Longitudinal axon guidance
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Our knowledge about molecular mechanisms underlying axon guidance along the antero–posterior axis in contrast to the dorso–ventral axis of the developing nervous system is very limited. During the past two years in vitro and in vivo studies have indicated that morphogens have a role in longitudinal axon guidance. Morphogens are secreted proteins that act in a concentration-dependent manner on susceptible groups of precursor cells and induce their differentiation to a specific cell fate. Thus, gradients of morphogens are responsible for the appropriate patterning of the nervous system during early phases of neural development. Therefore, it was surprising to find that gradients of two of these morphogens, Wnt4 and Shh, can be re-used for longitudinal axon guidance during later stages of nervous system development.

Introduction
Studies in a variety of organisms have contributed to our current understanding of the molecular mechanisms of axon guidance. Axons navigating through the pre-existing tissue towards their targets are guided by a combination of attractive and repulsive forces [1,2]. These can function over distances, in the case of long-range guidance cues (see glossary), or more locally, in contact-mediated fashion, in the case of short-range guidance cues. Over time, various molecules that act as axon guidance cues and their receptors have been discovered, such as the cell adhesion molecules of the immunoglobulin superfamily (IgSF CAMs), semaphorins, plexins, neuropilins, ephrins, Ephs, netrins, and slits.

To date, one of the most important systems used to study molecular mechanisms underlying axon guidance has been the midline of the nervous system [3,4]. Studies in both invertebrates and vertebrates have indicated that axons decide whether or not to cross the midline on the basis of a balance between positive and negative cues. These cues are derived from interactions between guidance molecules on the growth cone surface and their intermediate target, the midline [5]. In particular, axon guidance towards and across the floor plate, the ventral midline of the spinal cord, has been widely used to study molecular mechanisms of axon guidance [5,6]. By contrast, very little has been revealed about the guidance cues directing axons after midline crossing when they turn into the longitudinal axis of the spinal cord. Although some of the classical axon guidance cues were implicated in certain aspects of postcommissural axon guidance [7–9], and anatomical studies demonstrated that the majority of the axons turned rostrally along the anteroposterior axis [10], the molecular mechanisms underlying their decisions have remained unknown until recently.

Wnts as axon guidance cues for postcommissural axons
Two years ago, the first guidance cues directing postcommissural axons along the longitudinal axis of the spinal cord were reported on the basis of an in vitro assay [11]. By systematically culturing open-book preparations of rat spinal cord explants that were cut to different lengths, Zou and co-workers showed that commissural axons were guided along the anteroposterior axis by a diffusible guidance cue. Postcommissural axons were stalling or randomly turning into the longitudinal axis in short, but not long, spinal cord explants. Zou and co-workers reasoned that this was explained by the loss of a soluble guidance cue because of diffusion out of short, but not long, explants. Furthermore, they concluded that the guidance cue had to be attractive. This conclusion was reached because postcommissural axons located in the middle and close to the caudal end of long spinal cord explants were turning correctly in rostral direction, whereas those at the rostral end of the explant were stalling or making pathfinding mistakes. Wnt4 was identified in a candidate-based approach, in which candidate proteins were expressed in COS cells that were co-cultured with postcommissural axons in collagen gels and analyzed for an attractive effect on postcommissural axons. A role for Wnt4 in postcommissural axon guidance was supported by the detection of a Wnt4 gradient along the anteroposterior axis of an E11.5 mouse spinal cord [11].

The attractive effect of Wnt4 was blocked by Sfrps (secreted frizzled-related proteins) [11], consistent with their role as antagonists of Wnt signaling [12,13]. Interestingly, Sfrps were very recently shown to have an effect...
SHH was identified as one of the candidate genes [18**]. In the absence of Shh, postcommissural axons failed to turn rostrally along the contralateral floor-plate border in the lumbar sacral chicken spinal cord. Thus, Shh was the second morphogen shown to function as a guidance cue for postcommissural axons. Similar to the Wnts, an axon guidance effect for the morphogen Shh had been described for retinal ganglion axons [19] and for precommissural axons [20], before the discovery of its effect on longitudinal axon guidance [18**].

In contrast to the effect of Wnt4, the effect of Shh was not attracting but rather repelling postcommissural axons, as shown in vivo and in vitro [18**]. Accordingly, Shh is expressed in a rostral-low to caudal-high gradient in the lateral floor plate during the time window when commissural axons exit the floor plate and turn into the longitudinal axis.

Previously, Shh was found to cooperate with Netrin in attracting precommissural axons to the floor plate [20]. It was revealed that this effect was mediated by the well-known Shh receptor complex formed by Patched (Ptc) and Smoothened (Smo), but a different receptor, Hedgehog interacting protein (Hip), was found to mediate the repulsive effect of Shh on postcommissural axons [18**]. Blocking HIP by using in ovo RNAi resulted in the same rostro-caudal pathfinding errors of postcommissural axons as those found after blocking SHH function.

Do Shh and Wnt4 cooperate directly in postcommissural axon guidance?

The fact that both Wnt4 and Shh form a gradient along the rostro-caudal axis with orientations appropriate for their mode of action (attraction and repulsion, respectively) suggests that the two morphogens cooperate in axon guidance along the longitudinal axis of the spinal cord. However, the effects of Wnt4 and Shh have been demonstrated in different species, rodents and chicken (Figure 1). After midline crossing, commissural axons start to express Hip and, thus, switch their response to Shh from attraction to repulsion. According to the orientation of the gradient of Shh, postcommissural axons are repelled by high caudal levels of Shh and at the same time attracted rostrally by increasing Wnt4 levels.

Although this mechanism would suffice to explain the pathfinding behavior of postcommissural axons, there could be an even more direct link between the two morphogens in postcommissural axon guidance. Sfrp2, a secreted Wnt antagonist that was shown to affect the activity of Wnt as a postcommissural axon guidance cue [11], is upregulated by Shh [21] and is expressed in both mouse [22] and chicken embryos [23–25]. Thus, the gradient of Shh could influence the activity of Wnt4 indirectly by inducing high levels of Sfrps in the caudal spinal cord and, thereby, strengthen the gradient of Wnt4.

Commissural neurons located in the dorsolateral spinal cord do not express Ryk and were attracted to Wnt4 rather than repulsed. On the basis of these results it is tempting to speculate that Wnts act either as attractants in a Fz-dependent manner or as repellents using Drl/Ryk as a receptor. A recent follow-up study by the Zou laboratory [17*] provides additional evidence for this hypothesis. Interestingly, looking at corticospinal tract axons that express Ryk and that have to grow along the anteroposterior axis of the spinal cord in the opposite direction to the postcommissural axons studied previously, they found a repellent effect of Wnt4 that was mediated by Ryk, the vertebrate ortholog of Drl [17*].

More surprises: Shh — another morphogen functions as an axon guidance cue for the longitudinal axis

In a subtractive hybridization screen for axon guidance cues involved in the navigation of postcommissural axons, on neurite growth also in a Wnt-independent manner [14].

Wnts function as morphogens (see glossary) by activating signaling pathways downstream of Frizzleds (Fz). Therefore, an obvious next step in this investigation was to test for a role of Fz in longitudinal axon guidance. In fact, the phenotype seen in knockout mice was consistent with Fz3 being the receptor for Wnt4 in postcommissural axon guidance.

Although the study by Lyuksyutova et al. [11] provided the first evidence for Wnts acting as axon guidance cues for the longitudinal axis, Wnts had been implicated in axon guidance before. Wnt5 was shown to prevent axons expressing Derailed (drl), an atypical receptor tyrosine kinase of the RYK subfamily (see glossary), from acrossing the ventral midline of Drosophila ventral nerve cord through the posterior commissure [15]. Interestingly, although Ryk, the mammalian ortholog of Drl, was shown to be a co-receptor for Wnt together with Fz [16], no effect of Fz on Wnt5-dependent axon repulsion was detected [15].

Glossary

Guidance cue: Protein that conveys guidance information to an extending axon. Axons respond to these cues by means of surface receptors on their growth cones. Guidance cues are either secreted molecules (long-range guidance cues) or cell surface molecules (short-range guidance cues).

Morphogen: Morphogens are secreted proteins of the Hedgehog, Wnt, fibroblast growth factor (FGF), and transforming growth factor-β (TGF-β) families. They function in a concentration-dependent manner to induce the differentiation of responsive cells during early stages of nervous system development.

RYK subfamily: The receptor related to tyrosine kinases (Ryk) defines a subfamily of catalytically inactive receptor tyrosine kinases that share a glycosylated extracellular domain with structural similarity to the secreted Wnt inhibitory factor-1 (WIF-1).
by blocking its activity in the caudal spinal cord (Figure 2).

In addition to the link via Sfrps, there are other possibilities of interactions between shh and wnt, based on similarities in their signaling pathways (reviewed by Nusse and Kalderon [26,27]). In particular, the seven-pass transmembrane receptors for Wnt and Shh, Fzs and Smo, respectively, use members of the LRPs (LDL receptor-related proteins) as co-receptors [28]. Although an effect of LRP6 as co-receptor for Fz3 was excluded in the role of Wnt4 as longitudinal axon guidance cue [11], it remains to be seen whether other family members, in particular the closely related LRP5, could compensate for

Figure 2

Shh boosts expression levels of Sfrps, secreted antagonists of Wnt signaling, in a concentration-dependent manner. Thus, increasing levels of Shh in the lumbosacral floor plate could influence Wnt4 activity along the rostrocaudal axis of the spinal cord. Increasing levels of Sfrps strengthen or stabilize the Wnt4 gradient by blocking its activity at more caudal spinal cord levels.
loss of LRP6 function. Megalin/LRP2, another family member, was implicated in SHH function during nervous system development [29,30].

What about other morphogens?
Shh and Wnts are not the only morphogens with a role in axon guidance [31]. FGF receptors were implicated in neurite outgrowth more than 10 years ago in the context of axon growth promoted by IgSF CAMs [32,33] but also by activation via FGFs. For instance, FGF2 was shown to affect guidance of retinal ganglion cell axons in the Xenopus visual system [34–36]. More recently, an effect of FGF8 as attractant for trochlear motor axons was demonstrated in vitro and in vivo [37].

BMPs (bone morphogenetic proteins), antagonists of the effect of Shh during dorso-ventral patterning of the spinal cord [38], affect early aspects of commissural axon pathfinding [39,40]. BMP7, expressed in the roof plate, repels Shh, which attracts precommissural axons [20]. To date, nothing has been revealed about the receptors for BMPs in axon guidance.

Open questions
It will be interesting to see whether BMPs and FGFs also act in longitudinal axon guidance. And, if so, what are the receptors for BMPs? Can the hypothesis suggested by the observations made for Wnts and Shh be substantiated, that is, is the response to morphogen gradients, attraction versus repulsion, dependent upon the type of receptors expressed by a particular growth cone? What are the signaling pathways involved?

In addition to their effect on axonal pathfinding, Wnts have been implicated in target innervation [41] and synapse formation [42]. Is this also true for other morphogens? The implication of BMP type II receptors in the formation of Drosophila neuromuscular junctions suggests that at least for BMPs it is true (reviewed by Salinas [43]).

Conclusions
The identification of morphogens as axon guidance cues for the longitudinal axis has answered a longstanding question, but at the same time it has raised intriguing new ones about possible interactions between morphogens long after their initial function in early development in the patterning of the nervous system. Morphogens functioning as axon guidance cues are versatile, because they can switch from attractors to repellents depending on the receptors expressed by the neuronal subpopulations navigating along the morphogen gradients. Last but not least, the recent findings that morphogens have second or even third functions during later stages of neural development clearly demonstrate the importance of time in development. Timing is important not only for the developing embryo but obviously also for the analysis of developmental processes. Classical genetic tools are not suitable to study later time windows of embryonic development without affecting the earliest phase of gene function. Thus, the establishment of new approaches that enable temporal control of gene silencing, such as in ovo RNAi [44,45], will probably add more surprises to our discoveries of the functions of morphogens.

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References and recommended reading

Papers of particular interest, published within the annual period of review, have been highlighted as:

- of special interest
- of outstanding interest


The authors compared axonal pathfinding of Ryk-expressing corticospinal tract neurons with that of dorsolateral commissural neurons in the spinal cord. Dorsolateral commissural axons extend along the longitudinal axis in the opposite direction to Ryk-expressing corticospinal tract axons. The analysis of axonal pathfinding indicated that Ryk is required for a repulsive response to Wnt4. In the absence of Ryk Wnt4 is perceived as an attractant.


The authors identified Shh in a subtractive hybridization screen for floor-plate associated guidance cues that direct posterior commissural axons rostrally along the longitudinal axis of the spinal cord. In contrast to the function of Shh as a morphogen, its effect on posterior commissural axons was not mediated by the receptor complex formed by Patched and Smoothened, but rather by Hedgehog interacting protein.


