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# *Sonic hedgehog* in vertebrate neural tube development

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**ABSTRACT The formation and wiring of the vertebrate nervous system involves the spatially and temporally ordered production of diverse neuronal and glial subtypes that are molecularly and functionally distinct. The chick embryo has been the experimental model of choice for many of the studies that have led to our current understanding of this process, and has presaged and informed a wide range of complementary genetic studies, in particular in the mouse. The versatility and tractability of chick embryos means that it remains an important model system for many investigators in the field. Here we will focus on the role of Sonic hedgehog (Shh) signaling in coordinating the diversification, patterning, growth and differentiation of the vertebrate nervous system. We highlight how studies in chick led to the identification of the role Shh plays in the developing neural tube and how subsequent work, including studies in the chick and the mouse revealed details of the cell intrinsic programs controlling cell fate determination. We compare these mechanisms at different rostral-caudal positions along the neuraxis and discuss the particular experimental attributes of the chick that facilitated this work.**

KEY WORDS: *spinal cord, morphogen, chick, transcriptional network, central nervous system*

## Introduction

The vertebrate nervous system arises from the neural tube, the development of which is initiated early in embryogenesis during gastrulation. In amniotes, the neural tube primoridium first becomes recognizable as a thickened epithelium that forms over the midline of the embryo. As development progresses the centre of this epithelial sheet invaginates and its lateral edges rise, the eventual juxtaposition and fusion of these lateral edges forms the dorsal midline of the neural tube (for a full introduction see (Gilbert, 2016)). Hence the neural tube develops as a bilaterally symmetrical pseudostratified epithelium in which the basal surfaces of neural progenitor cells form the lateral edges of the neural tube and the apical surfaces are oriented towards the internal lumen, the central canal. Neural progenitors proliferate and their nuclei undergo a stereotypic interkinetic nuclear movement in which mitosis occurs apically and S phase basally (Lee and Norden, 2013). This results in a substantial expansion in the number of neural progenitors and the initial phase of neural tube development is marked by a considerable increase in tissue size (Kicheva *et al.*, 2014). As neural progenitors differentiate into post-mitotic neurons they detach from the apical surface of the neuroepithelium and migrate laterally to reside basal to the cell bodies of progenitors, a process that can be imaged in high resolution using slice cultures of the chick neural tube (Das and Storey, 2014). This identified a novel cell biological mechanism during neuronal differentiation in which the delamination of newly differentiatied neurons involves the abscission of the apical cell membrane through an actin-myosin–dependent cell constriction and dismantling of the primary cilium.

Although morphologically indistinguishable, neural progenitors rapidly acquire distinct transcriptional identities during development; this determines the mature cell type(s) a progenitor produces. In many regions of the nervous system, the transcriptional programmes depend on the position of the progenitor within the neural tube, (Dessaud *et al.*, 2008; Jessell, 2000; Briscoe and Small 2015). For example, in the ventral half of the forming spinal cord (perhaps the simplest and most conserved region of the neural tube) the spatially restricted expression of a set of homeodomain and bHLH transcription factors, which to a large extent were first defined in the chick (Ericson *et al.*, 1997, Briscoe *et al.*, 2000), divide the neuroepithelium into 6 discrete domains arrayed along the DV axis. Each domain expresses a distinct combination of transcription factors. Gain- and loss-of-function studies have shown that this code controls the differentiated cell type that each progenitor generates (reviewed in (Alaynick *et al.*, 2011; Dessaud

*Abbreviations used in this paper:* Shh, sonic hedgehog.

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*et al.*, 2008)). Thus, in the ventral half of the spinal cord, motor neurons and interneurons are formed. Analogous transcriptional codes are found in other regions of the neural tube and underlie the spatial pattern of neurogenesis in the dorsal half of the spinal cord (reviewed in Lai *et al.*, 2016) and in the brain (for reviews see (Guillemot, 2007; Pearson and Placzek, 2013; Scholpp and Lumsden, 2010). This principle, in which the spatially restricted expression of transcription factors in neural progenitors results in the spatially segregated generation of distinct neuronal subtypes, is the first step in the assembly of functional neuronal circuits. This facilitates the formation of the correct synaptic connections between neighbouring cell types and ensures that newly generated neurons are deposited in locations in which they are exposed to appropriate axon guidance signals. Thus, the later function of the vertebrate nervous system depends on the specific and reliable pattern of TF gene expression in neural progenitors.

The stereotypic patterns of neurogenesis in the neural tube raises the question of how neural progenitors obtain spatial information in order to establish the correct transcription factor expression profile. A series of embryological observations and surgical manipulations in chick embryos focused attention on the notochord, a specialised rod of axial mesoderm that underlies the posterior neural tube. Grafting an ectopic notochord next to the neural tube resulted in the induction of motor neurons and floor plate cells – a group of specialised glial cells occupying the ventral midline of the neural tube (van Straaten *et al.*, 1989; Yamada *et al.*, 1991). Conversely, notochord removal resulted in the absence of the floor plate and motor neurons (van Straaten *et al.*, 1988; Yamada *et al.*, 1991). Equivalent experiments with grafts of floor plate demonstrated that these cells also had a similar activity. The observation that ectopic floor plate cells differentiated immediately adjacent to grafted cells, whereas motor neurons were located at a characteristic distance (Yamada *et al.*, 1991) led to the conclusion that a secreted factor with a graded instructive role established the pattern of cell type generation in the ventral neural tube. This was confirmed and extended by a series of ex-vivo experiments in which explants of notochord/floor plate from chick were co-cultured with neural tissue (Yamada *et al.*, 1993). The use of explanted tissue from the chick neural tube has continued to provide an indispensable assay for the characterisation of patterning signals (e.g. Zagorski *et al.*, 2017) and it highlights some of the advantages of the chick, including the accessibility of embryos, the relative ease of micro-dissection and the ability to grow embryonic tissue *in vitro* in serum-free defined medium to test the direct effects of signalling factors on isolated tissue.

## Sonic hedgehog mediates ventral patterning in the posterior neural tube

The cloning of *Shh* in 1993/1994 offered the first insight into the molecular identity of the secreted signal responsible for ventral neural tube patterning (Chang *et al.*, 1994; Echelard *et al.*, 1993; Krauss *et al.*, 1993; Riddle *et al.*, 1993; Roelink *et al.*, 1994). *Shh* expression coincides with stages at which notochord and floor plate display their patterning activity. Strikingly, ectopic expression of Shh in the dorsal neural tube induces floor plate and motor neuron specification, recapitulating the activity of transplanted notochord and floor plate (Echelard *et al.*, 1993; Krauss *et al.*, 1993; Roelink *et al.*, 1994). Subsequently, Shh was shown to be sufficient for the induction of the cell types normally found in the ventral neural tube (Martí *et al.*, 1995; Roelink *et al.*, 1995). Demonstration of the necessity for Shh came a year later with the analysis of mice in which the Shh gene had been deleted using gene targeting – a technique not available in the chick, and an indication of the power in combining chick and mouse studies (Chiang *et al.*, 1996). Together, the embryological and molecular data suggested that Shh is initially expressed in the notochord and signals to the adjacent neural tube to induce floor plate cells that in turn synthesise and secrete Shh. Secreted Shh is then responsible for the patterning of the neural tube, and the eventual differentiation of its signature cell types, notably the motor neurons and interneurons that will form the characteristic circuits of the spinal cord.

Explant assays using chick neural tissue confirmed that a processed, secreted form of Shh, the ShhN isoform, was responsible for all the inducing activities of Shh (Martí *et al.*, 1995; Roelink *et al.*, 1995). These experiments also demonstrated that the induction of different cell types is controlled by different concentrations of ShhN, with higher concentrations of Shh required for the induction of more ventral cell types, such as floor plate, than for motor neurons (Ericson *et al.*, 1997; Roelink *et al.*, 1995).

Subsequent studies in the chick neural tube suggested that Patched1 (Ptch1) is the Shh receptor (Marigo and Tabin, 1996), an idea that was rapidly confirmed through biochemical binding studies (Marigo *et al.*, 1996). To test the range of Shh signaling *in vivo* a mutated form of Ptch1 that acted as a dominant inhibitor of Shh signaling was developed (Briscoe *et al.*, 2001). This was introduced into the chick neural tube by *in ovo* electroporation – a powerful technique that produces mosaic unilateral expression of a mutant protein allowing the cell autonomous and non-autonomous effects of a pertuburation to be assessed directly in individual embryos (eg Briscoe *et al.*, 2001; Kwong *et al.*, 2014). Analysis of the transfected regions demonstrated that inhibiting Shh signalling cell autonomously inhibited the generation of ventral cell types (Briscoe *et al.*, 2001). The cell types affected included not only floor plate and motor neurons, which had been identified by the earlier embryological studies, but also the progenitors of each of the four classes of interneurons generated in the ventral half of the neural tube. Together these studies confirmed that Shh acts in a graded manner over a long range to control the subtype identity and pattern of neurons along the D-V axis in the posterior ventral neural tube.

## Establishing a Shh gradient in the neural tube

The secretion, spread and reception of Shh within the neural tube depends on a large set of dedicated proteins, many of which are highly conserved (reviewed in Briscoe and Therond, 2013). Fatty acids covalently modify Shh to affect both its trafficking to lipid rafts, its secretion and its potency (Long *et al.*, 2015; Pepinsky *et al.*, 1998; Porter *et al.*, 1996). The route by which Shh protein is dispersed through the posterior neuroepithelium remains unclear. Immunological assays in both chick and mouse revealed Shh protein in a graded distribution within the ventral neural tube (Gritli-Linde *et al.*, 2001; Patten and Placzek, 2002; Cohen *et al.*, 2015) Analysis of a transgenic mouse strain containing a fluorescently labeled Shh protein (Shh-GFP), suggested that microtubule based transport traffics Shh from the notochord across cells in the midline of the forming neural tube (the prospective floor plate), possibly in vesicles, to their apical surface, where it is released (Chamberlain

*et al*., 2008). Consistent with this, although Shh protein can be observed basolaterally within the neuroepithelium (Gritli-Linde *et al*., 2001) it accumulates at the apical side of neural progenitors over several cell diameters from the ventral midline of the neural tube. This accumulation of Shh protein appears to be intracellular and associated with the basal body of the primary cilium (Chamberlain *et al*., 2008). Thus Shh protein might be trafficked apically following its internalization elsewhere on the cell.

Notwithstanding these uncertainties, it is clear that several extracellular and transmembrane proteins influence the spread of Shh protein through the neuroepithelium. Heparin sulphate proteoglycans (HSPGs) have been implicated in binding to many extracellular ligands including Shh, and may govern its rate of spread (Rubin *et al*., 2002). Moreover the expression of Sulf1, which catalyzes the sulfation of HSPGs, is induced in the ventral neural tube and associated with the accumulation of Shh protein (Danesin *et al*., 2006). This suggests that HSPGs modulate the distribution of Shh within the neural tube, although their diversity and pleiotropy has made their role difficult to determine.

Several proteins that are transcriptionally regulated by Shh signaling also bind to Shh protein to inhibit the activity and dissemination of Shh. These include Ptch1 and Hhip1, which are upregulated by Shh signaling (Chuang and McMahon, 1999; Goodrich *et al*., 1996). These block Shh signaling by binding to Shh, sequestering it and/or enhancing its degradation (Briscoe *et al*., 2001; Chuang *et al*., 2003; Jeong and McMahon, 2005). Moreover, while Ptch1 is a transmembrane protein, Hhip1 appears to be secreted and acts non–cell-autonomously to antagonize Shh signaling (Holtz *et al*., 2015; Kwong *et al*., 2014). Hence the upregulation of Ptch1 and Hhip1 attenuates Shh spread through the neural tissue, leading to a decrease in Shh at more distant, dorsal, positions in the neural tube.

By contrast, a second group of transmembrane proteins, including Cdon, Boc and Gas1, enhance Shh signaling in the posterior neural tube (Allen *et al*., 2011; 2007; Song *et al*., 2015; Tenzen *et al*., 2006). Cdon and Boc are conserved from *Drosophila* to mammals, while Gas appears to be mammalian-specific. These proteins appear to act as co-receptors for Shh since in mouse the removal of all three results in loss of ventral pattern in the neural tube (Allen *et al*., 2011). Gain-of-function approaches in the chick spinal cord show that although Cdon and Boc display functional redundancy, they appear to employ distinct molecular mechanisms to execute their HH-promoting effects (Song *et al*., 2015). The expression of this group of proteins is downregulated by Shh signaling. This has led to the suggestion that this set of proteins enhances Shh signaling during early stages of neural development when the level of Shh protein is low. As Shh production increases, their downregulation decreases the spread and stability of Shh, and in this way, reduces signaling (Allen *et al*., 2007; Jeong and McMahon, 2005, Song *et al*., 2015). Together these processes have been proposed to buffer fluctuations in the production or spread of Shh protein to add robustness to ventral patterning.

## Mechanism of Shh signaling in the neural tube

The patterning of the dorso-ventral axis of the posterior neural tube has served as a model for understanding how cells respond to a graded signal. The transmembrane protein Smoothened links the signaling pathway to its intracellular transduction in neural cells (Hynes *et al*., 2000). Deletion or inhibition of Smo activity abrogates ventral neural tube patterning (Wijgerde *et al*., 2002). Moreover the concentration effects of Shh protein can be recapitulated in chick neural tissue explants by the graded activation of Smo activity using small molecule antagonists and agonists (Dessaud *et al*., 2007; Frank-Kamenetsky *et al*., 2002).

Shh signaling depends on a cell's primary cilia. This was first noticed in mice with mutations in cilia components (Huangfu and Anderson, 2005; Huangfu *et al*., 2003). Subsequent analyses of ventral neural tube patterning in embryos lacking different ciliary components revealed that cilia are required for maintaining the signaling pathway in its 'off-state' as well as for transducing the active signal (reviewed in Goetz and Anderson, 2010). These studies included analysis of the Taplid3 chick mutant (Davey *et al*., 2006). This coiled-coiled domain containing protein is a component of the centrosome that forms the basal body of cilia and mutants fail to form cilia (Yin *et al*., 2009). Consistent with the importance of cilia, many of the Shh signaling components localize to cilia and dynamic changes in their localizations have been implicated in the mechanism of signaling (Corbit *et al*., 2005; Rohatgi *et al*., 2007, Stasiulewicz *et al*., 2015). Nevertheless, many of the molecular details of the signaling pathway, both within and outside the cilium, remain elusive, and patterning of the neural tube is likely to continue to be a valuable model for deciphering the identity, function localisation and regulation of components of the signaling pathway.

For the control of ventral neural tube patterning the pivotal event in the signaling pathway is the post-translational regulation of Gli protein activity (Briscoe and Therond., 2013). In mouse and chick this family consists of three genes, Gli1-3, which are translated into three proteins, two of which (Gli2 and Gli3) can be converted to a repressor form. Like other components of the signal transduction pathway, trafficking through the cilium appears to regulate the activity of Gli proteins, most likely determining their access to protein kinase A, which is important for the production of the repressor form of Gli3 and to restrain activation of Gli2 (Tuson *et al*., 2011). In the absence of Shh, full length forms of Gli2 and Gli3 proteins are proteolytically processed into repressive forms (GliR) in manner that depends on protein kinase A and the presence of a functioning primary cilium. In the presence of Shh, Gli processing is inhibited resulting in the production of transcriptionally active forms of Gli (GliA). This also depends on primary cilia. The net Gli activity that results from the amount of GliA and GliR in a cell regulates the expression of target genes, including the receptor Ptch1, Gli1 and several proneural transcription factors (Briscoe and Therond, 2013)

The precise contribution each Gli protein makes to ventral neural tube patterning differs. The forced expression of a dominant inhibitory form of Gli3 in the chick neural tube inhibits ventral neural tube patterning (Persson *et al*., 2002; Meyer and Roelink, 2003), consistent with the idea that the Shh-mediated removal of Gli3 repressor function is essential for ventral patterning. Indeed, the loss of Gli3 in mouse results in a dorsal expansion of the cell types specified by low levels of Shh signaling (Persson *et al*., 2002). Moreover, all but the most ventral cell types (floor plate and immediately-adjacent cells) are recovered in embryos lacking Shh in which Gli3 is also ablated (Litingtung and Chiang, 2000). By contrast, in mouse embryos lacking Gli2 the floor plate is no longer specified and there is a reduction and ventral shift in the formation of other ventral progenitor types (Matise *et al*., 1998). This indicates that Gli2 is required for the production of cell identi-

ties requiring the highest levels of Shh signaling.

Genetic experiments in mouse raised the possibility that graded Shh signaling establishes a gradient of Gli activity in the neural tube by progressively inhibiting Gli repressor activity and increasing Gli activator function. In support of this, gain-of-function experiments in the chick using *in ovo* electropooration indicated that mutated versions of the Gli proteins with different levels of transcriptional activity are sufficient to recapitulate the patterning activity of graded Shh signaling (Stamataki *et al*., 2005).

It is apparent, however, that there is not a simple relationship between the extracellular concentration of Shh and the level of transcriptional activation produced by Gli proteins. The amplitude of intracellular signaling is highly dynamic (Balaskas *et al*., 2012; Dessaud *et al*., 2007; 2010). A transgenic reporter in which the expression of a fluorescent protein is controlled by Gli binding sites indicated that neural progenitors are initially highly sensitive to Shh and the level of transcriptional activation induced by Gli proteins rises rapidly. With time, cells appear to adapt and become desensitized to ongoing Shh exposure, resulting in a decline in Gli transcriptional activity. A consequence of this adaptation is that different concentrations of Shh generate different durations of intracellular Gli activation, effectively creating a temporal dimension to the extent of Shh signalling. The induction of negative feedback, mediated by ligand-dependent antagonists such as Ptch1, might contribute to the desensitization of cells to Shh (Dessaud *et al*., 2007). Alternatively, features such as differential stability of activator and repressor forms of the Gli proteins and/or differences in the transcriptional regulation of the Gli genes could be responsible for the adaptation (Cohen *et al*., 2015).

## Interpretation of Shh signaling by a transcriptional network in the spinal cord

How do the observed dynamics of Gli activity result in the spatially restricted expression of the transcription factors that determine progenitor identity and control neuronal subtype identity? Initial studies divided the transcription factors that respond to Shh into two classes based on their mode of regulation: class I genes were defined by being repressed by Shh signaling, conversely class II genes were those 'induced' by Shh (Briscoe *et al*., 2000). A combination of gain-of-function experiments in chick and loss-of-function experiments in mouse identified selective cross-repressive interactions between pairs of class I and class II proteins (Briscoe *et al*., 2000; Ericson *et al*., 1997). Moreover, many of the transcription factors controlled by Shh were shown to directly interact with co-repressors of the Groucho/TLE family suggesting a prominent role for de-represson in the spatial regulation of gene expression (Muhr *et al*., 2001). The mutual repression between pairs of transcription factors provides a mechanism to produce the discrete switches in gene expression that delineate the progenitor domains in the neural tube.

In addition to generating discrete spatial switches in gene expression, the regulatory interactions between the Shh responsive transcription factors plays a major role in defining dynamics of the response to Gli activity and the temporal features of neural tube patterning (Balaskas *et al*., 2012; Cohen *et al*.,2014; for a review see Cohen *et al*., 2013). For example, during normal development cells destined to express Nkx2.2 transiently express Olig2 and Pax6 prior to Nkx2.2 (Dessaud *et al*., 2007). Gain of function experiments in chick embryos indicated that Nkx2.2 represses Olig2 (Novitch *et al*., 2001). Experimental observations, together with mathematical modeling, suggested that the repressive activity of Olig2 and Pax6 on Nkx2.2, and not solely the sensitivity of Nkx2.2 to Gli activity, is responsible for establishing the spatial pattern of Nkx2.2 gene expression (Balaskas *et al*., 2012). This same mechanism allows lower levels of Gli activity to maintain the expression Nkx2.2 once it has been induced suggesting a mechanism to maintain stable patterns of gene expression despite the changing levels of Gli activity. The presence of similar regulatory links between transcription factors that define other progenitor boundaries raises the possibility that similar mechanisms are used to interpret the dynamics of Gli activity to establish the ventral pattern of gene expression. In this view, the dynamics of the downstream transcriptional network is responsible for converting the evolving levels of Gli activity into stable patterns of gene expression.

Insight into how different gene regulatory inputs are integrated at the genomic level is beginning to emerge from studies of the cis-regulatory regions of the transcription factors induced by Shh signaling (Oosterveen *et al*., 2012; 2013; Peterson *et al*., 2012; Vokes *et al*., 2007; 2008; Nishi *et al*., 2015; Kutejova *et al*., 2016). ChIP and bioinformatic analyses have identified Gli binding sites associated with many of these transcription factors and transgenic reporter assays have confirmed the role of these sites in regulating gene expression (Peterson *et al*., 2012; Vokes *et al*., 2007). An unexpected correlation between the affinity of the Gli binding site and the distance from the ventral midline of gene induction was noted (Oosterveen *et al*., 2012). In conventional models of morphogen signaling, genes that are induced at lower concentrations of a morphogen are more sensitive because they have a high binding affinity for the morphogen-activated transcription factor. By contrast the induction of genes that require high levels of morphogen have a lower affinity binding site. For the targets of Shh-Gli signaling in neural cells the opposite was observed: high affinity Gli binding sites were found associated with genes that are normally induced in response to high levels of Shh-Gli activity and low affinity sites were found in more broadly induced genes. This observation, together with assays in chick embryos of Gli binding sites within cis-regulatory regions led to the suggestion that the mechanisms of short and long-range interpretation of Shh signaling differ. In this view, the context of the Gli binding site means that short range targets require Gli activator and high binding affinity whereas long range targets are regulated through low affinity sites by Gli repressor activity. This raises the question of how the response of cis-regulatory regions elements is determined.

In addition to Gli binding sites, many of the Shh responsive cis-regulatory in neural target genes are associated with binding sites for homeodomain proteins and SoxB proteins (Oosterveen *et al*., 2012; Peterson *et al*., 2012). Members of the SoxB family of transcriptional activators (Sox1-3) are broadly expressed throughout the neural tube and function as activators of target genes. Strikingly, the ectopic expression of Sox2 within cells of the limb is sufficient to allow the Shh-dependent induction of genes such as Nkx2.2 and Nkx6.1 that are normally restricted to neural progenitors (Oosterveen *et al*., 2013). Thus, SoxB binding appears to confer neural specificity to target genes. Moreover, the number, affinity or arrangement of SoxB binding sites within a regulatory element could influence its response to Gli input. By contrast to the Sox sites, mutation of the homeodomain binding sites in a cis-

regulatory element indicated that homeodomain protein binding normally mediates a repressive activity (Oosterveen *et al*., 2012). This supports and extends the genetic experiments that suggest a repressive function of the homeodomain transcription factors regulated by Shh-Gli signaling. Taken together the current model suggests that individual cis-regulatory elements integrate the transcriptional input from Gli proteins, with uniform activation provided by SoxB proteins, and transcriptional repression from the spatially controlled homeodomain transcription factors that comprise the gene regulatory network (Oosterveen *et al*., 2012; Peterson *et al*., 2012; Cohen *et al*., 2014). Together, these constituents control the spatial and temporal dynamics of gene expression in the neural tube.

## Floor plate induction

Although the graded activity of Shh signaling, decoded by the downstream transcriptional network, forms the basis of ventral neural tube patterning, additional mechanisms contribute to the diversification of cell fates. One example of this arises for the specification of the ventral midline floor plate (for a review see (Placzek and Briscoe, 2005). These cells are morphologically and functionally distinct from neural progenitors that reside in the rest of the neural tube. The constricted apical surfaces and basally localized nuclei of floor plate cells is responsible for the characteristic shape of the neural tube and these cells act as a secondary organizing centre by secreting Shh. By contrast to other neural progenitors, which acquire their characteristic transcriptional identities after neural tube closure, the induction of the floor plate requires exposure to Shh signaling at an earlier time point (Ribes *et al*., 2010; Sasai *et al*., 2014). Gain- and loss-of-function experiments indicate that only cells in the open neural plate are competent to form floor plate in response to Shh signaling. Moreover, following this early induction, components of Shh signaling are rapidly downregulated in presumptive floor plate cells, halting further signaling. This termination of signaling is necessary for floor plate differentiation and might also allow floor plate cells to secrete Shh efficiently, since the upregulation of Ptch1 and other Hh binding factors in cells responding to Shh would likely sequester the ligand. Thus, floor plate induction emphasizes the importance of timing and dynamics for the response of neural cells to Shh-Gli signaling.

## Shh signaling in anterior regions of the neural tube

*Shh* is expressed along the entire anterior-posterior (future rostro-caudal) length of the axial mesoderm and ventral midline of the neural tube (Riddle *et al*., 1993; Roelink *et al*., 1994). Exposure of chick neural explants dissected from different anterior-posterior positions to Shh, implantation of Shh-soaked beads at different axial positions or localised constitutive activation of Smo indicated that Shh induces a wide variety of ventral neurons of the brain, including interneurons of the telencephalon and diencephalon, dopaminergic neurons of the hypothalamus and midbrain and serotonergic neurons of the hindbrain (Ericson *et al*., 1995; Wang *et al*., 1995; Gunhaga *et al*., 2000; Craven *et al*., 2004; Ohyama *et al*., 2005). The importance of Shh throughout the neural tube was confirmed by the loss of ventral cell types in mouse embryos lacking the Shh gene (Chiang *et al*., 1996) and the discovery that HPE, a human congenital malformation in which ventral areas of the brain are not properly formed, results from disruption to Shh expression or signaling (Belloni *et al*., 1996; Roessler *et al*., 1996).

Targeted electroporation in chick, involving gain- and loss-of-function studies of homeodomain transcription factors, proved that the region specific outcome of Shh signaling depends on the expression of a set transcription factors that are established by earlier anterior-posterior cues. Six3, Irx3, and Otx2, for instance, promote expression of Nkx2.1, Nkx6.1, Dlx2 and Gbx2 in response to Shh, and hence hypothalamic, diencephalic and midbrain fates (Watanabe and Nakamura, 2000; Kobayashi *et al*., 2002; Ohyama *et al*., 2005; Kiecker and Lumsden, 2004)

For a brief early period, similar patterns of Gli genes are detected along the dorso-ventral axis in the anterior neural tube to those described posteriorly. Thus in both the chick and mouse anterior neural tube, Gli1, Gli2 and Gli3 are expressed in overlapping but distinct domains, Gli1 in ventral-midline cells, Gli2 in ventral and intermediate regions and Gli3, dorsally (Aglyamova and Agarwala, 2007; Ohyama *et al*., 2008). Further, some of the same homeodomain transcription factors as the prospective spinal cord are involved. For instance, Foxa1 is transiently expressed in the ventral midline, whilst Nkx2.2, Pax6 and Dbx1 are expressed more distantly (Chapman *et al*., 2002; Ferran *et al*., 2007). Analyses of Gli mouse mutants reveals that, similar to the prospective spinal cord, in the anterior neuraxis Gli2 performs the main GliA function and that Shh counteracts Gli3 (eg. Haddad-Tovolli *et al*., 2015). However, there are differences. In Gli2 mutant mice (that lack Shh expression in posterior floor plate cells), Shh-expressing ventral midline cells persist in the anterior neural tube (Ding *et al*., 1998; Matise *et al.,* 1998). Subsequent studies, analysing Shh enhancer elements, described a unique enhancer, SBE2 (Shh brain enhancer 2) that drives expression in rostral diencephalic ventral midline (RDVM) cells (Jeong *et al.,* 2006).

Studies in chick contributed to unravelling how Shh is regulated in the anterior neuraxis and highlighted differences to the prospective spinal cord. Fate mapping studies showed that RDVM cells are underlain by axial prechordal mesendoderm (PM), not notochord (Dale *et al*., 1997). RDVM cells are absent if PM is removed; conversely, grafting an ectopic PM next to the neural tube resulted in the induction of RDVM-like cells (Pera and Kessel., 1997; Patten *et al*., 2003; García-Calero *et al*., 2008). Ex vivo studies in which PM was cultured with neural tissue showed that PM can induce RDVM cells and initiate further ventral pattern (Dale *et al.,*. 1997; Dale *et al*., 1999; Ohyama *et al*., 2005; Hintze *et al*., 2017). PM, like notochord, expresses Shh (Patten *et al*., 2003; Ellis *et al*., 2015; Shimamura *et al*., 1995), and blockade or genetic ablation of Shh activity in the PM abrogates its ability to induce RDVM cells (Dale *et al*., 1997; Shimamura and Rubenstein, 1997; Patten *et al*., 2003; Geng *et al*., 2008; Aoto *et al*., 2009). Further, while mouse embryos in which Shh is conditionally deleted in the PM show cyclopia, those in which Shh is conditionally deleted in RDVM cells do not (Szabo *et al*., 2009). Therefore, the holoprosencephalic phenotypes that arise when Shh or Shh signaling are deregulated arise due to a failure of RDMV induction by PM (Roessler and Muenke, 2010). Nevertheless, exposure of neural tube explants to purified Shh protein revealed that Shh is not sufficient to induce RDVM cells. Instead, Shh acts co-operatively with the TGFb-ligand, Nodal, with which it is transiently expressed in the PM (Patten *et al.,* 2003; Ellis *et al*., 2015). Loss-of-function studies in mouse and zebrafish, and analyses of human patients, suggest that a Shh-Nodal co-operation may be widely conserved and that Shh and Nodal

signaling pathways are required cell-autonomously in RDVM cells (Mathieu *et al*., 2002; reviewed in (Placzek and Briscoe, 2005)). At present, the mechanism is not known, but this could be examined in chicks, for instance, by asking whether Shh and Nodal form part of a gene regulatory network that includes the TF, Six3, known to be responsive to Shh and to activate SBE2 (Geng *et al*., 2008; Jeong *et al*., 2008).

Studies in chick and mouse show that RDVM cells give rise to a highly proliferative hypothalamic progenitor population (Manning *et al*., 2006; Alvarez-Bolado *et al.,* 2012; Fu *et al*., 2017). The transition from RDVM to proliferating hypothalamic progenitors appears to be mediated by the PM. During the time the PM is adjacent to RDVM cells, Shh/Nodal expression declines and BMP expression increases (Dale *et al*., 1999; Ellis *et al*., 2015). Exposure of RDVM explants to BMPs upregulates the transcriptional repressor, Tbx2 and the signaling factor, Fgf10. Loss-of-function studies, electroporating a Tbx2 siRNA construct into chick RDVM cells showed that Tbx2 is required to downregulate Shh in RDVM cells and to promote cell cycle (Manning *et al*., 2006). Explant culture studies show that Fgf signals in an autocrine manner to promote proliferation of hypothalamic progenitors (Pearson *et al*., 2011). Studies in mice extended this work, showing that Tbx2 and Tbx3 repress Shh by sequestering Sox2 away from a Shh cis-regulatory element (Trowe *et al*., 2013), again, highlighting the importance of SoxB1 genes as activators of neural expression and their intimate association with Shh in the neural tube.

Recent fate-mapping experiments in chick and mouse show that RDVM-derived hypothalamic progenitors are a multipotent population and contribute widely to different subsets of hypothalamic progenitors (Alvarez-Bolado *et al*., 2012; Fu *et al.,* 2017). In chick, RDVM-derived hypothalamic progenitors give rise to anterior, tuberal and mamillary progenitors along the rostro-caudal axis,, each of which grows and differentiates sequentially over time (Fu *et al*., 2017). This shows an exquisite coupling of growth and fate in the hypothalamus, a process in which Shh signaling plays an essential role. Transient blockade of Shh signaling in the chick embryo prevents the growth of anterior progenitors (Fu *et al*., 2017). Studies in mouse and zebrafish suggests a potential mechanism, in which Shh induces the paired-like homeodomain TF, Rx/rx3 in hypothalamic progenitors, a determinant of anterior progenitor fate and growth. The subsequent down-regulation of Rx/rx3, by Shh, is required to realize hypothalamic fate. In the absence of Rx/rx3, anterior progenitors fail to grow and neurons characteristic of the anterior and tuberal hypothalamus fail to differentiate (Muthu *et al.,* 2015; Orquera *et al.,*. 2015). Amongst the genes upregulated or maintained in anterior progenitors by Rx/rx3 is Shh itself. Thus a regulatory loop between Shh and Rx/rx3, coupled to growth, may explain the complex pattern of expression of Shh in the hypothalamus, in which it is downregulated in central-most hypothalamic progenitors, but maintained/upregulated in emerging anterior progenitors. The notion that Shh-expressing anterior progenitors are a dynamic population provides an explanation for genetic lineage-tracing studies in the mouse, which reveal their extensive, but dynamic contribution to hypothalamic neurons (Szabo *et al*., 2009; Shimogori *et al*., 2010; Zhao *et al*., 2012; Haddad-Tovolli *et al*., 2015).

Central to this regulatory loop is the ability of Shh to non-autonomously induce Rx/rx3 and autonomously down-regulate it. In the posterior neural tube, autonomous and non-autonomous events are driven through the dyamic expression of Ptch1 and Hhip. In the hypothalamus, Ptch1 is rapidly downregulated in central hypothalamic progenitors, halting further signaling (Manning *et al*., 2006). Potentially, this enables them to secrete Shh efficiently, supporting its spread and the sustained maintenace/induction of Rx/rx3 anteriorly. Thus, as in the spinal cord, it is likely that Shh-activated genes can attenuate the effects of Shh signaling, confining the regional and temporal expression and actions of Shh. Whether the effects of Shh-activated co-receptors will operate in the same manner as in the spinal cord remains to be determined. Intriguingly, in the optic vesicle, in the absence of Ptc co-expression Cdon acts as a sink for Hh proteins, potentially limiting their spread (Cardozo *et al*., 2014). Additionally, Shh signaling may be attenuated by genes that are exclusive to the forebrain. The low-density lipoprotein receptor-related protein 2 (LRP2; megalin) has been implicated as a Shh coreceptor in the forebrain and has been suggested to concentrate Shh in the ventral forebrain at an appropriate developmental time (Christ *et al*., 2012).

The intricate regulation of induction and cessation of Shh signaling in sets of neighboring cells, coupled with the ability of Shh to integrate growth and differentiation, creates a temporal dimension that provides the opportunity to build increasingly complex arrays of neurons. The hypothalamus, for instance, contains a vast array of neurons that centrally regulate complex homeostatic processes that are essential to survival and species propagation.

Similarly, dynamic neural Shh-Gli activity underlies development of the thamalic complex, a region of the brain that processes and relays sensory and motor information to and from the cortex. The thalamic complex is composed of the pre-thalamus (anteriorly) and the thalamus (posteriorly), bissected through a narrow compartment, the zona limitans intrathalamica (ZLI) that expresses Shh. Studies of chimeric chick embryos generated through classic surgical manipulation, or explant apposition experiments, showed that the formation of the ZLI is due to an interaction between anterior and posterior neuroepithelia (Vieira *et al*., 2005; Guinazu *et al*., 2007). Targeted electoporation studies subsequently showed that the ZLI is induced through cross-repressive TF interactions (reviewed in Scholpp and Lumsden, 2010). Shh expression was found to be induced progressively in the ZLI, from ventral to dorsal. As in the hypothalamus, this appears to be mediated through an auto-induction mechanism (Vieira *et al*., 2005). Grafting and explant experiments in chick embryos revealed that the mid-diencephalic ZLI displays organizer activity, inducing thalamic and prethalamic characteristics (Guinazu *et al*., 2007; Vieira *et al*., 2005). Further, Shh-Gli was shown to mediate a significant extent of the organizing activity of the ZLI. Gain- and loss-of-function experiments using *in ovo* electroporation showed that Shh signaling is required for region-specific gene expression in the thalamus and prethalamus (Kiecker and Lumsden, 2004). Similarly, ectopic activation of the Shh pathway by misexpression of Smo, or GliAs induced the expression of thalamic markers (Vue *et al*., 2007; Hashimoto-Torii *et al*., 2003). Early indications suggested that Shh deriving from the MDO/ZLI spreads along the A-P axis, and acts as a morphogen to pattern the AP axis of the thalamus in a similar way to its actions along the dorsoventral axis of the spinal cord, generating 3 distinctive progenitor domains (pTH-R, closest to the zli, pTH-$C_2$ and pTH-$C_1$) whose transcriptional profiles are similar to those of the 3 ventral-most progenitor domains of the spinal cord (reviewed in Epstein, 2012; Scholpp and Lumsden, 2010). Recent studies,

however, suggest a more complex model, in which Shh arising from the basal plate plays a role in pTH-R progenitor specification, potentially contributing to increased levels or durations of signalling (Jeong *et al*., 2011; reviewed in Epstein, 2012). As in the hypothalamus, we are still some way from understanding the complex manner in which Shh integrates patterning, growth and differentiation to build the thalamic complex.

## Conclusion

Collectively, the studies of the last two decades have revealed the multiple roles that Shh plays in the development of the vertebrate nervous system. Reciprocally, the analysis of neural tube development has provided multiple insights into Shh signaling. The chick embryo has featured prominently in many of these studies and through this work we have gained new mechanistic insights into how a single signal can perform several functions and produce an ordered pattern of diverse cell types in a complex tissue. Not only have these insights deepened our understanding of fundamental developmental processes but they have aso been a major influence in the establishment of methods for the directed differentiation of specific neuronal subtypes from embryonic stem cells *in vitro* (Wichterle *et al*., 2002); Cundiff and Anderson, 2011; Liu and Zhang, 2010). Moreover the transplantation of stem cell-derived neurons back into chick has resulted in successful engraftment (Wichterle *et al*., 2002), raising the hope that this could provide an eventual route to cell based therapies for some neurodegenerative diseases. Despite the progress, much remains to be discovered about Shh signaling and neural tube development. Approaches that provide live, high-resolution measurements of the activity of key components of the pathway are necessary to decipher the signaling mechanism and provide insight into the dynamics of signal transmission through the pathway. Similarly, understanding how Shh signaling regulates differential gene expression to control cell fate decisions will benefit from the increased precision and resolution that new technologies are beginning to offer. It seems likely that the chick will continue to play a leading role in these approaches.

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