

## Review

# Wnt signaling: multiple functions in neural development

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**Abstract.** Wnt signaling has proven to be essential for neural development at various stages and across species. Wnts are involved in morphogenesis and patterning, and their proliferation-promoting role is a key function in stem cell maintenance and the expansion of progenitor pools. Moreover, Wnt signaling is involved in differentiation

processes and lineage decision events during both central and peripheral nervous system development. Additionally, several reports point to a role of Wnt signaling in axon guidance and neurite outgrowth. This article reviews and consolidates the existing evidence for the functions of Wnt signaling in neural development.

**Key words.** Wnt signaling;  $\beta$ -catenin; Frizzled receptor; proliferation; apoptosis; stem cell maintenance; differentiation; lineage decision; axon guidance; neurite outgrowth.

## Introduction

During the past few years, technical advances and improved methodology have helped to considerably increase our understanding of the mechanisms regulating neural development. However, our knowledge about neural development is still peppered with many unanswered questions. Various signaling molecules and signal transduction mechanisms, cell-cell interactions, as well as the extracellular matrix (ECM) have been implicated in neural development. Slowly, a complex scheme is emerging in which a plethora of factors and signaling cascades are orchestrated in a spatiotemporal manner. The signals involved include members of the transforming growth factor- $\beta$  (TGF- $\beta$ ) superfamily [1] such as the bone morphogenic proteins (BMPs) [2–4] and the growth and differentiation factors (GDFs) [5, 6]. In addition, members of the Hedgehog family, fibroblast growth factor (FGF), and many other cues are crucial for neural development [7–11].

In this review article, we focus on the role of Wnt proteins in vertebrate neural development. After a short introduction to the Wnt family, we describe the canonical Wnt signaling pathway and discuss the effects of Wnt signaling on key developmental processes like proliferation, apoptosis, stem cell maintenance, lineage decision, differentiation, and axon guidance. Much of the data on Wnt signaling are related to embryonic development, such as the formation of Spemann's organizer and dorsalization of the vertebrate central nervous system (CNS), which involve Wnt signaling [12–15]. Moreover, a great deal of effort has been put into studies on postnatal requirement and function of Wnt signaling. Various requirements for Wnt signaling in different cortical cell populations were recently reported for postnatal mouse brains [16]. Equally important are the effects of Wnt signaling in the adult organism in cases of signal deregulation or alteration. In such situations, aberrant Wnt signaling can act as a pathomechanism in tumorigenesis [17–20]. Furthermore, Wnt signaling is thought to play an important role in the onset of Alzheimer's disease [21–23].

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## The Wnt family

The Wnt family consists of a group of proteins encoded by 7 known genes in *Drosophila* and about 19 genes in vertebrates. The name Wnt is derived from the *Drosophila* gene wingless (*wg*), which plays a role in segment polarity, and the mouse gene *int-1*, which is required for midbrain and cerebellar formation as well as the generation of neural crest cells [24–27]. The size of Wnt proteins varies between 350 and 400 amino acids. They contain around 24 highly conserved cysteine residues most probably involved in disulfide bond formation, as is typical for extracellular proteins. The importance of Wnt proteins and their signaling pathways in development is reflected in the degree of conservation of protein structure across species [28–30]. Wnt proteins are mostly glycosylated prior to secretion. Their only lipid modification is palmitoylation, which is not strictly required for Wnt activity [31], but may be involved in tethering of the protein to the membrane thereby increasing its activity. Overexpression

of unpalmitoylated Wnt possibly overcomes the lack of membrane tethering [32]. Wnt proteins act in multiple disparate signaling pathways and bind to cell surface receptors to activate signaling cascades. In particular, three major pathways have been identified, all of which are thought to signal via Frizzled (Fz) receptors: (i) the Wnt/ $\beta$ -catenin pathway also referred to as the canonical Wnt signaling pathway, in which  $\beta$ -catenin – a *Drosophila* armadillo related protein – is crucially involved (fig. 1); (ii) the Frizzled/planar cell polarity (Fz/PCP) pathway, and (iii) the  $\text{Ca}^{2+}$  pathway [33, 34].

## The canonical Wnt signaling pathway

Extracellular Wnt molecules bind to Fz seven-pass transmembrane receptors and to low-density lipoprotein receptor-related proteins (LRP5 or LRP6) to form a ternary complex [35–38]. This receptor complex induces the phosphorylation of Dishevelled (Dsh). An alternative

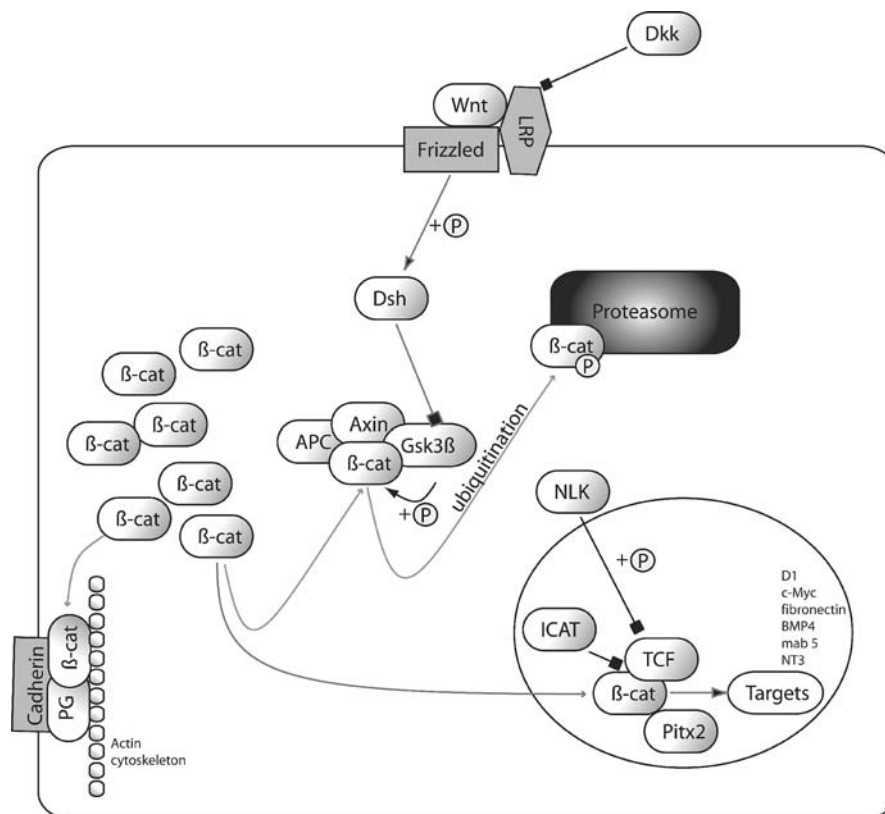


Figure 1. Model of canonical Wnt/ $\beta$ -catenin signaling pathway. Both adherens junctions and the canonical Wnt signaling pathway require  $\beta$ -catenin. By default,  $\beta$ -catenin is phosphorylated in the glycogen synthase kinase-3 $\beta$  (GSK3 $\beta$ ), Axin, APC complex and thus directed into the ubiquitin/proteasome degradation pathway. Wnt forms together with Frizzled and LRP 5/6 a trimeric complex and activates intracellular dishevelled (Dsh) by phosphorylation. Dickkopf (DKK) inhibits the formation of the Wnt/receptor complex. Activated Dsh inhibits GSK3 $\beta$  and thus leads to stabilization and accumulation of  $\beta$ -catenin in the cytoplasm. Stabilized  $\beta$ -catenin is transported to the nucleus in a concentration-dependent manner. There it activates, together with transcription factors of the TCF/LEF family, the transcription of target genes. Nemo-like kinase (NLK) phosphorylates TCF and regulates its DNA-binding affinity. Pitx2 acts as a transcriptional repressor when bound to  $\beta$ -catenin.

activation of the canonical Wnt signaling pathway via Fz dimerization only has been proposed from experiments with *Xenopus laevis* [39]. Moreover, there is evidence that Wnt proteins are able to induce phosphorylation of Dsh via LRP5/6 by pathways other than the canonical, since phosphorylation of Dsh did not necessarily lead to  $\beta$ -catenin stabilization [40].

Wnt signaling is regulated at the receptor level by various regulatory proteins, including LRP5/6-binding factors (Dickkopfs, DKKs) and secreted Fz-related proteins (SFRPs) [41–43]. DKKs inhibit the ternary complex formation between Wnt, Fz, and LRP5/6, and therefore inhibit canonical Wnt signaling [44]. Interestingly, DKK1 has been reported to be a direct transcriptional target of the canonical Wnt signaling pathway [45]. This might point to a feedback loop modulating canonical Wnt signaling. Moreover, Golan et al. [46] have shown that human Fz receptor 6 can act as a negative regulator for the canonical Wnt signaling pathway downstream of the  $\beta$ -catenin destruction complex (fig. 1).  $\beta$ -Catenin is not only a key component of the canonical Wnt signaling pathway, but also serves as a structural molecule that anchors the actin cytoskeleton to the intracellular domain of cadherins [47–49]. As such,  $\beta$ -catenin is also involved in cadherin-mediated cell-cell adhesion. Adherens junctions and canonical Wnt signaling possibly require distinct molecular forms of beta-catenin. While the cytosolic pool of  $\beta$ -catenin can bind to  $\alpha$ -catenin and cadherins, Wnt signaling is thought to promote a specific conformation of  $\beta$ -catenin which can bind to certain transcription factors but not to cadherins [50].

Dsh inhibits the complex formation of Axin, adenoma poliposis coli protein (APC), glycogen synthase kinase-3 $\beta$  (GSK3 $\beta$ ) and  $\beta$ -catenin. This complex is required for  $\beta$ -catenin phosphorylation, which directs the protein into the proteasome degradation pathway [51, 52]. If not degraded,  $\beta$ -catenin accumulates in the cytoplasm. Although  $\beta$ -catenin lacks a nuclear localization signal it can be translocated into the nucleus in a concentration-dependent manner. Translocation has been shown to be independent of the classical nuclear transport pathways that involve Ran or importin [53, 54]. However,  $\beta$ -catenin is too large for passive nuclear transport. A possible solution for the nuclear transport problem emanates from the fact that  $\beta$ -catenin interacts with the androgen receptor (AR), which might serve as a nuclear transporter. Indeed, AR agonists affected the nuclear transport kinetics of  $\beta$ -catenin and the AR in an analogous manner [55].

In the nucleus,  $\beta$ -catenin interacts with high-mobility group (HMG) box transcription factors, like the T cell factor (TCF, also known as lymphoid-enhancer factor LEF), and forms a transcriptional activator complex [56]. This activator complex targets genes such as cyclin D1, c-Myc, fibronectin, BMP4, mab-5, and NT-3 [27]. Complex formation between the TCF and  $\beta$ -catenin is inhibited

by a protein called inhibitor of  $\beta$ -catenin and TCF-4 (ICAT), which can bind to  $\beta$ -catenin armadillo repeats in a manner similar to that of the TCFs [57, 58]. In cells with elevated  $\beta$ -catenin, ICAT was proposed to sequester a subpopulation of  $\beta$ -catenin and thus to buffer increased  $\beta$ -catenin levels in the cytoplasm [59]. Phosphorylation of TCF/LEF by activated Nemo-like kinase (NLK) inhibits the DNA-binding affinity of the complex and thus indirectly regulates Wnt signaling in the nucleus [60]. Yet NLK has also been shown to act as a positive regulator of Wnt signaling in early zebrafish development [61]. Moreover, the factor Pitx2, which is involved in cell type-specific proliferation, has been shown to be converted from a transcriptional repressor into a transcriptional activator when bound to  $\beta$ -catenin [62]. Overall, the various levels of regulation of canonical Wnt signaling may indicate multiple possible interactions with other signaling cascades, required for differential signal integration.

### Proliferation and apoptosis

Precise regulation of proliferation/apoptosis ratios is essential in neural development [63]. Unbalance results in severe malformations during embryonic development, and promotes cancer formation postembryonically. In vitro as well as in vivo studies have shown that Wnt signaling is required to expand and maintain neural precursor populations in the brain and the spinal cord [64–66]. Wnt-1 regulates precursor populations in the mid/hindbrain and is necessary for its development [67, 68]. Wnt-3a signaling seems to be involved in hippocampal development by regulating the size of the caudomedial cortex through progenitor pool regulation and/or stem cell maintenance [69]. Moreover, Wnt signaling regulates the size of the cerebral cortex in the mammalian system [70]. In the spinal cord, progenitors are specified by BMP signaling that determines domains of Wnt ligand, receptor, and antagonist expression, resulting in spinal cord patterning [71]. In these BMP-defined progenitor populations, Wnt signaling is thought to regulate cell cycle exit and thus progenitor expansion [70, 72]. Similar proliferative effects of Wnt signaling have been described for stem cells and progenitors in various tissues like the skin, intestine, and the hematopoietic system [31, 73, 74].

While most results indicate a proliferative function of Wnt signaling, there are also reports which claim that Wnt signaling inhibits proliferation in certain cell types, such as human endothelial vein cells (HUVECs) [75]. This effect is thought to result from non-canonical Wnt/Ca<sup>2+</sup> pathway signaling that inhibits the proliferative effects of canonical Wnt signaling [76–78].

Wnt signaling not only affects proliferation but has also been implicated in apoptosis. In cancer research, for example, Wnt signaling has been related to drug resis-

tance in cancer therapy where vinblastine and vincristine are used as apoptosis-inducing drugs. The effect of these drugs has been overruled by Wnt signaling and cell survival maintained, while inhibition of Wnt signaling by expression of dominant-negative TCF4 rendered the cells responsive to the drugs [79]. Another study on 3T3-L1 cells revealed a TCF4-independent mechanism by which Wnt signaling inhibits apoptosis. While overexpression of dominant-negative TCF4 triggered the expression of apoptotic genes, Wnt signaling upregulated the expression of insulin-like growth factors (IGF I/II) which mediate antiapoptotic effects [80]. Moreover, conditional ablation of  $\beta$ -catenin in the dorsal spinal cord led to increased apoptosis, although whether this was due to impaired canonical Wnt signaling or to disturbed cell-cell interactions was difficult to assess [F. Ille, R. Kemler and L. Sommer, unpublished data]. Canonical Wnt signaling has been proposed to suppress apoptosis by inhibiting c-Myc-induced release of cytochrome c and caspase activation [81, 82]. In addition to the antiapoptotic effects of canonical Wnt signaling, apoptotic inhibition has been attributed to  $\beta$ -catenin-independent Wnt signaling via Janus kinase (Jnk) [83]. Despite the evidence for antiapoptotic effects of Wnt signaling, Wnt may also induce

apoptosis. In particular, Hasegawa and colleagues have shown that stabilizing  $\beta$ -catenin by conditional APC ablation leads to massive induction of apoptosis in neural crest cells in the mouse model [84].

### Stem cell maintenance, differentiation, and lineage decision

Stem cell maintenance and self-renewal are cellular processes closely associated with proliferation. Given the role of Wnt signaling in cell cycle regulation, Wnt signaling has not surprisingly also been implicated in the control of stem cell development [85]. Activation of Wnt/ $\beta$ -catenin signaling in human and mouse embryonic stem cells (ESCs) by administration of pharmacological GSK3 $\beta$  inhibitor maintains their self-renewal capacity as well as their pluripotency [86]. Exposure of hematopoietic stem cells to Wnt molecules and sustained expression of  $\beta$ -catenin in long-term cultures maintains self-renewal as well as the capacity of these cells to reconstitute the hematopoietic lineages in vivo [87]. Nonetheless, ablation of  $\beta$ -catenin seems to impair neither hematopoiesis nor lymphopoiesis under physiological conditions [88]. For

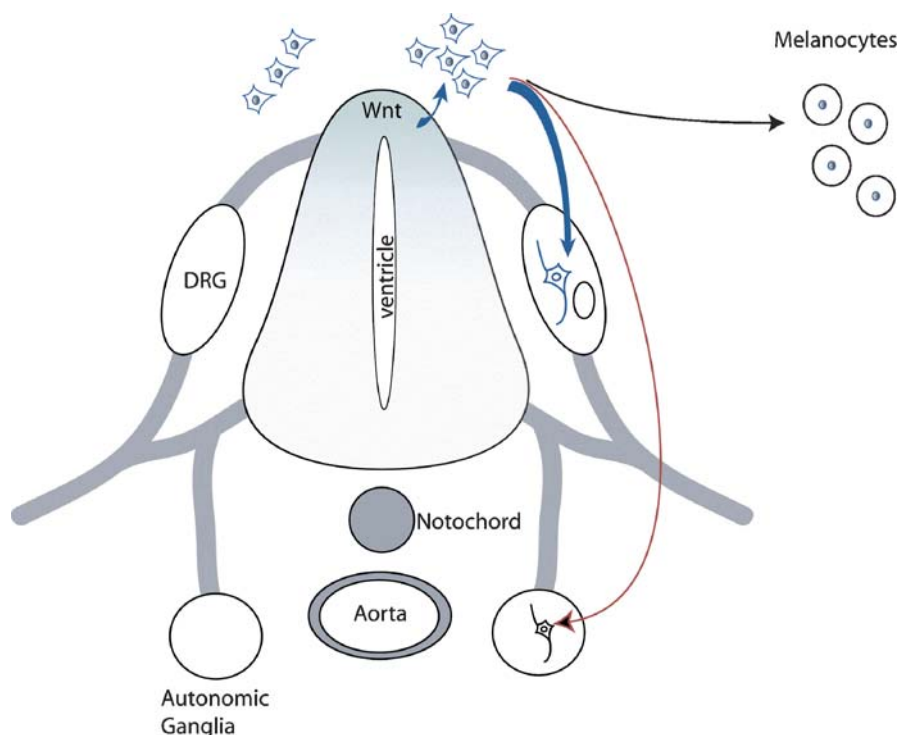


Figure 2. Wnt signaling in neural crest stem cell development. A gradient of Wnt molecules is established in the dorsal neural tube, which is involved in neural tube patterning and the generation of the neural crest cell population. Some of the early neural crest stem cells (eNCSC; blue-colored cells) are thought to become committed to the sensory lineage (blue arrow) in response to canonical Wnt/ $\beta$ -catenin signaling in the dorsal neural tube. Delaminated eNCSCs migrate toward their homing positions. At these positions, NCSCs respond to local signals and are involved in the generation of structures such as the dorsal root ganglia (DRG), the autonomic ganglia (red arrow), and structures of the skin (black arrow), and others.

these processes, the data suggest either an alternative rescue pathway or the involvement of a factor upstream of  $\beta$ -catenin in the Wnt signaling cascade.

The effects of Wnt signaling on stem cells seem to be diverse. While it inhibits neural differentiation and maintains pluripotency in ESCs [86, 89], it can also promote differentiation in other stem and progenitor cells [85]. Marretto et al. [90] identified a TCF-dependent reporter gene in differentiating cortical neurons during development, suggesting a potential role of Wnt/ $\beta$ -catenin signaling in the differentiation process. In agreement with this, Wnt-7a signaling induces differentiation in neural precursor cells (NPCs) of the neocortex [91]. This process reduces the NPC pool at late developmental stages (E13.5) whereas NPCs at earlier developmental stages (E10.5) do not seem to differentiate in the presence of Wnt. Recently, Wnt-3a has been reported to promote differentiation into the neural and astrocyte lineage by inhibiting neural stem cell maintenance [92]. Moreover Wnt/ $\beta$ -catenin signaling is required for neural differentiation in ESCs [93].

In neural crest stem cells (NCSCs), Wnt signaling has been linked to cell fate decision (fig. 2). Neural crest cells generate various cell types of the peripheral nervous system (PNS) as well as craniofacial, skin, and heart structures [94]. These cells are derived from the border of the neural plate adjacent to the ectoderm, and are strictly dependent on Wnt signaling [95, 96]. Wnt/ $\beta$ -catenin signaling regulates cell fate decisions in early neural crest stem cells (eNCSCs) by driving these cells into the sensory lineage, rather than affecting the population size. Hari et al. [97] have shown by cell type-specific gene ablation that loss of  $\beta$ -catenin-function in NCSCs prevents sensory

ganglia and melanocyte formation in vivo [97]. Intriguingly,  $\beta$ -catenin-deficient NCSCs emigrate and proliferate normally in culture, but they fail to acquire a sensory neuronal fate [97]. Conversely, a  $\beta$ -catenin gain-of-function study shows that, in vivo, continuous Wnt/ $\beta$ -catenin signaling in NCSCs leads to sensory neurogenesis at the expense of all other neural crest derivatives [98]. However, stabilized  $\beta$ -catenin does not affect NCSC migration and proliferation. Consistent with the in vivo data, practically all mutant cells lose NCSC features and adopt a sensory fate in cell culture. Although all eNCSCs are Wnt responsive, only a subpopulation of the cells generate sensory cells in vivo, indicating the presence of factors counteracting Wnt activity [85]. The development of the hippocampus, and in particular the generation of dentate gyrus granule cells, also appears to be regulated by LEF1/TCF transcription factors [99]. Similar effects have been reported for skin stem cells, in which  $\beta$ -catenin signaling determines differentiation into follicular and epidermal lineages [100, 101]. Taken together, these results underline the role of Wnt signaling in lineage decision, but the molecular mechanisms remain to be resolved.

### Axon guidance and neurite outgrowth

One essential aspect in CNS development is ‘wiring,’ a process which involves patterning, migration, axon guidance, and synapse formation. As mentioned before, Wnt molecules have been implicated in morphogenesis, where they establish tissue patterning together with other

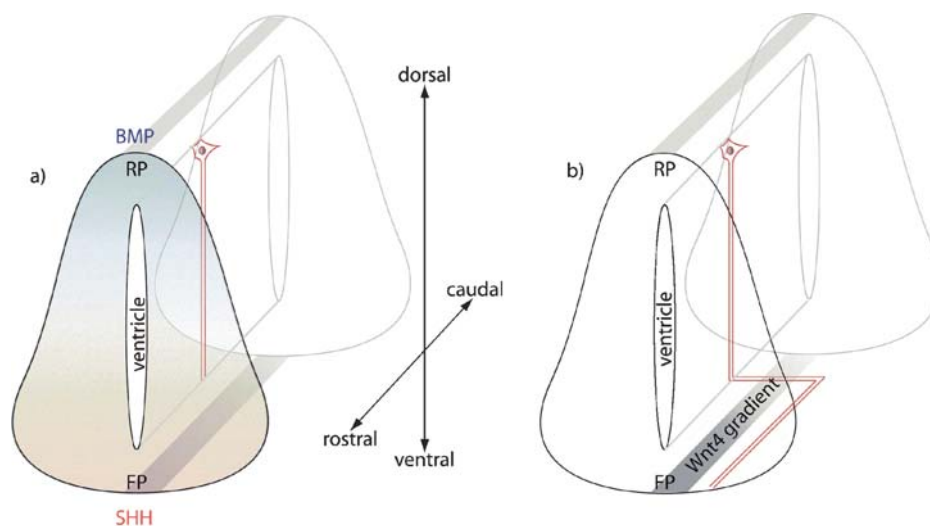


Figure 3. Guidance of commissural axons in the developing spinal cord. (a) Commissural axons of certain neurons are repelled by the dorsal bone morphogenetic protein (BMP) gradient (blue) that originates from the roofplate (RP). At the same time, the axons are attracted by the ventral sonic hedgehog (SHH) gradient in the floorplate (FP). Therefore, commissural axons extend ventrally toward the floorplate. (b) Once the axons reach the floorplate, they cross it and turn rostrally. After turning toward the head, the axons extend along a Wnt-4 gradient until they reach their target area and form synapses.

proteins like sonic hedgehog (SHH) and BMPs. In the early neural tube, for example, BMP4 expressed in the roofplate and SHH expressed in the floorplate generate two dorsoventral countergradients which influence proneural gene expression. Wnt-1 and Wnt-3a are expressed in the dorsal neural tube at later stages, and are thought to determine proneural domains of the dorsal neural tube by mechanisms which still remain to be elucidated [102, 103]. Wnt-4, however, is expressed in the ventral neural tube and has been related to axon guidance in commissural axons [104]. Commissural axon migration is repelled from the roofplate by BMP signaling, and at the same time, these axons are attracted toward the floorplate by SHH. Once these axons reach the floorplate, they cross it, turn rostrally, and extend toward the brain (fig. 3). Lyuksyutova et al. [104] have found Wnt-4 to be expressed in a rostrocaudal gradient in the floorplate, which attracts postcrossing commissural axons. Likewise, commissural axons which lack the Wnt receptor Fz3 exhibit rostrocaudal guidance defects in postcrossing commissural axons [104]. Since these results have not been tested in Wnt-4 knockout animals, the direct involvement of Wnt-4 in axon guidance remains to be proven. Nevertheless, evidence from invertebrate systems favors the hypothesis that Wnt molecules can act as guidance molecules. In *Drosophila*, Wnt-5 seems to fulfill a guidance function. By signaling through the Derailed receptor expressed in the growth cones, Wnt-5 acts as a chemorepellent [105–107].

In vertebrates, ablation of Ryk, the mammalian homologue of *Drosophila* Derailed, leads to axon guidance defects in vivo [108]. Unlike Derailed, Ryk acts as a Fz coreceptor and forms a ternary complex with Fz and Wnt-1. It also mediates TCF activation induced by Wnt-1 and is able to bind to Wnt-3a. Moreover, Ryk is required for neurite outgrowth in dorsal root ganglion neurons, induced by Wnt-3a [108]. Similarly, Wnt-7a has been described to remodel axon spreading and branching in developing cerebellar granule cells [109]. Axonal remodeling involves MAP-1B, a microtubule-associated protein that has been identified as substrate for GSK3 $\beta$  and implicated in axonal outgrowth [110]. Upon GSK3 $\beta$  inhibition, the amount of phosphorylated MAP-1B decreases in the cells, a process that naturally occurs prior to axonal remodeling and leads to changes in microtubule dynamics. Recently, Ciani et al. [111] have shown that the Wnt/ $\beta$ -catenin signaling component Dsh also induces axonal remodeling and stabilizes microtubules in developing neurons. Intriguingly, this process seems to involve neither  $\beta$ -catenin nor TCF factors, suggesting an alternative regulatory pathway for canonical Wnt signaling, downstream of GSK3 $\beta$ . Similar evidence comes from experiments in neuroblastoma [112]. Here, GSK3 $\beta$  and Axin have been reported to promote neurite outgrowth in a  $\beta$ -catenin-independent manner.

## Conclusion

The roles of Wnt signaling in neural development are manifold, and seem at times contradictory. While one of its most important functions is promotion of proliferation, in some contexts, Wnt signaling is able to inhibit it. Similarly, apoptosis is abolished by Wnt signaling in certain cases whereas in others it is induced. As much as Wnt signaling is responsible for stem cell maintenance, it is to a similar extent essential for differentiation and lineage decisions, axon guidance, and neurite outgrowth. With every newly described aspect of Wnt signaling, more questions arise. Some answers to these questions may lie in understanding the intrinsic status of a cell that reflects its 'history' and that is modified depending on the position of the cell in the organism at a given timepoint. In other words, the effects of Wnt signaling are tissue and cell type specific and represent the results of combinatorial signal integration in an environment of dynamic signaling networks.

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