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The role of Sonic hedgehog in neural tube patterning

I. Patten and M. Placzek*

MRC Intercellular Signalling Group, Developmental Genetics Programme, Firth Court, Western Bank, Sheffield S10 2TN (United Kingdom), e-mail: m.placzek@sheffield.ac.uk

Abstract. In the developing neural tube of vertebrate embryos, many types of neuronal and nonneuronal cells differentiate in response to the secreted signalling molecule, Shh. Shh shows a spatially restricted pattern of expression in cells located at the ventral midline, yet governs the differentiation of diverse cell types throughout the ventral half of the neural tube. Here, we describe how the distinct fate assumed by cells in response to Shh is dependent upon their position with respect to both the dorso-ventral and anterior-posterior axes of the neural tube and describe the ways in which a single factor, Shh, is able to pattern the developing nervous system. We first discuss the evidence that Shh does impose ventral identity on cells in the neural tube, then focus on the role of a graded Shh signal in patterning the neural tube and finally discuss the interaction of Shh with other factors that affect its signalling outcome.

Key words. Neural tube; development; neural cell differentiation.

Introduction

During vertebrate embryogenesis, the process of neural induction causes a sheet of epithelial cells to acquire specialised neural properties. This sheet, termed the neural plate, rapidly folds and gives rise to the neural tube. During this time and throughout the rest of embryogenesis, multipotent proliferating progenitor cells within the neural plate and neural tube undergo differentiation, generating neurons and glia. In this review we focus on amniote embryos, and describe how the secreted signalling molecule Sonic hedgehog (Shh) plays a key role in such differentiation events, patterning the early neural plate and neural tube by imparting neural progenitor cells with a particular regionalised character and inducing the differentiation of distinct cell types.

Shh imposes ventral identity on neural cells

Beneath, and immediately adjacent to, ventral-most regions of the neural tube is a specialised rod of axial mesodermal cells that extends along the length of the embryo (fig. 1a,b). This rod consists of notochord caudally and prechordal mesoderm rostrally, both of which express Shh from early neural plate stages [1, 2]. Many studies have suggested that the expression of Shh confers on axial mesoderm the ability to induce ventral character in the adjacent neural tube, manifest in the differentiation of a wide array of ventral cell types (fig. 1c,d). At the ventral midline of the neural tube, lying directly above the axial mesoderm, cells are induced which themselves go on to express Shh. Such cells are composed of floor plate cells throughout most of the neuraxis and of rostral diencephalic ventral midline (RDVM) cells in the forebrain [3–5]. Outside of the Shh-expressing ventral midline cells, a large number of diverse neuronal and nonneuronal cells are induced by the action of Shh. In the prospective spinal cord and hindbrain this includes motor neurons, a variety of ventral interneurons and, at later stages, oligodendrocytes [6–8]. Together these groups of neurons will function in the adult in the direct regulation of motor function and the integration of sensory information. In prospective midbrain and hindbrain regions Shh is involved in the induction of dopaminergic and serotonergic neurons [9–12]. Such neurons later have roles in both emotional regulation and higher-level control of movement, and are directly implicated in movement disorders such as Parkinson's disease and a variety of psychiatric conditions. In the prospective forebrain,

* Corresponding author.

cells of the medial and lateral ganglionic eminence which later form part of the adult basal ganglia [13] appear to be induced by Shh. In the most anterior part of the developing neural tube, Shh may play a role in the production of a bilateral eye field, its absence in this region leading to a cyclopic embryo with one medially positioned eye [14–16]. Clearly then Shh is necessary during development for the successful production of a wide variety of neural cells crucial to the function of the adult organism.

Shh induces ventral cell types in the neural tube

What is the evidence that Shh imposes ventral identity on cells within the neural tube? Transplantation experiments carried out in chick embryos revealed the notochord and floor plate to be potent sources of ventralising signals, able to suppress markers of dorsal spinal regional identity and to induce ventral midline floor plate cells and ventro-lateral motor neurons (fig. 2) [9, 17, 18]. Transplantation of the prechordal mesoderm in chick embryos similarly revealed its ability to induce the differentiation of ectopic forebrain-like ventral cells [19]. In converse experiments, the selective elimination of the notochord in vivo resulted in the failure of floor plate and motor neuron differentiation, and the expression of dorsal regional markers within ventral domains of the neural tube [9, 20, 21].

Cellular studies have provided strong evidence that Shh mediates the ventralising actions of the notochord, floor plate and prechordal mesoderm. First, Shh is expressed initially in notochord and prechordal mesoderm and is subsequently induced in the floor plate and RDVM cells [1, 2, 22, 23]. Second, experiments in vitro have revealed that when explants of neural plate tissue are cultured with a Shh-signalling source (notochord, floor plate or prechordal mesoderm), distinctive cell types are generated within the neural explant. Notochord and floor plate can induce the differentiation of floor plate [24], of dopaminergic [25], serotonergic [9] and motor neurons [9, 26], ventral interneurons [7] and glial-derived oligo-

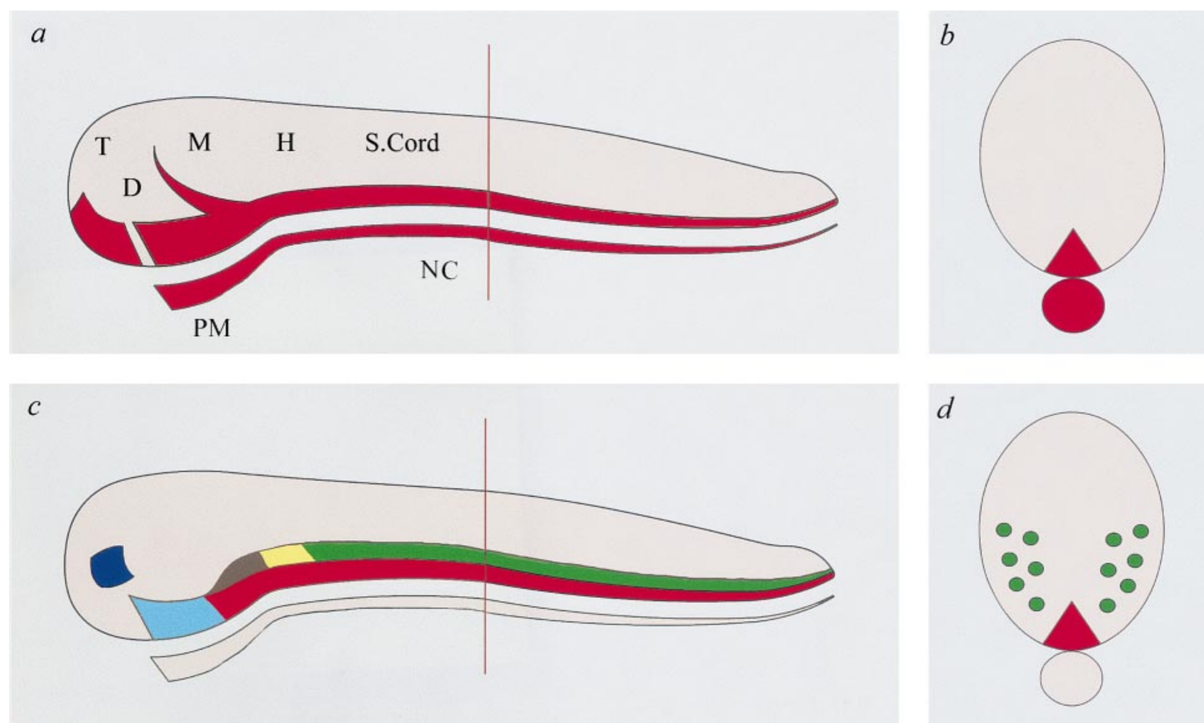

Figure 1. (*a*) Schematic representation of the neural tube and underlying axial mesoderm with regions expressing Shh indicated in red. Line shows position of transverse section shown in (*b*). T, telencephalon; D, diencephalon; M, midbrain; H, hindbrain; S.Cord, spinal cord; PM, prechordal mesoderm; NC, notochord. (*b*) Transverse section at the level of the spinal cord, showing expression of Shh in the notochord and floor plate. (*c*) Cell types induced by Shh vary according to their position along the anteroposterior axis. Different colours indicate regional differences in the cell types differentiating in response to Shh signalling. Dark blue, ganglionic eminence; pale blue, RDVM cells; brown, dopaminergic neurons; yellow, serotonergic neurons; green, motor neurons. (*d*) Transverse section at level of spinal cord (indicated in panel *c*) showing ventro-lateral cell types (green) arranged with bilateral symmetry around ventral midline floor plate cells (red).

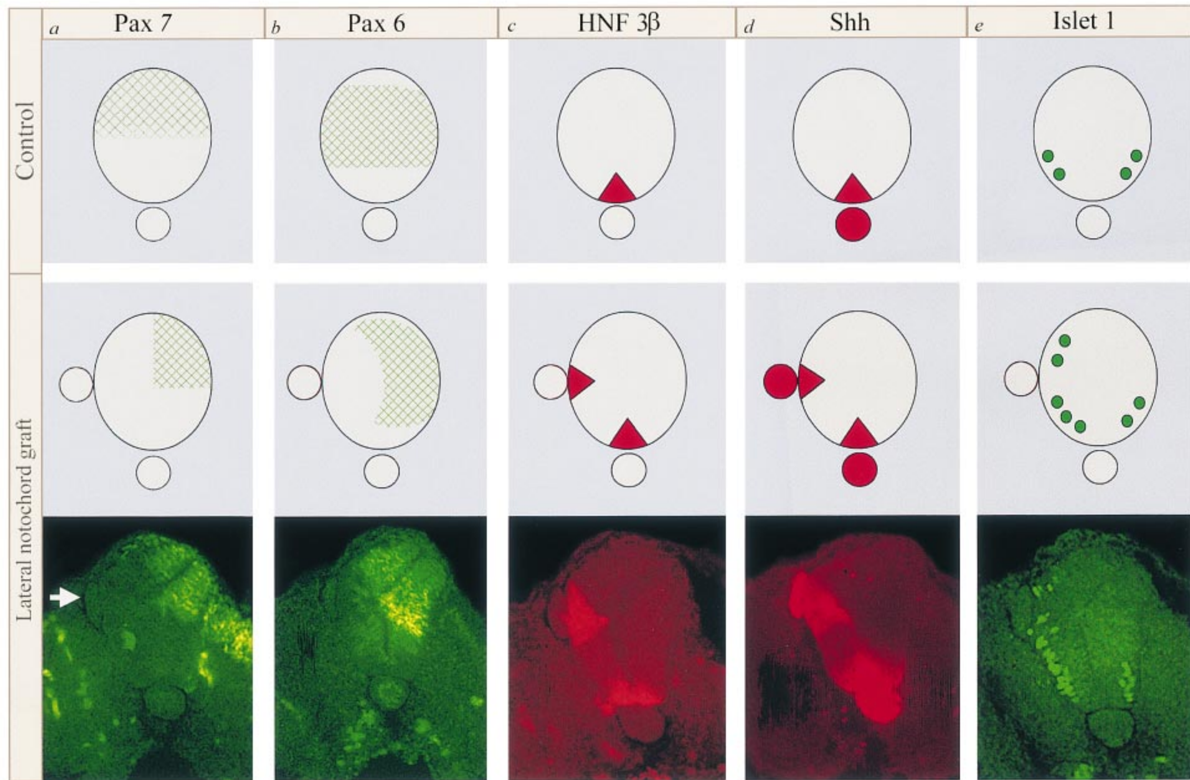

Figure 2. Notochord grafts ventralise the adjacent neuroepithelium. Serial adjacent transverse sections of a chick neural tube, after a lateral (left side) notochord graft (white arrow). Panels *a* and *b* show the repression of Pax7 and Pax6 in the neural tube adjacent to the graft; panels *c–e* show the induction of floor plate (HNF3*β* and Shh) and motor neurons (Islet 1).

dendrocytes [8]. Similarly, prechordal mesoderm induces the differentiation of RDVM cells [27]. Critically, the induction of floor plate, motor neurons, dopaminergic neurons and oligodendrocytes, and the prechordal mesoderm-mediated induction of RDVM cells are each inhibited by a monoclonal antibody (Mab) which specifically binds to Shh and prevents its function [8, 27–29].

Such cellular studies have been elegantly complemented by parallel genetic studies. In genetic gain-of-function studies, ectopic Shh signalling can lead to the ectopic differentiation of distinctive types of ventral cells from neural precursors in vivo [23, 30]. Moreover, ectopic expression of molecules that mediate Shh signalling elicits similar responses. Mis-expression of the zinc-finger transcription factor, gli1, and the winged helix-loop-helix transcription factor, HNF3*β* both downstream effectors of Shh-signalling, cause the ectopic differentiation of ventral floor plate cells [31, 32]. Likewise, transgenic mice in which the Shh signalling pathway is constitutively activated through expression of a dominant-negative form of protein kinase A (PKA) show the ectopic differentiation of both floor plate and motor neurons [33]. Such mis-expression studies have been complemented by loss-of-function studies. Most notably, the generation of Shh-null mice reveals that in the absence of Shh signalling in vivo, floor plate and motor neurons fail to differentiate [16]. Other than lacking expression of Shh, the notochord of Shh-null mice appears to initially develop normally, indicating a specific and essential role for Shh signalling in floor plate and motor neuron development. Analysis of these mice, moreover, indicates an essential role for prechordal mesoderm-derived Shh in patterning the forebrain: the mutant mice appear to lack the entire ventral forebrain, and develop a cyclopic eye [16]. Such holoprosencephaly mirrors human conditions in which the Shh gene is mutated, indicating a vital and conserved function of Shh signalling in development of the amniote

brain [34, 35 and see Incardona and Roelink, this issue].

Together, these studies establish that Shh signalling is both necessary and sufficient for the induction of ventral midline cells and motor neurons. The initial analysis of the Shh-null mouse was performed at a time before the availability of distinctive markers for ventral interneurons, leaving open the possibility that Shh may not function alone to cause the differentiation of these cells. Indeed, recent studies have shown that a retinoid-activated pathway of neurogenesis operates in parallel to that mediated by Shh to govern the differentiation of certain classes of ventral interneurons [36]. Nonetheless, whether operating alone or in parallel with other signalling pathways, the presence of Shh signalling appears to be of critical importance in the generation of a broad range of cell types within the ventral neural tube.

Patterning along the dorso-ventral axis: a graded Shh signal

A key question to arise is that of how a single factor can induce the differentiation of diverse cell types. The answer suggested by most experiments is that Shh acts as a morphogen, forming a gradient in the ventral neural tube, to which cells differentiate in a concentration-dependent fashion (fig. 3). This model derives largely through in vitro studies in which purified Shh has been shown to induce distinct cell types as a function of concentration [7]. Thus, neural explants that are exposed to twofold incremental increases in Shh concentration differentiate into specific ventral cell types in a concentration-dependent manner (fig. 3c). The highest concentrations of Shh induce ventral midline cells, whilst lower concentrations induce cell types found in vivo to lie further away from the notochord and ventral midline (fig. 3a,b).

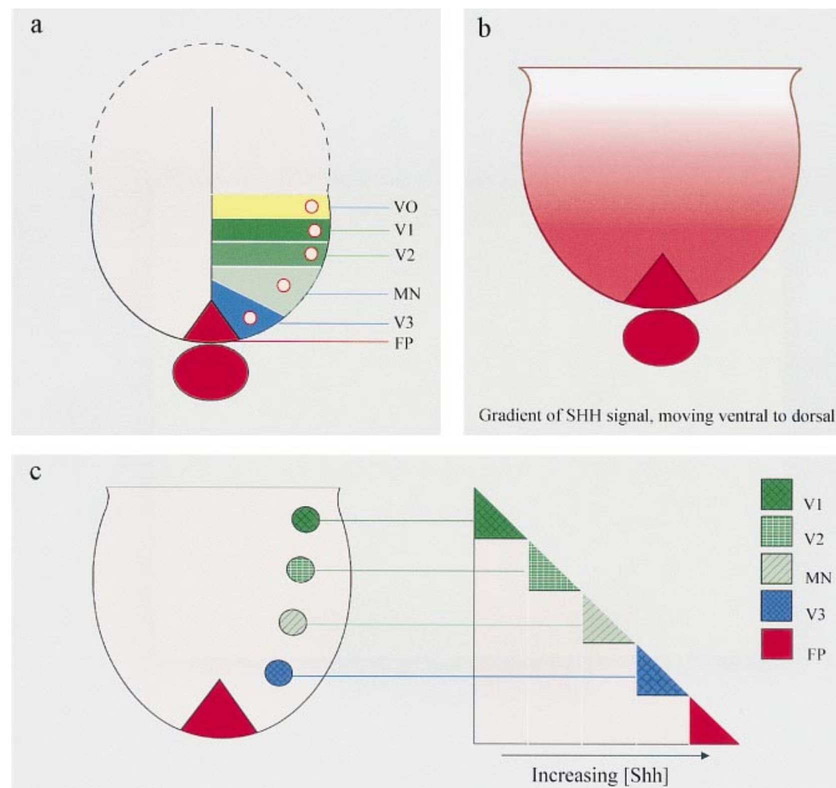

Figure 3. Gradient model for the induction of ventral cell types by Shh. (*a*) Distinct ventral cell types differentiate at stereotyped positions in the ventral neural tube. FP, floor plate; MN, motor neurons; V0–V3, classes of ventral interneurons generated at spinal cord levels. (*b*) Proposed gradient of Shh signal moving from its sources of expression in the ventral neural tube and notochord. (*c*) The concentration of Shh required to induce specific ventral cell types in vitro correlates directly with their dorso-ventral position in vivo.

Such a graded response to Shh signalling provides an explanation of how Shh not only induces different cell types, but how it can pattern neural tissue along the dorso-ventral axis such that distinct cells arise in a spatially predictable manner. When, though, does this graded signal operate to confer distinct identity upon neural precursor cells? Does it induce distinct neural progenitors at an early stage, control cell proliferation of limited stem cell populations, induce particular neuronal character, or does it play a role in all three of these events? As we describe below, a model for neuronal differentiation in response to Shh signalling has been proposed in which a graded Shh signal is primarily responsible for establishing the restricted expression of distinct homeodomain proteins that define progenitor territories along the dorso-ventral axis of the neural tube [37]. Evidence suggests that, once established, these progenitor cell domains can be maintained in a Shh-independent manner and are able to directly promote region-specific differentiation of individual ventral cell types [38].

Patterning and refinement of progenitor cell territories

Complementing earlier fate-mapping studies [39], analysis of the expression patterns of homeodomain transcription factor families, including members of the Pax, Dbx and Nkx families, suggests that progenitor cell territories exist in the ventral neural tube which prefigure the formation of specific cell types (fig. 3a and fig. 4a). Considerable evidence has accumulated for a Shh gradient in the ventral neural tube that initiates the formation of progenitor cell territories by controlling the pattern of expression of these transcription factors. When neural tissue is first induced, it displays no regional identity along the medio-lateral (future dorso-ventral) axis. Progenitor cells throughout the neural plate express, amongst others, members of the Pax and Msx families in a uniform manner. Shortly thereafter, however, these transcription factors become regionally restricted. Pax3 and Pax7 become confined to dorsal regions of the neural tube, prefiguring the generation of a variety of dorsal cell types [28, 40, 41]. The ability of low concentrations of Shh to abolish the expression of these genes in vitro suggests that their early and complete dorsal restriction in vivo likewise occurs in response to low-level Shh signalling [28]. Other genes, including Pax6, Dbx1, Dbx2 and Irx3, are only repressed by higher concentrations of Shh, and consistent with this their expression patterns extend further ventrally [36, 38, 42]. Indeed, the ventral limit of expression of each of these genes appears to correlate directly with the relative concentration of Shh required to abolish its expression in vitro (fig. 4b). Thus Pax6, which is only fully repressed by a high concentration of Shh, extends to a ventral limit lying just outside the floor plate, whilst Irx3, repressed by 'intermediate' levels of Shh, has a more dorsal limit. Graded activity of Shh thus results in the graded repression of genes that would otherwise be expressed thoughout the dorso-ventral extent of the neural tube; these genes have been termed class 1 genes [38].

As the class 1 genes Pax 6, Dbx1/2 and Irx3 expression are extinguished from ventral regions, these same regions start to express a second set of homeodomain transcription factors, including Nkx2.2 and Nkx6.1, which are referred to as class 2 genes. Thus, the dorsal limits of expression of Nkx2.2 and Nkx6.1 come to abut the ventral limits of Pax6 and Dbx2, respectively. The concentrations of Shh required to induce the expression of these genes in neural explants in vitro correlates both with their relative dorsal limits of expression, and with the concentration of Shh required to abolish the expression of the relevant class 1 gene [38]. Thus, Pax6 is completely repressed and Nkx2.2 induced by similar concentrations of Shh in vitro [42].

One question which remains to be clarified, however, is that of which actions of Shh in this early patterning process are direct and which indirect. Analysis of the small eye (Sey) mouse, which carries a point mutation in the Pax6 gene, shows a dorsally expanded domain of Nkx2.2 expression, indicating that the induction of Nkx 2.2 is at least partly an indirect response to Shh. Similarly, misexpression of Dbx2 in the ventral neural tube is able to suppress Nkx6.1 expression, suggesting that the dorsal limit of Nkx6.1 expression is controlled indirectly by the Shh-mediated repression of Dbx2 [38, 42]. In light of these observations, one favoured model is that the class 2 genes are expressed indirectly as a result of a direct and concentration-dependent repression of class 1 genes by Shh [37]. Interestingly, however, evidence is accumulating to suggest that such repression is reciprocal between pairs of class 1 and class 2 genes (fig. 4c). Although Nkx 2.2 null mice do not show a ventral expansion of Pax 6 this is most likely to be a result of redundancy between Nkx2.2 and Nkx2.9 [43]. Ectopic expression of either Nkx2.2, or the related gene Nkx2.9, in the chick neural tube results in the suppression of Pax6 expression [38]. Misexpression of Dbx2 and Nkx6.1 indicates a similar mutual repression between these two genes which define the boundary between the P1 and P2 progenitor domains [38]. It is possible then that the response of either or both of these classes of genes to Shh signalling in the ventral neural tube is at least partly indirect.

Experiments using function-blocking Shh antibodies in the chick indicate that, once established, patterns of class 1 and class 2 gene expression in ventral progenitor cell territories can be maintained in a Shh-independent manner [38, 43]. This finding, together with the exis-

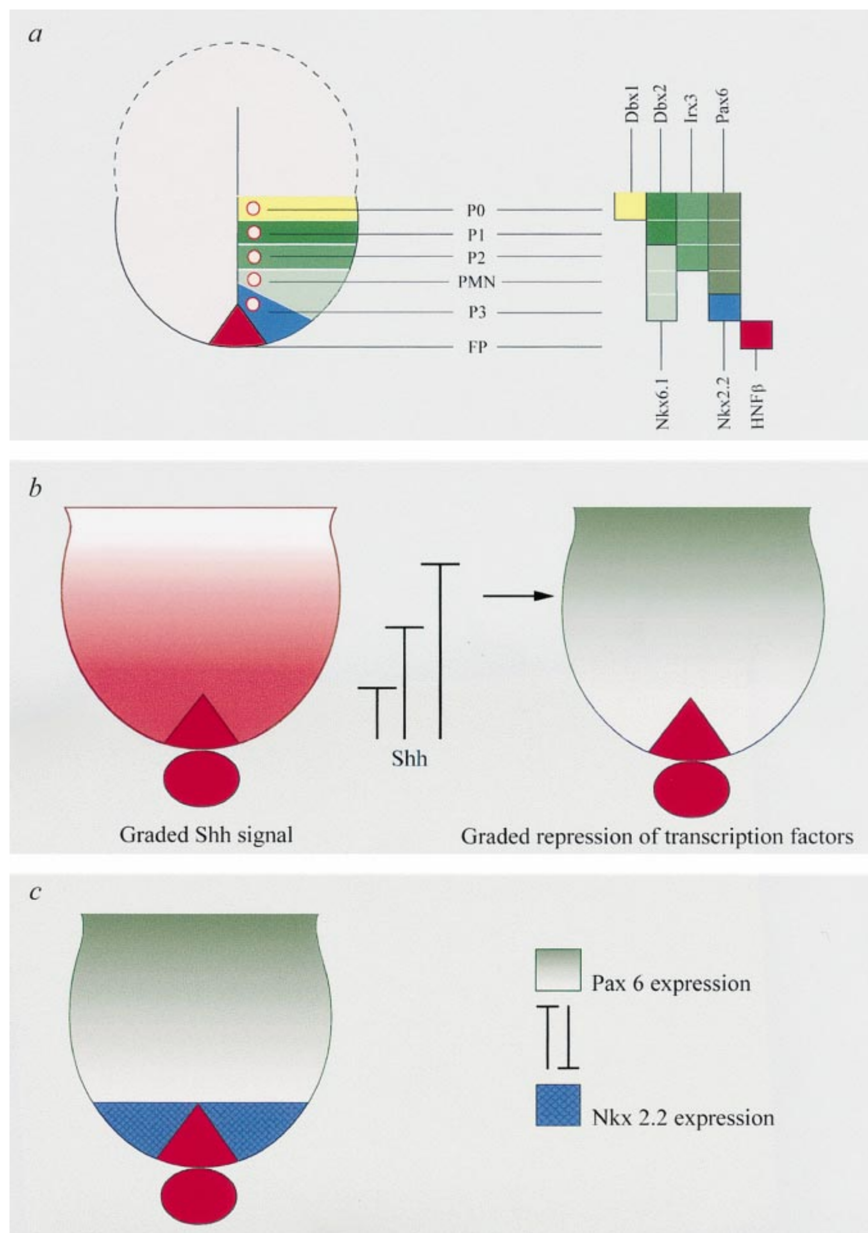

Figure 4. Establishment and maintenance of progenitor cell domains in the ventral neural tube. (*a*) Progenitor domains corresponding to the differentiation of specific ventral cell types (shown in fig. 4a) are shown on the left-hand side and indicated with a letter P. Each domain can be recognised by the combinatorial pattern of gene expression shown on the right. (*b*) Shh initiates the specification of progenitor cell domains by first exerting a graded repression of a number of genes which would otherwise be expressed more widely in the neural tube. These genes include Pax6, Irx3 and members of the Dbx family (see panel *a*). (*c*) The repression of Pax6 by Shh may indirectly allow the expression of Nkx2.2 in a discrete domain adjacent to the floor plate (P3). A reciprocal repression between these two genes may then act to refine and maintain the boundary between the P3 and PMN domains. Similar mechanisms are believed to occur at the boundaries between other ventral progenitor domains.

tence of reciprocal repressive interactions between adjacently expressed genes suggests that progenitor cell domains established initially in response to a graded Shh signal may be refined and maintained in a Shh-independent manner. Such a mechanism may serve to sharpen the boundaries between progenitor domains as they are specified and to maintain distinct cell territories in the rapidly proliferating neural tube.

Neuronal specification

As time progresses, the regionalised progenitor cells, their identities now defined by combinatorial patterns of transcription factor expression, begin to differentiate into distinct neuronal subtypes. Does Shh continue to affect cell character, altering the fate of neural progenitor cells? Or do progenitor cells established in response to a graded Shh signal differentiate into regionally specific ventral neurons in the absence of further Shh signalling?

Although initial observations implied that progenitors of somatic motor neurons require Shh signalling right up to their final cell division [7, 28], more recent genetic evidence suggests that this is unlikely to reflect a direct role for Shh in later aspects of motor neuron specification. In Nkx2.2 null mice somatic motor neurons develop in abnormally ventral regions, whilst in Sey mice, V3 interneurons which normally arise from Nkx2.2-expressing progenitors are generated in more dorsal positions at the expense of somatic motor neurons [42, 43]. Given that the levels of Shh in the V3 and motor neuron progenitor domains are unlikely to be altered in these mutant conditions, these data suggest that the Shh-dependent control of somatic motor neuron formation is not a function of a spatially restricted Shh signal but instead is negatively regulated by Nkx2.2. The negative regulation of motor neuron specification by Nkx2.2 is complemented by a promotion of motor neuron fate by Nkx6.1, such that the presence of Nkx6.1 in the absence of Nkx2.2 is sufficient to direct progenitors to a motor neuron fate. Ectopic expression studies of Nkx2.2, Nkx6.1 and Irx3 have in fact indicated that the combination of expression of these three genes is sufficient to independently initiate a programme of differentiation of three ventral cell types, namely V3 interneurons/visceral motor neurons, somatic motor neurons and V2 interneurons [37].

Evidence suggests that the way in which genes expressed in ventral progenitor cells, such as Nkx2.2, Nkx6.1 and Irx3, are able to promote the differentiation of specific neuronal cell types is through the regulation of other transcription factors such as MNR2/HB9 and Lim3 [38]. Once expressed in differentiating neurons, these genes are able to directly and independently promote specific neuronal fates and, at least in the case of motor neurons, to mark the transition from Shh dependence to Shh independence [44–48]. Given that the pattern of class 1 and class 2 gene expression can be maintained in a Shh-independent manner and can subsequently promote specific differentiation programs, it is unclear what the role of Shh might be following the initial establishment of the progenitor territories. One important function may be to regulate progenitor cell proliferation. Certainly, there is increasing evidence that Shh can regulate proliferation programmes in neural tissue [30]. Furthermore, when Shh function is blocked after the initial specification of progenitor domains, ventral cell types are reduced in number, consistent with a later role in the regulation of proliferation in limited stem cell populations [7, 28].

Interpretation of the Shh signal

The graded activity of Shh along the dorso-ventral axis indicates that this single signal can induce diverse cell types in a spatially organised manner. It remains unclear, however, how different levels of the signal are perceived and interpreted by responding cells. Almost certainly, the answer to this question lies in the details of Shh signal transduction. Genetic studies in *Drosophila* have identified a number of genes that are likely to transduce the Hedgehog (Hh: the *Drosophila* homologue of Shh) signal. At the cell surface, the transmembrane proteins patched (ptc) and smoothened (smo) exert opposing actions on normal Hh signalling such that smo activates the Hh pathway, whilst ptc inhibits it. Binding of Hh to its putative receptor ptc removes the inhibition of smo, thereby indirectly activating the pathway [49]. Evidence from the analysis of vertebrate homologues of these two genes suggests that they fulfil a similar role in neural tube patterning [50, 51]. Recent observations show that a constitutively active form of smo acts in a cell-autonomous fashion to induce the full range of ventral cell types, indicating that smo is at least potentially sufficient to mediate the response to Shh signalling [51].

A large body of evidence suggests that in *Drosophila*, the zinc-finger transcription factor Cubitus Interruptus (Ci) mediates Hh signalling. Under normal circumstances proteolytic cleavage of Ci leads to the repression of Hh target genes through the action of one of the cleavage products; however, in the presence of Hh, Ci is stabilised and Hh target genes are activated [49]. Three vertebrate homologues of Ci, termed Gli genes, have been identified [52]. Gli1 and Gli2 are both expressed in the ventral neural tube in response to Shh signalling, and a direct requirement for Gli2 in floor plate development has been demonstrated through the analysis of mice carrying a null mutation in this gene [31, 53, 54]. Misexpression of Gli1 in the neural tube is able to mimic Shh signalling, including the induction of floor plate markers and thence (probably indirectly) of dopaminergic and serotonergic neurons in the dorsal midbrain and hindbrain [31]. However, analysis of the Gli1 knockout mouse shows that Gli1 itself is not necessary for floor plate formation [54]. The induction of floor plate by Gli gene products may occur via the winged helix-transcription factor, HNF3β, which contains Gli binding sites in its promoter, and has been shown to

activate floor plate markers when expressed ectopically at high concentration [32, 55].
Interestingly, neither Gli1 nor Gli2 are required for the formation of motor neurons and ventral interneurons even in mice doubly homozygous for null mutations in both of these genes [54]. Given that Gli3 is unlikely to compensate for the absence of the other Glis in this region, its expression being restricted to the dorsal neural tube, the question remains as to what transcription factors mediate the Shh-mediated induction of these cells. It is possible that other, as yet unidentified, Gli gene products could mediate the Shh signal in nascent motor neurons. Alternatively, additional signalling pathways may operate to translate different signalling thresholds of Shh. Consistent with this, the motor neuron marker Coup-TF appears to be transcribed via a novel transcription factor that is activated in response to a Shh-responsive protein phosphatase [56]. This finding, together with emerging lines of evidence for alternative Hh signalling pathways in *Drosophila* and other organisms, raises the possibility that the response of neural cells to graded Shh signalling is effected by more than one intracellular transduction pathway [57–59].
In summary, although it seems likely that Shh signalling at all levels of the ventral neural tube is transduced by a single receptor complex, the pathways acting downstream of this are likely to be complex. The observation that different transcription factors are required depending upon the dorso-ventral position of the responding cell suggests that a high degree of regulation has already occurred upstream of this point, but little is yet understood as to how this occurs.

Evidence for a morphogen gradient

The finding that Shh acts as a graded morphogen raises the question of whether its graded activity is established temporally or spatially. In fact, evidence suggests that both mechanisms operate and that the integration of Shh signalling, in time and space, may determine cell fate. Evidence for a temporal gradient of Shh derives through observations that in the caudal spinal cord, many cells at the ventral midline transiently express Islet-1, a marker of motor neuron identity [21]. A plausible explanation is that, at these axial levels, the concentration of notochord-derived Shh to which ventral midline neural cells are exposed increases gradually over time. Evidence for a spatial gradient of Shh, in which Shh moves away from its source of expression and acts in vivo over a long range, has, until recently, been largely circumstantial, suggested by the regulation of expression of Shh-responsive genes at a distance from a source of Shh. More direct evidence, either to support the direct action of Shh at a distance from its source or for a Shh gradient in vivo remained elusive. Antibodies that recognise Shh in vivo label notochord and floor plate cells strongly and label ventro-lateral cells weakly, but do not appear to detect a gradient of Shh extending far beyond the floor plate into more dorsal regions (fig. 5). Nonetheless, recent evidence showing the cell-autonomous induction of ventral cell types in response to a constitutively active smo provides strong evidence that the patterning of the ventral neural tube is mediated directly by a Shh morphogen gradient without the induction of secondary signals [51].
The reasons for the poor apparent diffusion of Shh in vivo remain unclear. The levels of protein present outside the floor plate, whilst sufficient to exert morphogenetic effects, may be too low to detect. Alternatively, interactions with other proteins may mask the antibody-binding epitopes. A third possibility, suggested by the secretion of vacuoles from the floor plate into the neural tube lumen [60] is that Shh may be in part secreted into the lumen, there forming a gradient and acting on progenitors at the ventricular zone. The low levels of Shh apparently existing throughout the spinal

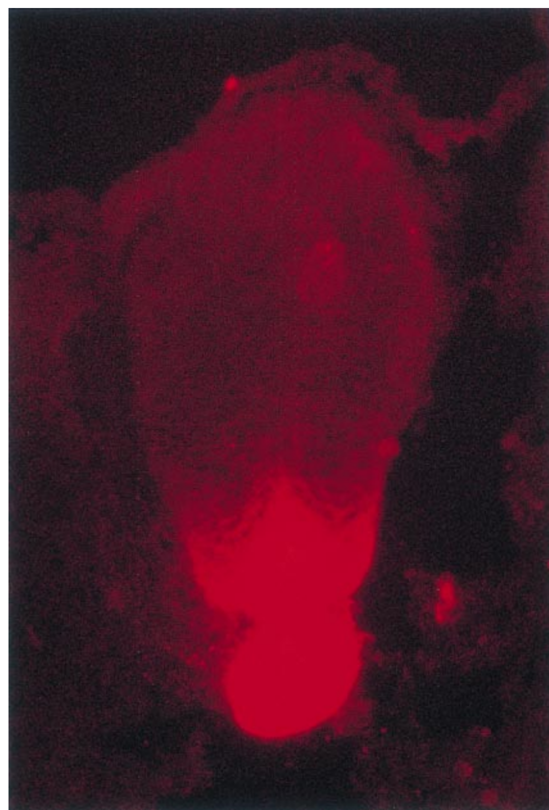

Figure 5. Transverse section through an E2.5 chick spinal cord, showing immunohistochemical visualisation of Shh protein.

cord contrast markedly with the abundant levels detected on the floor plate itself. Shh undergoes extensive posttranslational modifications (see Incardona and Roelink, this issue), including the addition of cholesterol as well as palmitoyl- and myristoyl-groups, that may act to limit the diffusion of Shh extracellularly and focus its concentration at the ventral midline as well as having possible roles in altering the potency of Shh [61].

How a gradient of Shh would be established is likewise currently unclear. In *Drosophila*, studies suggest that Hh movement is highly regulated. During normal processing Hh is cleaved to release the biologically active N-terminal fragment via a nucleophilic attack which adds a cholesterol moiety to the polypeptide. The addition of cholesterol to Hh-N, now referred to as Hh-Np (p standing for processed), results in its attachment to the cell membrane and a requirement for other factors to facilitate its movement away from expressing cells. The sterol-sensing domain protein dispatched is required for the the release of cholesterol linked Hh-N, as shown by loss-of-function mutations where Hh protein accumulates in the Hh-expressing cells [62]. A second gene product, toutvelu (ttv), is required for the movement of cholesterol-modified Hh-Np away from its source [63]. Ttv exerts this effect indirectly through synthesis of a heparan sulfate proteoglycan (HSPG) [64]. It is as yet unknown whether the HSPG resides in the extracellular matrix or is membrane attached. Conversely, the putative Hh receptor, ptc, has been demonstrated to limit the diffusion of Hh, a process which may also be dependent upon the cholesterol modification of Hh-N and may be separate from the signal transduction activity of ptc [65].

The extent to which such processes are involved in the regulation of Shh movement in the vertebrate neural tube remains to be seen. To date there are no known homologues of either ttv or dispatched. However, it appears that Shh does interact with extracellular matrix components to pattern cells within the neural tube. The extracellular matrix component vitronectin is induced in the ventral neural tube in response to Shh signalling at the time of motor neuron induction, and may be necessary for motor neuron induction, as demonstrated through the use of function-blocking antibodies in the chick embryo [66]. In vitro, vitronectin is able to synergise with Shh in the induction of motor neurons from dissociated neuroepithelial cells in culture [67]. These studies suggest a possible role for vitronectin in the presentation of Shh; however, other roles in the control of Shh movement remain possible.

Shh operates in conjunction with other signals to induce distinct cell types

The ability of a cell to differentiate according to the threshold concentration of Shh that it perceives does not appear to account solely for the diversity of cell types generated in response to Shh. In the intact embryo, neural cells are not exposed to Shh signalling alone, but instead to an ongoing array of signals that alter temporally and spatially as developing tissues migrate and grow. The final fate of a cell that is exposed to Shh appears to reflect the integration of Shh with many other signals. Indeed, a variety of experiments have shown that the outcome of Shh signalling can be modified through previous, simultaneous and subsequent events mediated by other signalling pathways. In particular, the ability of Shh to induce ventral cell types of particular anterio-posterior character appears to reflect that Shh signalling acts downstream of earlier patterning events.

Signals from anterior-posterior patterning centres affect Shh-mediated induction

Transplantation studies in chick embryos and in vitro explant experiments provided the first indication that the ventralising activity of Shh operates on neural tissue that is already prepatterned along its anterior-posterior axis [68, 69]. Indeed, many other studies have provided a wealth of evidence for tissues and signals that alter the early A-P character of the neural plate [70, 71]. The ability of Shh, or of notochord and floor plate, to induce cells with particular anterior-posterior character suggests that distinct forebrain, midbrain, hindbrain and spinal cord territories may be defined prior to the action of Shh, and that constraints and instructions exist that alter the outcome of Shh signalling. Thus, exposure to Shh leads to the induction of motor neurons and interneurons in spinal cord regions, to the induction of serotonergic neurons in the hindbrain, of dopaminergic neurons in the fore- and midbrain, and of basal ganglionic cells in the forebrain.

The specific induction of dopaminergic and serotonergic neurons within the brain is especially well understood. Dopaminergic neurons require Shh for their induction, yet are generated only in the dorsal forebrain and midbrain in reponse to ectopic application of Shh [11, 72]. These analyses suggest that Shh can induce dopaminergic neurons only in conjunction with a second signal, that is normally restricted to the prospective forebrain and midbrain. Two lines of evidence suggest that FGF8 may operate in vivo as this second signal [12]. First, early acting signals appear to limit expression of FGF8 to two specific dorso-ventral bands within the forebrain and midbrain, both regions intersecting

with Shh and prefiguring the sites of differentation of dopaminergic neurons. Second, although neither Shh not FGF8 can act alone in hindbrain tissue to induce dopaminergic neurons, exposure of hindbrain explants to Shh and FGF8 leads to the induction of dopaminergic neurons. Together, these observations suggest that signalling by FGF8 modifies the outcome of Shh signalling. As yet, it remains unclear whether the two signalling pathways impinge directly upon each other.
An even earlier involvement of fibroblast growth factors (FGFs) in affecting the reponse of cells to Shh signalling is suggested by the finding that preexposure of neural cells to FGF4 signalling may alter the outcome of FGF8/Shh signalling. When hindbrain explants are exposed to FGF4, FGF8 and Shh, dopaminergic neurons are no longer detected. Instead, serotonergic neurons are generated. In vivo, serotonergic neurons differentiate immediately caudal to the midbrain domapinergic neurons. This pattern of differentiation may reflect, therefore, the intersection of three signals, FGF4, FGF8 and Shh. Since FGF4 is expressed in vivo only early, in the primitive streak, these experiments suggest a model in which FGF4 acts as a caudalising prepatterning signal, upon which subsequent signalling by Shh and FGF8 impinges to generate, specifically, serotonergic neurons [12].

Temporally regulated changes regulate Shh responsiveness

In the spinal cord, the differentiation of ventral cells in response to Shh signalling appears to be temporally regulated. Ventral regions of the neural tube can generate either motor neurons or oligodendroctyes in reponse to similar concentrations of Shh. Both in vivo and under experimental conditions, Shh appears to promote the differentiation first of motor neurons and, subsequently, of oligodendrocytes [8]. Importantly, retroviral cell lineage studies have provided evidence that motor neurons and oligodendrocytes can derive from an early common precursor cell, suggesting that a change occurs in the responsiveness of cells to Shh signalling [39]. An intrinsic developmental programme, i.e. a cell-intrinsic 'clock', may operate in the common progenitor cell to regulate the fate of its progeny [73], so that motor neurons are preferentially generated early in the lineage, and oligodendrocytes generated later. Alternatively, temporally regulated environmental signals may operate in conjunction with Shh to dictate the fate of the progeny. Although little evidence exists to distinguish these possibilities, either mechanism suggests the operation of temporally controlled signalling pathway(s) that interact with Shh to specify distinct cell types.

Development of the neural tube ventral midline

As described above, Shh is able to induce the differentiation of both neuronal and glial-cell types. A third type of cell induced in response to Shh is that developing at the ventral midline of the neural tube; these cells give rise to floor plate and RDVM cells. These distinctive cells initially share with other neuroepithelial cells expression of 'pan-neural' markers, including members of the Sox gene family [74]. Subsequently, their exposure to Shh leads to the differentiation of specialised cells that display quite unique markers [27]. The very distinctive character of floor plate and RDVM cells has been suggested to reflect, particularly, that their progenitors are located apart from other neural plate progenitors, and develop in response to signals other than Shh [75]. However, this view is not supported by a wealth of studies, all of which suggest that the unique identity of ventral midline cells reflects their exposure to high levels of Shh [21]. Genetic studies show that ventral midline cells fail to differentiate in mice that lack the Shh gene and that floor plate cells fail to develop in mice that lack the gene encoding Gli-2. In vitro, ventral midline cells can be induced at concentrations of Shh two- to eightfold higher than those required to induce neuronal cells (fig. 3c); in vivo, antibody labelling confirms the presence of high levels of Shh on the notochord (fig. 5). Nonetheless, the requirement for Shh does not preclude that other signals may operate in concert with it to induce ventral midline cells. A key role for the transforming growth factor-β (TGF-β) family gene, nodal, in ventral midline development, is suggested through genetic analyses of zebrafish embryos [76, 77]. How nodal affects ventral midline development remains unclear. It is possible that nodal directly activates particular enhancers within the Shh gene, hence enhancing expression levels. Alternatively, nodal may sensitise cells to Shh signalling.

Heterogeneity in ventral midline cells

At gastrula stages of development, ventral midline cell precursors are situated in and immediately adjacent to the organiser. Shortly after axial mesoderm cells differentiate and undergo convergent extension, ventral midline cells of the neural tube undergo a similar process, extending forwards into the prospective forebrain [3]. The differential migration of ventral midline cells appears to result in their exposure to distinctive signals along the A-P axis and hence result in the development of different classes of ventral midline cells. Ventral midline cells that remain in posterior regions appear to be exposed only to notochord-derived Shh and differentiate into floor plate. In contrast, a subset of ventral midline cells (those at the leading edge) initially migrate

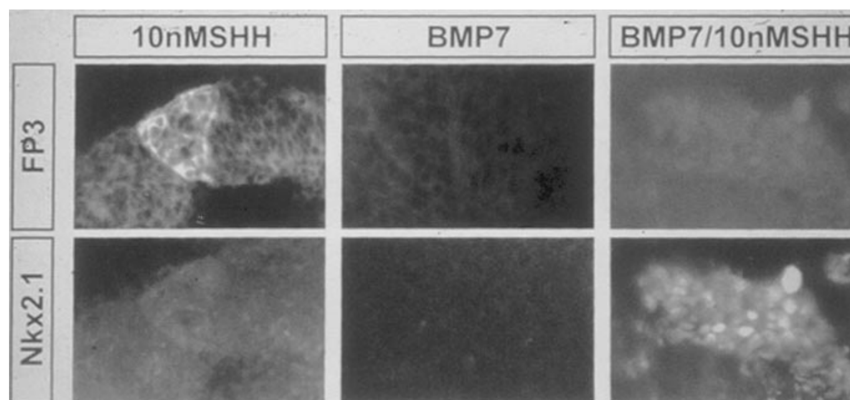

Figure 6. Cooperation of Shh and BMP7 in the induction of RDVM cells in vitro. 10 nM Shh induces floor plate cells. A combination of BMP7 and 10 nM Shh induces RDVM cells.

over the notochord, but then pass over the prechordal mesoderm. The prechordal mesoderm appears to alter the character of such ventral midline cells, causing them to differentiate into RDVM, rather than floor plate cells [3, 27].

The ability of prechordal mesoderm to induce RDVM cells appears to reflect its expression of BMP7 [3, 27]. Thus, while exposure of prospective hindbrain cells to Shh alone leads to the differentiation of floor plate cells, exposure of prospective hindbrain cells to Shh and BMP7 leads to the differentiation of RDVM cells (fig. 6). Thus the intersection of Shh with a BMP7-mediated signalling pathway appears to govern the formation of a specialised cell type. It remains unclear how these two pathways may impinge upon one another. Interestingly, however, two lines of evidence suggest that the effect of BMP7 on ventral midline cells may be to control the levels or the reception of Shh signalling, effectively sensitising cells to Shh. First, the ability of BMP7 to cooperate with Shh and induce RDVM cells can be mimicked in vitro by high concentrations of Shh alone [27]. Second, mutations in genes that are likely to result in a decrease, but not complete loss, of Shh, result in cyclopia (suggesting the loss of RDVM cells), without any noted loss of floor plate cells [34, 35, 78], suggesting that RDVM cells are particularly sensitive to the highest levels of Shh activity.

The downstream effectors of Shh signalling are thus likely to be very distinct in RDVM and floor plate cells. Support for such differences derives again from mouse mutant embryos. In Gli 2-null mice that lack floor plate cells, RDVM cells continue to express Shh [54]. Conversely, conditional Smad-2 mouse mutant embryos lack expression of Shh in the RDVM, develop holoprosencephaly and cyclopia, yet continue to express Shh in the floor plate [79]. Characterisation of the Shh promoter indicates that complex signalling events may govern expression of Shh messenger RNA (mRNA). Thus, in mouse, HNF3β-dependent and [80] -independent mechanisms seem to regulate the expression of *Shh* in different regions of the embryo, whilst in zebrafish, a FAST-1 binding site on the *Shh* promoter suggests again that Smad-2 mediated signalling may operate to govern regulation of Shh mRNA at particular axial levels [81]. Together, such analyses raise the possibility that differential regulation of the Shh promoter may govern the differentiation of cells into a floor plate or RDVM cell fate.

Conclusions

Much progress has been made in understanding how Shh can induce the differentiation of diverse cell types, both along the dorso-ventral and anterior-posterior axis, and in deciphering how the action of Shh is transduced. However, given the evidence that the absolute concentrations of Shh in vivo are likely to be of crucial importance to proper patterning of the nervous system, surprisingly little is known about the mechanisms that activate and maintain Shh expression, nor about the mechanisms that may maintain particular threshold levels of Shh. Any factor that can regulate its activity, its potency, its presentation or the way it is perceived, may potentially impinge upon its patterning function. Interactions with other signals, including complex feedback loops [82], are likely to alter the outcome of Shh signalling, yet the details of such interactions, and an understanding of whether they function directly or indirectly, remain elusive. Clearly, then, studies of

the role of Shh in neural patterning will remain at the centre of developmental studies for some years to come.

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