

REVIEW

Establishing neuronal diversity in the spinal cord: a time and a place

Andreas Sagner* and James Briscoe*

ABSTRACT

The vertebrate spinal cord comprises multiple functionally distinct neuronal cell types arranged in characteristic positions. During development, these different types of neurons differentiate from transcriptionally distinct neural progenitors that are arrayed in discrete domains along the dorsal-ventral and anterior-posterior axes of the embryonic spinal cord. This organization arises in response to morphogen gradients acting upstream of a gene regulatory network, the architecture of which determines the spatial and temporal pattern of gene expression. In recent years, substantial progress has been made in deciphering the regulatory network that underlies the specification of distinct progenitor and neuronal cell identities. In this Review, we outline how distinct neuronal cell identities are established in response to spatial and temporal patterning systems, and outline novel experimental approaches to study the emergence and function of neuronal diversity in the spinal cord.

KEY WORDS: Gene regulatory network, Hedgehog, Neural tube, Neurogenesis, Neuronal diversity, Patterning

Introduction

The spinal cord is crucial for how we perceive and interact with our environment. Perception of sensory information from our skin, muscles and internal organs depends on the activities of several classes of neurons, the cell bodies of which reside predominantly within the dorsal horns of the spinal cord. Conversely, our ability to respond to these peripheral cues depends on neuronal circuits located in the ventral spinal cord. These circuits convert motor commands from the brain and sensory inputs into precise patterns of motor activity and thus muscle contraction.

Functional characterization of the adult spinal cord, based on morphology, neuronal connectivity, axonal projections, neurotransmitter types and molecular markers, has revealed a modular organization with neurons performing specific tasks occupying stereotypical positions. For example, neurons in the dorsal horns are arranged in distinct laminae and each lamina receives sensory input from neurons involved in the transmission of distinct signals (reviewed by Lai et al., 2016). The outer laminae (laminae I and II) are innervated by afferents transmitting noxious stimuli, including pain, itch and thermosensation, whereas touch afferents project towards the deeper laminae (laminae II-V) and proprioceptive afferents project towards more ventral laminae or

directly onto motor neurons (MNs). In contrast to the laminar structure of the dorsal horns, MNs within the ventral spinal cord are organized into distinct clusters that occupy invariant positions. This is most obvious at limb levels, where MNs are organized into ~50 motor pools that innervate distinct limb muscles. The position of the neuronal cell body in the spinal cord matches the projection pattern of the MN, with proximally projecting neurons occupying more ventral positions, and distally projecting pools located in progressively more dorsal positions. Although neuronal settling position does not seem to be important for projecting to the right target muscle, it is essential for establishing the correct neuronal connections with sensory afferents (Balaskas et al., 2019; Demireva et al., 2011; Sürmeli et al., 2011). Similarly, the position of distinct subtypes of V1 neurons – inhibitory neurons that provide negative feedback to MNs and that are crucial for the alternating motor input to flexor and extensor muscles – seems to correlate with distinct proprioceptive input onto these neurons (Bikoff et al., 2016). These findings suggest that the spatial segregation of functionally distinct cell types at stereotypic positions underlies the specificity of circuit formation in the spinal cord.

This raises the question of how and when this organization arises during development. Work during the last 30 years revealed that this organization emerges early during development of the embryonic neural tube. Here, the activities of multiple morphogens that emanate from the dorsal and ventral poles of the spinal cord pattern the dorsal-ventral (DV) axis. These morphogens induce distinct basic helix-loop-helix (bHLH) and homeodomain (HD) transcription factors (TFs) in spatially delimited and combinatorially overlapping territories (Briscoe et al., 2000; Jessell, 2000). The resulting TF code defines 11 molecularly distinct types of neural progenitors (NPs), each of which gives rise to one or more distinct neuronal subtype (Alaynick et al., 2011; Lai et al., 2016; Lu et al., 2015). This exemplifies a recurring theme in animal development: morphogen signalling induces distinct spatial patterns of gene expression in progenitors, thereby specifying cell fates and resulting in the ordered and balanced production of terminally differentiated cell types (Rogers and Schier, 2011; Sagner and Briscoe, 2017). In fact, owing to its experimental accessibility, the vertebrate neural tube has come to serve as a leading model for understanding morphogen-mediated patterning and the principles of cell fate specification.

Recent technological advances have provided further insight into the molecular mechanisms underlying patterning and cell fate specification in this system. Several large-scale molecular profiling studies revealed an unexpectedly large number of molecularly distinct neuronal cell types. In particular, profiling of neuronal gene expression heterogeneity in embryonic and adult spinal cords by single cell RNA sequencing (scRNA-seq) suggested the existence of at least several dozen molecularly distinct neuronal subtypes (Delile et al., 2019; Häring et al., 2018; Hayashi et al., 2018; Sathiyamurthy et al., 2018). Furthermore, detailed characterization

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of V1 neuron heterogeneity at limb levels of the spinal cord has suggested much further diversification and the existence of ~50 distinct subtypes of one specific interneuron class (V1 neurons) based on the combinatorial expression of 19 TFs (Bikoff et al., 2016; Gabitto et al., 2016). Subsequent work suggested additional diversification of V1 neurons along the anterior-posterior (AP) axis of the spinal cord (Sweeney et al., 2018). Here, we review these recent findings and discuss how neuronal diversity emerges from the intersection of three orthogonal patterning systems that pattern the spinal cord in space and time. Although we do not provide a molecular mechanism for the formation of every neuronal subtype described in the literature, we argue that the framework is now established to achieve this goal. We also provide a brief overview of emerging model systems and experimental approaches that may

help us decipher the molecular mechanisms that underlie neuronal diversity and understand how this diversity contributes to the formation of functional neuronal circuitry.

Opposing morphogen gradients pattern the spinal cord along the DV axis

NPs acquire distinct identities along the DV axis of the neural tube in response to opposing morphogen systems (Briscoe and Small, 2015; Jessell, 2000; Le Dréau and Martí, 2012) (Fig. 1A). Sonic hedgehog (Shh), emanating first from the notochord and later from the floor plate, induces distinct ventral identities (Martí et al., 1995; Roelink et al., 1995), whereas ligands of the bone morphogenetic protein (BMP) and Wnt families, secreted from the roof plate, promote dorsal progenitor identities (Lee et al., 2000; Liem et al.,

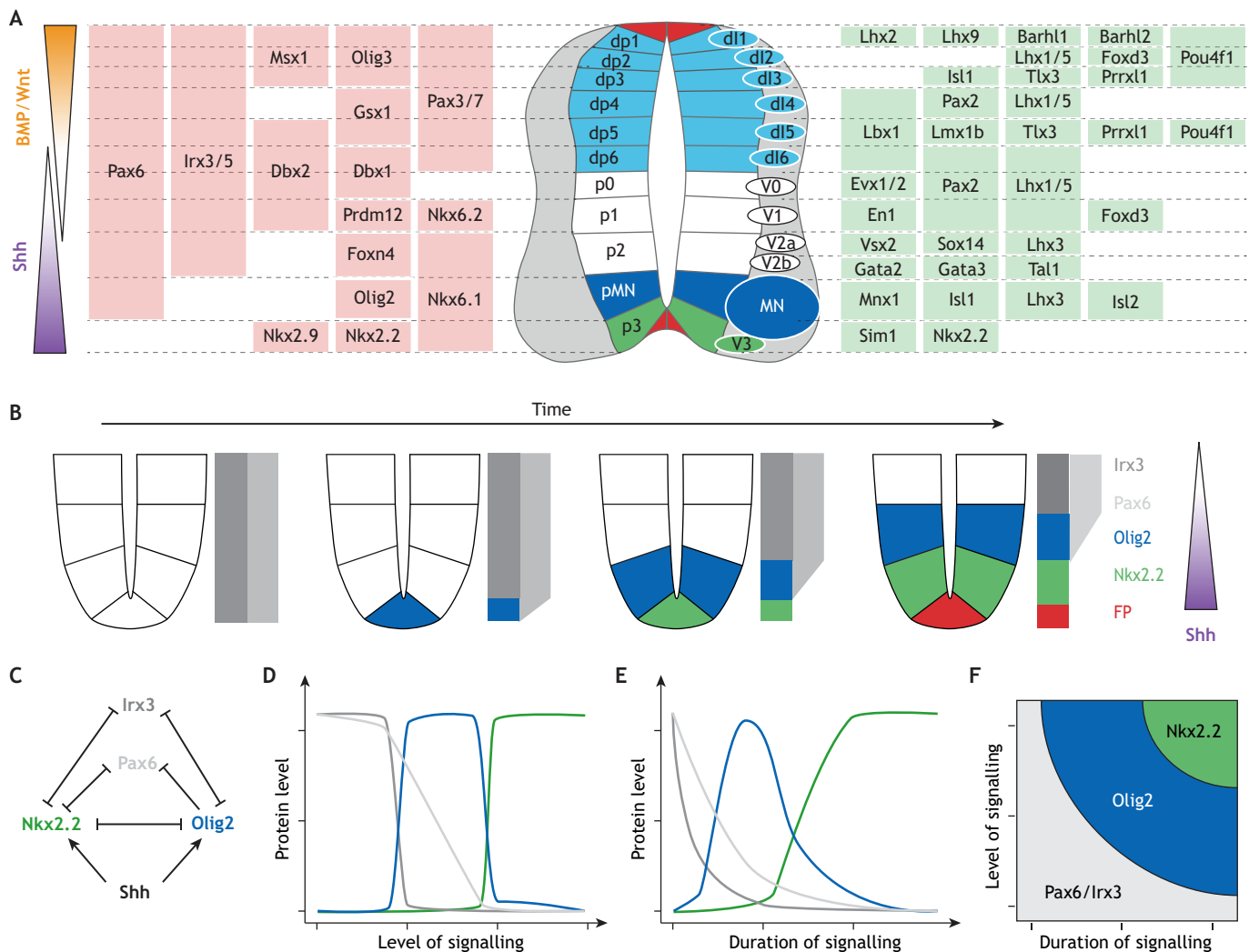


Fig. 1. Opposing morphogen gradients pattern the spinal cord along the DV axis. (A) Schematic cross-section of an embryonic spinal cord. Opposing morphogen gradients delineate specific TF expression domains (pink boxes) along the DV axis. The co-expression of TFs defines 11 distinct neural progenitor (NP) domains (dp1-dp6, p0-p2, pMN, p3) along the DV axis. Each of these NP domains gives rise to distinct neuronal subtypes (d11-d16, V0, V1, V2a, V2b, MN, V3), which are characterized by the expression of specific TFs (green boxes). (B) Sequential induction of progenitor markers in the ventral spinal cord. At early stages of development, NPs throughout the DV axis express Pax6 and Irx3 (grey). As development progresses, increasing levels and duration of Shh signalling result in the sequential induction of Olig2 (blue) and Nkx2.2 (green). FP, floor plate. (C) The GRN controlling spatial and temporal dynamics of gene expression in the ventral spinal cord. (D) As a consequence of the dynamics of this GRN, different levels of Shh lead to the expression of different TFs; lower levels and durations of Shh favour the expression of Olig2 (blue), whereas high levels of Shh signalling lead to expression of Nkx2.2 (green). (E) Dynamics of gene expression upon exposure of NPs to high levels of Shh. Although the transcriptional circuit favours Nkx2.2 expression at steady state, the system transiently produces Olig2 before Nkx2.2 induction. (F) Phase portrait depicting the relationship between levels and duration of Shh signalling for the induction of Pax6/Irx3, Olig2 and Nkx2.2 in NPs.

1997; Muroyama et al., 2002; Timmer et al., 2002; Wine-Lee et al., 2004). These morphogens induce the expression of HD and bHLH TFs in discrete but overlapping domains along the DV axis of the neural tube (Alaynick et al., 2011; Briscoe et al., 2000). The combination of TFs expressed by a progenitor determines the specific classes of neurons that it generates (Fig. 1A). Importantly, the TFs extensively cross-regulate each other's expression, forming a gene regulatory network (GRN), the architecture of which determines the spatial and temporal response to morphogen input. At the molecular level, this cross-regulation is mediated through modular enhancers that contain binding sites for multiple GRN components, for the transcriptional effectors of morphogen signalling pathways, and for broadly expressed TFs (Kutejova et al., 2016; Nishi et al., 2015; Oosterveen et al., 2013; Peterson et al., 2012). This allows these enhancers to integrate simultaneously the current state of the GRN and the signalling state of the cell. The dynamics of the GRN, which are determined by its architecture, then convert levels and duration of morphogen inputs into spatially delimited and partially overlapping domains of TF expression. The resulting pattern defines six dorsal (dp1-dp6) and five ventral (p0-p2, pMN, p3) NP domains (Fig. 1A). In addition to these transcriptional mechanisms, post-transcriptional regulation of mRNA and protein stability have also been implicated in establishing the correct pattern of NP domains in the spinal cord (Chen et al., 2011; Kim et al., 2018).

How morphogen input is converted into distinct progenitor identities is best understood in the context of the ventral neural tube, where Shh signalling specifies progenitor identities in a concentration- and duration-dependent manner (Balaskas et al., 2012; Cohen et al., 2014; Dessaud et al., 2007) (Fig. 1B-F). Here, the p3 domain is defined by Nkx2.2 expression, pMN by expression of Olig2 and low levels of Pax6, and p2 progenitors express Irx3 and high levels of Pax6. These four TFs are linked by cross-repressive interactions, with Nkx2.2 and Olig2 repressing all other TFs, Irx3 repressing Olig2 and Nkx2.2, and Pax6 repressing Nkx2.2 (Fig. 1C). This transcriptional circuit determines both the spatial and temporal dynamics of gene expression in response to Shh signalling (Briscoe and Small, 2015; Cohen et al., 2013) (Fig. 1B-F).

Although the role of Shh in promoting ventral cell identities is well established, the roles of BMP and Wnt signalling in the dorsal neural tube are not fully resolved. BMP and Wnt ligands are initially expressed in the roof plate but at later stages are also expressed in multiple dorsal NP populations (Andrews et al., 2017; Lee et al., 2000; Liem et al., 1997; Megason and McMahon, 2002). Genetic studies suggest that both signalling pathways are required for inducing the most dorsal progenitor and neuronal identities. Ablation of roof plate-derived BMPs and/or Wnts causes loss of most dorsal dI1 and dI2 neurons, and the production of intermediate positioned dI3 and dI4 neurons shifts to a more dorsal position (Lee et al., 2000; Muroyama et al., 2002; Wine-Lee et al., 2004). Furthermore, explant and *in vitro* cell culture assays have revealed the ability of both BMPs and Wnts to induce dorsal cell types (Andrews et al., 2017; Gupta et al., 2018; Liem et al., 1997; Muroyama et al., 2002; Tozer et al., 2013). These results indicate that both signalling pathways are required, but exposure of progenitors to either signal is sufficient for establishing dorsal progenitor identities. However, this interpretation is complicated by the observation that abrogation of BMP signalling affects the production of Wnt ligands (Lee et al., 2000; Wine-Lee et al., 2004), and exposure of NPs to BMPs (e.g. in explant cultures) probably induces the expression of Wnt ligands. Conversely, multiple dorsal

NP types express BMP ligands (Andrews et al., 2017). Thus, dorsalization by Wnts may also induce expression of BMP ligands in these cells.

Recent work has proposed that BMPs may not act as morphogens in the spinal cord and that instead the identity of BMP ligands is the main determinant for the generation of distinct interneuron types (Andrews et al., 2017). However, the combined activity of BMP ligands appears graded at early developmental stages, as assays for phosphorylated SMADs (the intracellular effectors of BMP signalling) reveal a dorsal-to-ventral gradient (Tozer et al., 2013; Zagorski et al., 2017). These seemingly contradictory findings could potentially be reconciled if the timing of exposure plays a role in the interpretation of BMP signalling, as has been suggested by Duval et al. (2019). In this view, the cell types generated further from the dorsal midline of the neural tube would require earlier and briefer exposure to BMP signalling than those arising closer to the source of BMP signalling.

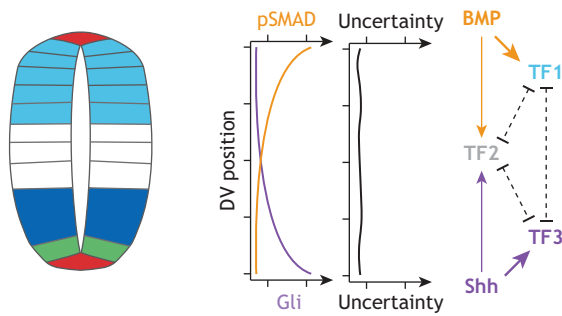
Taken together, these studies suggest that Wnt and BMP ligands are required for the establishment of dorsal progenitor identities in the spinal cord, but how these signals function to specify distinct identities, and the epistasis between them, is still unclear. Further work is required to discriminate between the molecular mechanisms through which BMPs and Wnts induce dorsal progenitor and neuronal identities. The recent development of novel stem cell-based differentiation protocols that allow ectopic manipulation of molecular signals and generation of large quantities of cells for molecular profiling assays provides a new avenue for tackling these questions (Duval et al., 2019; Gupta et al., 2018).

Growth and patterning dynamics of the spinal cord

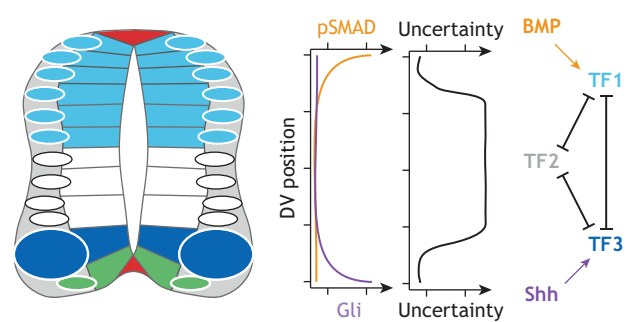
NPs in the mouse spinal cord generate neurons from approximately embryonic day (E) 9.0 until ~E13.5. During this time, the spinal cord continues to grow substantially along its DV axis. This raises the question of how the positions of gene expression boundaries and progenitor domains remain stable as the spinal cord exceeds the size that can be reliably patterned by morphogen signalling, and how neurogenesis and spinal cord patterning are integrated to ensure the generation of reproducible numbers of neurons. Recent work has addressed these questions in the mouse and chick spinal cord, revealing a two-phase model for growth and patterning (Kicheva and Briscoe, 2015; Kicheva et al., 2014). In short, these studies demonstrate that the pattern of NP domains is established at early developmental stages, when the size of the tissue is small and positions within morphogen gradients can be decoded with high precision (Fig. 2A). Although single morphogen gradients provide sufficient positional information to account for the observed precision in patterning close to the poles, only the combined ventral Shh and dorsal BMP signalling gradients appear to give sufficient accuracy to explain the observed precision of patterning along the entire DV length (Zagorski et al., 2017). Then, as the tissue grows and the signalling outputs of morphogen pathways become progressively refined to the poles (Balaskas et al., 2012; Zagorski et al., 2017), the pattern of progenitor domains is maintained by cross-repressive interactions between GRN components and relative domain sizes are mainly determined by their differentiation dynamics (Kicheva et al., 2014) (Fig. 2B,C).

The maintenance of progenitor domains does not mean that the identities of NPs are inflexible. Indeed, evidence from lineage-tracing studies suggests that cells can switch their identity, with cells in adjacent progenitor domains being frequently labelled by

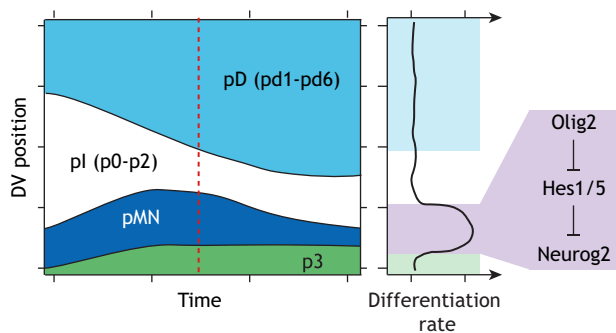
A Early: morphogen gradients delineate gene expression domains with high precision



B Late: morphogen gradients recede to the poles; cross-repression between TFs maintains progenitor domains



C Domain sizes are determined by unequal differentiation rates



D Upregulation of domain-specific progenitor markers prevents specification of mixed neuronal identities

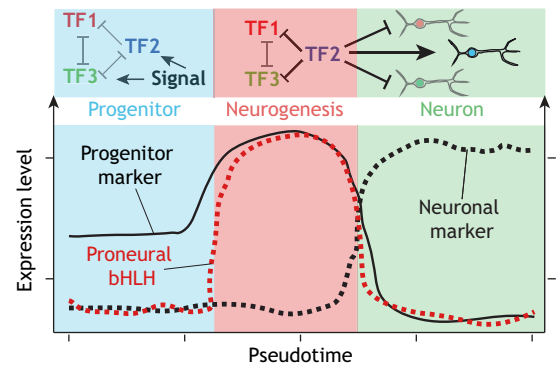


Fig. 2. Dynamics of patterning and growth of the developing spinal cord. (A) At early developmental stages, the combined BMP and Shh activity gradients provide sufficient positional information along the entire DV length of the spinal cord. (B) At later developmental stages, morphogen activity gradients recede to the poles of the spinal cord, and domain boundaries are maintained by cross-repressive interactions between patterning TFs. (C) Relative domain sizes in the spinal cord change over time (left plot). This is due to unequal rates of neuronal differentiation; for example, the terminal differentiation rate in MN progenitors is much higher than that of adjacent progenitor domains, due to Olig2 repressing Hes1/5, which promotes Neurog2 expression (shown in the plot on the right). (D) Scheme depicting gene expression dynamics along the neuronal differentiation trajectory. The upregulation of domain-specific progenitor markers during neurogenesis represses alternative progenitor and neuronal gene expression programmes and thereby prevents the specification of mixed neuronal identities.

the lineage tracer in such experiments (Dessaud et al., 2007, 2010). Furthermore, evidence in zebrafish and mouse has revealed that MNs and oligodendrocytes, which are both specified from Olig2-positive progenitors, seem to derive from different lineages in these systems (Ravanelli and Appel, 2015; Wu et al., 2006), suggesting that cells can switch their identities even at relatively late stages. This, however, poses a problem for the GRN in NPs. On the one hand, it has to be sufficiently flexible to permit cell-state transitions in response to changing extracellular cues, but on the other hand it has to be stable enough to prevent the specification of mixed neuronal identities. Recent characterization of the dynamics of gene expression during neurogenesis might provide a solution to this problem (Delile et al., 2019; Sagner et al., 2018). By reconstructing the gene expression dynamics underlying neurogenesis in multiple domains using scRNA-seq data, it was shown that domain-specific TFs (Olig3 in dp1-3, Dbx1 in p0, Olig2 in pMN and Nkx2.2 in p3 progenitors) undergo a characteristic upregulation at the onset of neurogenesis (Fig. 2D). Thus, lower levels of progenitor TFs in early non-neurogenic progenitors could provide sensitivity to morphogen inputs by facilitating progenitor-state transitions, whereas the increasing expression levels associated with the onset of neurogenesis consolidate progenitor identity and ensure robust differentiation into a specific neuronal subtype (Fig. 2D).

The rate and timing of neuronal differentiation have been linked to dynamics of morphogen signalling and the regulatory interactions downstream of patterning TFs in the spinal cord. This is best understood for MN progenitors (pMN) in the ventral spinal cord. The onset of neurogenesis in these cells seems to be directly controlled by the dynamics of Shh signalling (Saade et al., 2013). At early developmental stages, Shh signalling is high in pMN cells, which promotes symmetric cell divisions that expand the progenitor pool. Later, as levels of Shh signalling decrease, progenitors begin to differentiate. At the molecular level, high levels of Shh signalling maintain symmetric proliferative cell divisions by promoting the expression of the docking protein pericentrin, which allows the symmetric distribution of protein kinase A on both mother and daughter centrosomes (Saade et al., 2017). Whether similar principles apply to other NP domains and other morphogen pathways is currently unclear. Furthermore, although this mechanism explains the time of onset of neurogenesis in the spinal cord, it does not provide a mechanism for why pMN cells differentiate at a higher rate than progenitors in adjacent domains (Ericson et al., 1992; Kicheva et al., 2014; Novitsch et al., 2001) (Fig. 2C). In this regard, the TF Olig2, which defines pMN identity, appears to promote MN differentiation by directly repressing Hes TFs, which are known antagonists of neurogenesis. Repression of Hes TFs in turn favours expression of the proneural bHLH TF

Neurogenin2 (Neurog2) in these cells (Sagner et al., 2018) (Fig. 2C). In summary, global signalling dynamics and domain-specific transcriptional regulation downstream of GRN components dictate the timing and rates of neuronal differentiation in the developing spinal cord. Notably, similar to the Olig2-expressing pMN domain, the Olig3-expressing domain in the dorsal spinal cord shrinks in size between E9.5 and E11.5 in the mouse spinal cord (Müller et al., 2002), raising the possibility that Olig3 may play a similar role in these cells.

Taken together, opposing morphogen gradients pattern the spinal cord along its DV axis. Only at early developmental stages do these gradients provide sufficient accuracy to pattern the entire axis with the observed precision. At later developmental stages, the gene expression boundaries that delineate distinct progenitor domains are maintained by cross-repressive interactions between domain-specific TFs, whereas the size of the individual progenitor domains is mainly determined by their differentiation rates. These rates are, in turn, defined by the dynamics of morphogen signalling and domain-specific interactions between components of the patterning GRN and the neurogenic transcriptional programme.

Specificity of neuronal subtype identity is determined by TF codes

The TF codes that define distinct progenitor identities specify distinct types of neurons along the DV axis of the spinal cord. Neurogenesis throughout the nervous system depends on a small set of proneural bHLH TFs, in particular Ascl1 (also known as Mash1), neurogenins (Neurog1, Neurog2, Neurog3) and Atonal homologues (Atoh). In the embryonic spinal cord, these TFs are expressed in specific domains, and distinct progenitor to neuron transitions depend on the presence of different proneural bHLH TFs (Fig. 3A). This is best exemplified in the dorsal spinal cord (Lai et al., 2016). Here, the generation of dI1 neurons depends on Atoh1 whereas dI2 neuron generation depends on Neurog1. Furthermore, genetic ablation of Atoh1 leads to a loss of dI1 neurons and the expansion of dI2 neurons (Gowan et al., 2001). Similarly, the generation of inhibitory dI4 and excitatory dI5 neurons requires Ptf1a and Ascl1, respectively, and ablation of either TF results in the excess generation of the other neuronal cell type (Borromeo et al., 2017; Mizuguchi et al., 2006; Wildner et al., 2006). In the ventral spinal cord, the specification of neuronal identities is also mediated by

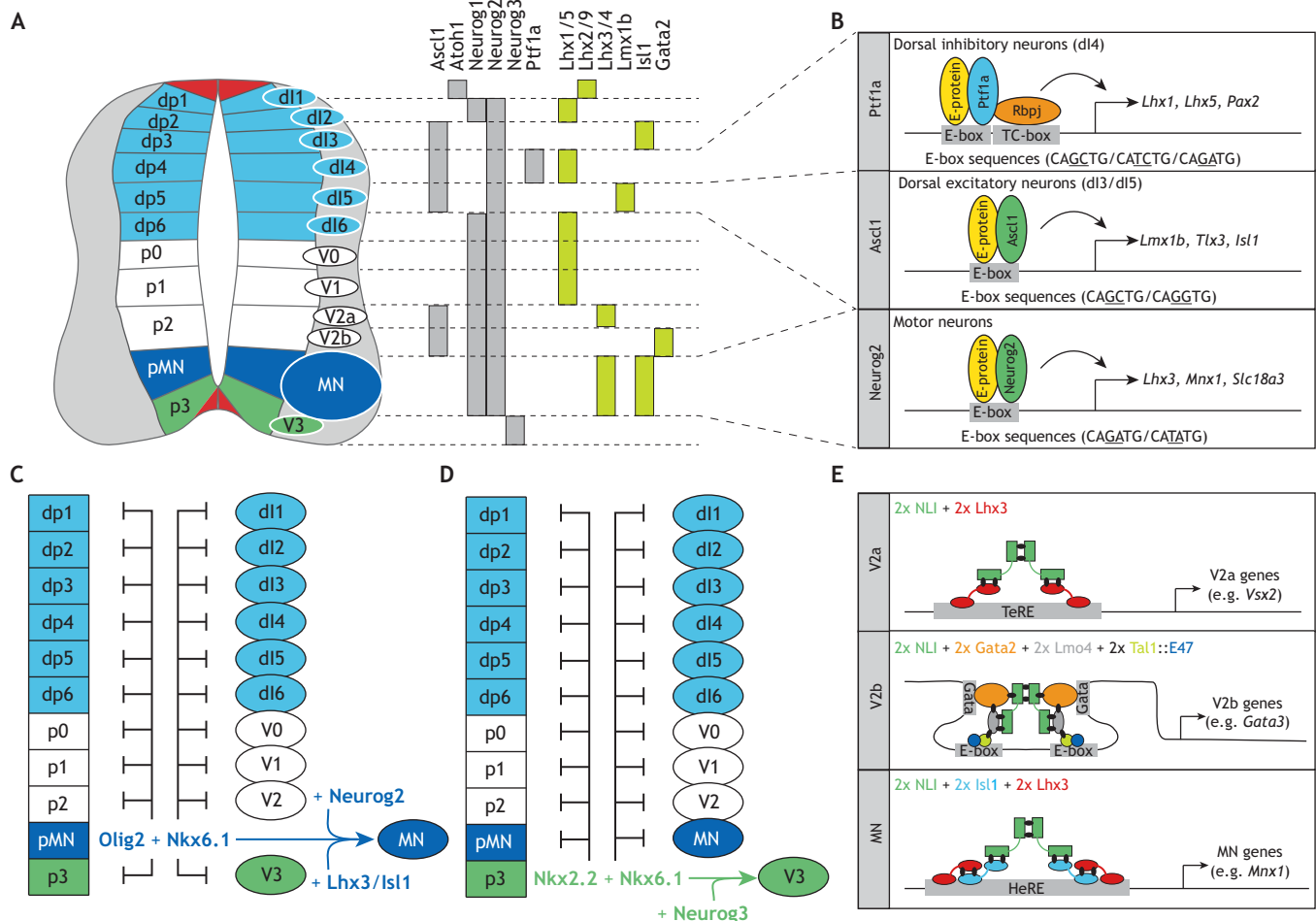


Fig. 3. Establishment of neuronal diversity in the spinal cord. (A) Schematic cross-section of an embryonic spinal cord. Patterned expression of proneural bHLH (grey) and LIM HD (green) TFs underlies the establishment of the cardinal types of neurons in the spinal cord. Note that Gata2 is not a LIM HD protein, but is depicted for completeness, as it is part of a LIM protein complex in differentiating V2b neurons. (B) These different proneural bHLH TFs show sequence specificity for different E-box sites and induce distinct downstream transcriptional programmes. E-box sequences according to Borromeo et al. (2017) and Aydin et al. (2019). (C,D) Widespread repression of alternative transcriptional programmes by Olig2/Nkx6.1 and Nkx2.2/Nkx6.1 provides competency to NPs to differentiate into MNs (C) or V3 interneurons (D), respectively. (E) Different LIM protein complexes [consisting of NLI and Lhx3 in V2a; NLI, Gata2, Lmo4, Tal1 and E47 (Tcf3) in V2b; and NLI, Lhx3 and Isl1 in MNs] bind to distinct enhancer elements and thereby induce the correct neuronal gene expression programmes in V2a, V2b and MNs.

distinct TFs: V2a neurons depend on *Ascl1*, MNs on *Neurog2*, and V3 neurons on *Neurog3* (Carcagno et al., 2014; Parras et al., 2002; Scardigli et al., 2001; Sommer et al., 1996). Furthermore, replacement of the *Ascl1* coding sequence with *Neurog2* leads to a specific depletion of V2a neurons, whereas replacement of the *Neurog2* coding sequence with *Ascl1* leads to a ventral expansion of V2a neurons (Parras et al., 2002). Taken together, these results demonstrate that proneural bHLH TFs act redundantly to induce neurogenesis, but have protein-specific functions for converting specific progenitors into neuronal cell identities. In line with this interpretation, different proneural bHLH TFs have been shown to display distinct sequence preferences and bind to distinct sites in the genome (Aydin et al., 2019; Borromeo et al., 2017) (Fig. 3B). These distinct binding patterns result in the activation of distinct neuronal expression programmes, including the direct activation of numerous TFs involved in neuronal subtype specification. Thus, proneural bHLH TFs have the intrinsic ability to activate distinct neuronal gene expression programmes and controlling the pattern of their expression is an important mechanism by which the TF code that defines progenitor domains determines which types of neurons can be formed from these domains.

Each proneural bHLH protein specifies neuronal cell identities at multiple places and times throughout animal development. Thus, additional molecular mechanisms must exist to ensure the context-dependent activation of the correct gene expression programmes. In this regard, techniques such as ChIP-seq have begun to uncover broader programmes of transcriptional regulation by the TFs that define progenitor identity in the spinal cord. For example, the TFs *Nkx6.1*, *Nkx2.2* and *Olig2*, which are essential for establishing the most ventral progenitor identities, repress not only the transcriptional programmes of adjacent progenitors and neuronal subtypes (Fig. 3C,D) but also the programmes associated with all alternative progenitor and neuron fates (Kutejova et al., 2016; Nishi et al., 2015). Similarly, activity of the TF *Prdm13* in the dorsal spinal cord is required for inhibiting the expression of ventral cell fate specification markers downstream of *Ptf1a* (Mona et al., 2017). This leads to a model in which proneural bHLH proteins have the ability to induce genes associated with a range of neuronal subtypes. This specificity is refined by the TF code that determines NP identity, which ensures the repression of genes associated with inappropriate progenitor and neuronal subtypes in each domain (Fig. 3C,D). The combined activity results in the initiation of domain-specific neuronal gene expression programmes that subsequently elaborate and consolidate the respective neuronal fates.

How this is achieved is arguably best understood for MNs and adjacent V2 neurons (Fig. 3E). Establishment of MN identity depends on a hexameric complex of NLI (nuclear LIM interactor; *LDB1*), *Islet 1* (*Isl1*) and *Lhx3*, whereas adjacent V2a neurons lack the expression of *Isl1*, and form a tetrameric complex consisting of NLI and *Lhx3* (Thaler et al., 2002) (Fig. 3E). These complexes activate the expression of distinct downstream target genes by binding to enhancers that are specific for the hexameric or tetrameric complexes (Lee et al., 2008; Thaler et al., 2002) (Fig. 3E). These targets include those encoding further TFs, such as *Mnx1* in MNs and *Vsx2* (also known as *Chx10*) in V2a neurons, that subsequently consolidate the respective neuronal identities (Arber et al., 1999; Clovis et al., 2016; Thaler et al., 1999). *Neurog2* has been demonstrated to interact with LIM-HD complexes in a phosphorylation-dependent manner (Ma et al., 2008), but the sites bound by these LIM protein complexes appear to be distinct from those bound by pro-neural bHLH TFs. A comparison of *Neurog2*- and *Lhx3/Isl1*-bound regions during reprogramming of embryonic

stem cells (ESCs) to MNs revealed that *Lhx3/Isl1* co-occupied sites specific for MN generation, whereas *Neurog2* controls genes more generally associated with neuronal differentiation (Velasco et al., 2017). Similar LIM protein complexes have been identified in multiple other neuron types, e.g. V2b or visceral motor neurons (Joshi et al., 2009; Mazzoni et al., 2013b), and most neuronal domains in the spinal cord express LIM HD TFs, suggesting this may be a common principle (Fig. 3A,E). Thus, the patterned induction of distinct transcriptional activator complexes results in the activation of distinct gene expression programmes in different neuronal domains.

The picture emerging from these analyses suggests that the TF code that specifies distinct progenitor identities promotes fidelity in the specification of neuronal identities by different mechanisms: via induction of domain-specific transcriptional activator complexes that activate domain-specific transcriptional programmes, and via the concomitant repression of alternative transcriptional programmes by the TFs that determine progenitor identity.

Further diversification of neuronal identity

The mechanisms guiding NP differentiation into post-mitotic neurons explain how the different cardinal types of neurons are formed, but these subsequently diversify further into multiple molecularly and functionally distinct subtypes. Recent studies suggest that a number of signalling pathways are involved in this diversification. Cell-cell interactions mediated by Notch signalling, for example, mediate the subdivision of V2 neurons into excitatory V2a and inhibitory V2b neurons. V2a neurons express the Notch ligand delta-like 4 (*Dll4*), and V2b neurons express Notch receptors and require Notch signalling for their specification (Peng et al., 2007; Skaggs et al., 2011). Recent evidence in zebrafish has revealed that V2s neurons, which, similar to mammalian V2c neurons, express *Sox1* orthologues, require Notch signalling for their generation from a combined postmitotic V2b/s precursor (Gerber et al., 2019). Thus, Notch-dependent cell-cell interactions sequentially subdivide the V2 lineage. Similarly, Notch signalling has been implicated in regulating the fate of excitatory and inhibitory neurons in the dorsal spinal cord at later developmental stages (Mizuguchi et al., 2006), the columnar identity of MNs (Tan et al., 2016), and V1 interneuron subtype formation (Hoang et al., 2018).

Diffusible signals have also been demonstrated to be important for the diversification of neuronal identities. For example, retinoic acid (RA) produced by early-born aldehyde dehydrogenase 1 family member A2 (*Aldh1a2*)-expressing lateral motor column (LMC) neurons recruits later-born MNs into the LMC, inducing downregulation of *Isl1* and induction of *Lhx1* in these neurons, dividing LMC neurons into medial and lateral identities (Sockanathan and Jessell, 1998). Thus, both cell-cell interactions and diffusible signals expand neuronal diversity in the spinal cord. However, these mechanisms have only been demonstrated to control the establishment of major subtypes in the spinal cord, and are probably not sufficient to account for the large number of molecularly and functionally distinct neuronal subtypes observed in recent studies. In the following sections, we describe how neuronal diversity in the spinal cord is increased by patterning systems that subdivide spinal cord neurons along two orthogonal axes: the AP axis and in time.

Hox genes pattern the spinal cord along the AP axis

NPs in the spinal cord acquire distinct identities along the AP axis. These identities are conferred by Hox genes, which subdivide progenitors and neurons in the spinal cord into three broad domains:

Hox4-7 genes define cervical, Hox9 genes thoracic and Hox10-11 genes lumbar cell identities (Philippidou and Dasen, 2013) (Fig. 4A,B). These identities are established in response to transient signals present in the early embryo, most notably Wnt, RA, fibroblast growth factors (FGFs) and growth differentiation factor 11 (Gdf11) (Bel-Vialar et al., 2002; Dasen et al., 2003; Ensini et al., 1998; Liu et al., 2001). Later, similar to the delineation of progenitor domains along the DV axis, they are maintained by cross-repressive interactions between these genes. Below, we focus on recent studies that have provided insights into how Hox genes function in MNs, in which Hox genes are essential for the acquisition of distinct columnar identities and for MN pool organization (Dasen et al., 2003, 2005). Although most other progenitor and neuronal populations in the spinal cord express distinct sets of Hox genes along the AP axis (Dasen et al., 2005), it is currently unclear to what extent similar mechanisms function in these cells. Notably, however, several recent studies have demonstrated that Hox genes also control the acquisition of distinct V1 and spinocerebellar tract subtype identities (Baek et al., 2019; Coughlan et al., 2019preprint; Sweeney et al., 2018), suggesting that similar principles may apply to most neuronal populations in the spinal cord.

RA promotes cervical cell identities by inducing Hox4-7 genes, whereas FGF promotes posteriorization by inducing thoracic Hox genes (Dasen et al., 2003). Lastly, the activity of both FGFs and Gdf11 is required for establishing the most posterior cell identities

(Liu et al., 2001). How do these signals act? RA induces ‘anterior’ Hox genes by recruiting retinoic acid receptors (RARs) to genomic sites located in the 3’ end of Hox clusters (Mahony et al., 2011), and promotes rapid removal of the repressive chromatin mark histone 3 lysine27 trimethylation (H3K27me3) and removal of polycomb repressive complex (PRC) 1 and PRC2 (Mazzoni et al., 2013a). Further posteriorization depends on FGF and Wnt signalling, most probably at a state before cells acquire a neural identity (Gouti et al., 2014; Lippmann et al., 2015; Mazzoni et al., 2013a; Metzis et al., 2018). However, ectopic activation of FGF signalling in NPs induces an anterior shift in the expression of ‘posterior’ Hox genes, suggesting that axial identity is, at least to some degree, plastic in neural cells (Bel-Vialar et al., 2002; Dasen et al., 2003). At the molecular level, these signals lead to the expression of Cdx TFs that, together with FGF signalling, lead to the removal of H3K27me3 from posterior Hox gene loci (Mazzoni et al., 2013a).

The transient nature of the posteriorizing signals raises the question of how axial identity is maintained at later stages. Distinct mechanisms have been described. Cross-repressive interactions between pairs of Hox genes delineate specific expression domains along the AP axis (Dasen et al., 2005). For different Hox gene pairs, the boundaries between these domains occur at distinct positions along the AP axis (Fig. 4B). Thus, similar to the delineation of specific progenitor identities by partially overlapping TF expression domains along the DV axis, Hox genes establish a combinatorial code in which AP position is defined by the co-expression of specific sets of Hox genes. These patterns of gene expression define distinct motor columns and pools that innervate specific muscles (Dasen and Jessell, 2009; Philippidou and Dasen, 2013) (Fig. 4B). These include the phrenic motor column (PMC), which depends on Hox5 genes at anterior cervical levels and innervates the diaphragm (Philippidou et al., 2012), the LMC at posterior cervical and lumbar levels, which innervates limb muscles and is characterized by the expression of Hox6 and Hox10/11 genes, respectively (Lacombe et al., 2013; Lin and Carpenter, 2003; Wu et al., 2007), and the preganglionic and hypaxial motor columns that innervate sympathetic ganglia and hypaxial muscles, which depend on Hoxc9 expression at thoracic levels (Jung et al., 2010). These columns can be further subdivided into specific pools. Co-expression of Hoxc6 and Hoxc8 in posterior cervical LMC neurons defines, for example, the *Etv4* (also known as *Pea3*)-positive motor pool that innervates specific muscles in the appendages (Lacombe et al., 2013; Livet et al., 2002; Vermot, 2005). Similarly, co-expression of Hoxc8 and Hoxc9 in the most posterior cervical LMC neurons defines digit-innervating MNs (Mendelsohn et al., 2017). Besides these cross-repressive interactions, maintenance of these boundaries depends on PRC1, but not PRC2 (Golden and Dasen, 2012). Depletion of the PRC1 component *Bmi1* leads to the ectopic expression of Hoxc9 and the conversion of cervical LMC neurons to thoracic preganglionic motor column (PGC) neurons, whereas elevating *Bmi1* levels extinguishes Hoxc9 expression at thoracic levels and converts PGC neurons to LMC neurons.

In summary, transient signals in the early embryo pattern the spinal cord by inducing Hox gene expression domains along its AP axis. Hox cross-repression and PRC1 activity subsequently refine and maintain the borders between these expression domains. The resulting combinatorial patterns of Hox gene co-expression define specific positions along the AP axis and lead to the sorting of MNs into distinct columns and pools, and confer distinct targeting specificities to them. Future experiments are needed to investigate whether similar principles apply to other neuronal populations in the spinal cord.

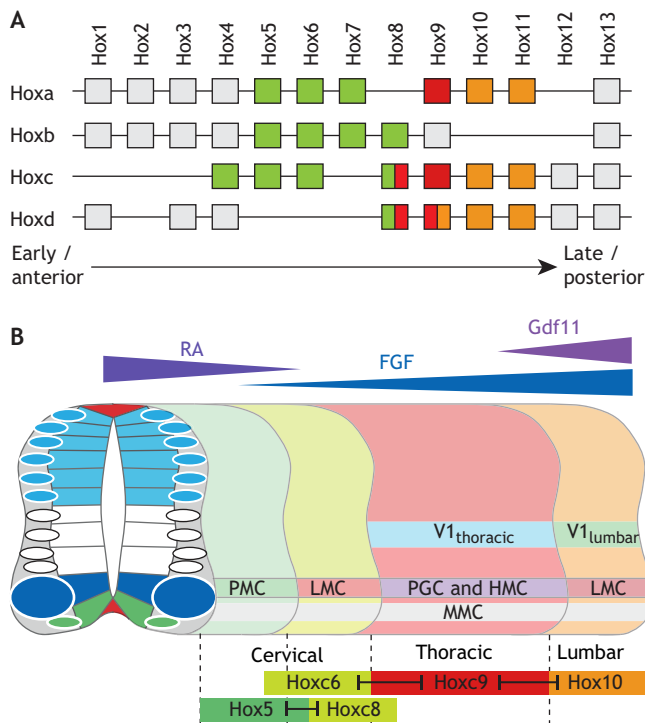


Fig. 4. Hox genes pattern the spinal cord along the AP axis.

(A) Scheme depicting the four Hox gene clusters and 13 paralogous groups. Colour-coding represents involvement of Hox genes in establishing distinct neuronal cell fates along the AP axis of the spinal cord. Green, red and orange boxes indicate Hox genes expressed at cervical, thoracic and lumbar regions of the spinal cord, respectively. (B) Opposing morphogen gradients induce distinct patterns of Hox gene expression along the AP axis of the spinal cord. Cross-repression between pairs of Hox genes delineates specific expression domains and defines distinct neuronal subtypes along the AP axis. HMC, hypaxial motor column; MMC, medial motor column.

Temporal mechanisms contribute to the generation of neuronal diversity

Temporal mechanisms underlie the diversification of neuronal subtypes in multiple systems throughout the animal kingdom (Holguera and Desplan, 2018). Furthermore, in multiple systems, the birth order of neurons has been demonstrated to underlie specificity in neuronal connectivity and circuit formation (Deguchi et al., 2011; Imamura et al., 2011; Tripodi et al., 2011). Thus, temporal mechanisms not only contribute to the generation of neuronal diversity, but also provide a means for the ordered establishment of neuronal circuitry.

Temporal mechanisms also play a key role in establishing distinct cell identities in the spinal cord. In general, the timing of developmental events varies along the AP axis of the spinal cord, with developmental events typically occurring at progressively later developmental stages in more posterior regions of the spinal cord. Furthermore, similar to other regions of the CNS, NPs in the spinal cord first give rise to neurons and later to glia. This temporal switch depends on the sequential induction of SoxE and NFI family members (Deneen et al., 2006; Kang et al., 2012; Stolt et al., 2003, 2005), and is regulated by multiple signalling pathways, most notably Notch (Sugimori et al., 2007; Taylor et al., 2007), FGF (Gaber et al., 2013; Guillemot and Zimmer, 2011) and TGF β signalling (Dias et al., 2014). Temporal stratification of neuronal subtypes has also been documented in multiple neuronal domains, e.g. in MNs (Hollyday and Hamburger, 1977; Sockanathan and Jessell, 1998), V1 neurons (Benito-Gonzalez and Alvarez, 2012; Stam et al., 2012), dorsal excitatory and inhibitory neurons (Müller et al., 2002), and premotor interneurons that synapse onto MNs innervating flexor and extensor muscles (Tripodi et al., 2011). Only in a few cases have the signals and molecular markers that subdivide these temporal populations been described. These include the expression of *Onecut* and *Foxp* family members in V1 neurons, which subdivide this population into Renshaw and non-Renshaw

cells (Stam et al., 2012), the RA-mediated induction of *Lhx1* in lateral LMC neurons (Sockanathan and Jessell, 1998), and the induction of *Gbx1* in late-born inhibitory neurons in the dorsal spinal cord (John et al., 2005).

Using single cell transcriptional profiling, cohorts of co-regulated TFs that appear to provide a temporal code that subdivides neurons throughout the DV axis were recently identified (Delile et al., 2019) (Fig. 5A,B). Early-born neurons (before E10 at cervical levels) from most domains express *Onecut* family TFs, whereas neurons born between E10 and E11 express *Pou2f2* and *Zfhx2-4*. In contrast, late-born neurons, generated after E11.5, express *Nfia*, *Nfib*, *Neurod2* and *Neurod6* (Fig. 5A). Notably, many of these TFs have previously been shown to label subpopulations of neurons that occupy specific positions in the spinal cord. Furthermore, in a few cases, phenotypes following their mutation have been described. For instance, *Onecut* TFs have been shown to label neuronal subsets in most domains in the spinal cord, and distinct functions for them in several neuronal classes have been described (Francius et al., 2013; Harris et al., 2019; Kabayiza et al., 2017; Roy et al., 2012; Stam et al., 2012). *Onecut* TFs are also required for the generation of Renshaw cells (Stam et al., 2012), to maintain *Isl1* expression in medial LMC neurons at limb levels, and to control the ratio of somatic and visceral MNs at thoracic levels of the spinal cord (Roy et al., 2012). Similarly, *Zfhx3* and *Nfib* have been demonstrated to divide V2a neurons into lateral and medial populations (Hayashi et al., 2018), and *Pou2f2* has been implicated in controlling the lateral migration of these neurons (Harris et al., 2019). Finally, *Neurod2* and *Neurod6* control the expression of neuropeptides in dorsal horn neurons (Bröhl et al., 2008). Taken together, these findings suggest that the temporal TF code is functionally important. Future studies will need to test how the TFs involved in this transcriptional code are linked to each other and how their activities are integrated with the transcriptional programmes that pattern the spinal cord along the DV and AP axes.

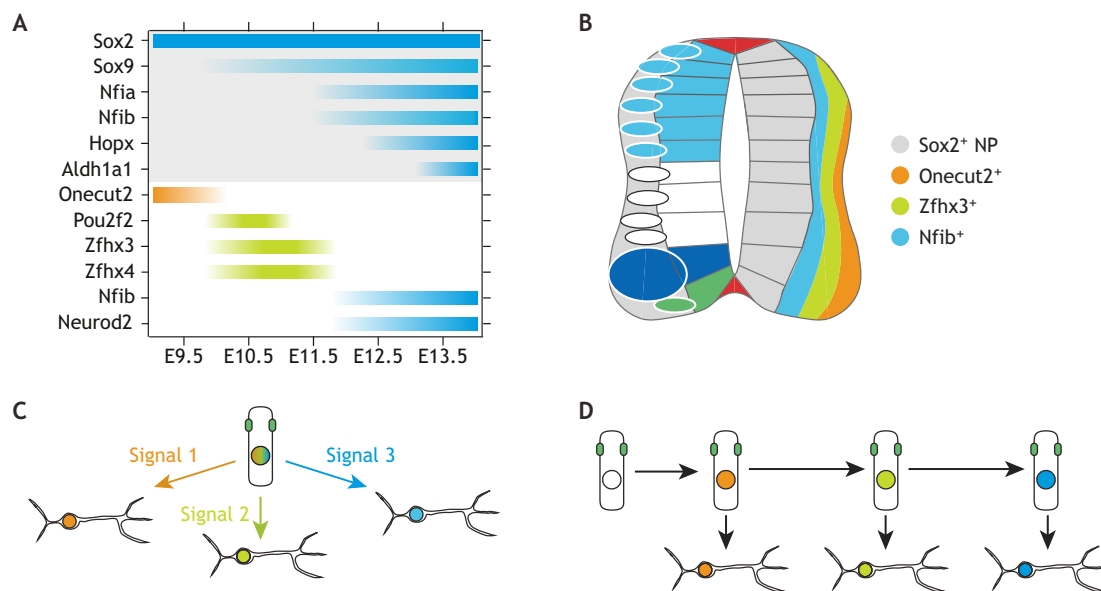


Fig. 5. Temporal stratification of neuronal subtypes. (A) Scheme depicting the sequential induction of the gliogenic programme in NPs (top; grey shading) and the temporal expression of TFs in neurons (bottom). The timings are based on the cervical portion of the spinal cord. (B) The temporal expression of TFs subdivides neurons in the spinal cord, independent of their DV domain of origin, based on their time point of birth. For instance, *Onecut2* labels neurons born before ~E10, *Zfhx3* labels neurons born between E10 and E11.5, and *Nfib* labels neurons born after E11.5. (C,D) Different models for the generation of neuronal diversity by temporal mechanisms. Different extracellular cues acting at distinct time points can induce the differentiation of an uncommitted NP into different neuronal cell types (C). Alternatively, NPs may progressively change their competence to give rise to specific neuronal subtypes over time (D).

These findings raise the question of how the temporal TF code is established. One possibility is that a temporal sequence of external signalling molecules could bias the differentiation of uncommitted NPs towards distinct neuronal cell identities (Fig. 5C). Such a model has been proposed for TGF β -mediated induction of the serotonergic programme in p3 progenitors in the vertebrate hindbrain (Dias et al., 2014). Indeed, multiple signalling pathways show transient activation throughout the neurogenic period in the spinal cord, and thus might contribute to the generation of distinct neuronal subtypes over time. For example, the RA-synthesizing enzyme *Aldh1a2* is downregulated in somites around E10 (Niederreither et al., 1997), which temporally coincides with the point at which neurons switch from expressing *Onecut* family members to expressing *Pou2f2* and *Zfhx2-4*. Consistent with this idea, RA has been shown to promote Renshaw cell identity over other V1 fates and LMC identity in MNs (Hoang et al., 2018; Sockanathan and Jessell, 1998), and both Renshaw neurons and LMC neurons are neuronal subtypes that specifically depend on *Onecut* TFs for their generation (Roy et al., 2012; Stam et al., 2012). Conversely, later neuronal identities may be favoured by signalling pathways that are involved in progenitor maintenance and promote gliogenesis (outlined above).

Alternatively, the gene expression profile of NPs could change over time, which could lead to a succession of competence states (Fig. 5D). Such a mechanism would resemble that observed in neuroblast lineages in the *Drosophila* embryo (Doe, 2017). Potential candidate mechanisms for mediating such a ‘competence cascade’ in NPs could involve the sequential activation of the gliogenic programme by *Sox9* and *Nfia/b*. *Sox9* starts to be expressed in NPs in the mouse neural tube between E9.5 and E10.5 (Stolt et al., 2003) and thus could be a good candidate for mediating the switch from *Onecut*- to *Pou2f2*-expressing neuronal subtypes (Fig. 5A). Similarly, onset of *Nfia/b* expression in NPs occurs between E10.5 and E11.5 (Deneen et al., 2006), and may terminate the phase during which *Zfhx* TFs are expressed in neurons and instead favour the generation of *Nfib*-positive neurons (Fig. 5A). Furthermore, because these TFs are involved in specifying glial cells and late neuronal subtypes, signalling pathways that promote gliogenesis may also favour the generation of *Nfib*-positive neurons, although it is currently unclear whether *Nfib*-expressing NPs generate *Nfib*-expressing neurons or whether additional signals are involved.

Overall, it appears that a wide range of signals and mechanisms may control the temporal progression of neuronal subtypes in the spinal cord. Future work will be required to investigate how these signalling molecules function and to determine the structure of the downstream GRN that controls the temporal progression of neuronal subtypes. Comparison with other temporal patterning systems may help to understand general design principles of this system and reveal the extent to which it is conserved in other regions of the CNS. In this respect, it is noteworthy that early-born neurons in the retina depend on *Onecut* TFs (Sapkota et al., 2014), whereas the latest-born neurons depend on *Nfia/b* for their generation (Clark et al., 2019). This raises the possibility that parts of this temporal patterning programme also apply to other regions of the CNS.

Novel techniques to study the emergence and function of neuronal diversity in the spinal cord

The emergence of novel sequencing methods that allow the profiling of transcriptomes and chromatin states from thousands of individual cells is revolutionizing cell and developmental biology. These techniques have enabled the generation of high-

resolution atlases of adult and developing tissues (Clark et al., 2019; Cusanovich et al., 2018; Muñoz-Manchado et al., 2018; Rosenberg et al., 2018; Saunders et al., 2018; Shekhar et al., 2016), and even entire developing embryos (Cao et al., 2019; Pijuan-Sala et al., 2019). Furthermore, the collection of time-course data has enabled the reconstruction of gene expression dynamics underlying cell state transitions and along differentiation trajectories with unprecedented resolution (Kester et al., 2018; La Manno et al., 2018; Trapnell et al., 2014; Wagner et al., 2016). Several large-scale molecular profiling studies have already revealed a much greater molecular diversity of cell types than previously anticipated in the spinal cord (Delile et al., 2019; Häring et al., 2018; Hayashi et al., 2018; Rosenberg et al., 2018; Sathiyamurthy et al., 2018; Zeisel et al., 2018). Similarly, several studies have resolved the gene expression dynamics that underlie neurogenesis of *in vitro* differentiated MNs at much greater resolution than previous embryological assays (Briggs et al., 2017; Rizvi et al., 2017; Sagner et al., 2018). Remarkably, these studies revealed that neurogenesis of *in vitro* generated MNs accurately recapitulates the known transcriptional changes that occur *in vivo* and even identified several previously overlooked features. Together, these studies have provided insight into the spatial and temporal patterns of gene expression in spinal cord cells, thereby establishing a framework for the analysis of perturbations that affect gene expression and function in these cells. These studies have also identified previously unknown neuronal subtypes, provided novel molecular markers for them, and implicated specific TFs in their specification, thereby identifying genetic handles with which to investigate their function.

Novel *in vitro* differentiation and reprogramming approaches have also greatly expanded our understanding of cell fate specification and neuronal circuit formation in the spinal cord. Stem cell-based *in vitro* differentiation and reprogramming assays have started to reveal the binding sites of TFs, how they affect chromatin dynamics, and how they specify cell fates (Aydin et al., 2019; Kutejova et al., 2016; Lai et al., 2011; Lee et al., 2008; Mazzoni et al., 2013b; Metzis et al., 2018; Nishi et al., 2015; Rhee et al., 2016; Velasco et al., 2017). Furthermore, protocols for the *in vitro* differentiation of neurons from most spinal cord domains have been established (Butts et al., 2017; Gouti et al., 2014; Gupta et al., 2018; Hoang et al., 2018; Sternfeld et al., 2017; Wichterle et al., 2002; Xu and Sakiyama-Elbert, 2015). These now provide the means to further elucidate how morphogen signalling dynamics and the binding patterns of morphogen effectors and patterning TFs specify distinct progenitor and neuronal cell identities. Furthermore, two recent studies that generated artificial locomotory circuits by mixing MNs with different spinal interneuron populations have revealed design principles that underlie the formation of spinal locomotory circuits and the function of different interneuron types within them (Hoang et al., 2018; Sternfeld et al., 2017). In the long run, differentiation and reprogramming protocols that generate cells that closely mimic their *in vivo* counterparts are likely to contribute to the development of novel *in vitro* disease models, e.g. for drug testing or to uncover the molecular basis of movement disorders such as amyotrophic lateral sclerosis (ALS), and may even enable novel therapeutic approaches.

In recent years, ‘organogenesis in a dish’ approaches that rely on the ability of stem cells to self-organize into three-dimensional structures resembling developing organs have further enabled the discovery of developmental design principles. These approaches also allow investigation of the molecular and cellular basis of complex pathologies, including microcephaly, autism spectrum disorders and schizophrenia (Di Lullo and Kriegstein, 2017; Fatehullah et al., 2016; Lancaster and Knoblich, 2014). Spinal

organoids have been described in the recent literature (Duval et al., 2019; Hor et al., 2018; Ogura et al., 2018), but so far do not appear to recapitulate the generation of the entire DV axis. Similarly, neural cysts have been described that spontaneously break symmetry by inducing Shh expression. These cysts generate a wide range of progenitor types along the DV axis (Meinhardt et al., 2014; Ranga et al., 2017); however, to what extent these produce the neuronal circuitry typical of the spinal cord is unclear. Finally, recently described ‘gastruloids’ have been shown to contain dorsal and ventral neural and mesodermal cell types, although these cells are not organized within structures that resemble the neural tube or somites (Beccari et al., 2018). Strikingly, gastruloids elongate and display colinear Hox gene expression *in vitro*, suggesting that these structures partially mimic the trunk of developing mammalian embryos. Future studies will undoubtedly reveal whether these structures are capable of forming functional neuronal networks or axonal projections from MNs to muscles. If they do, they will be promising candidates to study how neuronal circuitry is established during development and for *in vitro* modelling of the spinal cord.

Conclusions

The developing spinal cord has served as a leading model system to understand the molecular mechanisms underlying cell fate specification and the establishment and function of neuronal circuits. Ground-breaking work by multiple groups has revealed how neuronal cell identities are established by GRNs that delineate NP identities along the DV and AP axes of the embryonic spinal cord by integrating the levels, duration and dynamics of multiple signalling pathways. Neurons further diversify in response to cell-cell interactions, diffusible signals and along a temporal axis. These mechanisms provide a general framework for understanding neuronal subtype diversification, but they are probably just the tip of the iceberg and other mechanisms likely exist that drive further neuronal diversification. Molecular classification of V1 diversity has revealed the existence of ~50 distinct subtypes at lumbar and ~38 distinct subtypes at thoracic levels, with only a few V1 subtypes present at both axial levels (Bikoff et al., 2016; Gabitto et al., 2016; Sweeney et al., 2018). Similarly, characterization of neuronal diversity in the dorsal horns by scRNA-seq has revealed the existence of 15 distinct subtypes of inhibitory and excitatory neurons each (Häring et al., 2018). The precise position of neurons in the spinal cord serves as an important determinant for subtype specification, and the proximity of multiple neuronal subtypes may generate local signalling environments in which neurons experience a combination of signals that expands their diversity. In this view, the distribution of neuronal subtypes in the spinal cord is a consequence of an interplay between spatial and temporal patterns of neurogenesis that progressively constructs the spinal cord. Finally, the formation of synaptic contacts with specific types of neurons, and thus the formation of neuronal circuitry itself, could serve as a mechanism to establish distinct subtype identities. Further exploration of the molecular mechanisms underlying the formation of neuronal subtypes and characterization of their function within spinal neuronal networks may lay the foundation for the development of novel therapies for the treatment of movement disorders, spinal injuries and chronic pain conditions.

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Competing interests

The authors declare no competing or financial interests.

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