NEURONAL SPECIFICATION IN THE SPINAL CORD: INDUCTIVE SIGNALS AND TRANSCRIPTIONAL CODES

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Neural circuits are assembled with remarkable precision during embryonic development, and the selectivity inherent in their formation helps to define the behavioural repertoire of the mature organism. In the vertebrate central nervous system, this developmental program begins with the differentiation of distinct classes of neurons from progenitor cells located at defined positions within the neural tube. The mechanisms that specify the identity of neural cells have been examined in many regions of the nervous system and reveal a high degree of conservation in the specification of cell fate by key signalling molecules.

PROPRIOCEPTION
The part of the somatosensory system that relays information about trunk and limb position.

ROSTROCAUDAL
The axis of the vertebrate embryo that runs from head to tail. Also referred to as the anterior–posterior axis at early stages of neural development.

DORSOVENTRAL
The axis of the vertebrate embryo that runs from back to stomach.

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Many telling insights into vertebrate neuronal patterning have come from attempts to trace the pathways by which inductive signals commit cells to specific fates[1–5]. This article summarizes progress in defining some of these pathways through the analysis of cell fate specification in just one region of the central nervous system (CNS), the spinal cord. The physiology and connectivity of neurons within the mature spinal cord have been particularly well delineated[6], providing a clear end point for studies of the development of these circuits. Spinal neurons serve two main functions: they relay cutaneous sensory information to higher centres in the brain and they integrate proprioceptive input and motor output. These two functional systems are also segregated anatomically. The neurons and circuits that process cutaneous sensory input are concentrated in the dorsal spinal cord, whereas circuits involved in PROPRIOCEPTION and motor control are largely confined to the ventral spinal cord[6]. Progress in defining mechanisms of dorsal patterning has been discussed elsewhere[7], and so this article focuses solely on neuronal specification in the ventral spinal cord.

The allocation of cell fate in the spinal cord, as in other regions of the CNS, depends on two signalling systems that are activated together with the more basic program of neural induction[8]. These two signalling systems intersect along the ROSTROCAUDAL and DORSOVENTRAL axes of the neural tube to establish a grid-like set of positional cues[9,10]. The position of progenitor cells along these two axes is thought to influence their fate by defining the identity and concentration of inductive signals to which they are exposed. Signalling along the rostrocaudal axis of the neural tube establishes the main subdivisions of the CNS: the forebrain, midbrain, hindbrain and spinal cord[9]. The dorsoventral signalling system has a more prominent role in establishing cell type diversity within each of these rostrocaudal subdivisions[10]. But the diversity of neuronal cell types generated during embryonic development cannot be accounted for solely by the actions of these two signalling systems. In the spinal cord, for example, there is emerging evidence that signals transmitted locally between developing neurons are required to achieve the full repertoire of neuronal subtypes.

Acquisition of spinal cord character

The spinal cord is a caudal structure, but the neural cells from which it derives initially express rostral, forebrain-like characteristics[8,11]. The caudal character of neural cells emerges soon after neural induction, through the reprogramming of cell fates by a series of extrinsic signals. Many classes of secreted factors have been impli-

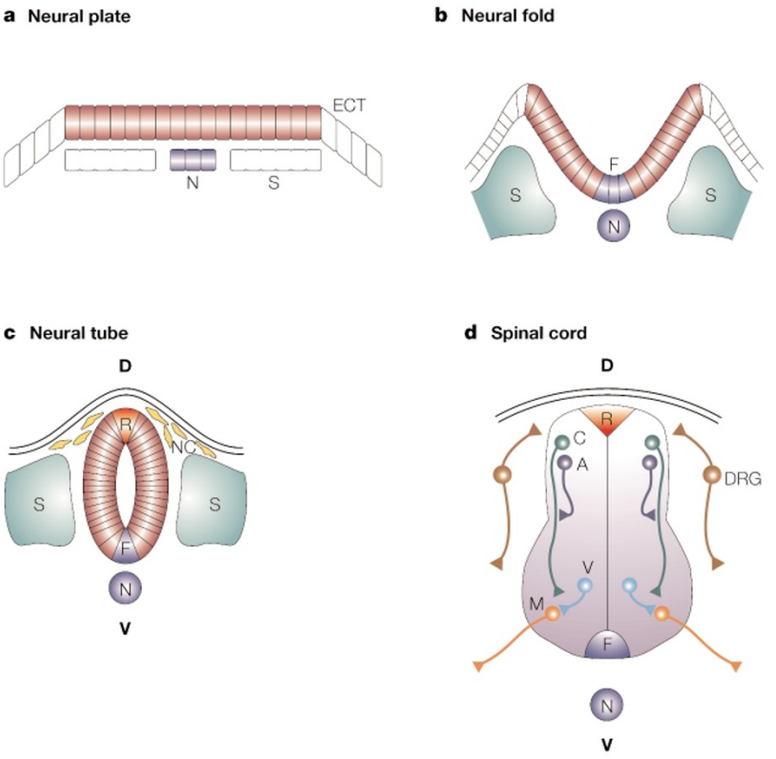

Figure 1 | **Four stages of spinal cord development.** Four successive stages in the development of the spinal cord are shown. **a** | At the neural plate stage, newly formed neural cells are flanked laterally by the epidermal ectoderm (ECT). Notochord cells (N) underlie the midline of the neural plate, and segmental plate mesoderm (S) underlies the lateral region of the neural plate. **b** | At the neural fold stage, floor plate cells (F) are evident at the ventral midline and the somitic mesoderm begins to develop. **c** | At the neural tube stage, roof plate cells (R) begin to differentiate at the dorsal midline, and neural crest cells (NC) start to delaminate from the dorsal neural tube. **d** | During the embryonic development of the spinal cord, distinct sets of commissural (C) and association (A) neurons differentiate in the dorsal half of the spinal cord, and motor neurons (M) and ventral interneurons (V) develop in the ventral half of the neural tube. Dorsal root ganglion (DRG) neurons differentiate from neural crest progenitors. The dorsal (D) and ventral (V) axes are shown in bold.

cated in the acquisition of caudal neural character: fibroblast growth factors (FGFs), retinoids, bone morphogenetic proteins (BMPs), Wnts and a PARAXIAL MESODERM caudalizing (PMC) activity[11–14]. These signals derive from cells in the PRIMITIVE STREAK of gastrula stage embryos or from the posterior paraxial mesoderm.

Many details of the interplay between these factors during rostrocaudal patterning remain obscure, but the emergence of cells of spinal cord character in the chick has been proposed to involve a three-step signalling pathway[14]. In this scheme, the exposure of prospective neural cells to FGFs derived from the primitive streak and to PMC activity imposes a generic caudal neural character. The specification of neural tissue of midbrain or hindbrain character seems to depend in part on the concentration of PMC activity to which cells are exposed. The differentiation of cells of spinal cord character, however, requires the action of a retinoid-mediated signal provided by the prospective caudal paraxial mesoderm[14]. The capacity of the paraxial mesoderm to synthesize retinoids is reflected by its expression of a key retinoid synthetic enzyme, retinaldehyde dehydrogenase-2 (RALDH-2)[15,16]. The RALDH-2 dependent restriction in retinoid synthesis to the caudal paraxial mesoderm seems to be a critical step in establishing the early distinction between neural cells of spinal cord and hindbrain character. Nevertheless, retinoids clearly have later roles in patterning the rostrocaudal axis of the hindbrain[17].

Less is known about the steps that establish rostrocaudal distinctions in cell identity at different segmental levels of the spinal cord. Members of the *Hox-c* and *Hox-d* gene clusters are expressed at different rostrocaudal levels of the spinal cord[18,19], indicating that neural cells at different segmental positions may possess distinct positional values. However, most of the neuronal subtypes generated within the spinal cord are represented at all segmental levels, raising the issue of whether rostrocaudal positional information contributes significantly to the establishment of neuronal subtype identity at spinal levels. Motor neurons represent a striking exception to the apparent uniformity in neuronal subtype identity at different segmental levels, and the signalling pathways that control motor neuron diversity are discussed later in this article.

Cell specification along the dorsoventral axis

The specification of neuronal subtypes in the spinal cord becomes evident with the appearance of distinct cell types at defined positions along the dorsoventral axis of the neural tube (FIG. 1). At early stages of ventral neural tube development, three main classes of cells are generated: floor plate cells — a specialized class of glial cell — differentiate at the ventral midline soon after NEURAL PLATE formation (FIG. 1a, b), whereas motor neurons and interneurons are generated at more dorsal positions (FIG. 1d).

The differentiation of these ventral cell types is triggered by signals provided initially by an axial mesodermal cell group, the notochord, and later by floor plate cells themselves[20] (FIG. 1d). As the floor plate serves as a secondary source of ventral inductive signals and is generated before any neuronal cell type, there has been interest in whether the mechanisms that underlie floor plate differentiation are distinct from those of other ventral cell types. Many studies support the view that floor plate differentiation is mediated by inductive signalling from the notochord[20,21]. An alternative view, however, argues that the floor plate emerges not by induction, but through insertion into the neural plate of a group of floor plate precursors that are set aside in the axial mesoderm before neural plate formation[22]. The merits of these two views have been discussed elsewhere[23,24].

The main signalling activities of the notochord and floor plate are mediated by a secreted protein, Sonic hedgehog (Shh)[21] (FIG. 2a, b). Ectopic expression of *Shh in vivo* and *in vitro* can induce the differentiation of floor plate cells, motor neurons and ventral interneurons[25–27]. Conversely, elimination of Shh signalling from the notochord by antibody blockade *in vitro*[25,27], or through gene targeting in mice[28], prevents the differentiation of floor plate cells, motor neurons and most classes of ventral interneurons[28,29]. Even though Shh can induce

PARAXIAL MESODERM
Mesodermal cells that derive from the segmental plate mesoderm that flanks the midline axial mesoderm.

PRIMITIVE STREAK
A group of cells in gastrula-stage chick and mouse embryos that actively ingress from the epiblast layer to form mesodermal cell types.

NEURAL PLATE
The initial group of columnar neuroepithelial cells that forms as a result of neural induction.

all ventral cell types, the generation of certain sets of interneurons in the dorsal-most region of the ventral neural tube does not depend on Shh signalling[29]. These interneuron subtypes can be induced by a parallel signalling pathway that is mediated by retinoids derived from the paraxial mesoderm and possibly also from neural plate cells[29]. So retinoid signalling seems to have sequential roles in spinal cord development, initially imposing spinal cord identity and later specifying the identity of some of its component neurons.

Graded Shh signalling. Progressive two- to threefold changes in Shh concentration generate five molecularly distinct classes of ventral neurons from neural progenitor cells *in vitro*[30,31] (FIG. 2c). Moreover, the position of generation of each of these neuronal classes *in vivo* is predicted by the concentration of Shh required for their induction *in vitro*. Neurons generated in progressively more ventral regions of the neural tube require correspondingly higher concentrations of Shh for their induction[31] (FIG. 2c).

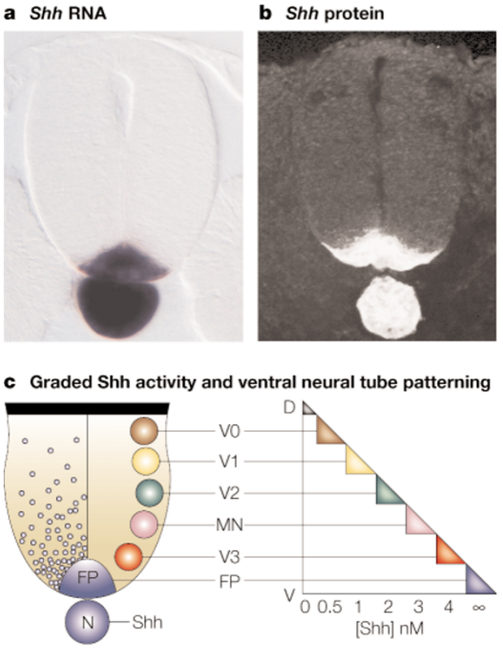

Figure 2 | **Shh expression by notochord and floor plate controls ventral pattern. a** | Cross-section through stage-18 chick spinal cord showing the expression of *Shh* RNA by the notochord (N) and floor plate (FP) (see panel c). **b** | Cross-section through chick neural tube showing the expression of Shh protein by the notochord and floor plate. **c** | A model for the influence of Shh on the specification of ventral neuronal fates. To the left is shown the presumed gradient of Shh activity in the ventral neural tube (blue dots), distributed in a ventral-high, dorsal-low profile within the ventral neural epithelium, and the position of five classes of neurons that are generated in response to graded Shh signalling. V0–V3 indicate four different classes of ventral interneurons. MN indicates motor neurons, and FP the floor plate. To the right is shown the profile of neuronal generation in intermediate neural plate explants grown in different concentrations of the recombinant amino-terminal fragment of Shh, termed Shh-N. D, dorsal neural tube; V, ventral neural tube. The more dorsal the position of neuronal subtype generation *in vivo*, the lower the concentration of Shh required to induce the same neuronal subpopulation *in vitro*. (Modified from REFS 32,33.)

Although these findings support the idea that the position of a progenitor cell within a ventral-to-dorsal gradient of Shh signalling activity directs its differentiation into specific neuronal subtypes, they pose the problem of how neural progenitor cells interpret graded Shh signals. Recent studies have provided evidence that a group of homeodomain proteins expressed by ventral progenitor cells act as intermediary factors in the interpretation of graded Shh signalling[29,32,33]. These homeodomain proteins can be divided into two classes on the basis of their pattern of expression and mode of regulation by Shh[33] (FIG. 3a). The expression of each class I protein is repressed at a distinct Shh threshold concentration and, as a consequence, their ventral boundaries of expression delineate progenitor domains. Conversely, the expression of each class II protein requires Shh signalling and is achieved at a distinct Shh threshold concentration. So their dorsal boundaries delineate progenitor domains. The combinatorial expression profile of these two classes of homeodomain proteins defines five cardinal progenitor cell domains within the ventral neural tube (FIG. 3c).

How do these homeodomain proteins convert a gradient of extracellular Shh signalling activity into discrete progenitor domains? This feat is achieved through selective cross-repressive interactions between the complementary pairs of class I and class II homeodomain proteins that abut the same progenitor domain boundary[33] (FIG. 3b). Such interactions seem to have three main roles. First, they establish the initial dorsoventral domains of expression of class I and class II proteins. Second, they ensure the existence of sharp boundaries between progenitor domains. Third, they help to relieve progenitor cells of a requirement for ongoing Shh signalling, consolidating progenitor domain identity[33].

The central role of cross-repression between transcription factors in ventral neural patterning has parallels in other neural and non-neural tissues. In the developing brain, cross-repressive interactions between the homeodomain proteins Pax6 and Pax2 help to delineate the diencephalic–midbrain boundary[34], and interactions between Otx2 and Gbx2 define the midbrain–hindbrain boundary[35]. Cross-repression between other classes of transcription factors have been implicated in regionalization in the embryonic mesoderm[36] and pituitary gland[37]. The general principles of ventral neural patterning also seem similar to those used to subdivide the *Drosophila* embryo along its anteroposterior axis[38]. So cross-regulatory interactions between transcription factors seem to be a prevalent strategy for the regional allocation of cell fate in response to graded inductive signals.

Homeodomain proteins and neuronal fate. Homeodomain proteins expressed by progenitor cells seem to specify the identity of each of the classes of post-mitotic neurons that derive from individual progenitor domains. The misexpression of individual homeodomain proteins in chick neural tube changes the fate

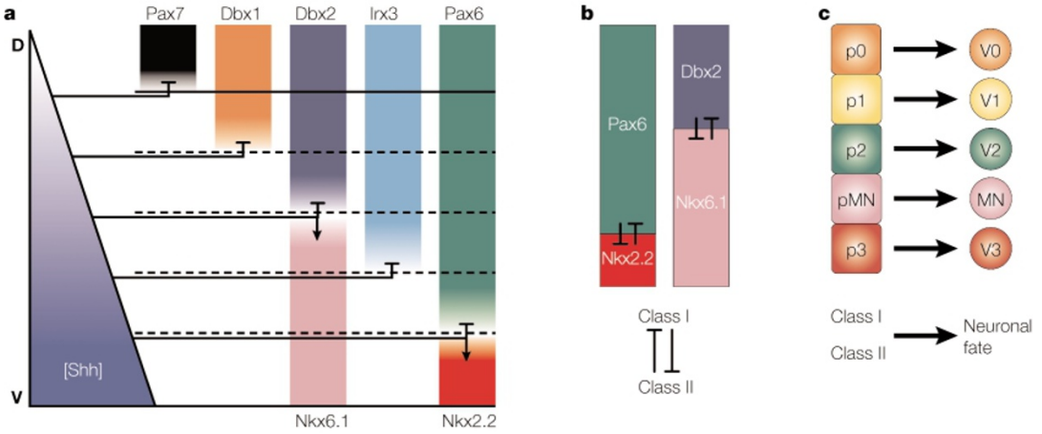

Figure 3 | **Three phases of Shh-mediated ventral neural patterning. a** | Shh mediates the repression of class I homeodomain proteins (Pax7, Dbx1, Dbx2, Irx3 and Pax6) at different threshold concentrations and the induction of expression of class II proteins (Nkx6.1 and Nkx2.2) at different threshold concentrations. Class I and class II proteins that abut a common progenitor domain boundary have similar Shh concentration thresholds for repression and activation of protein expression, respectively. Shh signalling defines five progenitor domains in the ventral neural tube. **b** | The pairs of homeodomain proteins that abut a common progenitor domain boundary (Pax6 and Nkx2.2; Dbx2 and Nkx6.1) repress each other's expression. **c** | The relationship between neural progenitor (p) domains and the positions at which post-mitotic neurons are generated along the dorsoventral axis of the ventral spinal cord. (For details see REF. 33.)

and position at which individual classes of neurons are generated, as predicted by the normal profile of homeodomain protein expression (FIG. 3c)[33]. Conversely, there are predictable switches in progenitor domain identity and neuronal fate in mice in which individual class I and class II homeodomain proteins have been inactivated by gene targeting[30,32,39,40].

These studies have also provided an initial framework for defining Shh-regulated transcriptional cascades that direct neural progenitor cells along specific pathways of neurogenesis. For example, Shh-regulated homeodomain proteins can be ordered into a pathway that helps to explain how motor neurons acquire an identity distinct from that of adjacent interneurons[33,41] (FIG. 4). The combinatorial actions of three homeodomain proteins — Nkx6.1, Nkx2.2 and Irx3 — restrict the generation of motor neurons to a single (pMN) progenitor domain. Within this domain, Nkx6.1 activity directs the domain-restricted expression of downstream factors, such as the homeodomain protein MNR2 (REF. 41). MNR2 is first expressed during the final division cycle of motor neuron progenitors and functions as a dedicated determinant of motor neuron identity (FIG. 4). Ectopic dorsal expression of MNR2 does not change the pattern of expression of class I and class II proteins, but is sufficient to subvert their activity and elicit a coherent program of post-mitotic motor neuron differentiation[41]. Moreover, once induced, MNR2 positively regulates its own expression[41], further consolidating the progression of progenitor cells to a motor neuron fate (FIG. 4).

Ectopic expression of other progenitor transcription factors that function downstream of the class I and class II proteins can similarly direct ventral cell fates in the spinal cord independently of the prior developmental history of the progenitor cell[33,41–43]. The fates of neurons in other regions of the CNS may therefore be determined through the actions of neuronal subtype-dedicated transcription factors. Defining such factors may aid studies that aim to direct neural stem cells along specific pathways of neuronal differentiation.

Missing links in neural Shh signalling. Several aspects of neural Shh signalling remain unresolved. First, the pathway through which graded Shh signalling initially regulates class I and class II homeodomain protein expression has not been defined. Some components of

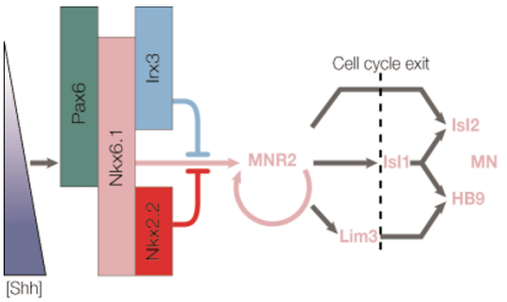

Figure 4 | **A molecular pathway for motor neuron generation.** Homeodomain proteins that function downstream of Shh in the pathway of motor neuron (MN) generation in the chick embryo. Graded Shh signalling establishes an initial progenitor domain profile in which Nkx6.1 expression, in the absence of Nkx2.2 and Irx3 expression, delineates the domain from which motor neurons are generated. The activity of Nkx6.1, when unconstrained by the inhibitory effects of Irx3 and Nkx2.2, is sufficient to induce the expression of the homeodomain protein MNR2. MNR2 induces the expression of downstream transcription factors, including Lim3, Isl1, Isl2 and HB9. MNR2 also positively autoactivates its own expression, so consolidating the decision of progenitor cells to select a motor neuron fate. The timing of onset of homeodomain protein expression with respect to cell cycle exit is indicated. (Modified from REFS 33,41.)

the hedgehog signalling pathway operate in different tissues and organisms. In particular, the Gli class of zinc-finger transcription factors have been proposed as key intermediaries in vertebrate hedgehog signalling[44]. The idea that different levels of Gli activity repress or activate different class I and class II homeodomain proteins is, therefore, attractive. However, ventral neuronal pattern remains almost unchanged in mice that carry mutations in both the *Gli1* and *Gli2* genes[45,46], indicating that *Gli3* may have as prominent a role in ventral neuronal patterning as it has in limb patterning[47]. Alternatively, other Shh-regulated transcription factors, such as COUP-TFII[48], could participate in the initial interpretation of graded Shh signals within ventral progenitor cells. Second, because the complementary class I and class II protein pairs that form domain boundaries are potent repressors of each other's expression, it remains unclear if Shh signalling initially represses class I or activates class II proteins. Third, it is not known whether these progenitor homeodomain proteins refine domain boundaries through their actions as direct repressors, or indirectly through inducing expression of a distinct set of intermediary repressor proteins.

Another elusive issue is the process by which long-range Shh signalling is achieved. There is evidence, albeit indirect, that the secretion of Shh from the notochord and floor plate creates a long range ventral-to-dorsal gradient of signalling activity and exerts a direct influence on ventral cell fate and pattern. First, extracellular Shh activity is detectable throughout the ventral neural tube, well away from ventral sources of *Shh* synthesis[27]. This finding implies that the active amino-terminal fragment of Shh, termed Shh-N[49], is somehow transferred over many cell diameters through the ventral neural epithelium. Second, the *Patched* (*Ptc*)gene, which encodes the ligand binding subunit of the Shh receptor[50,51], is expressed in a relatively smooth ventral-to-dorsal gradient within the ventral neural tube[50,52]. The level of *Ptc* expression seems to reflect the intensity of Shh signalling[53], and so the detection of a *Ptc* gradient is indicative of a corresponding gradient of Shh activity. Third, ectopic expression of an activated form of smoothened (*Smo*), the gene encoding the signal transducing subunit of the Shh receptor[54], seems to induce ventral cell types in a cell-autonomous manner[55], consistent with other evidence[27] that Shh acts directly on target cells to specify ventral cell fates.

Although the gradient of Shh activity could, in principle, reflect the local concentration of active Shh protein, there is increasing evidence for the involvement of accessory factors that modulate the Shh signalling pathway. The response of ventral neural progenitors to specific levels of Shh signalling activity, for example, seems to be dependent on ambient BMP signalling[56]. Exposure of neural progenitor cells *in vitro* to a fixed concentration of Shh in the presence of BMPs results in a ventral-to-dorsal shift in the identity of neural progenitor cells and post-mitotic neurons. Conversely, proteins that bind to BMPs or BMP-receptor complexes and attenuate BMP signalling, such as follistatin, markedly ventralize the response of neural plate cells to a given concentration of Shh[56].

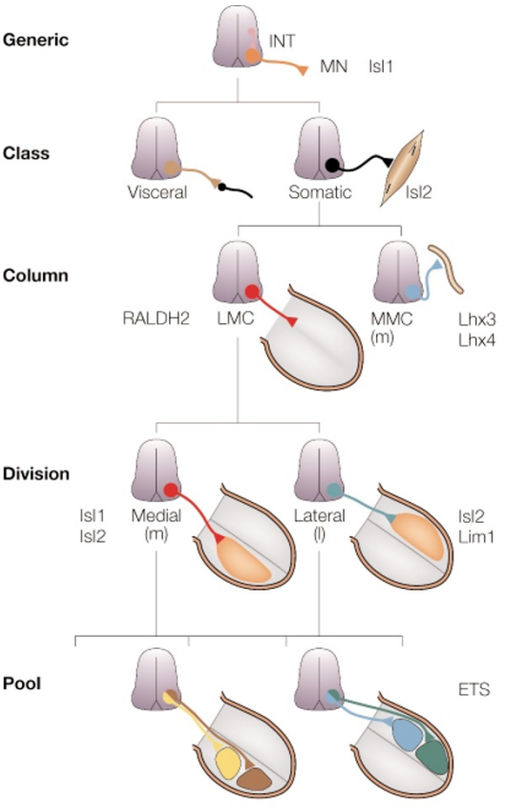

Figure 5 | **A hierarchy of motor neuron identities.** Motor neuron subtype organization in the developing spinal cord, on the basis of cell body position, axonal projections and gene expression. Generic: features common to all motor neurons (MN), such as the projection of axons out of the spinal cord, that distinguish them from interneurons (INT). Class: subdivision of motor neurons on the basis of the innervation of skeletal muscle targets (somatic) or neuronal or glandular targets (visceral). Visceral and somatic motor neurons are generated from the same ventral progenitor domain at spinal levels but from distinct progenitor domains at cranial levels. Column: sets of motor neurons arrayed in longitudinal columns and projecting to distinct regions in the periphery. Lateral motor column (LMC) neurons are generated only at limb levels and send axons into the limb mesenchyme. The median motor column (MMC) can be divided into a medial (m) group, which is found at all rostrocaudal levels and projects to axial muscles, and a lateral group (not shown), found only at thoracic levels and projecting to body wall muscles. Division: binary subdivision of main columns, based on cell body position and differences in axonal projection pattern. Pool: subsets of motor neurons within the LMC that innervate a single muscle group in the limb.

Genetic studies also support the idea that BMP antagonists have roles in ventral patterning *in vivo*. Mice with a disruption in the gene encoding the notochord-derived BMP antagonist noggin lack floor plate cells and motor neurons at caudal levels of the spinal cord[57]. This ventral patterning defect is accompanied by the ectopic expression of *BMP4* by ventral neural cells. So the secretion of noggin by the notochord may normally prevent ventral neural expression of *BMP*s, in effect sensitizing neural cells to Shh signals. Furthermore, analysis of *BMP* mutant phenotypes in zebrafish embryos has revealed an

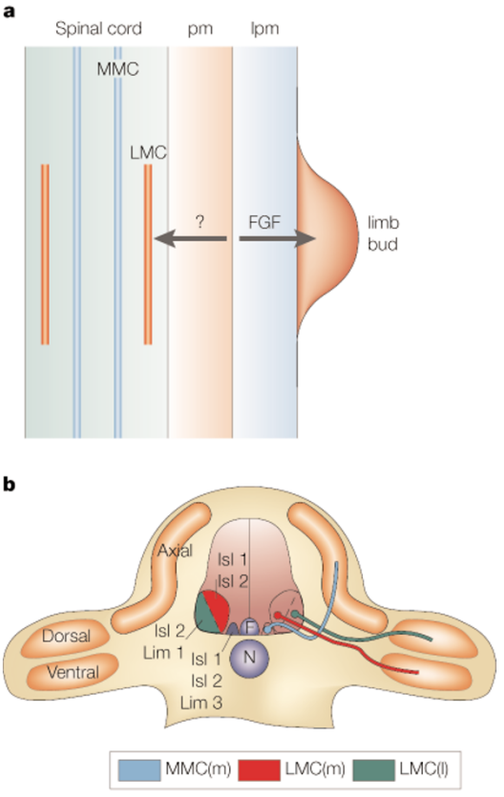

Figure 6 | **Spatial organization of motor neurons in the developing spinal cord. a** | Top down view of the embryonic spinal cord showing the rostrocaudal position of generation of motor neurons of the medial division of the median motor column (MMC(m)) and of the lateral motor column (LMC). An unknown (?) signal from the paraxial mesoderm (pm) has been implicated in the specification of LMC neuronal identity. Similarly, a signal from the paraxial mesoderm has been suggested to initiate the differentiation of the limb bud, in part through activation of expression of FGFs in the prospective limb field of the lateral plate mesoderm (lpm). **b** | Transverse section of the chick embryo at limb levels (both forelimb and hindlimb), showing the position of motor columns in relation to the axonal projection pattern in the periphery and to LIM homeodomain protein expression. N, notochord; F, floor plate; LMC(m), medial division of the LMC; LMC(l), lateral division of the LMC. Brown regions indicate the positions of muscle targets.

expansion in the expression domain of ventral neural markers[58,59], consistent with the idea that the fate of cells in prospective ventral regions of the neural plate is regulated by BMP signalling. So regulated BMP signalling may be involved in establishing a ventral-to-dorsal gradient of Shh signalling activity within the ventral neural tube, as well as in patterning the dorsal neural tube[7]. Factors other than BMPs may also influence neural cell responsivity to Shh signalling. Shh induces the ventral neural expression of Hedgehog-interacting protein (Hhip), a surface membrane protein that binds to Shh and attenuates its signalling activity[60]. Shh also induces ventral neural expression of vitronectin, an extracellular matrix protein that can bind Shh and has been suggested to act as an obligate cofactor in neural Shh signalling[61].

How the neural patterning role of Shh is integrated with other more general regulators of neurogenesis also remains unclear. In vertebrates, as in insects, neurogenesis is regulated by signalling pathways that involve Notch and basic helix–loop–helix (bHLH) proteins[62]. Notch ligands, and many bHLH proteins, are expressed within discrete domains along the dorsoventral axis of the ventral spinal cord[63–65], and in some regions of the CNS bHLH factors have been suggested to influence neuronal subtype identity[66]. It will therefore be important to determine whether individual Notch ligands and bHLH proteins with distinct patterns of expression in the spinal cord have equivalent functions in neuronal specification. It is also unclear whether the regional expression of Notch regulators and bHLH proteins is imposed by the homeodomain proteins that establish cardinal progenitor domains.

Beyond Shh signalling

Although studies of Shh signalling have provided many insights into mechanisms of neuronal specification and patterning, it is evident that further signalling pathways are necessary to enhance the diversity of cell types that populate the ventral spinal cord. In some instances, a single progenitor domain is known to generate distinct cell types at different developmental stages[32,67], implying a temporal control of cell fate that is still poorly understood. The same progenitor domain can also generate distinct classes of neurons at spinal cord and hindbrain levels[30,32], emphasizing the idea that rostrocaudal positional cues function in concert with dorsoventral patterning mechanisms to specify individual neuronal fates[68]. Moreover, there is evidence that more than one class of neuron can be generated from a single progenitor domain over the same developmental period. Each of these points can be illustrated through the analysis of motor neuron diversity in the spinal cord.

All spinal motor neurons derive from a single ventral progenitor domain[30,33], but they acquire many distinct subtype identities, which have traditionally been based on the position of their cell bodies in the spinal cord, and by their axonal projection patterns in the periphery (FIG. 5). In higher vertebrates, one level of organization is evident in the alignment of motor neurons with common target projections into longitudinally oriented columns[69]. These columns occupy distinct and discontinuous domains along the rostrocaudal axis of the spinal cord. For example, motor neurons of the lateral motor column (LMC) that innervate target muscles in the limb are generated only at limb levels[70] (FIG. 6a). At a second level of organization, neurons within the main motor columns are segregated into medial and lateral divisions and project axons along different trajectories. Within the LMC, for example, motor neurons in the medial and lateral divisions project to ventral and dorsal limb muscles, respectively[70] (FIG. 6b). At a third level, discrete pools of motor neurons exist within each division of the LMC and innervate specific muscles in the limb (FIG. 5)[69]. In lower vertebrates, such as zebrafish, there is also evidence for an intrasegmental specificity in motor

neuron subtype[71]. Three main types of 'primary' motor neurons can be identified by their rostrocaudal position within a single segment of the neural tube, and by their selective projections to different axial muscle domains in the periphery.

Anatomically defined motor neuron subclasses are also molecularly distinct, as defined by the restricted expression pattern of transcription factors. The main columnar subclasses of motor neurons found in higher vertebrates and in zebrafish primary motor neurons can be distinguished by the combinatorial expression of LIM homeodomain proteins (FIG. 6b)[72,73], and individual motor neuron pools within the LMC can be defined by expression of members of the ETS and forkhead classes of transcription factors (FIG. 5)[74,75].

The use of transcription factors as markers of motor neuron subtype identity has helped to define the origin of extrinsic signals that control motor neuron diversity, and has emphasized the idea that motor neuron differentiation depends on sequentially acting mesoderm-derived signals. For example, as discussed below, progressive steps in the specification of LMC neuron identity seem to depend on three distinct mesodermal signals. Axial mesodermal cells of the notochord provide a signal (Shh) that specifies the generic identity of motor neurons. Signals from the paraxial mesoderm help to specify LMC identity and position, and a later signal from the lateral plate mesoderm is required for some of the differentiated features of individual motor pools. The following sections summarize progress in defining some of these signals and discuss the role of transcription factors in specifying functional aspects of motor neuron subtype identity.

Control of motor neuron columnar identity. Motor neuron diversification along the rostrocaudal axis of the spinal cord seems to depend on positionally-restricted signals derived from the paraxial mesoderm. Transplanting segments of the chick neural tube, or of the paraxial mesoderm itself, to different rostrocaudal positions results in a transformation in the columnar identity of motor neurons, as assessed by LIM homeodomain protein expression[76]. Similarly in zebrafish, transplanting individual primary motor neurons to different intrasegmental locations produces a change in their identity, as defined both by altered LIM homeodomain protein expression and by the respecification of axonal trajectory[71,73]. The identity of paraxial mesoderm-derived signals that control these aspects of motor neuron identity along the rostrocaudal axis of the neural tube, however, remains unknown.

LIM homeodomain protein function is required to establish both the generic and columnar identities of motor neurons. Isl1 function is required initially for the generation of all motor neurons[77], whereas Lhx3 (Lim3) and Lhx4 (Gsh4) seem to have more selective roles in specifying motor neuron columnar identity[78]. Motor neurons generated in mice lacking both these genes acquire a cranial visceral identity, as assessed by their intraspinal settling position and the dorsal position at which their axons exit the neural tube[78]. This finding can be explained by the fact that, at cranial levels, visceral motor neurons normally derive from a distinct ventral progenitor domain that excludes Lhx3 and Lhx4 expression[30]. So Lhx3 and Lhx4 seem to have an early role in establishing spinal motor neuron identity. In addition, ectopic expression of Lhx3 in all spinal motor neurons converts LMC and preganglionic autonomic motor neurons to an MMC-like identity, and re-routes motor axons towards axial muscles — the normal targets of medial MMC neurons[79] (FIG. 7a,d). So the selective expression of LIM homeodomain proteins within subsets of motor neurons in vertebrates, as in *Drosophila*[80], helps to establish distinct motor axon projection patterns.

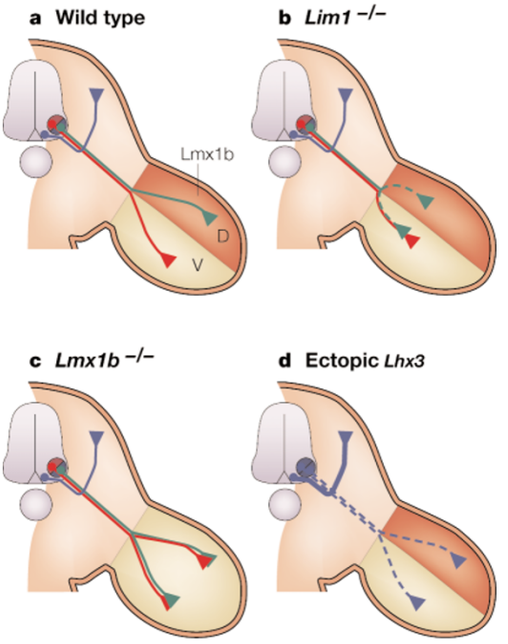

Figure 7 | **LIM homeodomain proteins control motor axon trajectory. a** | Wild type: projections of MMC(m) (blue), LMC(m) (red) and LMC(l) (green) axons from the spinal cord at limb levels. **b** | *Lim1*$^{-/-}$: *Lim1* mutant LMC(l) neurons show aberrant ventral axonal projections. **c** | *Lmx1b*$^{-/-}$: loss of *Lmx1b* expression results in a ventral duplication of the limb mesenchyme. Consequently, motor axons from both divisions of the LMC randomly project axons into the dorsal (D) and ventral (V) limb mesenchyme. **d** | Ectopic *Lhx3*: misexpression of *Lhx3* in all motor neurons results in the conversion of most or all spinal LMC and visceral neurons to an MMC(m)-like identity. Many, but not all, motor axons now select a dorsal pathway to axial muscles. However, only a small increase in the net number of motor axons projecting into the axial pathway is permitted. (For further details see REFS 79,85.)

Subdivision of motor neurons within columns. Although the columnar identity of motor neurons is controlled by inductive signals from the axial and paraxial mesoderm, other features of motor neuron differentiation seem to involve signalling systems that originate within the spinal cord itself, as is evident in the specification of neurons within the medial and lateral subdivisions of the LMC. Both sets of LMC neurons are generated from progenitor cells that occupy the same dorsoventral and

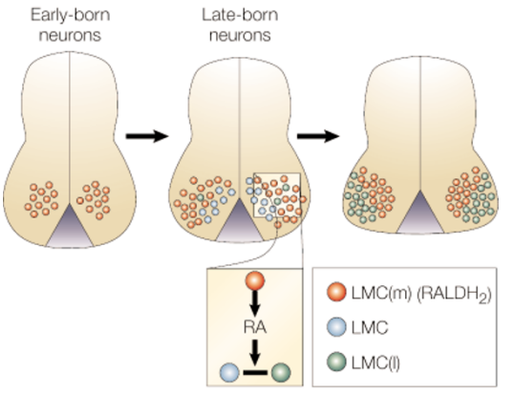

Figure 8 | **Retinoid signalling by early born LMC neurons and the control of lateral LMC neuronal identity.** The possible influence of retinoid signals provided by early-born, prospective medial (m) LMC neurons (red) on the fate of later-born, prospective lateral (l) LMC neurons. RALDH-2 expression in newly generated LMC neurons results in the synthesis and secretion of retinoic acid (RA), which acts on late-born LMC neurons to specify lateral LMC identity. (Modified from REF. 82.)

rostrocaudal positions within the spinal cord, making it difficult to imagine how signals from the axial or paraxial mesoderm could impose this distinction. One feature that does distinguish the development of these two sets of LMC neurons, however, is a difference in their birthdates. Motor neurons destined to form the medial LMC leave the cell cycle before lateral LMC neurons[81]. Consequently, when late-born prospective lateral LMC neurons emerge from the ventricular zone, they are required to migrate past medial LMC neurons to their final settling position.

These findings indicate that a signal provided by early-born LMC neurons helps to specify the fate of later-born lateral LMC neurons, and other studies have implicated retinoid signalling in this aspect of motor neuron subtype diversification. LMC neurons selectively express RALDH-2 and can synthesize biologically active retinoids[82–84]. Moreover, retinoids provided by early LMC neurons can function in a non-cell-autonomous manner to induce the expression of a defining marker of lateral LMC identity, the LIM homeodomain protein Lim1 (FIG. 8)[82]. So at limb levels of the spinal cord, motor neuron diversification seems to arise, in part, by inductive signalling between post-mitotic motor neurons themselves.

Selective expression of Lim1 by lateral LMC neurons helps establish the differential dorsoventral trajectory of LMC axons as they enter the limb. In mice lacking Lim1 function the specification of the lateral LMC proceeds normally, but the axons of lateral LMC neurons project into the dorsal and ventral halves of the limb mesenchyme at equal incidence, apparently selecting their trajectories at random (FIG. 7a, b)[85]. In a complementary manner, the expression of the LIM homeodomain protein Lmx1b by dorsal limb mesenchymal cells controls the dorsoventral trajectory of both medial and lateral LMC neurons (FIG. 7a, c)[85]. So the activity of LIM homeodomain proteins within cells that guide motor axons, as well as in motor neurons themselves, is required to establish appropriate motor axon trajectories.

The downstream targets of LIM homeodomain proteins that mediate motor axon guidance in vertebrates remain poorly defined. There are few cell-surface or secreted proteins that distinguish subsets of motor neurons or surrounding cells that have been implicated in motor axon guidance. The most promising candidates are members of the Ephrin–Eph signalling system, some of which are differentially expressed by subsets of motor neurons[86–89]. EphA4, in particular, is expressed preferentially by the axons of lateral LMC neurons and also by a proximal group of dorsal limb mesenchymal cells[90]. Moreover, mice lacking EphA4 function show a defect in the dorsal projection of lateral LMC axons within the limb[90]. These findings invite closer examination of the idea that LIM homeodomain proteins control motor axon pathfinding through the regulation of Ephrin signalling.

Control of motor pool identity. How the pool identity of motor neurons within the LMC is determined is unknown. The onset of expression of *ETS* genes by individual motor neuron pools occurs at a comparatively late developmental stage and coincides with the arrival of motor axons at the base of the limb[70]. Early removal of the limb prevents the onset of *ETS* gene expression by motor neurons[74], indicating that a limb target-derived signal is required for *ETS* gene expression by motor pools. Nevertheless, this signal is likely to function in a permissive manner, rather than by imposing a precise pattern of *ETS* gene expression on individual motor pools. Motor pool identity can also be respecified by inversion of the neural tube at limb levels, as assessed by changes in the pattern of motor axon projections to the limb and by *ETS* gene expression[74,91]. So signals from the paraxial mesoderm probably influence both the pool and columnar subtype identity of motor neurons. Moreover, as neurons in individual motor pools have coherent birthdates[92], it is possible that the timing of motor neuron generation is involved in establishing pool as well as divisional identities within the LMC.

The role of transcription factors in the differentiation of motor neuron pools is also poorly understood. The specificity of motor axon projections to muscle targets seems to be unaffected by inactivation of the mouse *ETS* gene *Er81*, although proprioceptive afferent ingrowth into the ventral spinal cord is blocked[93]. The late onset and specificity of *ETS* gene expression by motor neurons[74] leaves open the possibility that these genes control the pattern of innervation of motor pools by functionally related proprioceptive afferents[94]. Mice lacking the forkhead protein TWH show a disruption of LMC development[75], and certain *Hox-c* and *Hox-d* class gene mutations result in defects in the development of LMC neurons[19,95], but the cellular basis of these defects remains unclear.

Delving deeper into ventral patterning

The studies described in this article reflect some progress

in defining strategies of neuronal fate specification in the ventral spinal cord. However, they have also revealed aspects of this problem that remain poorly understood; for example, how the patterning mechanisms controlled by Shh, retinoids and other extrinsic signals are integrated with cell proliferation and cell survival control. Both Shh and retinoids enhance cell proliferation in the ventral neural tube[82,96], but are these actions direct or mediated by the induction of secondary mitogenic factors? Furthermore, the time at which ventral neural progenitors exit the cell cycle is likely to influence the final number of each neuronal subtype[3]. But it remains unclear how the onset and duration of expression of homeobox genes and other intrinsic determinants of neuronal identity are integrated with factors that control the decision of progenitors to exit the cell cycle.

Finally, this article has focused on the motor neuron as an exemplar of neuronal subtype identity, but motor neurons represent only a minor fraction of the neurons that populate the ventral spinal cord. Local circuit and projection interneurons predominate[6], and are critical in integrating motor output. Defining functional subsets of interneurons at early stages of spinal cord development is a more challenging task than identifying motor neuron subtype, and so much less is known about the extent of spinal interneuron diversity or of the development of patterns of connectivity. But important advances have been made towards this goal[97–100]. Any satisfying understanding of the development of neuronal circuits in the ventral spinal cord demands a detailed accounting both of local interneurons and of inputs from supraspinal neurons. Although these are still formidable challenges, there is renewed interest in the use of the spinal cord as a model system for addressing neural circuit formation, accelerating the rate of progress in tackling these issues. Combined with the recent methodological advances in the cellular and genetic analysis of neural development, some of the classical and once intractable questions may soon have informative answers.

Links

DATABASE LINKS RALDH-2 | Shh | Nkx6.1 | Nkx2.2 | Irx3 | *gli1* | *gli2* | *gli3* | COUP-TFII | *Ptc* | *Smo* | noggin | *BMP4* | Hhip | Notch | vitronectin | Isl1 | Lhx3 | Lhx4 | Lim1 | Lmx1b | Er81

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