Neuronal subtype specification in the vertebrate neural tube is one of the best-studied examples of embryonic pattern formation. Distinct neuronal subtypes are generated in a precise spatial order from progenitor cells according to their location along the anterior-posterior and dorsal-ventral axes. Underpinning this organization is a complex network of multiple extrinsic and intrinsic factors. This review focuses on the molecular mechanisms and general strategies at play in ventral regions of the forming spinal cord, where sonic hedgehog-based morphogen signaling is a key determinant. We discuss recent advances in our understanding of these events and highlight unresolved questions.

Introduction

In ventral regions of the presumptive spinal cord, the secreted molecule sonic hedgehog (Shh) acts as a long-range morphogen (Box 1) that directs the pattern of neurogenesis by conferring positional information to ventral neural progenitors (see Jessell, 2000; Lupo et al., 2006). Here, the Shh signaling pathway (Fig. 1) functions by regulating the expression of transcription factors, including members of the homeodomain (HD) protein and basic helix-loop-helix (bHLH) families (Fig. 2). These produce a combinatorial transcriptional code that delineates spatially distinct progenitor domains along the dorsal-ventral (DV) axis of the neural tube (Fig. 2B). Each domain generates one or more distinct neuronal subtypes, the identity of which is determined by the combination of transcription factors expressed by the progenitors. Establishment of the gene expression territories is a dynamic process that requires the progressive repression and induction of genes along the DV axis (Fig. 2C). As a consequence, the neural tube undergoes a progressive ventralization, with the sequential emergence of increasingly more ventral transcriptional codes. How then is graded Shh signaling translated into spatial- and temporal-specific profiles of gene activity in neural progenitors?

We divide this problem into three parts: (1) production and spread of Shh – the regulation of Shh distribution in the neural tube; (2) transduction of the Shh signal – the conversion of the extracellular gradient into intracellular signals that initiate cellular responses; and (3) regulation of gene expression – the molecular mechanisms that determine the transcriptional code in progenitors to control the identity of differentiating neurons.

These processes are not entirely independent as Shh-controlled feedback mechanisms modulate ligand distribution, transduction and progenitor specification within the ventral neural target field. Nevertheless, this division provides a convenient framework for the organization of this review.

Establishing an extracellular gradient of Shh

Shh is initially produced by the notochord, a rod-like population of mesodermal cells that acts as an organizing center for the overlying neural tissue and establishes an equivalent neural pattern on the left and right sides of the developing spinal cord (Echelard et al., 1993; Roelink et al., 1994). In amniotes, notochordal Shh induces a second center of Shh production within floor plate cells at the midline of the neural tube (Fig. 2A) (Marti et al., 1995; Roelink et al., 1995). In other vertebrates, the mechanism of floor plate induction appears to be less dependent on notochord-derived hedgehog (Hh) signaling (reviewed by Placzek and Briscoe, 2005). Several lines of evidence...
Biologically active Shh (ShhNp) is cholesterol modified at the C-terminus (Bumcrot et al., 1995; Lee et al., 1994; Porter et al., 1995). The precursor protein is auto-catalytically cleaved and lipid modified under goes a series of post-translational modifications in which the cells is a unique feature of the Hh signaling pathway (Fig. 3). Shh lipidation affects its production and spread dedicated molecular machinery. Each step is tightly regulated and involves

indicate that the spread of Shh through the ventral neural tube of the mouse and chick embryo establishes a gradient of activity that provides crucial spatial information necessary for pattern formation. As with other morphogens, the formation of a Shh gradient depends on three processes: (1) Shh production and secretion into the target field; (2) its spread through the tissue; and (3) its degradation and removal from the tissue. Each step is tightly regulated and involves dedicated molecular machinery.

**Shh lipation affects its production and spread**

The mechanism by which Shh protein is produced and released from cells is a unique feature of the Hh signaling pathway (Fig. 3). Shh undergoes a series of post-translational modifications in which the precursor protein is auto-catalytically cleaved and lipid modified (Bumcrot et al., 1995; Lee et al., 1994; Porter et al., 1995). Biologically active Shh (ShhNp) is cholesterol modified at the C-terminus, palmitoylated at the N terminus and forms a high molecular weight complex (Chen et al., 2004; Pepinsky et al., 1998; Porter et al., 1996a; Porter et al., 1996b). Both lipid attachments are essential for assembly of this complex (Chen et al., 2004) and affect the activity of the secreted protein.

The release of active ShhNp from producing cells requires a multi-pass transmembrane protein, dispatched 1 (Disp1). In Disp1−/− mouse embryos, ventral neural tube patterning is severely disrupted and ShhNp is absent from the target field (Casparry et al., 2002; Kawakami et al., 2002; Ma et al., 2002). How Disp1 promotes the release of ShhNp remains to be determined, but its requirement is limited to lipidated Shh; Shh lacking cholesterol (ShhN) undergoes Disp1-independent secretion (Fig. 3) (Tian et al., 2005). One possible action of Disp1 is to facilitate the assembly of soluble ShhNp multimers that are long-range, high-activity signaling complexes (Goetz et al., 2006; Zeng et al., 2001).
The formation of the high molecular weight ShhNp complexes is likely to influence the ability of Shh to diffuse (Zeng et al., 2001). Thus, ShhN, which lacks the cholesterol moiety, would be expected to spread more rapidly and to penetrate further into the tissue. Surprisingly then, ventral neural tube patterning is compacted in mice that express ShhN in place of wild-type Shh (Huang et al., 2002; Tian et al., 2005). This might reflect a decreased range for the ShhN as compared with fully processed ShhNp and/or a reduced activity of the monomeric form. Alternatively, computational modeling predicts that the high diffusion rate for an extracellular protein of the size of monomeric ShhN results in the rapid diffusion of ligand away from the site of synthesis (Saha and Schaffer, 2006). Consequently, less ShhN could be retained in the target field (as discussed below).

Palmitoylation, the second lipid modification of Shh, is catalyzed by the acyltransferase Skn (also known as Hhat). Skn<sup>−/−</sup> mouse embryos resemble Shh and Indian hedgehog (Ihh) compound mouse mutants (Chen et al., 2004; Zhang et al., 2001). Similar to ShhN, the non-palmitoylated form of Shh fails to multimerize; however, in contrast to the ShhN cholesterol-deficient signal, the non-palmitoylated form has dramatically reduced bioactivity (Chen et al., 2004; Taylor et al., 2001; Williams et al., 1999). Thus, palmitoylation might play a role beyond Shh multimerization. For example, this hydrophobic moiety might enable a high affinity interaction with Ptch1, the Shh receptor, a model consistent with hydrophobic amino acid and lipid substitutions at the N terminus of ShhN (Pepinsky et al., 1998; Taylor et al., 2001). Alternatively, the activity difference in vivo between cholesterol- and palmitoylate-deficient ShhN might reflect differential cellular release through Disp1-independent and Disp1-dependent mechanisms, respectively.

Extracellular proteins modulate Shh spread

The spread of Shh in the neural target field is influenced by the expression of extracellular and transmembrane proteins. Several classes of proteins bind to extracellular Shh protein and either restrict its diffusion or alter the rate of Shh degradation (Fig. 4). Heparan sulfate proteoglycans (HSPGs), components of the extracellular matrix, bind ShhNp at a conserved site (Rubin et al., 2002). Developmentally regulated enzymes that catalyze post-translational modifications of HSPGs (glycosylation or sulfation) might regulate ligand binding to HSPGs at different stages of development. Expression of sulfatase 1, which catalyzes the sulfation of HSPGs, correlates with the accumulation of Shh protein in the ventral lumen of the neural tube and with the dorsal expansion of Nkx2.2 expression associated with the onset of oligodendrocyte production (Danesin et al., 2006). Exploring the role of HSPGs during earlier stages of DV patterning might reveal additional functions for these proteins.

The binding of Shh to a diverse set of membrane-linked proteins has also been shown to influence the response of neural progenitors to Shh. On the basis of their effect on Shh signaling,
these proteins can be grouped into two classes (Fig. 4). The first class is encoded by genes that are transcriptionally upregulated by Shh signaling and includes Ptch1, the Shh receptor, and Hhip1, which encodes an EGF-repeat-containing membrane-linked protein. Their expression results in a cell-autonomous inhibition of Shh signal transduction by sequestration of ligand and, in the case of Ptch1, by endocytosis and degradation of Shh (Incardona et al., 2002). In addition, as ligand availability is decreased by sequestration, Shh signal transduction is attenuated non-cell-autonomously at distant positions in the target field (Chuang et al., 2003; Chuang and McMahon, 1999; Goodrich et al., 1997; Jeong and McMahon, 2005). Thus, a Ptch1- and Hhip1-mediated negative-feedback loop, or ligand-dependent antagonism (LDA), modulates the activity and spread of signal (Fig. 4, Fig. 5A) (Jeong and McMahon, 2005). Indeed, when LDA is inhibited, more-ventral p3 and pMN progenitors expand at the expense of more-intermediate (p0-p2) progenitors (Fig. 5A) (Jeong and McMahon, 2005). Thus, an individual cell makes a more ventral response than it should for its position in the target field. Furthermore, ectopic Shh response is observed in the dorsal region of the neural tube shortly after the initiation of Shh patterning, highlighting the importance of negative feedback in the progressive specification of a full complement of ventral cell identities. In addition, the sharpness of progenitor subtype boundaries is lost (Jeong and McMahon, 2005). Thus, upregulation of Ptch1 and Hhip1 contributes to both the spatio-temporal dynamics of patterning and the reliability of that pattern. Consistent with this, the modeling of negative-feedback mechanisms that result in enhanced ligand degradation predicts an increased robustness in the morphogen gradient (Eldar et al., 2003). Thus, LDA mediated by Ptch1- and Hhip1-dependent negative feedback might buffer fluctuations in the production and distribution of ligand and might limit the cellular response to Shh.

The effectiveness of buffering might be productively explored in embryos with decreased Shh production. For example, in Disp1 hypomorphs, a reduction in Shh protein would be predicted to reduce the dorsal expansion of ventral cell identities observed when negative feedback is attenuated, but at the same time it may exacerbate the loss of precision that is associated with fluctuations in ligand distribution.

A second class of Shh-binding cell surface proteins enhance Shh signaling in a cell-autonomous manner, opposing the inhibitory activity of Ptch1 and Hhip1. These include the related transmembrane proteins Cdo (also known as Cdon) and Boc, and the glycosylphosphatidylinositol (GPI)-linked protein Gas1 (Fig. 4) (Allen et al., 2007; Martinelli and Fan, 2007; Tenzen et al., 2006). This suggests that Cdo might act to boost the response to Shh signaling and that this increased signal is necessary for normal floor plate induction. The deletion of Gas1 results in a loss of cell identities in the ventral neural tube, a defect that is enhanced in Shh+/− embryos (Allen et al., 2007; Martinelli and Fan, 2007). Furthermore, when Gas1 and Cdo gene dosage is progressively lowered, the neural phenotype becomes progressively
more severe to the point that Gas1<sup>−/−</sup>; Cdo<sup>−/−</sup> mouse embryos have no floor plate, p3 or pMN domains (Allen et al., 2007). Thus, these two proteins have cooperative, semi-redundant roles.

Together, these studies suggest a model for the role of cell surface Shh-binding proteins (Fig. 4) in which molecules, such as Cdo and Gas1, enhance Shh signaling in the target field, sensitizing cells to low levels of Shh ligand. As cells respond to Shh, these positive factors are transcriptionally inhibited while negative-feedback components are upregulated. A striking feature of this model is that the signal-dependent regulation of these proteins results in dynamic changes in the target field that influence the spread and degradation of the ligand, as well as the response of cells to the signal. The exchange enhances signaling when Shh levels are low and reduces signaling in cells exposed to high levels of ligand. This outcome might compensate for fluctuations in ligand availability, rendering a degree of robustness to the process of gradient formation. Additional molecules, such as Scube2 (Hollway et al., 2006; Kawakami et al., 2005), have been implicated in regulating the spread or activity of Shh; however, their function remains to be verified. Moreover, as no systematic effort has been made to identify all Shh binding partners, it is likely that more remain to be discovered. Both experimental testing and mathematical modeling will be necessary to gain a fuller understanding of how individual proteins shape the Shh gradient and regulate neural tube patterning.

**Route and timing of Shh spread**

Many of the experiments described above rely on changes in the expression of Shh target genes to infer an effect on the distribution of Shh protein. This has limitations, particularly when the proteins being studied influence both the extracellular movement of Shh and the intracellular transduction of the Shh signal. It is therefore crucial to directly visualize Shh protein in the neural tube. Several studies have convincingly demonstrated the presence of Shh ligand and several cell diameters from the floor plate, overlapping regions where Shh-feedback components are expressed, consistent with active, long-range signaling (Gritli-Linde et al., 2001; Huang et al., 2007). However, these studies are limited in their cellular resolution and depend on fixed tissue. The recent development of a biologically active fluorescently tagged allele of Shh (Shh-GFP), a product of the endogenous Shh locus, has significantly extended these initial observations (Chamberlain et al., 2008).

Shh-GFP protein generates a dynamic gradient along the DV axis of the neural tube (Fig. 5C). In Smo<sup>−/−</sup> mouse embryos, which lack a Shh response, Shh-GFP diffuses more dorsally, demonstrating the importance of negative feedback in restricting the range of Shh in the neural tube. Conversely, limited Shh-GFP was detected in Shh<sup>−/−</sup> mouse embryos, in which Shh release and/or receptor binding is diminished (Chamberlain et al., 2008). The analysis also revealed temporal changes in the level of Shh-GFP protein along the DV axis of the neural tube. Thus, cells close to the ventral midline are exposed to progressively increasing amounts of ligand, while at the same time the ligand spreads dorsally. Analyzing ligand levels in progenitors suggest that the duration of Shh exposure, as well as its concentration, influence the response of neural cells (see below).

Shh accumulates at the ventricular, apical pole of neural progenitors (Chamberlain et al., 2008) (Fig. 5C), an observation that raises several questions. How does a basally derived signal reach an apical position in the neural epithelium? In neural cells located in the midline of the neural tube, physically adjacent to the notochord, Shh protein is observed in small punctae closely associated with microtubules (Fig. 5C). This suggests that transcytosis might move Shh ligand received at the basal surface to an apical location (Fig. 4). The trafficking appears to be specific for Shh because a second, GFP-tagged, palmitoylated ligand (Wnt1) accumulates in basolateral positions (Chamberlain et al., 2008). Interestingly, a Displ homolog in *C. elegans* (CHE-14) is associated with the apical release of proteins (Michaux et al., 2000). Whether Displ plays a

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**Fig. 4. Multiple proteins modulate Shh spread in the neural tube.**

Schematic of proteins that affect Shh spreading in the ventral neural tube. Several cell surface proteins, including HSPGs, Ptc1, Hhip1, Gas1, Cdo and Boc, interact with extracellular Shh, decreasing its spread (blue arrow) through the neural tube and either enhancing or blocking signaling in a cell-autonomous manner. HSPGs possibly bind extracellular Shh protein and slow its spread through tissue. Ptc1 and Hhip1 both bind and sequester Shh protein, and act as cell-autonomous inhibitors of signal transduction. Gas1, Cdo and Boc also bind and sequester Shh, but act cell-autonomously to promote Shh signaling. Ptc1 and Hhip1 are transcriptional targets of Shh signaling and, consequently, are upregulated in the ventral neural tube as development progresses. By contrast, the positive regulators of signaling, Gas1, Cdo and Boc, are transcriptionally repressed by Shh signaling. Unlike Boc, Gas1 and Cdo are initially expressed in ventral regions of the neural tube, but are subsequently downregulated in response to Shh.
role in the release and movement of ShhNp in neural tube cells remains to be addressed. Does the apical accumulation require cells to respond to ligand? The answer appears to be no, as apical accumulation is still evident in Smo–/– embryos in which the entire target field is non-responsive. Where does ShhNp accumulate apically? Immunolocalization shows that GFP-labeled ShhNp is closely associated with the basal body, beneath the primary cilium (Fig. 5C). The association of Shh protein with cilia components might be significant given that the intracellular transduction of Shh signal requires the primary cilium (see below). Moreover, Shh basal body localization is not limited to the cells that contact the notochord, suggesting that it might represent a general mechanism...
for the spread and intracellular trafficking of Shh. Alternatively, ShhNp might extend its range of action as dividing cells, which contain Shh protein, are displaced dorsally with growth of the neural tube.

The availability of the Shh-GFP allele also confirmed that the induction of most primary ventral neural progenitor types occurs in the absence of floor plate-derived Shh (Ding et al., 1998; Matise et al., 1999; Chamberlain et al., 2008). The role of floor plate-derived Shh might therefore be limited to later functions, such as axon guidance or glial cell-type specification (Bourikas et al., 2005; Charron et al., 2003; Danesin et al., 2006; Hochstimm et al., 2008; Okada et al., 2006). Alternatively, floor plate-derived Shh might maintain DV gene expression patterns after their initial induction from a notochordal source (Danesin et al., 2006; Hochstimm et al., 2008). A thorough analysis of where and for how long Shh signaling is necessary will be required.

Several additional questions remain to be addressed. Does the Shh gradient reach a steady state in which a gradient is maintained by balancing the production and degradation of Shh protein? Alternatively, does the gradient continue to evolve and, if so, is this regulated by changes in the production or spread of Shh protein? Moreover, despite the importance of ligand degradation in the establishment and maintenance of a gradient, these systems have not been thoroughly investigated. Quantitative, high-resolution, dynamic imaging of ligand production, trafficking, spread and turnover should provide new insights into these questions.

The intracellular transduction of graded Shh signaling

Once Shh has reached responding cells, how do cells perceive and respond to the signal? Despite the significant gaps in our knowledge about this signaling pathway, an understanding of how the graded signal is communicated is beginning to emerge. In particular, both the amount of Shh and the duration of signaling control pattern in the neural tube, leading to a model of how cells dynamically interpret a gradient of Shh signal.

Smoothened activation transmits the signal intracellularly

The transmembrane protein Smo molecularly links extracellular Shh ligand to the activation of its intracellular signaling pathway (see Fig. 1). Use of small-molecule antagonists and Smo agonists in neural cells indicate that a graded activation of Smo recapitulates the graded cellular response to a Shh concentration gradient (Chen et al., 2002a; Chen et al., 2002b; Dessaud et al., 2007; Frankne et al., 2007; Frumence et al., 2007). The signaling pathway culminates in the regulation of three members of the Gli family of zinc-finger-containing transcription regulators (reviewed by Matise and Joyner, 1999). An attractive model of Shh morphogen activity envisages graded Shh signaling evoking a gradient of Gli activity in the neural tube by progressively inhibiting Gli repressor (GliR) activity and potentiating Gli activator (GliA) function (Jacob and Briscoe, 2003). Consistent with this model, gain-of-function experiments suggest that progressive changes in the level of Gli activity are sufficient to recapitulate the patterning activity of graded Shh signaling (Lei et al., 2004; Stamataki et al., 2005). In this view, each Gli protein contributes to the production of a gradient of Gli activity that is proportional to the level of signal transduction. This implies that the sum of the activity of individual Gli proteins in the cell determines its gene expression response to Shh signaling. Therefore, the specific involvement of individual Gli proteins to the gradient depends on their intrinsic transcriptional regulatory activity, their level of expression and their post-translation regulation by Shh signaling. The changes in DV patterning in the neural tube of embryos that lack individual Gli proteins can be viewed from this perspective.

In Gli2−/− mouse embryos, the most ventral cells (the floor plate) are not specified. This suggests that Gli2 is required to generate the highest Shh signaling response, consistent with the notion that Gli2 acts predominately as an activator (Ding et al., 1998; Matise et al., 1999; Park et al., 2000). Gli3, conversely, appears to function mainly as a transcriptional repressor as mouse embryos mutant for Gli3 exhibit a dorsal expansion of intermediate neural tube progenitors, indicating that a dorsal extension of Shh signaling has occurred (Persson et al., 2002). Moreover, all but the most ventral cell
identities (p3 and floor plate) are recovered when Gli3R function is abrogated in Shh- and Smo-null embryos (Litingtung and Chiang, 2000; Wijgerde et al., 2002). Thus, derepression via the removal of Gli3 is crucial for the specification of MN and more-dorsal Shh-dependent progenitor domains. Interestingly, an abnormal intermixing of different progenitors is observed in both these embryos (Litingtung and Chiang, 2000; Wijgerde et al., 2002) and those lacking all Gli activity (Bai et al., 2004).

A temporal adaptation mechanism integrates the duration of Shh signaling

In addition to concentration, the duration of Shh signaling also influences DV patterning (Dessaud et al., 2007; Jeong and McMahon, 2005; Stamatakis et al., 2005). A model for how both the ligand concentration and the duration of Shh signaling can control differential gene expression has been proposed (Fig. 6) (Dessaud et al., 2007). This "temporal adaptation" model relies on a progressive decrease in the sensitivity of receiving cells to ongoing Shh signaling. Cells first appear to be highly sensitive to Shh signal. Consequently, low concentrations of Shh are sufficient to evoke high levels of Gli activity. With increasing time, cells become desensitized to ongoing Shh signaling; thus, the concentration of Shh necessary to achieve the highest levels of Gli activity increases. As a result, different concentrations of Shh generate an intracellular signal for different periods of time, such that the duration of signaling is proportional to Shh concentration. Accordingly, increasing concentrations of Shh sustain intracellular signal transduction for increasing periods of time.

The response and function of Ptc1, and perhaps other negative-feedback inhibitors, can explain the gradual desensitization of cells to ongoing Shh signaling. In response to Shh signaling, cells steadily upregulate inhibitors such as Ptc1 (Goodrich et al., 1996; Marigo and Tabin, 1996). This suggests that increasing concentrations of Shh are necessary to block the inhibitory activity of accumulating Ptc1 (Dessaud et al., 2007). As discussed earlier, negative feedback is likely to have both cell-autonomous and non-autonomous roles in regulating the spread and response of cells to Shh (Figs 4-6). Distinguishing the relative contribution that cell-autonomous and non-autonomous processes make to normal patterning is difficult and highlights the importance of understanding the dynamics of signal transmission through the pathway. Methods that provide an ongoing measure of the activity of key components of the pathway will be essential, as will approaches for manipulating the duration of the activity of these components. Understanding how the levels and kinetics of Gli activity are altered in mutants deficient in individual Gli proteins will also be important. A key issue is to assess how the duration of signaling influences the establishment of normal DV patterning. As discussed earlier, analyses using Shh-GFP protein suggest a close correlation exists between gene induction in neural cells and the duration of their exposure to Shh (Chamberlain et al., 2008).

Dissecting the genetic network regulated by graded Shh signaling

The positional information supplied by Shh signaling regulates the spatial expression of a set of transcription factors in ventral progenitor cells (Fig. 2). These comprise a genetic network, which we will term the neural tube gene regulatory network (GRN). In common with other GRNs, there are three key issues to address. First, the members of the GRN must be identified and the molecular mechanism of their regulation uncovered. Second, we must understand how the structure and topology of the regulatory network generates the profile of gene expression observed in the neural tube. Third, the downstream targets of the network that ultimately control cell behavior and the identity of neural subtypes need to be determined. Initial progress towards these goals has been made by conventional developmental biological approaches. These have revealed specific details of the network and have suggested certain general features of its operation. The recent deployment of newer genomic and bioinformatic techniques is expanding our detailed knowledge of the neural tube GRN and is providing a broader picture of how the network functions.

Regulation of neural tube GRN genes

The initial studies that identified the HD and bHLH transcription factors that are expressed in restricted DV domains of progenitors, subdivided these regulatory factors into two classes based on their mode of regulation by Shh: class I genes are repressed by Shh, whereas class II genes require Shh exposure for their expression (Briscoe and Ericson, 2001). Many, although not all, of these factors function as transcriptional repressors (Muhr et al., 2001), and selective repressive interactions between pairs of class I and class II proteins have been defined (Figs 2, 7). As a consequence, pairs of neural tube GRN proteins delineate boundaries of progenitor domains (Fig. 7A). For example, Pax6 and Nkx2.2 comprise a cross-repressive pair that delineates the p3-pMN boundary. In mouse embryos mutant for Pax6, Nkx2.2 expands dorsally (Ericson et al., 1997). Conversely, Nkx2.2 expression is sufficient to inhibit Pax6 expression, although Nkx2.2 might share this role in vivo with Nkx2.9 (Briscoe et al., 1999; Ericson et al., 1997). Similar relationships are observed between other pairs of class I and class II proteins in the neural tube. Moreover, regulatory interactions between pairs of class II proteins (Fig. 7A) have also been identified (Novitch et al., 2001; Vallstedt et al., 2001). Together, these findings reveal that an elaborate network of cross-regulation exists between members of the neural tube GRN, although how the specificity of these interactions is determined remains to be defined.

More recently, an in silico approach by Bailey et al. (Bailey et al., 2006) has extended these ideas (Fig. 7B). An analysis of evolutionarily conserved regions (ECRs) of non-coding DNA from vertebrate genomes, presumed to encode regulatory elements, found several hundred ECRs that were enriched in binding sites for HD, Sox and POU transcription factors (Bailey et al., 2006). Remarkably, many of these ECRs are close to genes that are expressed in the developing CNS, with several adjacent to established members of the neural tube GRN. This led the authors to propose that the HD transcription factor members of the neural tube GRN provide specific, direct inhibitory activity through the ECRs (Fig. 7B). Concomitantly, Sox and POU factors, which are broadly expressed in neural progenitors, provide a positive transcriptional input to confer neural expression on the genes associated with the ECRs. The distinct spatial patterns of neural tube GRN gene expression would therefore be achieved by a spatially uniform, positive transcriptional input combined with temporally and spatially restricted specific negative inputs. This mechanism is in agreement with a genetic analysis of mice mutant for certain HD transcription factors (Fig. 7C). What remains to be determined is how the DV positional information that regulates the spatial pattern of gene expression in the neural tube is incorporated into this model. The presence of other binding sites within these ECRs will doubtless be one determinant. The existence of Gli binding sites (GBSs) would presumably be important for explaining the action of graded Shh signaling (see below).
The repressive activities of neural tube GRN proteins might partly explain the temporal profiles of neural tube GRN genes. Members of the network display dynamic changes in their patterns of expression during neural tube development (Fig. 2C, Fig. 6). For individual genes, this parallels their dependence on different levels and durations of Shh signaling – that is, class I proteins are initially expressed more ventrally in the neural tube but are progressively repressed in a ventral-to-dorsal manner. The order in which class I genes are repressed is inversely related to their sensitivity to Shh signaling (Dessaud et al., 2007; Ericson et al., 1997). Thus, Shh signaling progressively defines the ventral expression boundaries of class I proteins. Class II proteins, conversely, are sequentially induced in the ventral neural tube in an order that corresponds to their increasing requirement for Shh signaling (Dessaud et al., 2007; Jeong and McMahon, 2005; Stamataki et al., 2005). These temporal features of the neural tube GRN are consistent with the importance of the duration of Shh signaling in ventral neural tube patterning. Together, the data suggest that the spatio-temporal pattern of expression for an individual neural tube GRN gene is determined by a combination of uniform positive inputs (e.g. Sox and/or POU proteins) and the duration and level of Shh signaling.

**Fig. 6. A ‘temporal adaptation’ model for interpreting graded Shh signaling.** (A) A model for the spatial and temporal specification of progenitor cells during exposure to Shh secreted from a ventral source. At early time points (t₀), Ptch1 expression levels (brown receptor) in neural progenitors are low; consequently low levels of Shh protein (blue) are sufficient to bind the available Ptch1. This produces high levels of Smo signal transduction (green) and, consequently, high levels of positive Gli activity (GliA, red), even in cells that are exposed to a low concentration of Shh (cell 3, t₀). The upregulation of Ptch1 (and possibly other negative regulators of the pathway) by Shh signaling (t₁) increases the level of Ptch1 in responding cells. As a result, the concentration of Shh necessary to sustain high levels of signal transduction increases with time (t₁). In cells exposed to concentrations of Shh insufficient to bind all of the raised level of Ptch1 (cell 3, t₁), the level of GliA declines (GliA, orange). This process of cell-autonomous desensitization continues (t₂), resulting in distinct temporal profiles of Gli activity in cells arrayed along the DV axis. In addition, the upregulation of ligand-binding inhibitors of Shh signaling, including Ptch1, results in the sequestration of Shh protein in more-ventral regions of the neural tube (cell 1). Both the level and the duration of Shh-Gli activity influence the gene expression profile in responding cells. Low levels of Gli activity, for example produced by the partial inhibition of the generation of GliR activity, are sufficient to repress Pax7 (cell 4, t₁). The duration of Shh signaling is partially responsible for the distinction between Olig2 and Nkx2.2 induction. High levels of Gli activity induce Olig2 expression (cells 1-2, t₁). If the levels of signaling are sustained (cell 1, t₂), Nkx2.2 is induced and Olig2 is repressed. By contrast, if the levels of signaling in a cell decline prior to this time point, Olig2 expression is consolidated (cell 2, t₂). (B) The response of the indicated cells in A to Shh can be represented as a function of both Gli activity and the duration of Shh exposure (time). The adaptation of cells to ongoing Shh signaling results in different concentrations of Shh producing distinct profiles of Gli activity. Hence, temporal adaptation transforms different concentrations of morphogen into corresponding durations of increased Gli activity. In this view, the induction of each progenitor state requires exposure to a concentration of Shh above a defined threshold for a distinct period of time.
proteins) and spatio-temporally restricted inputs from Shh signaling, as well as positive or negative input from members of the neural tube GRN (Fig. 7).

Shh regulation of the neural tube GRN

Although graded Shh signaling and Gli activity are essential for DV neural tube patterning, the direct targets of Gli regulation in this network are poorly defined. Binding sites for Gli proteins have been investigated in only two Shh-dependent neural tube GRN genes, Nkx2.2 and Foxa2. In both cases, there is evidence that GBSs are required for the expression of these genes in the neural tube (Lei et al., 2006; Sasaki et al., 1997; Vokes et al., 2007). A recent ChIP-on-chip screen has begun to expand the number and location of GBSs (Vokes et al., 2007). A custom array of genomic regions surrounding 122 genes that appear to be regulated by Shh signaling, in neural and non-neural contexts, was screened using Gli1 chromatin immunoprecipitation products from ventralized neural cells, recovering a small number of GBSs, including some close to Nkx2.2/Nkx2.9 (V3 progenitors) and Foxa2 (floor plate progenitors). These findings are indicative of, and consistent with, a direct Shh input into localized expression of these determinants (Lei et al., 2006; Sasaki and Hogan, 1996). A whole-genome interrogation is likely to reveal a more complete Gli neural tube GRN. Furthermore, identifying sites where Gli3 binding is lost upon Shh signaling might

Fig. 7. The neural tube gene regulatory network. (A) Selective cross-repressive interactions contribute to the spatial-temporal regulation of gene expression in the neural tube. (Upper panel) Cross-repression between Nkx6 and Dbx proteins generate distinct gene expression patterns in p0, p1 and p2 domains of progenitors. (Lower panel) The generation of p2, pMN and p3 domains depends on selective cross-repressive interactions between Pax6 and Nkx2.2, and Olig2 and Irx3, and on the repression of Olig2 by Nkx2.2. (B) Schematic of DV patterning through repressive interactions. Representative transverse sections through mouse spinal cord are shown (RF, roof plate; FP, floor plate). The identification of regulatory modules that contain binding sites for POU, Sox and HD transcription factors adjacent to many genes of the neural tube GRN has led to a model for the regulation of these genes. POU (green) and Sox (blue) factors are transcriptional activators that are broadly expressed in neural progenitors (bottom panel, green), thus they are believed to provide spatially unrestricted activation to the regulatory modules. The HD factors (red), which have spatially and temporally restricted expression patterns in neural progenitors (example in bottom panel), mostly function as transcriptional repressors and have therefore been proposed to provide repressive input to the modules. The combination of uniform positive input and spatially restricted negative input on a target gene (yellow) would generate a spatially restricted pattern of expression (bottom panel). In this model, the consequences of mutating the repressive HD gene would be the derepression of the target gene (bottom panel, right). (C) Alterations in the pattern of gene expression in embryos lacking individual members of the neural tube GRN confirm the importance of cross-repressive interactions for DV pattern formation. The changes in gene expression in mouse embryos mutant for the indicated genes are summarized in the diagrams. In Nkx6.1−/− embryos, Nkx6.2 (6.2) and Dbx2 (D2) are expressed in more-ventral progenitors. Dbx1 (D1) expands ventrally in Nkx6.2−/− mutants. Deletion of both Nkx6.1 and Nkx6.2 results in the ventral expansion of both Dbx1 and Dbx2. In Olig2−/− mouse embryos, Irx3 expands into the domain normally occupied by MN progenitors. Olig2 (O2) expands ventrally in Nkx6.2−/− embryos. In embryos mutant for Pax6, Nkx2.2 (2.2) expands dorsally, repressing Olig2 expression (Irx3 expression has not been determined).
reveal a different set of targets to those bound by Gli1 activator. The low number of GBSs in the Shh-regulated gene set raises the possibility that only a limited number of genes within the neural tube GRN require a direct Gli input. If so, a direct Shh input might create a ‘pioneer’ patterning response, while cross-regulatory interactions within the neural tube GRN refine DV pattern through additional transcriptional inputs.

In silico methods to predict GBSs in the genome have also been employed with different degrees of success (Hallikas et al., 2006; Vokes et al., 2007). However, these approaches make significant numbers of false-positive and false-negative predictions. A better understanding of the regulatory logic that underlies the binding and activity of transcription factors will generally facilitate de novo predictions of regulatory mechanisms from primary genomic sequence. These approaches are not able to predict in which target tissue a particular GBS operates. Thus, predictions need to be intersected with other data (e.g. transcriptional profiling) to unravel networks in a specific cell population.

The basic question of how graded Shh signaling is interpreted at the genomic level to control differential gene expression remains to be answered. In other systems, differential gene expression is regulated in response to the positioning, affinity or number of binding sites for a morphogen-regulated transcription factor (Driever et al., 1989; Gaudet and Mango, 2002; Stathopoulos et al., 2002). Genes that contain several binding sites (GBSs) in a regulatory module might explain differential sensitivity of genes to the level of Shh signaling: (B) The number or affinity of Gli binding sites (GBSs) in a regulatory module might explain differential gene expression. Genes with several high affinity GBSs (yellow gene C) would respond to low concentrations of Shh, resulting in a broad expression domain, with a dorsal boundary far from the ventral midline. Genes with fewer high affinity GBSs (orange gene B) or low affinity GBSs (green gene A) would require higher levels of Gli activity and therefore would be expressed in correspondingly more restricted regions of the neural tube. (C) Gli activity may act in conjunction with other repressor and activator signals. In this case, the presence of other transcription factors influences the response of individual genes to Shh signaling. For example, in yellow C and orange gene B, the presence of binding sites for transcription factors acting as activators (TF+) sensitizes the response of these genes, facilitating induction at lower levels of signaling. By contrast, the red gene A, which contains binding sites for a transcriptional inhibitor (TF–), requires higher levels Gli activity and, consequently, higher levels of signaling to overcome the repressive activity. (D) The addition of cross-regulation between Shh-dependent genes (see Fig. 7) is likely to refine specific domains of expression in the ventral neural tube. Adding an inhibitory input between two genes from the network in panel C restricts the expression of gene C to a specific domain of progenitors. Such a mechanism could account for the regulation of genes such as Nkx2.2 and Olig2. RF, roof plate; FP, floor plate; Gli, Gli transcription factor; TF, transcription factor.
and Hogan, 1996; Sasaki et al., 1997; Vokes et al., 2007). Whether the difference in the sequence of the two GBSs explains the difference in Foxa2 and Nkx2.2 expression has not been tested, but seems unlikely. Interestingly, both genes are initially activated by Shh signaling with similar temporal and spatial kinetics (Jeong and McMahon, 2005). Thus, some secondary event generates cells that exclusively express each determinant. More generally, it seems likely that the integration of a direct Gli transcriptional input with other inputs (positive and negative) establishes the threshold response for genes in the neural tube GRN (Fig. 8). Thus, the response threshold of a gene is determined by the sum of regulatory inputs on the gene, not solely by the affinity or number of GBSs. This combinatorial mechanism for setting response thresholds is consistent with the identification in neural tube GRN genes of ECRs that contain binding sites for a suite of transcriptional regulators. In this view, the differential integration of positive and negative transcriptional inputs on an individual target gene determines its threshold response to Shh signaling. A similar mechanism has also been suggested for gene regulation by the Drosophila Bicoid morphogen (Ochoa-Espinosa and Small, 2006). This regulatory strategy permits mechanisms, such as feed-forward loops (Mangan and Alon, 2003), that would provide an explanation for the dynamic nature of gene expression in the neural tube, as well as the requirement for prolonged duration of signaling for the induction of some neural tube GRN genes.

The regulation of the neural tube GRN

Combined experimental and bioinformatic approaches are beginning to piece together the neural tube GRN, although the details are still fragmentary. Two key ideas emerge. First, specific regulatory interactions between genes in the neural tube GRNs are important for defining the spatio-temporal profiles of gene expression. This aspect of the network is yet to be addressed in detail at the molecular level. Second, the role of graded Shh signaling within the neural tube GRN provides a positional basis to the network by regulating the extent and timing of expression of certain transcription factors within the neural tube GRN. In this view, graded Shh signaling imparts positional information by providing one of a number of inputs into the regulation of members of the neural tube GRN (Fig. 8). The predetermined regulatory interactions within the neural tube GRN transpose this positional information to the tightly regulated patterns of gene expression characteristic of the neural tube. This regulatory strategy might also shed light on the tightly regulated patterns of gene expression characteristic of the neural tube GRN (Fig. 8). The predetermined regulatory interactions network by regulating the extent and timing of expression of certain GRN genes of ECRs that contain binding sites for a suite of transcriptional regulators. In this view, the differential integration of positive and negative transcriptional inputs on an individual target gene determines its threshold response to Shh signaling. A similar mechanism has also been suggested for gene regulation by the Drosophila Bicoid morphogen (Ochoa-Espinosa and Small, 2006). This regulatory strategy permits mechanisms, such as feed-forward loops (Mangan and Alon, 2003), that would provide an explanation for the dynamic nature of gene expression in the neural tube, as well as the requirement for prolonged duration of signaling for the induction of some neural tube GRN genes.

Conclusions

The progress towards understanding DV patterning of the neural tube provides insight into some of the underlying principles that determine the exquisite pattern of vertebrate neurogenesis. What has been revealed is a complex, integrated network of molecular and genetic interactions that receives and interprets the positional information supplied by graded Shh signaling. Importantly, neural target cells are not passive recipients of a graded Shh signal; instead, the cells dynamically influence the spread of the signal through the tissue and alter their response to the signal. This suggests that in place of the conventional view of a morphogen (Kerszberg and Wolpert, 2007), a definition needs to be considered in which the concentration and duration of signaling, together with the response of the tissue, are critical for the formation and interpretation of the morphogen gradient (Box 1). Thus, signal and tissue collaborate to produce a specific pattern. Our understanding will be enhanced if these events can be dynamically imaged and quantified and the complete neural tube GRN defined. A diversity of experimental approaches – genetic, molecular, imaging and modeling – are required to provide a clearer picture of this dynamic, multi-dimensional system.

In this review, we have focused on one crucial component, Shh. However, a similar patterning strategy is thought to underlie neural specification in the dorsal neural tube (Chizhikov and Millen, 2005; Liu and Niswander, 2005). Here, transforming growth factor (TGF) β and Wnt family members are the dominant positional cues, but regulatory interactions between transcription factors controlled by extrinsic and intrinsic signals remain central to the control of positional identity. Furthermore, in addition to their role in the dorsal neural tube, bone morphogenetic protein (BMP) and Wnt signaling also appear to influence patterning in the ventral neural tube (Alvarez-Medina et al., 2008; Lei et al., 2006). How cells integrate the effects of multiple co-incident signals in order to generate an appropriate response will be a crucial question for future studies.

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