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Axon guidance and injury – lessons from Wnts and Wnt signaling

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Abstract

Many studies in the past decade have revealed the role and mechanisms of Wnt signaling in axon guidance during development and the reinduction of Wnt signaling in adult axons upon traumatic injury, which has profound influences on axon plasticity. With 19 Wnts and 14 known receptors (10 Frizzleds (Fzds), Ryk, Ror1/2 and PTK7), the Wnt family signaling molecules contribute significantly to the wiring specificity of the complex brain and spinal cord circuitry. Subsequent investigation into the signaling mechanisms showed that conserved cell polarity pathways mediate growth cone steering. These cell polarity pathways may unveil general principles of growth cone guidance. The reappeared Wnt signaling system after spinal cord injury limits the regrowth of both descending and ascending motor and sensory axons. Therefore, the knowledge of Wnt signaling mechanisms learned from axon development can be applied to axon repair in adulthood.

Wnts are conserved guidance molecules for a large numbers of central nervous system axons

Vertebrate Wnts were first found to be axon guidance molecules from studies on the guidance mechanisms along the anterior-posterior axis of the spinal cord after midline crossing of the well-known commissural axons [1]. The commissural axons, originating from the dorsal spinal cord, turn anteriorly after they have reached and crossed the midline (Fig. 1a). A decreasing anterior-to-posterior expression gradient of several Wnt family secreted signaling proteins (Wnt4, Wnt5a, Wnt7a and Wnt7b) controls the appropriate anterior turning of post-crossing commissural axons through an attractive mechanism via Frizzled3, one of the Wnt receptors. Disrupting Wnt gradients by secreted Frizzled-related proteins (sFRPs) or Wnt signaling in *Frizzled3, Celsr3 or Vangl2* mutant mice resulted in randomized growth of post-crossing commissural axons along the anterior-posterior axis (Fig. 1b). In the same year, *Drosophila* Wnt5 was found to repel axons via another Wnt receptor, Derailed [2] [3].

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The vertebrate homology of Derailed, Ryk (receptor tyrosine kinase-related tyrosine kinase), was subsequently shown to be a repulsive Wnt receptor promoting the posterior-directed growth of descending corticospinal tract axons along the anterior-posterior axis of the spinal cord [4]. Wnt1 and Wnt5 are expressed in an anterior-high-posterior-low gradient in the dorsal spinal cord in neonatal rodents when corticospinal tract axons are pathfinding from the brain down through the spinal cord (Fig. 2a). These studies suggest that along the anterior-posterior axis of the spinal cord, Wnt gradients may be important for wiring many longitudinal tracts, an economic way to wiring many axons with a few molecular cues [5].

In addition to the corticospinal tract axons, the Wnt-Ryk signaling system also repels corpus callosum after they have crossed the midline to ensure that they pathfind correctly in mice [6]. During midline crossing of callosal axons, Wnt5a expressed in indusium griseum and in the glial wedge functions as a repellant, resulting in the tightly fasciculated axon tract in the corpus callosum. In Ryk knockout mice, callosal axons are able to cross midline, but are unable to project in the tight bundle (Fig. 2b). Further more, cortical neurons in hamsters are also repelled by Wnt-Ryk signaling [7] [8]. Therefore, a large number of brain axons are regulated by Wnts as guidance cues during circuit wiring.

In addition to the Wnt gradients in the forebrain and the spinal cord, Wnt5a and Wnt7b also show graded expression along A-P axis in the developing hindbrain and midbrain, although these gradients are more complex and change directions at important embryonic tissue boundaries (Fig. 2c). The Wnt5a and Wnt7b gradients along the A-P axis determine the orientation of ventral midbrain dopaminergic axons (DA axons) and hindbrain serotonergic axons in a Wnt-PCP pathway-dependent manner [9] [10].

The fact that Wnts have a general role in A-P guidance of axons in vertebrate raises the question whether Wnts are evolutionarily conserved cues for the A-P axis. Indeed, mutations of Wnts and Wnt signaling components affect the anterior-posterior guidance or patterning of many axons in *C elegans* [11] [12] [13] [14]. In addition to pathfinding, Wnts are also conserved topographic mapping molecules in target selection, which is beyond the scope of this review [15] [16]. It should be noted that this function of Wnts is very different from the pathfinding, whereby Wnts provide directional information. In topographic mapping, Wnt concentration represents positional information to determine axon termination in topographic mapping. The mechanisms of how Wnt gradients specify topographic positions are not known.

While the roles of Wnts in axon guidance of different neuronal types and in different species are continuing to be discovered, several key questions are looming larger and larger: 1) How are various Wnt gradients established and maintained? 2) How does Wnts coordinate and integrate with other axon guidance cues to establish patterns of connections? 3) Do Wnts also regulate direction of axonal growth in the peripheral nervous system? The role of Wnts in controlling the directions of peripheral axon growth has not been rigorously tested. In fact, from the published studies, Wnts often do not appear to play important roles in guidance but may rather control the extension and survival of motor axons in the periphery [17]. In addition, spiral ganglion axons were found not responsive to several Wnts that are present in the environment [18]. More studies will need to be carried out to fully understand

the role of Wnt signaling system in axon guidance. This will give rise to a comprehensive view about a major genetic program governing brain wiring and how this program interact with the rest of the molecular program.

Cell polarity signaling pathways mediate Wnt function in growth cones

The sharp 90° anterior turn of postcrossing commissural axons provides an outstanding experimental system to understand signaling mechanisms leading to growth cone steering. As described in previous sections, this anterior turn is mediated by an anterior-high-posterior-low expression gradient of several Wnts [19]. The first step is to identify which Wnt signaling pathway controls the direction of turning.

One of the Wnt signaling pathway is the planar cell polarity pathway (PCP), which establishes planar polarity in many tissues [20] [21]. This important polarity pathway is conserved from invertebrate to vertebrate. Frizzled, Dishevelled (Dvl), Diego, Vangl (Van Gogh in Drosophila), Celsr (Flamingo in *Drosophila*) and Prickle (Pk) are the six “core components” of this signaling pathway. In addition to Frizzled3, additional PCP components, Celsr3 and Vangl2, are also required for normal anterior turning of commissural axons after midline crossing, suggesting that commissural axon growth cones use PCP signaling to mediate turning [22].

PCP signaling is mediated by a series of largely not-yet-well-understood biochemical reactions or cellular processes. These processes are likely spatially and temporally organized in cells or cellular structures to drive polarization. A recent study revealed a novel antagonistic interaction between PCP components, Vangl2 and Dishevelled1 [22]. Dishevelled1 induces Frizzled3 hyperphosphorylation and inhibits Frizzled3 endocytosis, whereas Vangl2 inhibits Dishevelled1 induced Frizzled3 hyperphosphorylation and promotes Frizzled3 endocytosis. Antagonistic interactions among cell polarity signaling components are likely the key mechanisms to introduce or maintain asymmetry. In live growth cones, Vangl2 is asymmetrically localized to filopodia tips that are elongating. The tips of shrinking filopodia have either low levels or no Vangl2 protein, suggesting that Vangl2 may promote Frizzled3 endocytosis in a subset of filopodia to cause asymmetry of signaling for steering and that the growth cone is not equally sensitive everywhere [22].

Because Frizzled endocytosis is a critical step for Wnt-PCP signaling, it provides an important handle to understand growth cone signaling mechanisms. The next useful step is to understand how Frizzled3 phosphorylation and endocytosis is regulated. Surprisingly, Dishevelled1 and 2, which are cytoplasmic adaptor proteins and mediate Wnt-PCP signaling, act in opposition to regulate Frizzled3 endocytosis [23]. Dishevelled2 antagonizes Dishevelled1 effects on Frizzled3 (hyperphosphorylation and membrane accumulation) and is essential for Wnt-PCP pathway activation as measured by JNK activation. Once Dishevelled2 is activated by Wnt-Frizzled3 and Frizzled3 is endocytosed, Dishevelled1 is inhibited, allowing for more Frizzled3 endocyotosis. More Frizzled3 endocytosis further activates Dishevelled2 [23]. Therefore, it is plausible that this biochemical amplification loop may underlie the rapid polarization of growth cones to quickly select a subset of filopodia over the rest, which is commonly observed in growth cone guidance (Fig. 3a).

A previous study identified atypical Protein Kinase C (aPKC), a key components for apical-basal polarity (A-BP) pathway, which mediates Wnt attraction of post-crossing commissural axons and thus mediates anterior turning [24]. aPKC was found to suppress Dishevelled1's effects on Frizzled3 independent of Vangl2. Furthermore, Dishevelled2, but not Dishevelled1, activates aPKC. These results suggest that aPKC is not only intimately associated with PCP signaling but may also be a key component of this biochemical amplification loop [23] (Fig. 3a). This finding may have general implications in cell and tissue polarity signaling beyond axon guidance. PCP signaling is only active on the apical side of epithelial tissues where aPKC is localized at the adherence junctions, but not on the basal-lateral sides where aPKC is absent [20] [25] [21].

In addition to commissural axons, dopaminergic and serotonergic axons also respond to Wnts via PCP signaling [9] [10]. In invertebrates, PCP signaling has also been shown to mediate guidance of several classes of axons [26] [27]. Because apical-basal polarity and planar cell polarity signaling pathways are universal in all neuronal growth cones, the widespread use of these cell polarity proteins in growth cone steering inspires a question whether this could be a universal signaling logic for directionality in axon guidance in general [28].

These studies broke open new avenues to address the long-standing questions of how growth cones respond to shallow concentration gradients of guidance cues. Further elucidating how Frizzled3 trafficking and phosphorylation is regulated will shed important insights. Frizzled3 trafficking appears to be through a specific route by a small GTPase, Arf6, a separate trafficking “channel” from many other vesicles mediated by a number Rabs, which may mediate the trafficking of other PCP components (Fig. 3a) [23]. Sorting out the exact trafficking routes will be essential for fully understanding the signaling mechanism. Identifying Frizzled3 kinases will also be highly informative.

Cytoskeletal structures play pivotal roles in growth cone steering and axon initiation. How extracellular cues coordinate the dynamics of actin and microtubules to achieve directed growth is not fully understood [29] [30] [31] [32]. Asking whether and how Frizzled3 endocytosis leads to changes in polymerization and stability of actin and microtubules in our near the filopodia where Frizzled3 is internalized may provide valuable clues [23]. aPKC activation and Frizzled3 endocytosis are both concentrated on the sides of growth cones facing higher Wnt concentration (Fig. 3b). One of the PCP signaling out put, JNK, is likely activated in select subsets of filopodia [23]. The role of aPKC and JNK on cytoskeletal structures and membrane trafficking in growth cones are not yet understood. Additional signaling effects of endocytosed Frizzled3 will also need to be investigated to understand how cell biological events are regulated during turning.

Wnt5a-Ryk repulsive signaling

Wnt5a-Ryk repulsive signal is also mediated by calcium signaling in the hamster cortical neuron culture [7] [8] [33]. Inhibition of IP3 receptors and TRP channels reduces the rate of axon outgrowth. Inhibition of TRP channels shows guidance defects. A recent study showed that Tau phosphorylation by CaMKII influences microtubule dynamics in the growth cones

and is necessary for Wnt5a-repulsive responsiveness [34]. Because both Frizzled3 and Ryk are required for proper corpus callosum axon guidance, it is possible that Wnt-Frizzled signaling may affect Wnt-Ryk signaling pathway or vice versa. Indeed, sFRP2 pretreatment blocks Wnt5a-induced growth cone repulsion, but not axon elongation, suggesting Frizzled(s) works together with Ryk and TRP channels (Fig. 4). However, it's still unclear which Frizzled(s) is/are involved in this repulsive signaling, how Ryk and Frizzled(s) regulate calcium influx through TRP channels, and how calcium signal from different sources separately regulates axon elongation and repulsion. Two recent papers may provide important clue. Ryk turned out to be a regulator of PCP signaling, suggesting that PCP signaling is the core of guidance signaling and Ryk mediates Wnt repulsion by inhibiting PCP signaling [35] [36].

Injured axons in adult spinal cord respond to Wnts in similar fashion as in development

Research on axon regeneration after adult spinal cord injury has been focused on the molecular environment in the adult nervous system. The role of axon guidance molecules has never been the center of attention. However, recent work began to reveal a somewhat unexpected principle: injured adult axons still respond to axon guidance molecules in ways similar to development [37]. Wnts are either not expressed or expressed at undetectable levels in adult spinal cord. However, upon spinal cord injury, Wnt transcripts are quickly elevated in multiple cell types at and around the lesion site [38-43] (Fig. 5a). Subsequently, the induction of Wnt signaling molecules in the spinal cord has been confirmed in multiple models of spinal cord injury and observed on the transcript and protein level [38,44,45]. Concomitant with the up regulation of Wnts after dorsal column injury, Ryk was found quickly induced as well [38,46]. Just as Ryk acts as a repulsive Wnt receptor in developing corticospinal neurons, its causes axon retractions and limits plasticity after injury. Inhibition of Wnt-Ryk signaling with polyclonal antibody infusion reduces corticospinal axon retraction and promotes sprouting of collaterals within the spared spinal cord tissue [38] (Fig. 5a). Another study confirmed the induction of Ryk in the CST and repulsive function of Wnt-Ryk signaling to corticospinal tract axons [46].

In addition to the descending motor axons, the regenerative ascending sensory axons are also inhibited by Wnts in adulthood [47]. Conditioning of the peripheral nerve by nerve crush can initiates a cascade of growth signals in primary sensory dorsal root ganglia (DRG) neurons. This growth state promotes regeneration following either a subsequent peripheral injury proximal to the original, or, remarkably, following an injury to the central sensory axon within the spinal cord [48,49]. Curiously, peripheral conditioning induces several Wnt signaling components in the DRG, including the repulsive receptor Ryk [47,50,51]. These induced Wnt signaling components sensitize dorsal column sensory axons to spinal cord injury induced Wnts, limiting conditioning-mediated regeneration [47] (Fig. 5b). Exogenously applied Wnts are capable of causing long-range retraction of sensory axons after peripheral conditioning injury, providing anatomical evidence for the repulsive action of Wnts on injured sensory axons [47]. Therefore, similar to development, Wnts can repel injured axons if they express Ryk in adulthood.

Although these findings are exciting, the research on the roles of Wnt signaling in spinal cord injury is at its infancy. Due to the complexity of Wnt signaling pathway and multiple functions of Wnt signaling in different cell types, the interpretation of results require extra care. For example, we do not fully understand the biological function of the reinduced Wnt signaling system in adult spinal cord, particularly in glial responses. Canonical Wnt signaling has been shown activated in $NG2^+$ progenitors and astrocytes after brain injury by the β-catenin reporter BATgal mouse line [52]. Interestingly, β-catenin activity induced in NG2 proteoglycan-expressing glial precursors in the brain has not been observed in the injured spinal cord [45,52]. Demyelinating lesions of the spinal cord induce β-catenin activity in Oligodendrocyte Lineate Transcription Factor 2 (Olig2) cells and increasing canonical Wnt signaling results in hypomyelination after injury [53]. Therefore, manipulating Wnt signaling may affect myelin repair. In addition to axon regeneration and glial responses, Wnt signaling is likely important in regulating axon survival and/or degeneration, as robust changes of Wnt signaling components in animal models of neurodegeneration have been observed [54]. Therefore, functional improvements may also be contributed by increase of neuronal survival after spinal cord injury, which need to be characterized.

On one hand, it may appear an impossibly daunting task to fully understand the injury response in the adult spinal cord. On the other hand, this may be the only way to be successful. Genetic approaches to knockout genes in a spatial and temporal way in different neuronal and glial cell types will be an important step to tease out the intricate molecular details that are necessary. Clever and clean ways to deliver interventions in an appropriate spatial and temporal manner will be need to achieve repair without causing adverse effects.

Summary

Studies on the role and mechanisms of Wnt signaling in axon guidance and spinal cord injury has taught us that important axon guidance cues are often highly conserved and that the same molecular cues can be used to wire many different neurons. Signaling mechanisms of growth cone steering may be universally shared and may be similar to how cells are polarized in epithelia. Axon guidance molecules are reinduced in spinal cord injury and play profound roles in the capacity for axon regeneration in the central nervous system. Axon guidance molecules, such as Wnts, may also have functions on the glial and fibroblast environment and may regulate immune responses as well as growth of axons. Teasing out the detailed mechanisms of Wnts in all these processes will be necessary to develop effective treatments.

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Highlights

- Wnts are conserved axon guidance molecules for many axons.
- Conserved cell polarity signaling components mediate Wnt functions in steering.
- Wnts are reinduced in spinal cord injury and regulate axon regeneration.

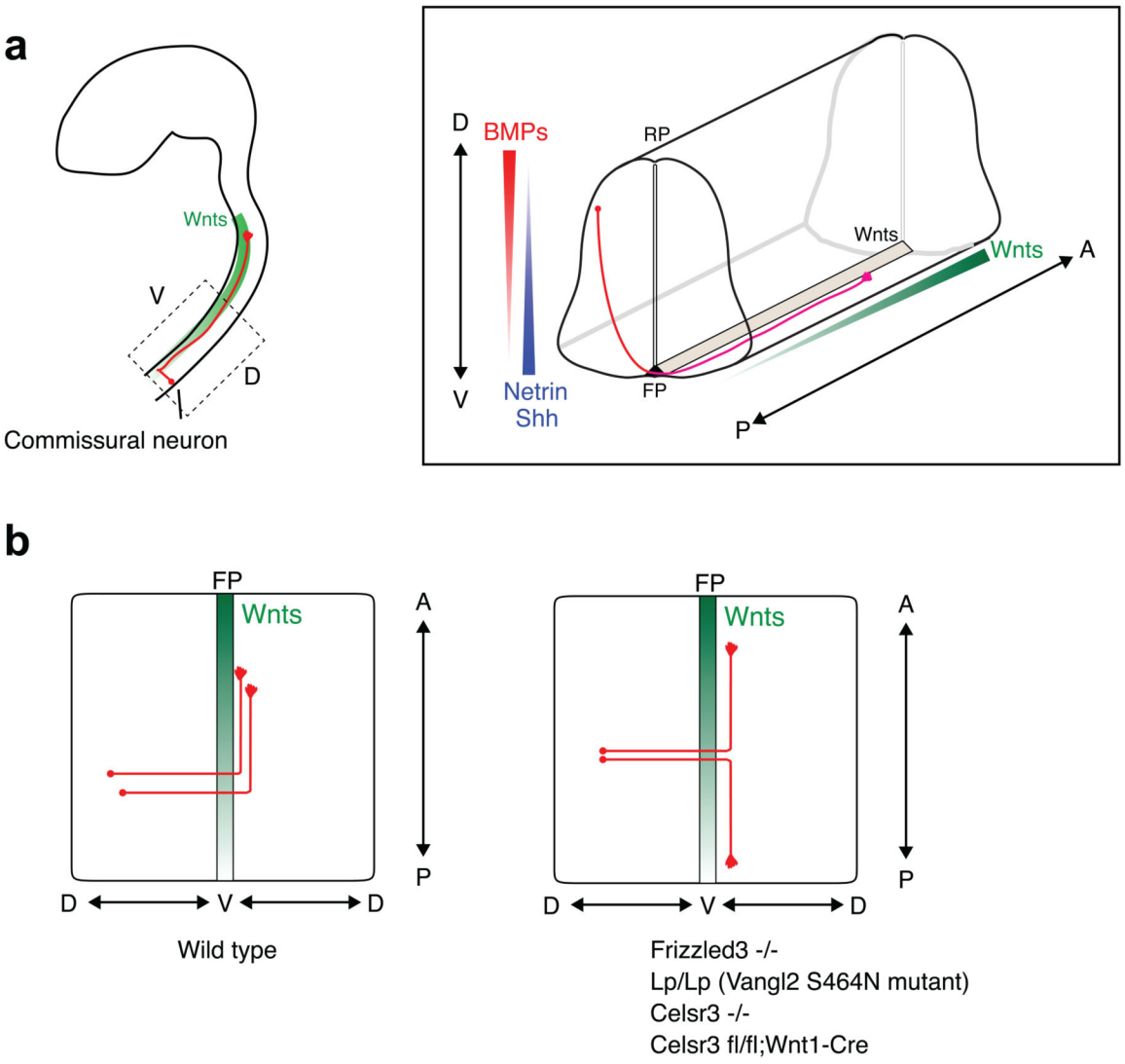

Figure 1.

Spinal commissural axon A-P guidance is dependent on Wnt-PCP signaling.

a. A depiction of dorsal spinal commissural axonal tract in the developing (E11.5) spinal cord. Commissural neurons extend the axons toward the floor plate according to Netrin and Shh gradient. After midline crossing, commissural axons turn anteriorly following Wnts gradient (anterior-high to posterior-low).

b. A typical phenotype of commissural axon A-P guidance defect caused by PCP gene disruption. In wild type spinal cord, dorsal commissural axons strictly turn anteriorly after midline crossing. On the other hand, Frizzled3 knockout randomizes post-crossing commissural axon guidance, meaning axons randomly turn toward anterior or posterior. Same phenotype is observed in Vangl2 mutant (looptail mutation; S464N) mice, Celsr3 straight knockout mice, and Celsr3 conditional knockout (Celsr3 fl/fl;Wnt1-Cre) mice. A, anterior; P, posterior; D, dorsal; V, ventral; RP, roof plate; FP, flor plate.

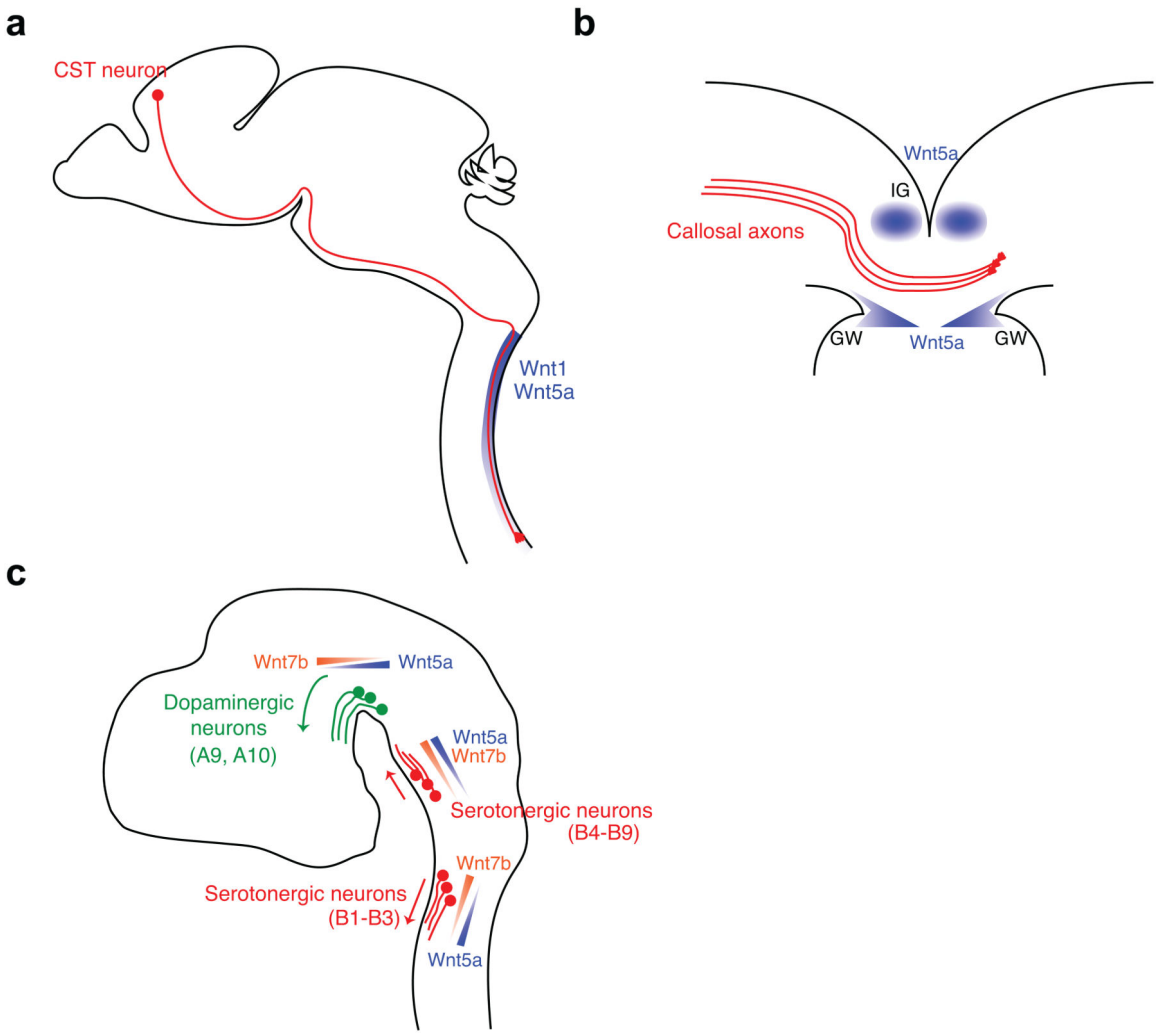

Figure 2.

Axon guidance mediated by Wnts.

a. A depiction of dopaminergic and serotonergic axon guidance in the developing midbrain and hindbrain. In the ventral midbrain, Wnt5a is expressed in increasing anterior to posterior gradient, and Wnt7b is expressed in opposite gradient. The dopaminergic neurons in A9 and A10 nuclei extend the axons toward anterior due to Wnt5a repulsion and Wnt7b attraction. On the other hand, the serotonergic neurons in the hindbrain (B4∼9 and B1∼3) sense attractive Wnt5a gradient.

b. A depiction of corticospinal axon tract (CST) in the developing (∼P0) central nervous system. Wnt1 and Wnr5a are expressed in decreasing anterior to posterior gradient within the dorsal spinal cord. CST neurons express Ryk, and Wnt-Ryk signaling repels axons, causing descending CST axon tract in the dorsal spinal cord.

c. Wnt5a-Ryk repulsive signal in corpus callosum. In the cortex, Wnt5a is expressed in indusium griseum (IG) and in the glial wedge (GW). Callosal axons express Ryk and are guided by Wnt5a repulsive signal.

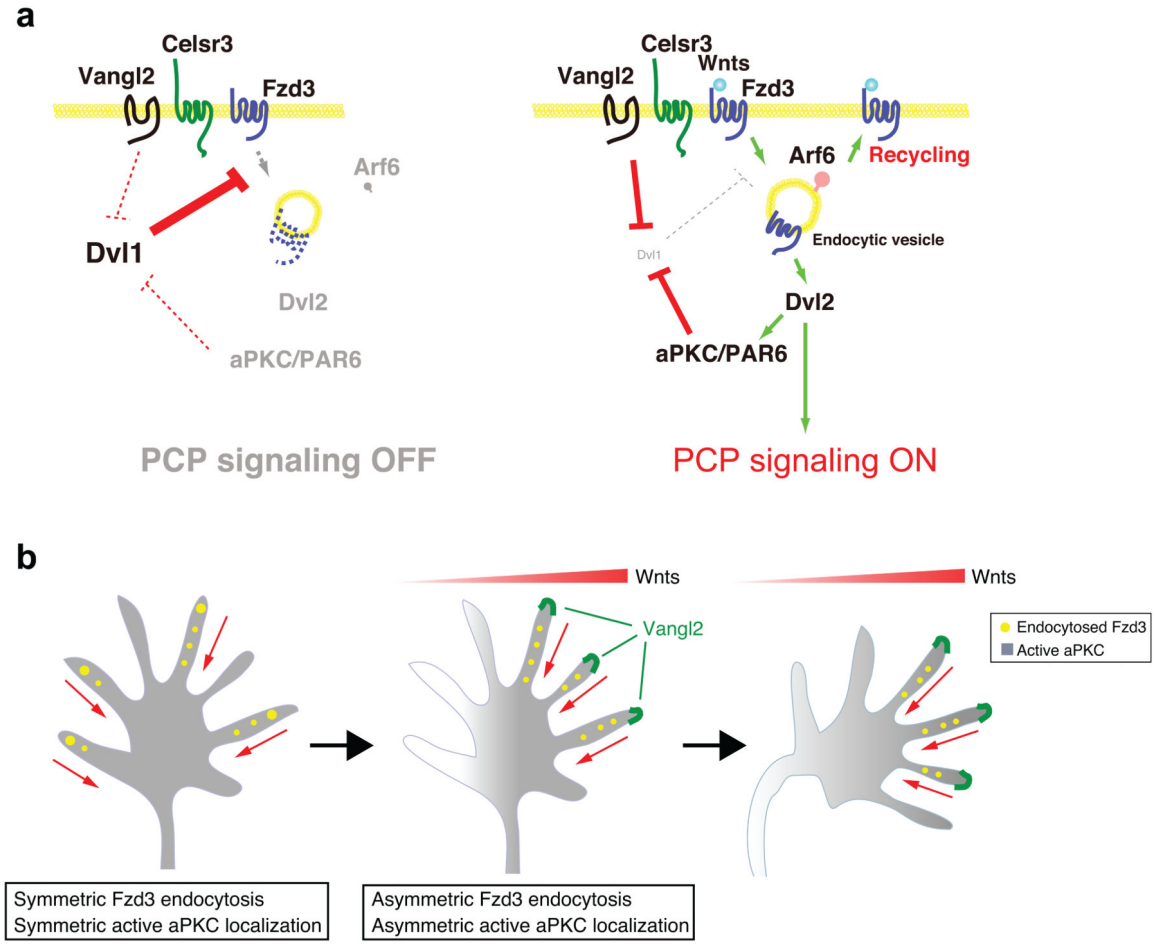

Figure 3.

A working hypothesis for Wnt-PCP signaling-mediated axon guidance.

a. Scheme of molecular mechanism in Wnt-PCP signaling. In the absence of Wnt, Dishevelled1 inhibits Frizzled3 endocytosis by inducing Frizzled3 hyperphosphorylation. Upon Wnt binding, Frizzled3 is endocytosed and activates the Dishevelled2-aPKC/PAR6 axis. aPKC then inhibits Dishevelled1. In the meantime, Vangl2 also inhibits Dishevelled1. As a result, more Frizzled3 is endocytosed and activates downstream signaling, which then in turn further removes inhibition by Dishevelled1. Arf6 mediates Frizzled3 endocytosis and its essential roles in PCP signaling activation.

b. A working hypothesis of commissural axonal growth cone turning according to Wnt gradients. In the absence of Wnts gradient, Frizzled3 is endocytosed randomly through the filopodia tips and active aPKC is distributed uniformly (left growth cone). In a Wnt gradient, Frizzled3 endocytosis occurs more frequently in the proximal side and causes more aPKC activation (middle growth cone). Those asymmetry may help to steer growth cone turning (right growth cone).

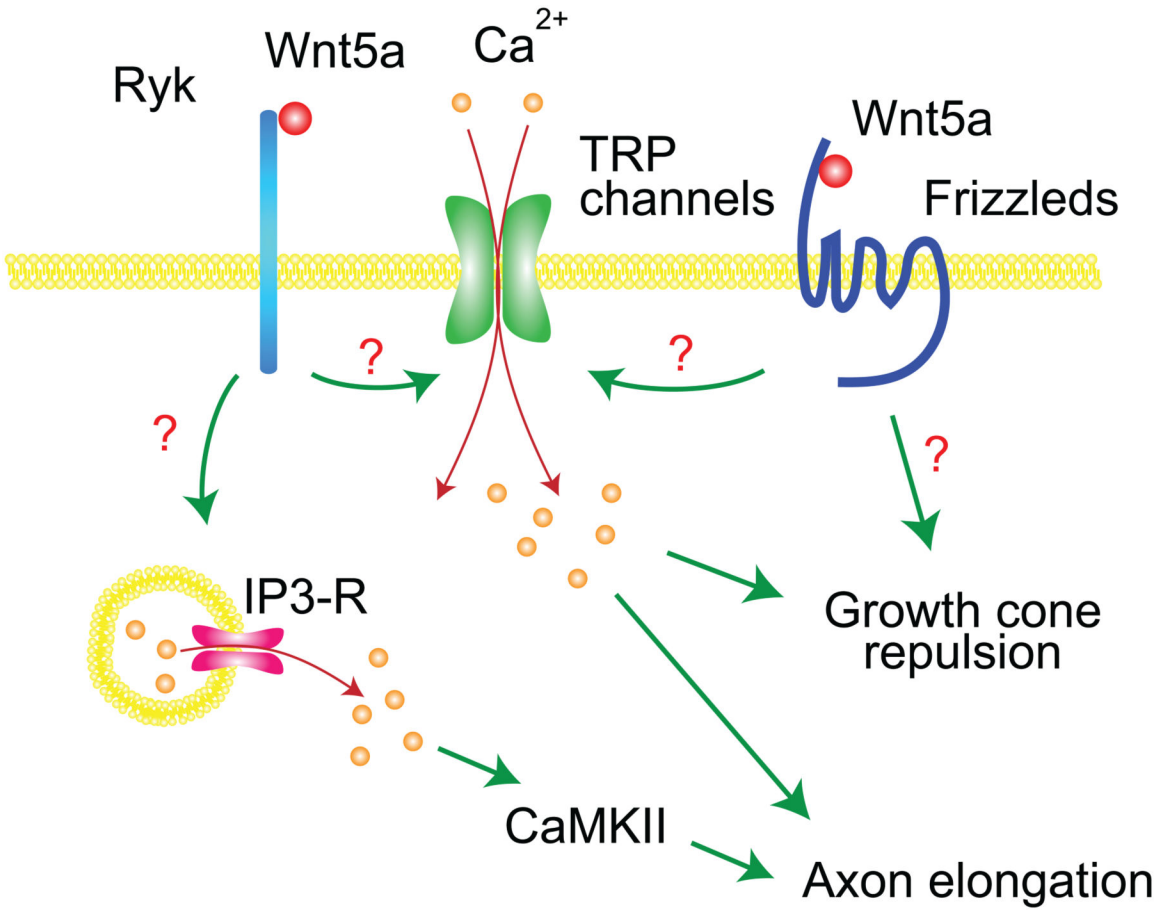

Figure 4.

A model of Wnt5a-Ryk repulsive signaling. Wnt5a evokes calcium activity through Ryk and Frizzled(s), which involves calcium entry through TRP channels and calcium release from intracellular stores through IP3 receptor on the ER. Ryk and TRP channels activity are required for both Wnt5a-induced axon elongation and repulsion. On the other hand, Frizzled(s) and IP3 receptor activity are required for Wnt5a-induced axon repulsion and elongation, respectively. It remains unclear how Ryk and Frizzled regulate TRP channels, and how calcium signal from different source regulates different process.

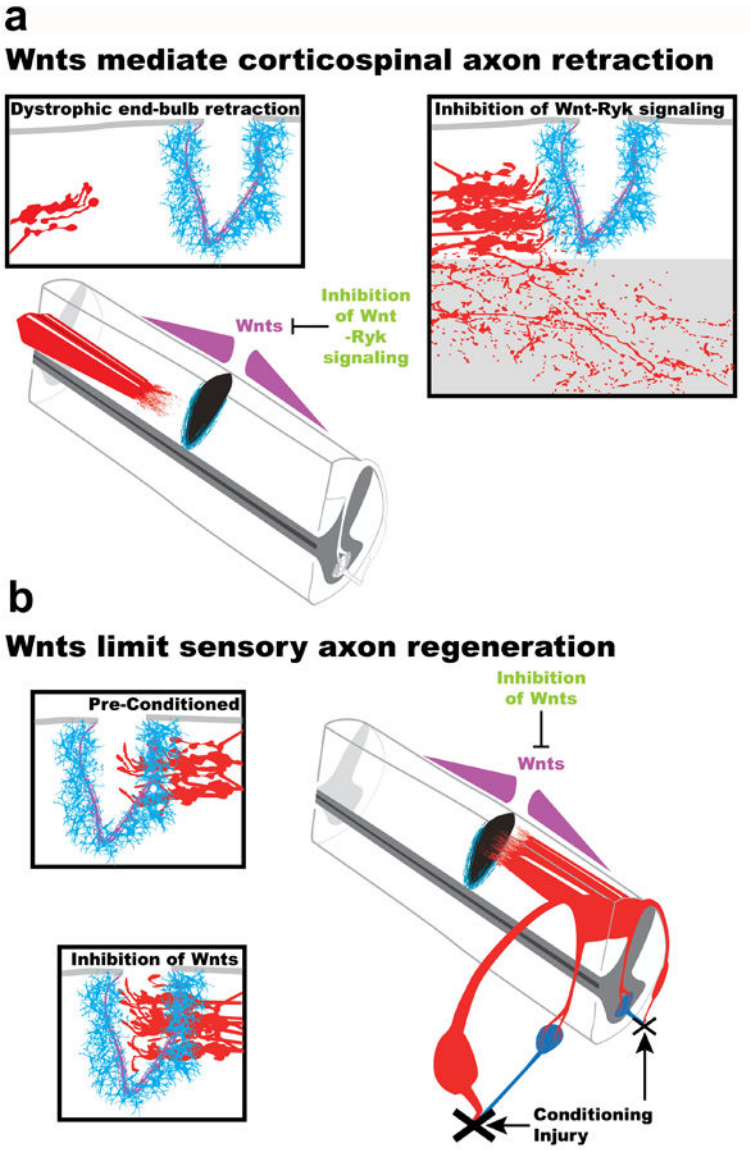

Figure 5.
Reinduced Wnts repel both motor and sensory axons in injured spinal cord.