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RESEARCH HIGHLIGHT

Shh goes multidirectional in axon guidance

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Shh and Wnts, secreted by the floor and roof plate of the spinal cord, direct longitudinal growth of the axons from the adjacent ventral funiculus and cortico-spinal tract. Whether these midline cues influence the directionality of axons elongating in more lateral positions of the spinal cord is unexplored. Song and colleagues investigate this possibility and demonstrate that the location of descending raphe-spinal tract in the ventrolateral spinal cord is dictated by the simultaneous repellent activity of Shh gradients in both the anterior-to-posterior (A-P) and medial-to-lateral (M-L) axis.

The spinal cord is the main pathway for exchange of information between the brain and the rest of the body. Sensory information collected in the body periphery is conveyed to the brain by axonal tracts that ascend along the spinal cord whereas motor information travels from the brain to the periphery in descending tracts. Precise spatial organization of these fiber tracts is thus essential for animal behavior and survival.

Axonal tract assembly is established early in embryonic development largely in response to spatio-temporally regulated sets of guidance cues that axons encounter and interpret as they grow along their stereotyped pathway. However, the molecular nature of the specific cues that determine axon directionality along the longitudinal axis of the spinal cord remained poorly explored until the recent discovery that Sonic hedgehog (Shh) and Wnt ligands play a prominent role in this event [1, 2]. In mouse, Wnt molecules, expressed in an anterior^{high} to posterior^{low} gradient at the dorsal midline of the neural tube, act as repellents and force cortico-spinal axons to descend the spinal cord [3]. A similar gradient of Wnt ligands, but this time derived from ventral midline cells, attract post-crossing commissural axons as they ascend along the ventral funiculus [4] (Figure 1A). In chick instead, Shh, expressed in a caudal^{high} to rostral^{low} gradient, forces the anterior growth of post-crossing commissural axons [5] and, at the same time, controls the formation of a functionally attractive gradient of Wnt proteins [6] (Figure 1B).

Both of these tracts develop in close contact with the floor or roof plate, which are the cellular sources of Shh and/or Wnt proteins. This raises the question of whether midline-derived morphogens only influences the growth of axons in the immediate surroundings or can also signal to those elongating in more lateral positions of the spinal cord. In a recent paper published in *Cell Research*, Song and colleagues [7] addressed this question and demonstrated that in mouse embryos Shh signaling provides ventrolateral positional information to the descending raphe-spinal tract by simultaneously repelling its axons in the anterior-to-posterior (A-P) and medial-to-lateral (M-L) direction (Figure 1C).

The serotoninergic nuclei of the caudal raphe in the brainstem participate in the regulation of different functional systems [8]. Their axons grow ventrally and ipsilaterally along the brainstem and extend up to the caudal spinal cord. Song and colleagues [7] first demonstrated that, in contrast to what were observed in chick embryos [5], Shh protein formed a high-to-low gradient along the A-P axis of the mouse spinal cord. With conventional *in vitro* assays, they thereafter tested the response of caudal raphe explants to Shh-secreting cells. Similarly to what were observed for retinal ganglion [9, 10] and the post-crossing commissural [5] axons, they reported that Shh strongly inhibits serotoninergic axon outgrowth. They thus hypothesized that the A-P Shh gradient could force axons to descend along the spinal cord. To test this hypothesis, they developed the "A-P graded assay", where the ventral side of a hemisected spinal cord was co-cultured with three equivalent caudal raphe explants placed at different A-P levels. In this assay, outgrowth of serotoninergic axons was strongly impaired but with a progressive lower intensity in explants facing the caudal neural tube. Outgrowth was restored in the presence of Shh block-

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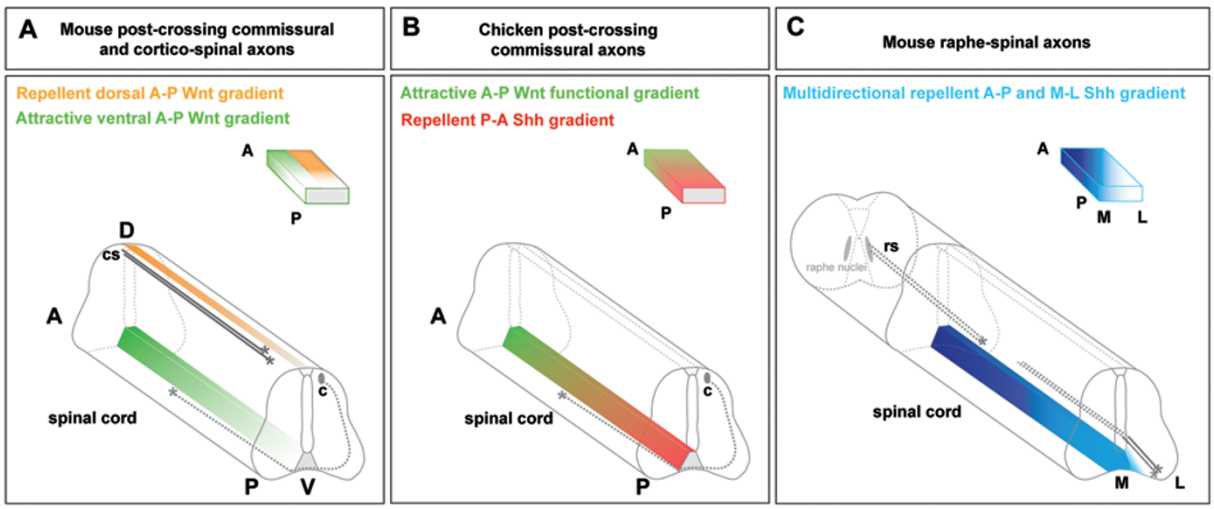

Figure 1 Schematic representation of longitudinal axon guidance in the vertebrate spinal cord. **(A)** In mouse, an anterior^{high} to caudal^{low} gradient of Wnt molecules, expressed in the roof plate (orange) or floor plate (green) repels descending cortico-spinal (cs) axons (grey line) and attracts post-crossing commissural (c) axons (dotted grey line), respectively. **(B)** In chick, a caudal^{high} to rostral^{low} gradient of Shh (red) repels post-crossing commissural axons in the anterior direction and controls the formation of a functionally attractive Wnt gradient (green). **(C)** In mouse, an anterior^{high} to caudal^{low} (dark blue) and medial^{high} to lateral^{low} (pale blue) gradient of Shh repels axons from the raphe caudal nuclei of the brainstem (grey line) in the posterior and lateral direction.

ing antibodies, establishing a clear link between Shh activity and serotoninergic axon repulsion.

This observation *per se* identified an additional type of Shh-sensitive axons, reinforcing the idea that morphogens are important and widely-used regulators of axon guidance [11]. Song *et al*. [7] however went a step forward and asked whether the lateral position occupied by serotoninergic axons in the ventral spinal cord could be similarly dictated by a graded Shh repulsive activity, but now in the M-L direction. In line with previous reports [12], they found that Shh protein diffused throughout the ventral spinal cord. Thus, the medial region of the ventral neural tube contained higher Shh levels than those present in the lateral regions. When raphe nuclei explants were co-cultured with different medial to lateral neural tube slices, serotoninergic axon outgrowth was differentially suppressed, with more pronounced effects in the presence of medial slices.

Transduction of Shh signaling involves a number of different transmembrane proteins, including Hhip and Megalin, the cell adhesion molecules Cdo and Boc, the GPI-anchored cell surface protein Gas1, the seven-pass Patched (Ptch) receptor and the G-protein-coupled like-receptor Smoothened (Smo). Although interaction among these proteins might be important for efficient Shh reception [13], it is well established that Shh binding to Ptch activates signal transduction in Shh-receiving cells. In basal conditions, Ptch inhibits Smo, but upon ligand binding, this inhibition is released and Smo activates a cascade that leads to control of gene transcription and likely to the activation of local second messengers in growth cones [14]. Song *et al*. [7] observed that Ptch and Smo localized at the surface of serotoninergic caudal raphe axons as they descend along the spinal cord and demonstrated that Ptch and Smo function is required for multidirectional Shh repellent activity. Indeed, electroporation of a Shh-insensitive version of Ptch, which dominantly represses Shh signal transduction, or pharmacological interference with Smo activity, efficiently abrogated the repellent activity observed in both the A-P and M-L graded assays, strongly supporting that Shh signaling is responsible for multidirectional guidance of serotoninergic axons. More importantly, Song *et al*. [7] analyzed pathfinding of the raphe-spinal axons after *in utero* electroporation of a Shh-insensitive version of the Ptch receptor and investigated the raphe-spinal phenotype in *Shh* hypomorphic and *Smo* conditionally-inactivated embryos, where patterning of the neural tube was normal. Supporting their *in vitro* studies, they found that the formation of the raphe-spinal tract was impaired in the three conditions, although the phenotypes were not identical. In *Shh* hypomorphs, the number of serotoninergic axons extending into the spinal cord was reduced, likely because a less steep Shh gradient interfered with efficient axon extension. Alterations in Ptch and Smo function was instead associated with stalling of serotoninergic axons, which failed to reach the caudal spinal cord and invaded the medial region of the neural tube. Furthermore,

in transgenic mouse embryos, where Shh signaling is over-activated by the expression of a constitutively active form of Smo, raphe-spinal axons barely reached the cervical spinal cord looping back into the brainstem, likely because of over-interpretation of the repulsive information.

Cooperation among different axon guidance cues, each one providing information in a single direction, has best explained axon pathfinding in three-dimensional space [15]. The novel findings of Song *et al*. [7] instead delineates an alternative and efficient mechanism, in which a single guidance cue can simultaneously provide multidirectional information, somewhat resembling morphogens endowing cells with positional information during patterning events [16]. This novel view raises additional questions, for example, whether other morphogenetic signaling pathways have similar multidirectional guidance activity. This possibility is particularly attractive in the case of Wnt signaling because the outgrowth of the raphe-spinal axons along the brainstem apparently occurs under the influence of an attractive Wnt5 gradient [17]. An anterior^{high} to posterior^{low} gradient of Wnt ligands is also present in the ventral spinal cord (Figure 1A), making worthwhile investigating whether Wnts cooperate with Shh in positioning raphe-spinal axons within the spinal cord. Wnt/Shh cooperation has a precedent in the guidance of chick post-crossing commissural axons (Figure 1B). Furthermore, Wnt participation could explain why a proportion of raphe-spinal axons still follow the right path in embryos, in which Shh signaling is altered.

Multidirectional guidance by a single signal also raises the perhaps general question of how each individual axon senses the absolute concentration of a given guidance cue and interprets it accordingly. An important contributing factor might be the type and level of surface receptors present in each growth cone [14]. As mentioned above, Shh reception involves different transmembrane proteins that appear to interact with Ptch [13], even though it is unclear whether and how this interaction modifies signal transduction. Expression of Boc, Hip or Gas1, which have also been implicated in axon guidance [14], might participate in Shh-mediated repulsion of caudal raphe neurons, providing a possible alternative explanation for the incomplete penetrance of the raphe-spinal tract phenotype after alteration of Shh signaling *in vivo*.

Independently of multidirectionality, the study of Song *et al*. [7] points to a puzzling species-specific difference in Shh-mediated guidance of longitudinal axon tracts in the spinal cord. In chick, post-crossing commissural axons grow anteriorly forced by a caudal^{high} Shh gradient [5], whereas raphe-spinal axons descend the spinal cord under a similar repellent activity but mediated by an opposite Shh gradient [7] (Figure 1B and 1C). Although temporal dynamics may explain these differences, as suggested by Song *et al*. [7], it will be interesting to undertake a simultaneous evaluation of commissural and raphe-spinal axon behavior in response to Shh signaling manipulations in both chick and mouse embryos.

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