2.2* (yellow), gradually restricting the *Pax6/Irx3* domain (grey). **D**) Activation by Sox2 and positive/negative inputs from GliA/GliR results in cross-repressive interactions between ventral domain-specific TFs Nkx2.2, Olig2, Pax6 and Irx3. Arrows, activating interactions; T bars, repressive interactions. (see Delás and Briscoe, 2020). **E**) Three types of inputs are

integrated into CREs: broad activators that promote multiple fates (Sox2), signalling inputs (GliA/GliR), and cell type specific repressors (Olig2, Nkx2.2, etc), which repress all alternative fates. **F**) Most of the ventral domains are established by differential binding of domain-specific TFs on a shared chromatin landscape. However, the ventral-most p3 domain exhibits distinct chromatin accessibility, established by the pioneer TF FoxA2. **G**) Zebrafish NPCs are spatially intermixed downstream of a heterogeneous response to Shh. NPCs sort into precise domains with sharp boundaries due to a specific adhesion code, controlled by the GRN components. **H**) Boundary precision in amniotes is encoded in the GRN dynamics, as shown by phenotypes caused by alterations in the nodes or edges of the network (e.g., *Pax6-/-*).

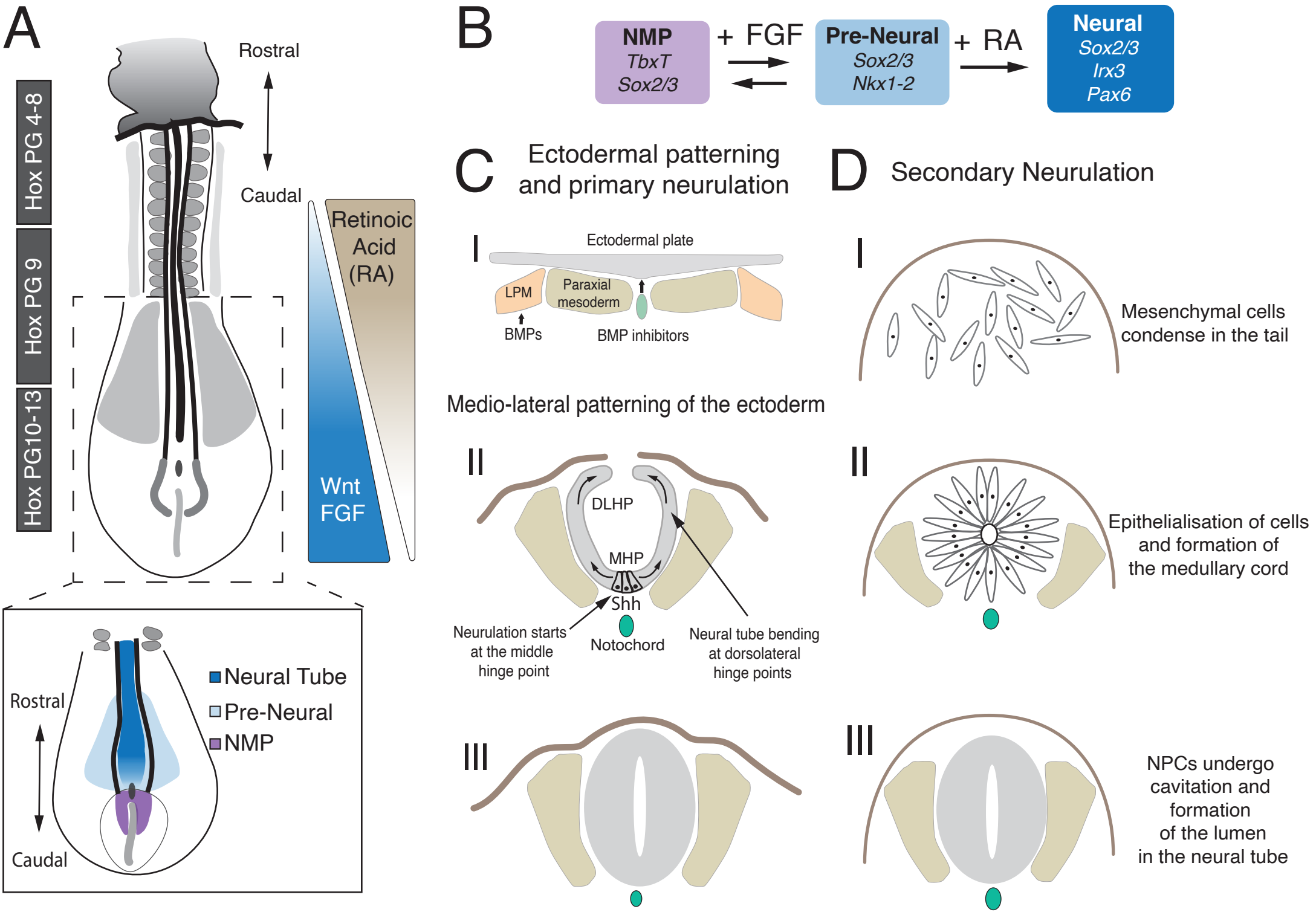
Figure 3: Growth and diversification of cell types in the spinal cord.

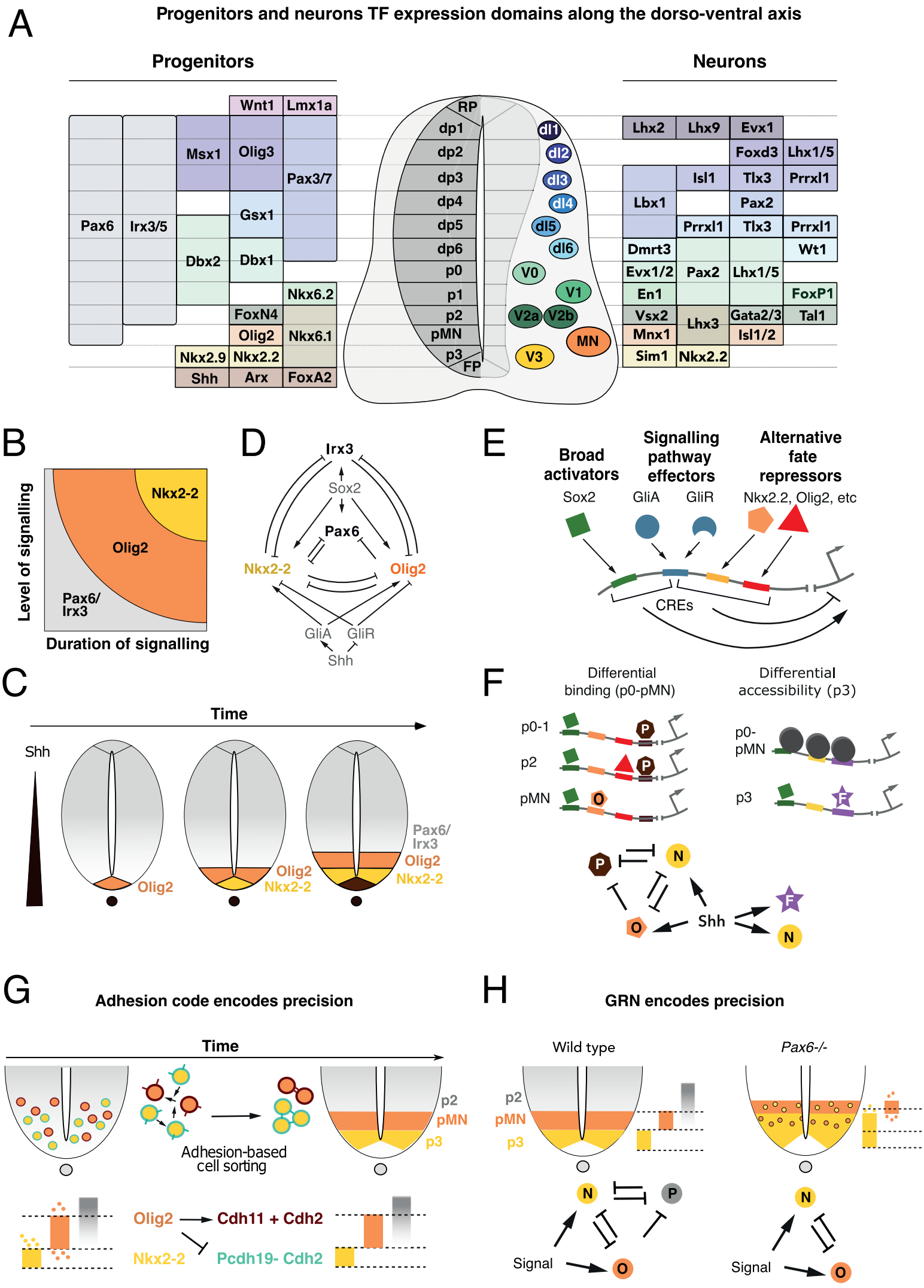
(A) (I) Spinal cord development can be separated into two distinct phases. During the specification phase, domains of neural progenitor cells (NPCs) are specified and grow in proportion with tissue size. In the growth phase, differentiation of NPCs occurs at domain specific rates, resulting in differences in domain proportions over time. In general, ventral NPCs, particularly MN progenitors, undergo differentiation at a higher rate than their dorsal counterparts. **(II)** Graphs depicting the change in proportion of NPCs and expansion of neurons with a ventral identity. **(B)** Interkinetic nuclear movement of NPCs in the VZ. Nuclei of cells in G1/S-phase are located basally. They move toward the apical surface during G2 phase and undergo mitosis at the apical surface. During asymmetric cell division, the differentiating daughter cell (blue) undergoes apical abscission **(C)**. The differentiating cell detaches from the apical membrane and leaves the VZ from the basal surface. Apical abscission is outlined in **(C)** (1) NPCs are attached to the apical surface by N-cadherins. During apical abscission (2), N-cadherins break down and actomyosin cables contract, leaving behind the primary cilium (3). **(D)** Cell type identity that is initiated in NPCs is transmitted to neurons during differentiation by a spatial code of bHLH pro-neural genes, and LIM-Homeodomain genes. **(E)** NPC identity is transmitted to neurons, initially by the repression of alternate neuronal identities and activation of specific downstream differentiation programs by NPC identity genes, resulting in the expression of specific neuronal identity genes.

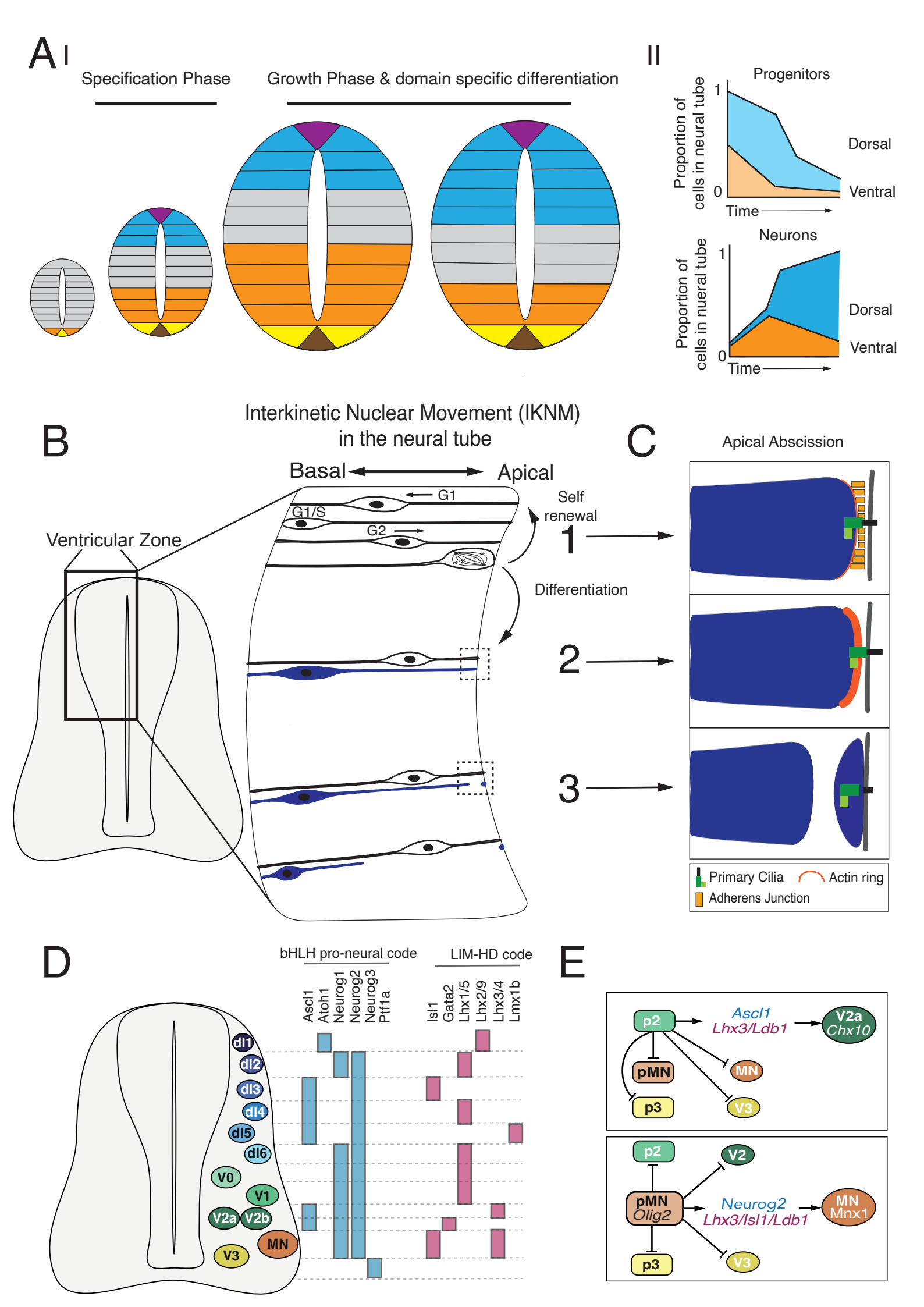
Figure 4: Temporal patterning of the spinal cord: neurons and glia

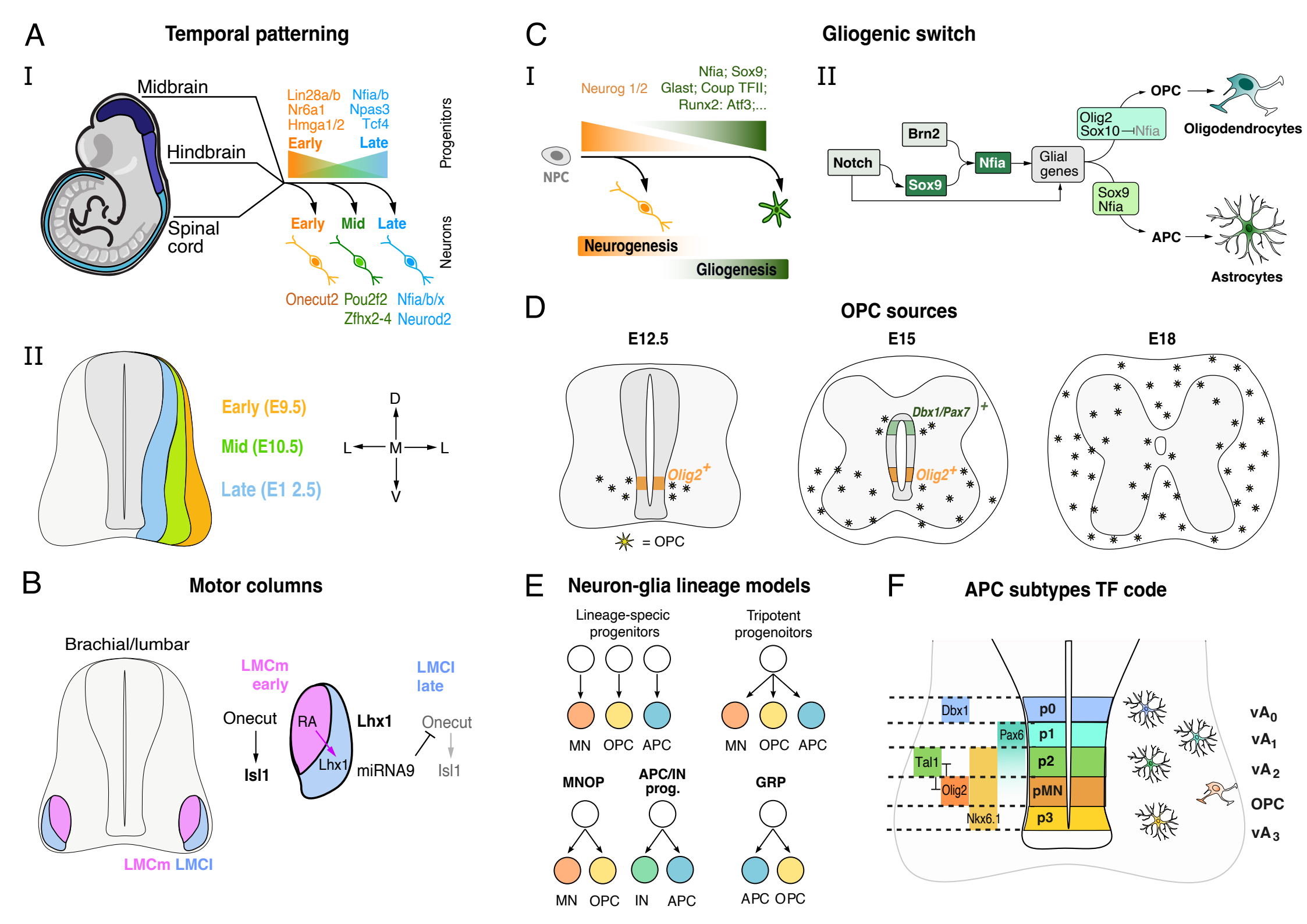
A) Conserved temporal patterning of neural progenitors and neurons throughout the CNS is characterized by the expression of specific temporal transcription factors (**I**). In the spinal cord early born neurons (orange) are initially located laterally compared to mid and late born ones (**II**). M, medial; L, lateral; D, dorsal; V, ventral. (Adapted from Sagner et al, 2021 and Delás and Briscoe, 2020) **B**) Spinal motor columns as an example of temporal patterning and related functional heterogeneity. The lateral motor column is divided in medial (LMCm), born first, and lateral (LMCl), born later, characterized by specific gene expression shown in the inset. **C**) Neurogenesis and gliogenesis are characterized by a specific and gradually transitioning gene

expression program **(I)**. **(II)** The GRN controlling the gliogenic switch and the further specification of glial cells into oligodendrocyte precursor cells (OPCs) and astrocyte precursor cells (APCs). **D**) Schematics of OPC origin from different sources: early OPCs derive from *Olig2+* ventral NPCs (orange) and late OPCs from *Pax7/Dbx1+* dorsal NPCs (green). By E18 the two populations of OPCs are spatially intermingled in the NT. **E**) Diagrams depicting different models of neuron and glia lineage relationships. MNOP, motor neuron oligodendrocytes progenitors; GRP, glial restricted progenitor; IN, interneurons; MN, motor neurons; OPC, oligodendrocyte precursor cells; APC, astrocyte precursor cells. **F**) Schematic cross-section of a ventral spinal cord depicting the diversification of astrocytes subtypes (vA_0-vA_3) based on their domain of origin. Cross-repressive interactions and combinatorial expression of TFs in NPCs (left) creates a transcriptional code driving molecular identity acquisition and spatial location in astrocytes.


A
Rostral
Caudal
Hox PG 4-8
Hox PG 9
Hox PG10-13
Retinoic Acid (RA)
Wnt
FGF
Neural Tube
Pre-Neural
NMP
Rostral
Caudal
B
NMP
TbxT
Sox2/3
+ FGF
Pre-Neural
Sox2/3
Nkx1-2
+ RA
Neural
Sox2/3
Irx3
Pax6
C
Ectodermal patterning and primary neurulation
I
Ectodermal plate
LPM
Paraxial mesoderm
BMPs
BMP inhibitors
Medio-lateral patterning of the ectoderm
II
DLHP
MHP
Shh
Notochord
Neurulation starts at the middle hinge point
Neural tube bending at dorsolateral hinge points
III
D
Secondary Neurulation
I
Mesenchymal cells condense in the tail
II
Epithelialisation of cells and formation of the medullary cord
III
NPCs undergo cavitation and formation of the lumen in the neural tube


A
Progenitors and neurons TF expression domains along the dorso-ventral axis
Progenitors
Neurons
Wnt1
Lmx1a
Msx1
Olig3
Pax3/7
Gsx1
Pax6
Irx3/5
Dbx2
Dbx1
Nkx6.2
FoxN4
Olig2
Nkx6.1
Nkx2.9
Nkx2.2
Shh
Arx
FoxA2
RP
dp1
dp2
dp3
dp4
dp5
dp6
p0
p1
p2
pMN
p3
FP
dI1
dI2
dI3
dI4
dI5
dI6
V0
V1
V2a
V2b
MN
V3
Lhx2
Lhx9
Evx1
Foxd3
Lhx1/5
Isl1
Tlx3
Prrxl1
Lbx1
Pax2
Prrxl1
Tlx3
Prrxl1
Dmrt3
Wt1
Evx1/2
Pax2
Lhx1/5
En1
FoxP1
Vsx2
Lhx3
Gata2/3
Tal1
Mnx1
Isl1/2
Sim1
Nkx2.2
B
Level of signalling
Nkx2-2
Olig2
Pax6/
Irx3
Duration of signalling
D
Irx3
Sox2
Pax6
Nkx2-2
Olig2
GliA
GliR
Shh
E
Broad
activators
Signalling
pathway
effectors
Alternative
fate
repressors
Sox2
GliA
GliR
Nkx2.2, Olig2, etc
CREs
C
Time
Shh
Olig2
Olig2
Nkx2-2
Pax6/
Irx3
Olig2
Nkx2-2
F
Differential
binding (p0-pMN)
Differential
accessibility (p3)
p0-1
p2
pMN
p0-
pMN
p3
P
O
F
N
Shh
G
Adhesion code encodes precision
Time
Adhesion-based
cell sorting
p2
pMN
p3
Olig2
Cdh11 + Cdh2
Nkx2-2
Pcdh19- Cdh2
H
GRN encodes precision
Wild type
Pax6-/-
p2
pMN
p3
N
P
Signal
O
N
Signal
O


A I
Specification Phase
Growth Phase & domain specific differentiation
II
Progenitors
Proportion of cells in neural tube
1
0
Dorsal
Ventral
Time
Neurons
Proportion of cells in nueral tube
1
0
Dorsal
Ventral
Time
B
Interkinetic Nuclear Movement (IKNM) in the neural tube
Basal
Apical
Ventricular Zone
G1
G1/S
G2
Self renewal
1
Differentiation
2
3
C
Apical Abscission
Primary Cilia
Actin ring
Adherens Junction
D
bHLH pro-neural code
LIM-HD code
Ascl1
Atoh1
Neurog1
Neurog2
Neurog3
Ptf1a
Isl1
Gata2
Lhx1/5
Lhx2/9
Lhx3/4
Lmx1b
dI1
dI2
dI3
dI4
dI5
dI6
V0
V1
V2a
V2b
V3
MN
E
p2
pMN
p3
Ascl1
Lhx3/Ldb1
V2a
Chx10
MN
V3
p2
pMN
Olig2
p3
V2
Neurog2
Lhx3/Isl1/Ldb1
MN
Mnx1
V3


A
Temporal patterning
I
Midbrain
Hindbrain
Spinal cord
Lin28a/b Nfia/b
Nr6a1 Npas3
Hmga1/2 Tcf4
Early Late
Progenitors
Early Mid Late
Neurons
Onecut2 Pou2f2 Nfia/b/x
Zfhx2-4 Neurod2
II
Early (E9.5)
Mid (E10.5)
Late (E1 2.5)
D
L M L
V
B
Motor columns
Brachial/lumbar
LMCm LMCl
LMCm early
LMCl late
Onecut
Isl1
RA
Lhx1
Lhx1
miRNA9
Onecut
Isl1
C
Gliogenic switch
I
Nfia; Sox9;
Neurog 1/2 Glast; Coup TFII;
Runx2: Atf3;...
NPC
Neurogenesis
Gliogenesis
II
Notch
Brn2
Sox9
Nfia
Glial genes
Olig2
Sox10 Nfia
OPC
Oligodendrocytes
Sox9
Nfia
APC
Astrocytes
D
OPC sources
E12.5
E15
E18
Olig2+
Dbx1/Pax7+
Olig2+
= OPC
E
Neuron-glia lineage models
Lineage-specic progenitors
Tripotent progenoitors
MN OPC APC
MN OPC APC
MNOP
APC/IN prog.
GRP
MN OPC IN APC
APC OPC
F
APC subtypes TF code
Dbx1
Pax6
Tal1
Olig2
Nkx6.1
p0
p1
p2
pMN
p3
vA0
vA1
vA2
OPC
vA3