SIGNALLING IN DEVELOPMENT

# Morphogen to mitogen: the multiple roles of hedgehog signalling in vertebrate neural development

*Marc Fuccillo, Alexandra L. Joyner and Gord Fishell*

Abstract | Sonic hedgehog has received an enormous amount of attention since its role as a morphogen that directs ventral patterning in the spinal cord was discovered a decade ago. Since that time, a bewildering array of information has been generated concerning both the components of the hedgehog signalling pathway and the remarkable number of contexts in which it functions. Nowhere is this more evident than in the nervous system, where hedgehog signalling has been implicated in events as disparate as axonal guidance and stem cell maintenance. Here we review our present knowledge of the hedgehog signalling pathway and speculate about areas in which further insights into this versatile pathway might be forthcoming.

Morphogen
A secreted factor that can induce more than two different cell fates over a sheet of cells in a concentration-dependent manner by forming a gradient.

Mitogen
Any factor that promotes proliferation.

*Developmental Genetics Program and the Department of Cell Biology, Skirball Institute of Biomolecular Medicine, New York University Medical Center, 540 First Avenue, New York, NY 10016, USA. Correspondence to G.F. email: fishell@saturn.med.nyu.edu*
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The best-studied functions of the members of the hedgehog protein family in the nervous system are their roles as morphogens; each protein acts at a distance from the source of the signal to establish cell identities in the ventral spinal cord in a concentration-dependent manner. However, during the past decade it has become apparent that the functions of the members of the hedgehog protein family in development are diverse, with their specific actions changing with both space and time. This has proven to be true with respect to the molecular components that modulate and transduce hedgehog signalling, and the specific cellular processes that it controls. The increasing number of both intra- and intercellular modulators of hedgehog signalling have been comprehensively discussed in recent reviews[1,2].

Here, we largely confine our discussion to the roles of hedgehog signalling in the mammalian nervous system. We first discuss the function of hedgehog proteins in directing neural progenitors to acquire specific cell identities. In particular, we focus on how members of the GLI family of transcription factors act as effectors of hedgehog signalling in the spinal cord, allowing an extracellular concentration gradient of hedgehog to be translated into different cell identities. We then discuss how iterations of this molecular pathway at more anterior levels of the nervous system lead to the induction of cell types appropriate for the telencephalon, diencephalon, midbrain and anterior hindbrain. In addition to this role in patterning, we review the many developmental events that hedgehog signalling mediates, including the generation of oligodendrocytes and its mitogenic role in controlling the proliferation of neural progenitors. Finally, we discuss some more recently recognized roles for hedgehog signalling in axonal pathfinding and the maintenance of adult stem cells.

## Molecular mechanisms of hedgehog signalling

The molecular mechanisms by which extracellular hedgehog signals are received, interpreted and transformed into intracellular cell fate decisions have received a great deal of attention[3,4]. Work from both vertebrate and invertebrate species has delineated a canonical hedgehog signalling pathway, in which cell-surface binding of hedgehog ligands leads ultimately to the formation of repressor or activator forms of members of the GLI/CI family of zinc-finger transcription factors (FIG. 1)[5]. In vertebrates, the functions of the protein encoded by the ancestral invertebrate gene cubitus interruptus (*ci*) have been divided between the three GLI proteins (GLI1, GLI2 and GLI3)[6]. Hedgehog signalling in mammals is initiated by one of three homologues of the Drosophila hedgehog protein[7] — sonic hedgehog (SHH), indian hedgehog (IHH) and desert hedgehog (DHH), of which the first two have been implicated in neural development[8,9].

The transduction of hedgehog signalling in cells is mediated by interactions between two proteins, the twelve-pass membrane hedgehog receptor patched (PTC1)[10,11] and the seven-pass G-protein-coupled receptor smoothened (SMO)[2,12]. Genetic and biochemical data indicate that, in the absence of hedgehog ligand, PTC1

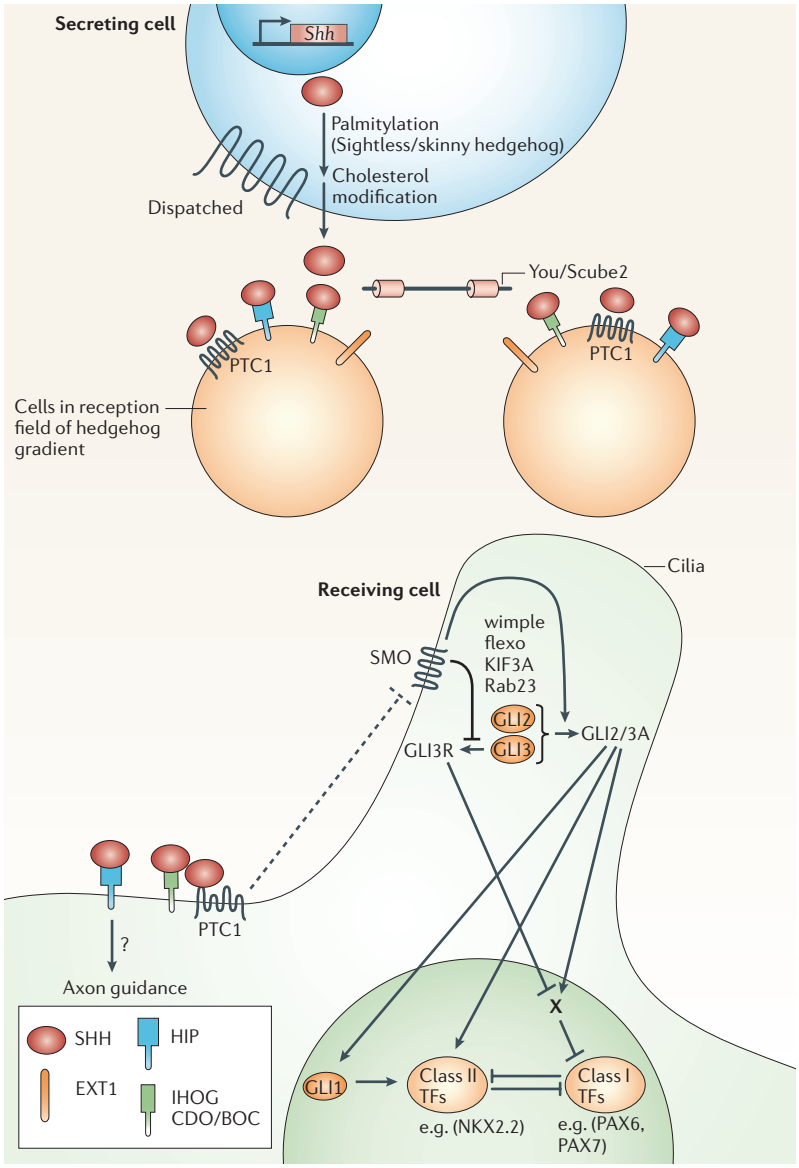


Figure 1 | **The vertebrate hedgehog signalling pathway.** The figure shows the canonical hedgehog signalling pathway, from the release of hedgehog protein to the interpretation of hedgehog signalling by downstream transcriptional mediators. In the cell producing the ligand, sonic hedgehog protein (SHH) is acetylated (palmitylation is mediated by the acetylase skinny hedgehog and the cleavage-mediated addition of cholesterol) and released through the action of the membrane-transporter protein dispatched. The SHH gradient is regulated by extracellular proteins (such as You/Scube2 in zebrafish), as well as proteins on the surface of cells situated between those producing and receiving hedgehog signals (for example, patched (PTC1), hedgehog interacting protein (HIP), exostosin (EXT1), CDO (interference hedgehog (IHOG) in *Drosophila*) and its relative BOC). In the cell receiving the hedgehog ligand, hedgehog signalling components are sequestered to the primary cilia. The hedgehog ligand relieves the constitutive repression of the seven-pass receptor smoothened (SMO) by the twelve-pass receptor PTC in mice. The output of hedgehog signalling is mediated by the modulation of the GLI transcriptional activators GLI2 and GLI3 (GLI2/3A), and the GLI repressor GLI3 (GLI3R). Intracellular modulation of the hedgehog transcriptional cascade includes both positive (wimple, flexo, KIF3A) and negative (Rab23) regulators of hedgehog signalling, components that are also involved in cilia function. The final output of the hedgehog signalling cascade is mediated through the repression of Class I transcription factors (TFs), such as PAX6 and PAX 7 and the activation of Class II transcription factors, such as NKX2.2. The X in the figure refers to the as yet unknown transcriptional repressor that inhibits Class I proteins, which is presumably induced by GLI2/3A and inhibited by GLI3R.

constitutively represses SMO activity[120]. This repression is non-stoichiometric, implying that PTC1 acts by a catalytic mechanism[13]. The details of this interaction are currently unknown, although the homology of PTC1 to bacterial proton-driven transmembrane molecular transporters offers the intriguing possibility that PTC1 regulates the distribution of a small molecule that alters SMO activity[14]. Hedgehog ligand binding to PTC1 relieves this inhibition through a mechanism that is also poorly understood.

In *Drosophila*, the pathway from SMO activation to the processing, activation and translocalization of the CI transcriptional mediator involves the activity of a large protein complex including the kinesin-like protein costal 2 (COS2), the serine/threonine protein kinase fused (FU), and suppressor of fused (SUFU)[1,15]. However, work involving species from fish to mice shows that these components might not be utilized in a conserved manner in mediating vertebrate intracellular hedgehog signalling, even between these relatively close species. For example, the function of COS2 as a negative regulator of hedgehog signalling in the absence of hedgehog ligand seems to be conserved in fish[16] but not in mice[17] (for a review, see REF. 1).

In addition, numerous unforeseen intracellular components that modify hedgehog signalling have been revealed through forward genetic screens. In vertebrates, mutations in three separate intraflagellar transport proteins — wimple (IFT172), flexo (hypomorphic allele of polaris, IFT88) and the intraflagellar transport motor protein KIF3a — result in similar neural phenotypes in mice, with characteristics of both reduced and excess hedgehog signalling[18,19]. Another link between neural hedgehog signalling and intracellular vesicle transport was revealed by the *open-brain* mouse mutant, which has the loss-of-function *Rab23* allele, a member of the Rab family of GTPases[20]. Interestingly, many of the proteins mentioned above are associated with cilia function, suggesting a role for this structure in hedgehog signalling[19,21]. Mutants that result in the loss of cilia, such as those associated with oral-facial-digital syndrome 1 (*OFD1*) loss-of-function and Bardet-Biedl syndrome, have defects in patterning reminiscent of reduced hedgehog signalling[22] and, consistent with this hypothesis, a recent study has demonstrated that SMO can localize to cilia in response to hedgehog pathway stimulation[23]. Moreover, mutations of amino acid residues in SMO that prevent this localization result in a functionally inactive form of SMO. With the discovery that the three GLI proteins and the negative regulator SUFU are located at the distal tips of the cilia in addition to the nucleus, it seems likely that the cilia function as localized mediators of hedgehog signal propagation[24]. Certainly the presence of primary non-motile cilia on most CNS neurons allows for the possibility that hedgehog signalling utilizes this vestigial structure[25].

## Patterning the ventral spinal cord

Our greatest understanding of the function of hedgehog signalling as a morphogen in the vertebrate nervous system has come from analysis of the mouse and chick

V0–V3 interneuron domains
Subdomains of the ventral spinal cord that give rise to specific populations of interneurons.

spinal cord, where SHH is expressed by the most ventral cells, the floor plate (FIG. 2). In this setting, SHH shows all the characteristics of a morphogen: it acts at a distance from the point source in a concentration-dependent manner and acts directly on the recipient cell without any relay mechanism[9,26–29].

The first studies supporting the role of SHH as a morphogen came from *in vitro* culture assays utilizing intermediate regions of chick embryo spinal cord that were collected prior to the obvious establishment of dorsoventral identity. When these explants were exposed to controlled levels of recombinant SHH, it was found that particular ventral spinal cord identities could be induced, based on the concentration of SHH[27]. Importantly, different concentrations of SHH were sufficient to induce specific ventral spinal cord neuronal populations. For example, whereas the lowest levels of SHH could induce intermediate ventral identities, such as V0, V1 and V2 interneurons (FIG. 2), higher concentrations were required to induce motor neurons and the highest concentration was necessary to induce the most ventral neuronal cell types: the V3 interneurons and the floor plate.

*In vivo* evidence for SHH's action as a morphogen during neural development has been best demonstrated by two studies employing cell-autonomous methods to render cells unresponsive to SHH in mice and chicks[9,28]. In these experiments, SHH responsiveness has been ablated either through the expression of a delta-loop deletion mutant of PTC1 that renders cells expressing this mutant protein insensitive to the endogenous ligand, or through the generation of *Smo*-null chimaeras, in which SHH non-responsive cells were intermingled with wild-type cells in the neural tube. In the delta-loop PTC1 studies, in addition to the cell-autonomous effects expected in ventral progenitors lacking SHH signalling, there was also a marked non-cell-autonomous repatterning around mutant groups of cells. This phenomenon is probably due to a local skewing in the gradient of SHH, because it is able to travel unimpeded across cells that express the mutant PTC1 receptor.

The SHH gradient in the spinal cord is not a passive source–sink gradient but is instead maintained by active mechanisms that regulate the activity, release, transport and retention of SHH protein across the neuroepithelium (FIG. 1). These modulators of SHH signalling include sightless/skinny hedgehog, an acetyltransferase that modifies SHH by adding palmitic acid to its amino (N) terminus[30,31], as well as proteins such as dispatched, You/Scube2 and tout-velu (the mouse homologue of this gene is exostosin (EXT1)) that control the release and transport of hedgehog proteins in different species[32–35]. In addition, several other extracellular proteins limit the range of SHH movement such as PTC1 and hedgehog interacting protein (HIP)[10,36] (for a more detailed discussion of these components of SHH signalling, see REF. 2). More recently, a novel extracellular protein CDO (interference hedgehog (IHOG) in *Drosophila*) and its relative BOC have been found to positively regulate hedgehog signalling through the binding and sequestration of hedgehog ligand, resulting in locally enhanced signalling[37–39].

A fundamental question in development is how the extracellular concentration gradient of a morphogen is translated into the intracellular specification of multiple cell identities (FIG. 2). It is now clear that all the effects of SHH in ventral spinal cord patterning in mammals are mediated by the combined activity of the three GLI transcription factors[6,40]. Elegant genetic studies have now exposed both the partially redundant and mainly unique functions of each mouse *Gli* gene in an *in vivo* context. Genetic loss-of-function and knock-in studies in which the *Gli* genes have been ablated[41–44] or genetically substituted[6,45] have shown that, at least in the context of ventral neural tube specification, GLI3 functions primarily as a transcriptional repressor whereas GLI1 and GLI2 function as activators[6,41–47]. Positive SHH signalling induces the formation of GLI2 and (to a lesser extent) GLI3 activators, which induce transcription of the constitutive activator GLI1. By contrast, absence of the ligand allows for the formation of the GLI3 repressor. Other regions of the nervous system employ these same activator/repressor combinations to different extents in transforming SHH signals into cellular responses.

Primarily GLI2 activator function is essential for the induction of the ventral-most cell types, including the floor plate and V3 interneurons[41,43,44], whereas the proper regulation of GLI3 repressor levels controls the number and location of V0, V1 and V2 progenitors[6,42]. Situated between these extremes are motor neurons, the differentiation of which is regulated by a combination of GLI2 and GLI3. In contrast to the positive activity of SHH signalling in the floor plate and V3 interneurons[6,45], SHH seems to function primarily as a negative regulator in intermediate

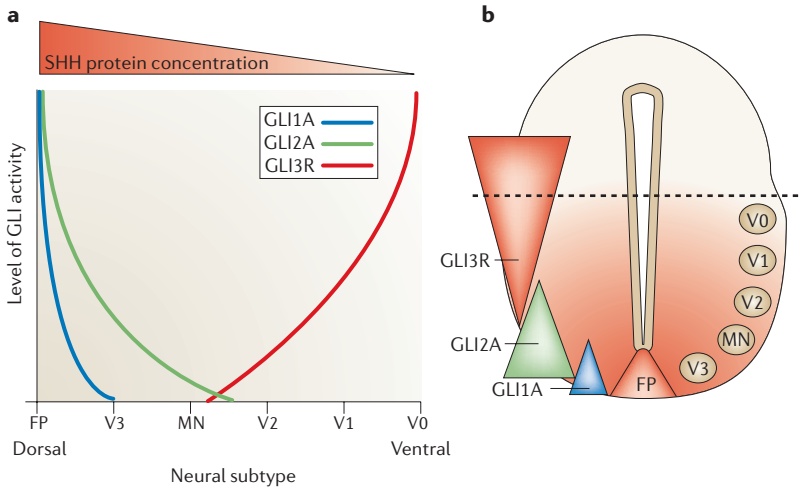


Figure 2 | **Sonic hedgehog gradients are translated into unique cell identities through the modulation of GLI activators and repressors.** The highest concentrations of sonic hedgehog (SHH) result in the production of the GLI transcriptional activator GLI1 (GLI1A) through effects on transcription, the activator GLI2 (GLI2A) through effects on post-transcriptional activation, and the removal of the repressor GLI3 (GLI3R) by inhibition of post-translational cleavage of the full-length form of GLI3. This results in the specification of the floor plate (FP) and V3 interneurons. Lower levels of GLI2A, as well as the removal of GLI3R result in the induction of V2 interneurons and motor neurons (MN). V0 and V1 patterning is controlled largely by the levels of GLI3R. **a** | Hypothetical relative activities of the primary GLI activators and repressors in the mammalian spinal cord. **b** | Schematic diagram illustrating (left) the activity of the GLI activators and repressors as well as (right) the position of the ventral populations of neurons generated through the action of hedgehog signalling.

ventral spinal cord patterning[42]. Interestingly, it does so by suppressing cues that would otherwise direct progenitors to adopt a dorsal fate, rather than by specifying ventral fate directly[52].

Downstream of the GLI proteins, hedgehog signalling mediates cell specification through the simultaneous repression of Class I homeodomain transcription factors (for example, *Pax6*, *Pax7*, *Irx3* and *Dbx1/2*) and the induction of Class II homeodomain transcription factors (for example, *Nkx2.2*, *Nkx6.1*) in ventral spinal cord progenitors[48–50]. Cross-repressive interactions between these resultant homeodomain transcription factors then act to sharpen the expression boundaries and subsequently direct cells to differentiate into specific subtypes[48]. The mechanistic link between the GLI transcriptional activators and repressors and the resultant homeodomain pattern has yet to be elucidated. It seems likely that differential promoter affinities and region-specific cofactors aid in translating the overlying combinatorial GLI transcription factor gradients into cellular decisions. In addition, recent electroporation studies in chick embryos have suggested that GLI transcriptional mediators can be cumulatively integrated over time to achieve appropriate homeodomain transcription factor patterning[51].

That positive SHH signalling is not required for the generation of some ventral cell types is evident from compound mutants of *Shh* or *Smo* and *Gli3* (REFS 9,52), as well as mouse embryos deficient in all three GLI proteins[6,40]. In these mutants, some aspects of ventral spinal cord patterning are maintained, with the exception of the floor plate, V3 interneurons and some motor neuron populations. Therefore, GLI activators are not required to induce these intermediate ventral cell types. With regard to the potential signalling pathways responsible for the induction of the cell types for which SHH signalling is not required, both fibroblast growth factor (FGF) and retinoid signalling have been proposed to have a role in ventral patterning in the neural tube. FGF signalling seems to regulate the timing of ventral cell type specification. Electroporation studies and explant work from chick embryos have suggested that retinoids provide the positive regulation of class I homeodomain proteins that complement the actions of SHH in repressing dorsal neural identities and inducing class II gene expression ventrally[53].

A surprising set of findings indicate that hedgehog signalling might not only control ventral progenitor specification, but could also act to maintain the proper stratification of these distinct progenitor domains in their appropriate neuronal pools. Genetic removal of hedgehog signalling results in disorganization of the ventral progenitor pools, with extensive cell mixing disrupting the normal segregation of progenitors along the dorsoventral extent of the neural tube[6,9,40]. This suggests that, in addition to a role in ventral subtype specification, hedgehog signalling through GLI transcriptional mediators controls sorting of distinct progenitor groups, perhaps by modulating cell affinities. At present, however, there is only evidence from *in vitro* neuroepithelial cultures to connect SHH signalling with known adhesion mechanisms associated with hetero- and homotopic cell sorting[54].

Homotopic cell sorting
The sorting of populations of cells that possess similar adhesion properties.

Medial ganglionic eminence
(MGE). A transient proliferative zone in the ventral telencephalon that is both patterned by and ultimately expresses sonic hedgehog. It seems to be the origin of most cortical interneurons, as well as a subpopulation of oligodendrocytes.

Zona limitans intrathalamica
(ZLI). An embryonic structure that is positioned at the boundary between the dorsal and ventral thalamus and acts as an organizing centre, at least partially due to its expression of SHH.

Adult proliferative niche
Select regions within the telencephalon in which postnatal neurogenesis occurs.

## Cell specification throughout the neuraxis

Beyond the spinal cord, the role of hedgehog signalling in ventral patterning in the nervous system has been examined in several different regions of the developing vertebrate CNS, including the forebrain, midbrain and cerebellum. Work from each of these fields is reviewed here with a focus on novel insights into regional differences in the patterning functions of SHH (FIG. 3).

***Forebrain.*** The developing forebrain, comprising the telencephalon and diencephalon, has multiple sources of SHH signal that influence patterning and specification in this region. In the embryonic telencephalon, SHH is expressed within the mantle of the medial ganglionic eminence (MGE), the preoptic area and the amygdala, whereas the expression of SHH in the embryonic diencephalon is found within the hypothalamus and in the zona limitans interthalamica (ZLI), a band of cells at the border between the ventral and dorsal thalami[7,55]. Interestingly, the onset of the expression of *Nkx2.1*, an early marker of ventral forebrain specification, precedes the expression of SHH in the forebrain, implying that ventral specification in these structures is the result of a non-neural source of SHH signalling[56]. *In vitro* work with chick embryos and conditional gene ablation in mice have supported this notion, suggesting that SHH sources from the node and the prechordal plate, which underlies the developing telencephalic primorium, pattern the ventral forebrain[57,58]. Furthermore, it seems that by the time SHH is expressed in the telencephalon, the initial patterning of this structure is complete and SHH signalling is required for the maintenance of ventral-most telencephalic gene expression[59]. Abrogation of SHH signalling after this initial patterning stage leads to a decrease in *Nkx2.1* expression in MGE progenitors and a resulting reduction in the two major populations of cortical interneurons derived from this region[60]. The reiterative use of SHH signalling to effect diverse telencephalic functions highlights the importance of the specific competence of the neural progenitors receiving and integrating the signal. In the telencephalon, temporal changes in the competence of cells to respond to SHH signals seem to be the rule rather than the exception. In basic ventral telencephalic patterning, it seems that the timing of exposure to SHH is crucial in establishing medial versus lateral ganglionic eminence fates[61]. Likewise, in oligodendrocyte specification and population of the adult proliferative niche, only specific cohorts of cells that respond to SHH within discrete time windows follow these maturation programs, as will be described below.

Recently, the protein Megalin, which is a low-density lipoprotein receptor-related protein, was shown to specifically effect ventral telencephalic patterning through what seems to be a SHH mediated mechanism[62]. Loss of *Megalin* results in the loss of ventrally-derived interneurons and oligodendrocytes in the mouse forebrain. The phenotype seen in these mice is reminiscent of that observed in mutants in which SHH signalling in the telencephalon was conditionally ablated at embryonic day (E) 9 by the removal of *Smo* using a Cre/LoxP system driven by the telencephalon-specific gene *Foxg1* (REF. 57).

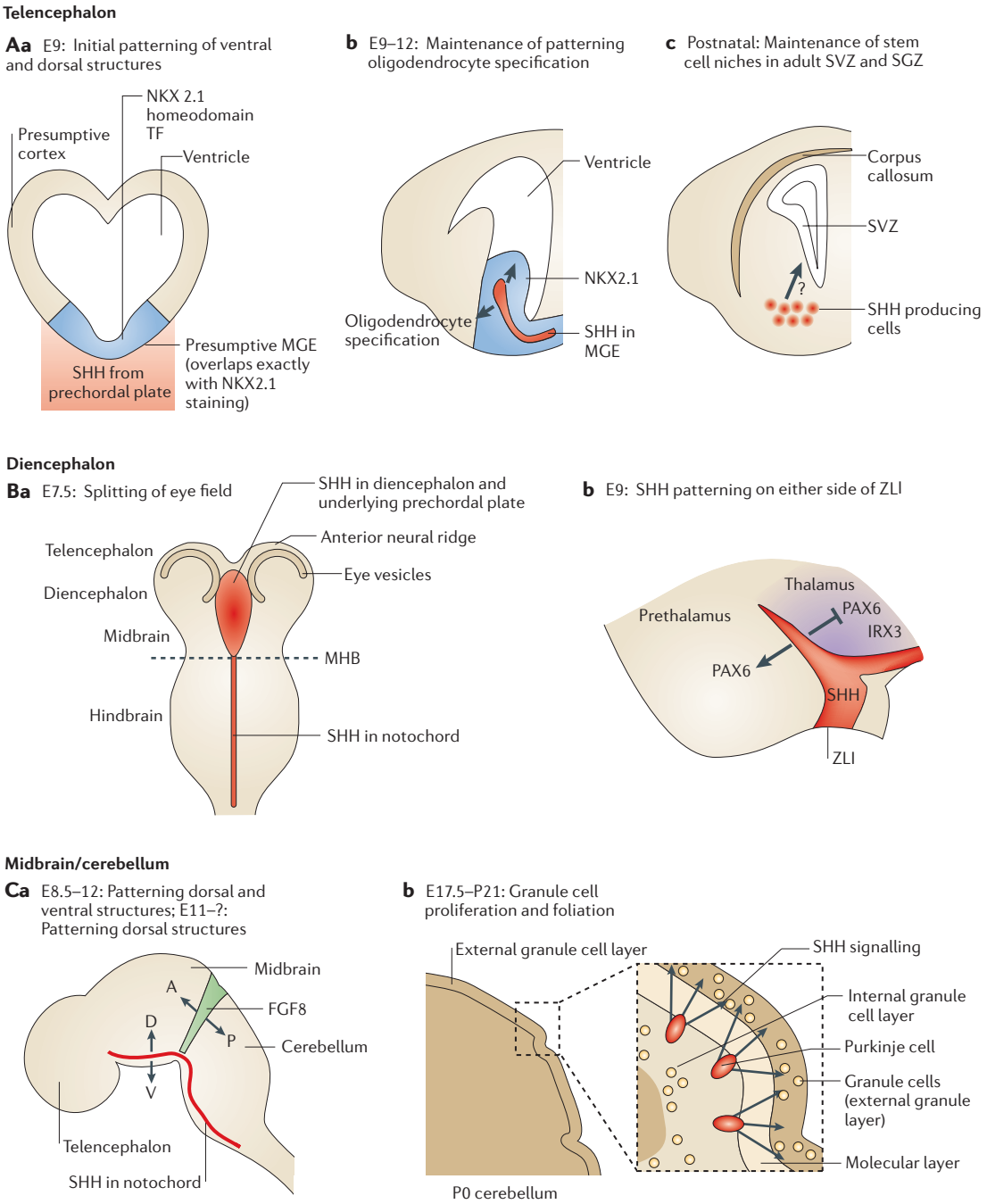


Figure 3 | **The utilization of sonic hedgehog differs across the developing neuraxis.** The figures depict the effects of sonic hedgehog (SHH) expression (red) in the developing mouse CNS. **A** | Coronal sections through the telencephalon. Throughout embryonic development, SHH signalling expressed in the ventral telencephalon (the medial ganglionic eminence (MGE)) maintains ventral patterning (through NKX2.1 expression) (**a**), oligodendrocyte specification (**b**) and proliferation and stem cell maintenance in the subventricular zone (SVZ) and subgranular zone (SGZ) (**c**). Whether SHH ligand moves from source cells to responding cells by diffusion, active transport or axonal trafficking is presently unclear. **Ba** | Dorsal (D) view of the neural plate prior to neural tube closure. Early (embryonic day (E) 7.5) SHH signalling is required for splitting the bilateral eye fields. **Bb** | Sagittal view of the diencephalon. At a slightly later embryonic timepoint, SHH expressed within the zona limitans intrathalamica (ZLI) differentially patterns the anterior–posterior axis of the diencephalon. This is achieved in conjunction with posteriorly expressed IRX3, which alters the competence to respond to SHH. Posterior (P) to the ZLI, SHH inhibits PAX6 transcription, whereas anterior (A) to the ZLI SHH is required for the maintenance of PAX6 expression[74]. **Ca** | Side view of the developing brain. In the midbrain and cerebellum, SHH expressed in the ventral (V) midline regulates dorsoventral patterning and the expression of the midbrain-hindbrain organizer FGF8 during early development (E8.5–E12). **Cb** | Saggital section of the cerebellum. From E17.5, SHH expressed by Purkinje cells (inset) regulates proliferation of the granule cells and is required for cerebellar foliation. MHB, midbrain–hindbrain boundary; TF, transcription factor.

The group that originally identified Megalin proposed that its role in SHH signalling might be to function as a SHH co-receptor[63]. More recently, others have argued that its effects are indirect and act through an increase in bone morphogenetic protein signalling, which then acts to attenuate SHH signalling[62]. Nevertheless, whether as a result of direct or indirect effects, these findings show that SHH signalling in the telencephalon is specialized at least in its requirement for Megalin.

Another unique characteristic of SHH signalling in telencephalic development is its role in the specification of the dorsal cortical midline, which later gives rise to the hippocampal primordium and midline choroid plexus epithelium[64,65]. This activity is in marked contrast to the role of SHH in the spinal cord, in which loss-of-function mutations in the hedgehog pathway have no obvious effects on patterning of the dorsal progenitor domains[45]. The mechanism of SHH's dorsal action in the telencephalon, in addition to whether its actions are direct or indirect, through the modulation of other key signalling centres, is presently unclear.

Another interesting aspect of telencephalic hedgehog signalling that contrasts with its action in the spinal cord is the apparent absence of GLI activator function in the specification of most telencephalic progenitors. *Gli1*$^{-/-}$;*Gli2*$^{-/-}$ double-null mutant mice have no major telencephalic defects[41], and most of the patterning defects of the *Shh*-null mice can be rescued by removing the repressor form of GLI3 (REF. 65). This implies that a gradient of the repressor form of GLI3 is primarily important for telencephalic cell specification, whereas the spinal cord uses the full complement of GLI activator and repressor forms to specify ventral and intermediate cell types. Perhaps this is not surprising given that the telencephalon is exclusively derived from the alar plate, whereas the GLI activators seem generally to be linked to the specification of basal plate progenitors. The fact that the dorsoventral patterning is rescued in *Shh*$^{-/-}$;*Gli3*$^{-/-}$ and *Smo*$^{-/-}$;*Gli3*$^{-/-}$ compound mutants suggests that, as in the spinal cord, positive regulators independent of SHH must also regulate ventral telencephalic patterning. *Fgf8* and retinoids seem to both be likely candidates to mediate this patterning[66,67]. Reminiscent of SHH's role in controlling FGF8 expression in the midbrain–hindbrain boundary, recent work suggests that FGF signalling can mediate ventral patterning independently of hedgehog signalling in the telencephalon[68]. Specifically, unlike *Shh*$^{-/-}$ mice, in which the loss of ventral patterning can be reversed by the compound removal of *Gli3* (REF. 65), no improvement in the ventral telencephalic pattern is seen in fibroblast growth factor receptor 1/3 conditional mutants when *Gli3* gene function is also reduced (that is, *Fgfr1*$^{-/-}$;*Fgfr3*$^{-/-}$;*Gli3*$^{+/-}$)[68]. Several other papers have shown that SHH indirectly regulates *Fgf8* transcription through modulation of the GLI3 repressor. This suggests that FGF signalling functions as a positive regulator of ventral telencephalic patterning, whereas SHH signalling functions negatively to prevent dorsalization of ventral telencephalic domains.

SHH signalling in the ventral diencephalon has long been associated with the ventral separation of the eye fields, most obviously demonstrated by the cyclopia seen in SHH loss-of-function mutants[8,69]. Numerous studies have implicated SHH in patterning multiple aspects of the visual system, including the retina, optic chiasm and optic stalk[70–72]. With regards to dorsoventral patterning in the diencephalon, SHH signalling has been associated with the ventral expression of PAX2, as well as the ventral repression of PAX6 — a master regulator of eye development, which is required for the separation of the bilaterally positioned eye fields[73]. That GLI activity is the mediator of patterning in this region is indicated by the partial absence of the diencephalon seen in *Gli1*$^{-/-}$;*Gli2*$^{-/-}$ double mutants[41].

With regard to more dorsal regions of the diencephalon, recent studies of *SHH* in the chicken ZLI have elucidated a novel mechanism of SHH-induced neural cell specification as well as a role for SHH signalling in patterning. Interestingly, the mechanisms of SHH signalling at the ZLI seem akin to the actions of *Drosophila* hedgehog in anterior–posterior compartments of the wing imaginal disc. The ZLI, which expresses *SHH* along its entire dorsoventral extent, splits the prethalamus anteriorly from the posterior thalamus. These abutting diencephalic regions are patterned by the same ZLI SHH source, yet they acquire distinct regional gene expression fates, implying that the two diencephalic regions have a differential competence. *In ovo* electroporation studies in chick embryos have shown that the homeobox gene *IRX3*, expressed posterior to the ZLI, creates a state of thalamic competence, such that misexpression of *IRX3* in the prethalamic compartment allowed ZLI-derived *SHH* to induce dorsal thalamic gene expression profiles ectopically[74]. The diverse responses of these two adjacent compartments to an identical SHH source illustrates the importance of region-specific factors in generating functional diversity from a single protein expressed widely across the neuraxis. So, just as the interpretation of SHH signals seems in some contexts to rely on changes in temporal competence, here differential regional gene expression mediates differential spatial competence in the manner in which specific tissues respond to SHH.

***Midbrain, hindbrain, cerebellum.*** Although the role of the isthmic organizer molecule FGF8 in anterior–posterior patterning of the mid/hindbrain region has been studied extensively, the role of SHH in dorsoventral patterning of the region and its later functions in cerebellum development have only recently been addressed. Studies of SHH and GLI function in the midbrain and rhombomere 1 (r1), a segment of the hindbrain, have uncovered the temporal requirements for SHH signalling and shown a distinct differential regulation of the GLI2/3 activators and the GLI3 repressor in patterning this region. Based on gain-of-function studies mainly carried out in the chick and analysis of the early embryonic (before E11) defects in *Shh*-null mutant mice it became clear that, as in the spinal cord, SHH signalling through GLI2 is both necessary for the induction of particular ventral cell types in the midbrain/r1, and sufficient to induce certain cell types, at least in combination with FGF signalling[43,75–79].

Alar plate
The dorsal aspect of the developing neural plate/tube.

A recent study used conditional mutagenesis of *Smo* and *Gli2* to determine the functional repertoire of SHH at different stages of mid/hindbrain development[80]. SHH signalling through the GLI2 activator was found to be required before E11 to induce many, but not all ventral cell types. GLI1 and GLI3 probably also contribute to the induction of ventral cell types. Unlike the spinal cord, in which GLI3 has only a small role in patterning intermediate regions, in the tectum (dorsal midbrain) and cerebellum (derived from dorsal r1), regulation of the GLI3 repressor by SHH signalling was found to be crucial, and this requirement continues past E11. At least part of the function of SHH signalling through GLI3 in these regions is to maintain *Fgf8* expression, thereby providing a mechanism to coordinate patterning in the anterior–posterior axis as well.

In the developing cerebellum, *Shh* begins to be expressed in Purkinje cells at around E17, and signals both to the outer granule layer and to nearby Bergmann glia[81]. Furthermore, SHH is both necessary and sufficient for the induction of granule cell proliferation[81–86], which is a largely postnatal event that is required for cerebellum foliation. GLI2 was found to be the primary GLI protein required in this process, although GLI1 probably has a minor redundant role[81,86]. In a recent study, an allelic series of viable conditional mutants in *Gli2* and *Smo* were constructed to address the role of SHH in regulating morphogenesis of the cerebellum by controlling growth of the granule cell layer[86]. It was found that mutants with reduced SHH signalling have reduced foliation, with a pattern that reflects the normal progression of folia formation, whereas a mutant with excess SHH signalling forms an extra fold, the position of which is conserved in the normal rat cerebellum. These results indicate that SHH might act as a permissive factor for foliation by inducing granule cell proliferation, whereas a different pathway could determine the pattern of folia.

## Additional functions of hedgehog signalling

***The generation of oligodendrocytes.*** Hedgehog signalling has been implicated in the specification and subsequent development of oligodendrocytes (FIG. 4), the glial component of the CNS responsible for the myelination of axon tracts. In accordance with the requirement for hedgehog signalling in ventral patterning, studies have noted that the earliest identifiable cells of the oligodendrocyte precursor cell (OPC) lineage are derived from the ventral ventricular zone in the spinal cord and forebrain[87–89]. Ectopic expression of recombinant SHH protein in the chick dorsal spinal cord induces the formation of ectopic OPCs[87,89]. Furthermore, using function-blocking antibodies, a window of development during which SHH is required for OPC specification was delineated[87]. A possible molecular link between SHH signalling and OPC specification was revealed with the cloning of the basic helix-loop-helix (bHLH) transcription factors *Olig1* and *Olig2* (REFS 90,91). These two genes were not only induced by SHH, but were also closely associated with oligodendrocyte development and, in gain-of-function mutants, acted to promote OPC formation[121]. *Olig1*;*Olig2* double knockout mice have no oligodendrocytes at E18.5, indicating that these two SHH-induced genes are both necessary and sufficient for oligodendrocyte formation[92,93].

Studies of the telencephalic regulation of oligodendrogenesis have added further details to the picture of hedgehog signalling in OPC specification. *Nkx2.1*-null mice, which lack most telencephalic *Shh* expression, have a complete loss of early oligodendrocyte markers, with the exception of the amygdala region that continues to express *Shh* in the mutant background[55,94]. Gain-of-function experiments using a *Shh*-expressing retrovirus at E9.5 gave rise to mature oligodendrocytes at postnatal day 21 (REF. 55). Similar experiments performed in *Nkx2.1* nulls were capable of rescuing the absence of early OPC markers, suggesting that telencephalic *Shh* is indeed important for oligodendrocyte development. These findings also indicate that hedgehog signalling might work independently of *Nkx2.1* function to specify oligodendrocytes, possibly acting directly by continued upregulation of the *Olig* genes.

As in the spinal cord, where the ventral neuronal patterning activity of SHH requires the presence of additional inductive cues, recent evidence indicates that FGF might act positively in the induction of OPCs. Two separate *in vitro* studies have suggested a role for SHH and FGF in inducing oligodendrocytes. *In vitro* analysis indicated that both SHH and FGFs could induce oligodendrocytes in neocortical cultures[95]. Blocking the hedgehog and FGF pathways using cyclopamine and PD173074 respectively showed that, whereas the FGF pathway leading to OPC formation is hedgehog-independent, the hedgehog pathway requires a basal level of FGF pathway activation. Similarly, neurocortical neurosphere cultures from E13.5 progenitors, which do not give rise to OPCs *in vivo*, can give rise to these cells in the presence of an FGF-containing medium[96]. At least part of this effect seems to be the result of an FGF-mediated upregulation of endogenous *Shh* transcripts in neurosphere cultures. As for other instances of interaction between these two pathways, the complex regulatory interactions involved have yet to be fully appreciated, although their function seems to be intertwined in many contexts.

***Control of embryonic proliferation.*** Overexpression studies have demonstrated the ability of SHH to promote proliferation in both neural and non-neural tissues[97–99]. Notably, the competence of neuroblasts to proliferate in response to hedgehog signalling seems to be regulated both temporally and spatially. Transgenic-mediated ectopic expression of SHH in the mouse early embryonic dorsal neural tube led to neural hyperplasia at E12.5 but was unable to increase proliferation above wild-type levels at E18.5 (REF. 99). Furthermore, comparison of conditional *Smo* mutants with *Shh* nulls in the mid/hindbrain has shown that SHH is mainly required for proliferation before E9 (REF. 80). The precise response of neural cells to SHH seems to be related to their mitotic state. Exposure to hedgehog signalling during the peak of neurogenesis in mice further augmented progenitor proliferation, whereas exposure in the post-neurogenic period seemed to maintain responding cells in an undifferentiated state[99]. During

Bergmann glia
A specialized form of radial glia found in the cerebellum that, unlike most radial glia, persists throughout life.

Foliation
The process by which neural tissue is folded into gyri and sulci. This occurs most prominently in the cerebellum of mice and in the cerebral cortex of higher vertebrates, such as ferrets, monkeys and humans.

Hyperplasia
Exuberant proliferation that might or might not be cancerous in nature.

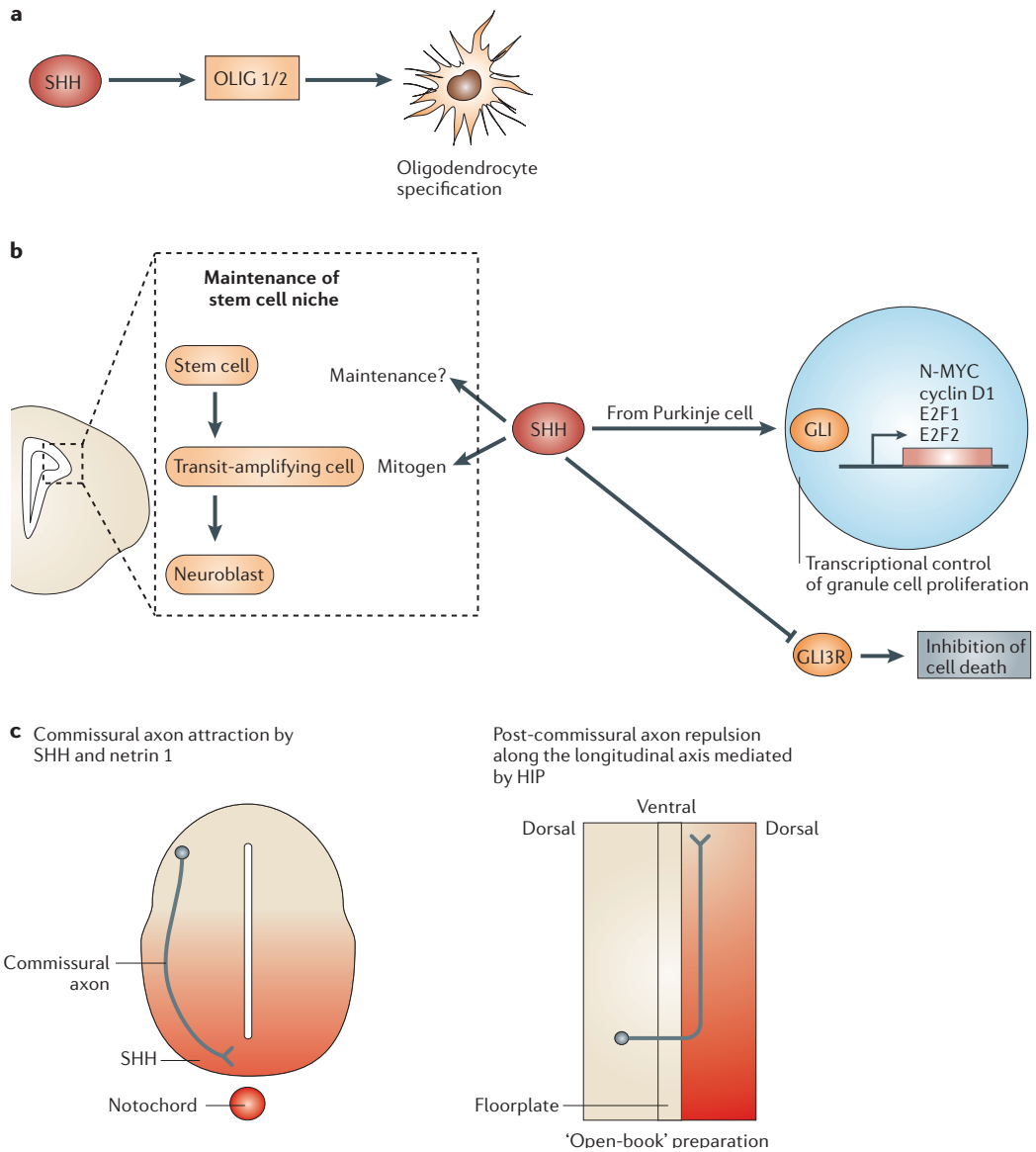


Figure 4 | **SHH signalling functions in a number of distinct contexts in neural development. a** | SHH expression is required for the specification of oligodendrocytes through its induction of the basic helix-loop-helix (bHLH) transcription factors OLIG1 and OLIG2. **b** | The SHH ligand also controls multiple aspects of cell proliferation and the survival of neural progenitors. Adult neural stem cells and transit amplifying cells respond to SHH signalling and SHH is required for maintenance of the adult stem cell niche. SHH from Purkinje cells transcriptionally controls the proliferation of granule cells through upregulation of the cell cycle, as well as other proliferation-related proteins, such as N-MYC, cyclin D1 and E2F1/E2F2. SHH suppresses cell death through removal of the GLI repressor GLI3 (GLI3R), and inhibits patched-mediated apoptosis. **c** | Finally, SHH (red) has recently been shown to have a role in axonal guidance by mediating midline crossing and the longitudinal extension of axons. HIP, hedgehog interacting protein.

perinatal periods, hedgehog signalling can still stimulate proliferation in cerebellar granule cells and in retinal ganglion cells, long after it can stimulate proliferation in the neural tube. That SHH signalling has a biological role in the regulation of proliferation is suggested by its involvement in disease states such as Gorlin's syndrome, in which mutations in *Ptc1* lead to uncontrolled signalling activity and a predisposition for medulloblastomas[10].

Recent studies have begun to explore how hedgehog signalling influences the molecular machinery that controls proliferation. In particular, several new studies have reported how specific downstream effectors are modulated by SHH signalling to regulate the cell cycle. In this regard, work in the mouse cerebellum has implicated *N-myc*[100], *cyclin D1* (REF. 101), *E2f1* and *E2f2* (REF. 102) as possible links between SHH signalling and cell cycle regulators. Work on the medulloblastoma-inducing effects of *N-myc* have illustrated in detail the molecular synergy between the SHH and insulin-like growth factor (IGF) signalling pathways, as SHH acts to induce *N-myc* transcription through GLI proteins, and phosphatidylinositol-3-kinase (PI3-K) stabilizes the *N-myc* protein product[103,104].

In addition to a role in proliferation and differentiation, SHH signalling also seems to control cell death. Evidence from the spinal cord, forebrain and mid/hindbrain indicate that the apoptosis caused by the removal of *Shh* gene function can be suppressed by the removal of *Gli3*, indicating that the repressor fragment might act as an inducer of apoptosis[52,80]. In addition, it has recently been suggested that *Ptc* has a role in mediating cell death through a SMO-independent mechanism[105].

***Shh and axon guidance.*** The expression of *Shh*, ventrally located and spanning the entire anterior–posterior extent of the neural tube, puts it in an ideal place for mediating axonal pathfinding events (FIG. 4). An early link between hedgehog signalling and ventral midline crossing was noted for retinal ganglion cell axons, which normally form the optic chiasm as they cross the midline of the diencephalon (for a review, see REF. 106). Mutations in SHH downstream pathway members disrupt proper crossing in the optic chiasm; at present, however, it is unclear whether this is a direct effect of SHH[107] or is instead a result of the disruption in ventral midline patterning common to many of these mutants. Evidence supporting the latter hypothesis comes from work in zebrafish, where Shh seems to act indirectly through its ability to restrict the expression of various chemorepellent Slit proteins that impede the crossing of several commissural populations[108]. Furthermore, the commissural crossing phenotype resulting from the reduction of hedgehog signalling due to the loss of *yot* (a zebrafish homologue of *Gli2)* can be rescued by attenuating *slit* expression in midline glial cells in the ventral forebrain using morpholinos[109].

So far, the most compelling evidence of a role for SHH as a chemoattractant has come from the identification of its function as a floorplate-derived midline guidance cue for commissural axons, working in conjunction with netrin signalling[110]. Conditional inactivation of *Smo*, selectively in commissural neurons or following cyclopamine treatment of explant cultures, clearly demonstrates that this guidance effect is mediated through a *Smo*-dependent mechanism. However, it is not yet apparent whether its role in this context requires the GLI proteins or is mediated through a transcription-independent mechanism. The ability of an acutely presented focal source of SHH to redirect the growth cone of *Xenopus* spinal axons *in vitro* in one hour might argue against a requirement for transcription in this context.

In a striking example of parsimony, recent studies have suggested that immediately after commissural axons have traversed the floorplate, SHH has a subsequent role in directing these axons anteriorly, along the longitudinal axis[111]. This work provides the first example of SHH acting as a chemorepellent. In this context, the investigators argue that this chemorepellent activity is mediated neither through PTC nor SMO. Instead, they suggest this mechanism is dependent on non-canonical signalling mediated by HIP, because the removal of HIP by RNA interference in a chick 'open-book' explant assay prevents commissural axons from being directed anteriorly. It seems inevitable that further examples of SHH functioning in axonal guidance will be forthcoming. This aside, perhaps the most pressing unanswered question is how the receipt of a hedgehog signal acts to remodel the cytoskeletal organization of the growth cone in these guidance events. In this regard, it will be interesting to determine whether instances of SHH acting as a chemoattractant versus a chemorepellent represent the modulation of a single transduction mechanism or whether distinct pathways function in these two contexts. The fact that, in a single population of commissurally projecting neurons, chemoattraction requires *SMO* while chemorepellence requires *HIP* seems to support the latter hypothesis.

***SHH and adult neural stem cells.*** Although the connection between hedgehog signalling and embryonic proliferation has been extensively studied, exciting recent work has pointed to an intriguing role for hedgehog signalling in regulating the proliferation of adult neural progenitors (FIG. 4). The mammalian forebrain is an excellent system for the analysis of adult neural stem cells because it has two localized areas of postnatal neurogenesis — the dentate gyrus of the hippocampus and the subventricular zone (SVZ) of the lateral ventricles. Whereas the hippocampal progenitors locally populate the hippocampus, the SVZ progenitors migrate along the rostral migratory stream to populate the olfactory bulb. Work in mice has shown that this SVZ niche consists of quiescent (slow-cycling) progenitors, transit-amplifying cells and migrating neuroblasts. Using AraC anti-mitotic treatment to ablate rapidly dividing cells in the precursor and neuroblast lineages, it was shown that astrocyte-like cells could divide to replenish these two populations, indicating that a slowly dividing glial population serves as the SVZ stem cell[112].

*In vivo* evidence highlighting the importance of hedgehog signalling in these two postnatal proliferative niches came from conditional genetic loss-of-function studies, viral gain-of-function studies and genetic fate mapping. Conditional removal of *Smo* in the brain at mid-gestation circumvents the early embryonic patterning defects seen in the *Shh* nulls and allows postnatal examination of the effects of SHH signalling in the dentate gyrus subgranular zone (SGZ) and SVZ proliferative regions[59]. Both regions showed a marked reduction in the number of proliferating progenitors at 2–3 weeks after birth in these mutants, and the SVZ also showed increased cell death. As a result of these perturbations of the postnatal proliferative niches, the granule cell populations of both the hippocampus and the olfactory bulb were severely depleted. A similar decrease in proliferation was observed in the SVZ of juvenile mice that were administered a one week course of the SHH signalling inhibitor cyclopamine, and in the hippocampus of mice that had this inhibitor injected directly into the same region[113,114].

In a series of experiments with interesting clinical implications, artificial stimulation of the hedgehog signalling pathway in the rodent forebrain led to increases in neural progenitor proliferation in both the SVZ

Morpholinos
A modified form of RNA that interferes with translation of the complementary RNA.

and the dentate gyrus of the hippocampus. Activation of hedgehog signalling was achieved either through adenoviral delivery of the N-terminal active fragment of SHH[113], or through the use of a small-molecule hedgehog agonist that upregulates signalling in hedgehog responsive tissues through interactions with *Smo*[59]. This suggests that enhancing hedgehog signalling might be effective as a means of promoting neural repair.

Although it seems clear that hedgehog signalling has an essential role in maintaining adult neural progenitor proliferation, the downstream mechanistic details are still largely unknown. Genetic inducible fate mapping (GIFM) studies in mice[115] have begun to illuminate the developmental history and cell-type specificity of SHH signalling in these proliferative niches[116]. Using *Gli1* as a marker of hedgehog responding cells, it was definitively shown that quiescent and transit-amplifying stem cells in the adult SVZ and SGZ normally receive GLI activator signalling. Furthermore, the quiescent cells expressing GLI1 continue to give rise to neurons that populate the olfactory bulb and hippocampus for over a year and are multipotent. In addition, it seems that the SVZ and SGZ niches are formed sequentially towards the end of embryonic forebrain development. With regard to the cellular mediators of this process, analysis has shown that periventricular astrocytes, the characterized SVZ stem cell population, express not only *Gli1* but also other components of the hedgehog signalling cascade[114]. *In vitro* analysis of dissociated cortices of *Gli2* and *Gli3* mutants indicates that these genes could in part mediate the role of hedgehog in adult progenitor proliferation[117]; however, further *in vivo* experiments will be necessary to accurately assign function to these genes. In addition to these issues, it will be interesting to better understand the source of the hedgehog signals that act in the two proliferative niches, as well as the instructive events in receiving cells that are directed by these signals. Finally, given the dichotomy between the proliferative response to SHH and its role in maintaining the adult stem cell niche, it will be interesting to determine whether this differential response reflects different mechanisms of SHH action or different functional effects in discrete lineages within the stem cell niche.

## Concluding remarks

Hedgehog signalling in the mammalian nervous system has now been studied for over a decade. During this period our mechanistic understanding of the extrinsic and intrinsic regulators of hedgehog signalling has increased exponentially. Similarly, beyond its role in ventral patterning, hedgehog signalling is now known to have multiple iterative roles throughout development, including (as described above) its role in dorsal fate specification, the regulation of oligodendrogenesis, stem cell maintenance and axon pathfinding. It seems likely that a great deal more information will be forthcoming regarding the role of this multifaceted signalling pathway. Three areas of future research that are likely to prove interesting are: further understanding how the levels and duration of hedgehog signalling are modulated by recipient cells; understanding how hedgehog signalling interacts with other signalling pathways; and examining how hedgehog signalling influences brain function in the adult animal.

Given the role of hedgehog proteins as both morphogens and mitogens, the fact that this pathway is under exquisite regulation is perhaps not surprising. That this regulation seems to be intimately tied in vertebrates to proteins that control transport in cilia was, however, not anticipated. Whether this indicates that the vestigial cilia found on many cell types are required for hedgehog signalling is a particularly interesting conundrum to solve.

Considering the multifunctional uses of the hedgehog signalling pathway, it seems likely that it will interact with others in a context dependent fashion. For example, evidence has already suggested an interaction between the hedgehog and Notch signalling pathways in the developing cerebellum[118].

Several groups have reported that the expression of hedgehog ligands, most notably SHH, are not limited to development. The patterns of hedgehog ligand expression in the adult are intriguing and could reveal a role for this signalling pathway in the mature animal. Indeed, in *Drosophila*, a recent report reveals that hedgehog has a role in sexual dimorphism, suggesting that male and female brains might be partially distinct in the way that they utilize hedgehog signalling[119]. Whatever the future holds, it seems certain that further research into hedgehog signalling will uncover more surprises.


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The authors declare no competing financial interests.

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