Many telling insights into vertebrate neuronal patterning have come from attempts to trace the pathways by which inductive signals commit cells to specific fates. 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, 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. Progress in defining mechanisms of dorsal patterning has been discussed elsewhere, 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. These two signalling systems intersect along the rostrocaudal and dorsoventral axes of the neural tube to establish a grid-like set of positional cues. 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. The dorsoventral signalling system has a more prominent role in establishing cell type diversity within each of these rostrocaudal subdivisions. 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.
as a result of neural induction.

neuroepithelial cells that forms

The initial group of columnar

NEURAL PLATE

mesodermal cell types.

stage chick and mouse embryos

A group of cells in gastrula-

PRIMITIVE STREAK

mesoderm that flanks the

from the segmental plate

PARAXIAL MESODERM

the dorsal neural tube.

Dorsal root ganglion (DRG) neurons differentiate from neural crest progenitors. The dorsal

differentiat at the ventral midline, and neural crest cells (NC) start to delaminate from

the neural plate.

Cells are flanked laterally by the epidermal ectoderm (ECT). Notochord cells (N) underlie the

midline axial 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

esomesial cell types.

NEURAL PLATE

The initial group of columnar

neuroepithelial cells that forms

as a result of neural induction.

cated in the acquisition of caudal neural character: fibroblast growth factors (FGFs), retinoids, bone mor-

phogenetic proteins (BMPs), Wnts and a PARAXIAL MESO-

derm Caudalizing (PMC) activity11-14. 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 pathway22. 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 mesoderm23. The capacity of the paraxial mesoderm to synthesize retinoids is reflected by its expression of a key retinoid synthetic enzyme, retinalde-

hyde 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 estab-

lishing the early distinction between neural cells of spinal cord and hindbrain character. Nevertheless, retinoids clearly have later roles in patterning the rostro-

caudal axis of the hindbrain24.

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 cord25,26, 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 represent-
ed at all segmental levels, raising the issue of whether rostrocaudal positional information contributes significa-
tantly 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 neu-

rons 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 meso-
dermal cell group, the notochord, and later by floor plate cells themselves25 (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 notochord26,27. 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 formation27. The merits of these two views have been discussed elsewhere28,29.

The main signalling activities of the notochord and floor plate are mediated by a secreted protein, Sonic hedgehog (Shh)28 (Fig. 2a, b). Ectopic expression of Shh in vivo and in vitro can induce the differentiation of floor plate cells, motor neurons and ventral interneurons28-30. Conversely, elimination of Shh signalling from the notochord by antibody blockade in vitro31,32, or through gene targeting in mice33, prevents the differentiation of floor plate cells, motor neurons and most classes of ventral interneurons34,35. Even though Shh can induce

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.
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. 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. 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 (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 (Fig. 2c).

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. These homeodomain proteins can be divided into two classes on the basis of their pattern of expression and mode of regulation by Shh (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 delimitate 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 delimitate 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 (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.

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, and interactions between Otx2 and Gbx2 define the midbrain-hindbrain boundary. Cross-repression between other classes of transcription factors have been implicated in regionalization in the embryonic mesoderm and pituitary gland. The general principles of ventral neural patterning seem similar to those used to subdivide the Drosophila embryo along its anteroposterior axis. 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...
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...
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\(^4\). 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\(^46\), indicating that Gli3 may have as prominent a role in ventral neuronal patterning as it has in limb patterning\(^6\). Alternatively, other Shh-regulated transcription factors, such as COUP-TFII\(^4\), 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\(^2\). This finding implies that the active amino-terminal fragment of Shh, termed Shh-N\(^\ast\), 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\(^31\), is expressed in a relatively smooth ventral-to-dorsal gradient within the ventral neural tube\(^32\). The level of Ptc expression seems to reflect the intensity of Shh signalling\(^3\), 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\(^34\), seems to induce ventral cell types in a cell-autonomous manner\(^2\), consistent with other evidence\(^2\) 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\(^46\). Exposure of neural progenitor cells in vitro to a fixed concentration of Shh in the presence of BMP results in a ventral-to-dorsal shift in the identity of neural progenitor cells and post-mitotic neurons. Conversely, proteins that bind to BMP 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\(^2\).

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\(^9\). 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 BMP4, in effect sensitizing neural cells to Shh signals. Furthermore, analysis of BMP mutant phenotypes in zebrafish embryos has revealed an
expansion in the expression domain of ventral neural markers,
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.

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. 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.

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. Notch ligands, and many bHLH proteins, are expressed within discrete domains along the dorsoventral axis of the ventral spinal cord, and in some regions of the CNS bHLH factors have been suggested to influence neuronal subtype identity. 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, 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, emphasizing the idea that rostrocaudal positional cues function in concert with dorsoventral patterning mechanisms to specify individual neuronal fates. 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, 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.

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.
neuron subtype. 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. 6); 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).

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. 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. 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 proteins control motor neuron subtype identity. LIM homeodomain proteins control motor neuron subtype identity. 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.

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both medial and lateral LMC neurons (FIG. 7a, c)85. So enchymal cells controls the dorsoventral trajectory of homeodomain protein Lmx1b by dorsal limb mesenchyme in a complementary manner, the expression of the LIM proteins selecting their trajectories at random (FIG. 7a, b)85. In a comparable manner, the expression of the LIM homeodomain protein Lim1 (FIG. 8)82. So at limb levels the control of lateral LMC neuronal identity.

The studies described in this article reflect some progress delving deeper into ventral patterning. 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 signaling system, some of which are differentially expressed by subsets of motor neurons86–89. EphA4, in particular, is expressed preferentially by the axons of lateral LMC neurons and also by a proximal group of dorsal limb mesenchymal cells85. Moreover, mice lacking EphA4 function show a defect in the dorsal projection of lateral LMC axons within the limb85. These findings invite closer examination of the idea that LIM homeodomain proteins control motor axon pathfinding through the regulation of Ephrin signaling.

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 limb85. Early removal of the limb prevents the onset of ETS gene expression by motor neurons85, 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 specified 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 expression84–85. 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 birthdates86, 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 blocked85. The late onset and specificity of ETS gene expression by motor neurons84 leaves open the possibility that these genes control the pattern of innervation of motor pools by functionally related proprioceptive afferents84. Mice lacking the forkhead protein TWH show a disruption of LMC development85, and certain Hox-C and Hox-D class gene mutations result in defects in the development of LMC neurons82,85, 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, 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. 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 neurons predominate, 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. 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 | BM4 | Hhip | Notch | vitronectin | Isl1 | Lhx3 | Lhx4 | Lim1 | Lim3b | Erb1

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Motor neurons and spinal cord movement of...


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