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WNT SIGNALING IN THE ANTEROPOSTERIOR PATTERNING OF NERVOUS SYSTEMS

Master Thesis

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SUMMARY

Nervous systems are amongst the most astonishing and intriguing outcomes of metazoan evolution. Wnt signaling encompasses a complex mechanism of cell-cell communication which has been extensively implicated in correct embryonic development and adult functioning of these systems. Moreover, impairments in these communication mechanisms have been associated with debilitating neurodegenerative and neuropsychiatric diseases, as well as with severe birth defects. In bilaterian animals, Wnt signaling is also crucial in the embryonic anteroposterior polarization which establishes the primary body axis. In this review, I will highlight our current understanding of the role of Wnt signaling in the polarized establishment and patterning of the nervous system along the anteroposterior body axis, by integrating evidence from both vertebrate and invertebrate model organisms.

1. INTRODUCTION

The development of any multicellular animal, from a single-cell zygote to its mature form, is a complex and fascinating process where growth and patterning must be tightly coupled to guarantee proper morphogenesis. This process is dependent on inherited cell-autonomous factors and on the ability of cells to sense and adequately respond to stimuli from their surroundings. At the cell surface, extracellular signals are continuously perceived and communicated to the intracellular compartment through ligand-receptor interactions which activate different signal transduction cascades. The resulting intricate temporal and spatial control of gene expression underlies the timely regulation of cellular proliferation, differentiation, migration and programmed death which are pivotal in complex developmental processes such as axis establishment and tissue morphogenesis, cornerstones in the genesis of a functional and healthy organism (Gilbert, 2010). The evidence accumulated so far shows that a small group of signaling pathways, including the Wnt, Hedgehog (Hh), transforming growth factor-β (TGF-β), receptor tyrosine kinase (RTK), Janus kinase/signal transducer and activator of transcription (JAK/STAT), Notch and nuclear hormone pathways, are responsible for cooperatively orchestrating most of animal development (Pires-daSilva and Sommer, 2003). It also revealed that these pathways are brought into play repeatedly in different developmental processes and stages where they trigger specific responses. This suggests that these may not be rigid signal transduction pathways but instead developmental "building blocks" which have a remarkably flexible capacity of generating distinct responses according to the specific history and context of the cell. Moreover, these signaling systems seem to be essential throughout the development of virtually all metazoan groups, from the relatively simple sponges to the sophisticated vertebrates (Adamska et al., 2011; Pires-daSilva and Sommer, 2003), thus suggesting that these were already operating in the last common ancestor of most animals and have been highly conserved during metazoan evolution.

Since its discovery in the 1980's, the highly conserved Wnt signaling pathway has been extensively studied leading to the current general acceptance of its crucial role in several processes of metazoan development (van Amerongen and Nusse, 2009). Wnt signaling regulates a variety of cell behaviors including stem cell maintenance, cell fate, proliferation and migration, and have been implicated in a multitude of developmental events such as axis specification, gastrulation and heart and limb formation (Croce and McClay, 2008; van Amerongen and Nusse, 2009). Naturally, as a consequence of this prominent role in embryogenesis, defective Wnt signaling recurrently leads to anomalous development (Cadigan and Peifer, 2009). Furthermore, these cellular processes controlled by Wnt

signaling are essential mechanisms in tumorigenesis and tumor metastasis, and deregulation of the Wnt signaling pathway has been involved in a considerable number of cancers (Clevers, 2006; Ford et al., 2013; Ying and Tao, 2009). As a matter of fact, the field of Wnt signaling research has been mainly driven by its potential in the development of cancer therapeutics.

More recently, a growing body of evidence brought to light the key role of Wnt signaling in the formation and modulation of nervous systems. Wnt proteins have been implicated in early developmental patterning where they act as posteriorizing signals and are involved in processes such as neural crest cell induction, neural cell proliferation and neurogenesis (Amoyel et al., 2005; Dorsky et al., 1998; Kiecker and Niehrs, 2001). Furthermore, Wnt signaling has also been reported as decisive in processes which are fundamental for correct brain formation and wiring, for instance, neuronal polarization, neuronal migration, axon guidance, dendrite morphogenesis and synapse establishment (Hall et al., 2000; Lyuksyutova et al., 2003; Rosso et al., 2005; Vivancos et al., 2009). Accordingly, it has been proposed that Wnt signaling is involved in several neurodegenerative and neuropsychiatric diseases such as Alzheimer's disease and schizophrenia (Inestrosa et al., 2012).

In this thesis, I will review the function of Wnt signaling in various aspects of the formation and patterning of nervous systems along the anteroposterior (A-P) axis, the primary axis of all bilaterian metazoans.

2. THE PATHWAYS OF WNT SIGNALING

The first reported Wnt gene was identified through a mutation that causes the lack of wing and haltere in *Drosophila* and, therefore, it was named *wingless* (*wg*) (Sharma and Chopra, 1976). Conversely, *Int1* (currently *Wnt1*) was identified as a putative oncogene of murine mammary carcinoma induced by integration of mouse mammary tumor virus (MMTV) (Nusse and Varmus, 1982). Through comparative genomic analyses, these two genes were found to be homologous (Rijsewijk et al., 1987), and their names (*wg* and *Int*) were subsequently combined as Wnt, which now terms most of the members of this multi-gene family (Nusse et al., 1991).

The Wnt protein family encompasses a group of secreted glycoproteins which mediate cell-to-cell signaling. The advent of DNA sequencing revealed multiple and evolutionarily conserved Wnt genes which are present in the genome of a broad range of metazoans (Logan and Nusse, 2004). For instance, 19 Wnt genes were identified in human and mouse genomes, 5 in *C. elegans* and 7 in *Drosophila*. Wnts have also been discovered in cnidarians, evidencing their remarkable conservation along metazoan evolution. In order to generate a cellular response, these ligands can bind to several transmembrane receptors at their target cells. On the top of this list come the Frizzled (Fz or Fzd) proteins, a seven-pass membrane receptor family which is considered the prototypical Wnt receptor (Bhanot et al., 1996). Several other receptors and co-receptors have further been identified as mediators of Wnt signaling, among which are the low-density lipoprotein receptor-related protein 5/6 (LRP5/6), the receptor tyrosine kinase-like orphan receptor (Ror), and the receptor-like tyrosine kinase (Ryk) (Fradkin et al., 2010; He et al., 2004; Minami et al., 2010).

Historically, Wnt signaling pathways have been classified in two distinct categories, canonical and non-canonical, based on presumed intrinsic properties of the Wnt molecules, and which correlated with the intracellular outcome of the ligand-receptor interaction (van Amerongen and Nusse, 2009). The canonical pathway is characterized by an increase in the translocation of β-catenin to the nucleus which results in the regulation of downstream target gene transcription, thus it is also known as the β-catenin-dependent pathway **(Figure 1A)**. On the other hand, the non-canonical pathways trigger intracellular responses which do not involve β-catenin activation and, therefore, are also classified as β-catenin-independent pathways **(Figure 1B,C)**. Wnt1, Wnt3a and Wnt8 have been commonly regarded as canonical Wnt ligands, whereas Wnt5a and Wnt11 have been considered non-canonical (van Amerongen et al., 2008).

This classification was driven by an early interpretation of Wnt signaling responses as the outcome of discrete and linear pathways. However, the accumulated evidence during the last 30 years of research revealed that Wnt signaling is neither discrete nor linear. At all levels of the signaling cascades, crosstalk and feedback mechanisms between the different pathways are observed (van Amerongen and Nusse, 2009). For instance, Wnt5a is capable of inducing β-catenin-dependent signaling in HEK 293 cultured cells which express Fz4 and LRP5 (Mikels and Nusse, 2006). Another typical non-canonical Wnt, Wnt11, is implicated in β-catenin-independent convergent extension (CE) movements during *Xenopus* and zebrafish gastrulation (Marlow et al., 2002). However, Wnt11 has also been reported to activate signaling through β-catenin in dorsal axis formation of the early *Xenopus* embryo (Tao et al., 2005). In addition, canonical and non-canonical Wnts may also compete for Fz binding, triggering the inhibition of the reciprocal pathway (Grumolato et al., 2010). Moreover, the specificity of the activated pathway is not only dependent on the Wnt ligand but also on the co-receptor which is recruited to transduce the signal upon Wnt-Fz binding (Grumolato et al., 2010). These observations corroborate an emerging paradigm where the specific outcome of Wnt signaling induction is not completely dependent on the properties of the ligand, as once postulated, but is tightly related with the nature and combination of the membrane receptors, and the context and history of the cell.

The Disheveled (Dvl or Dsh) proteins are an intriguing intracellular point of convergence and a potential paradigm of crosstalk shared by possibly all Wnt pathways **(Figure 1)**. Dvl proteins are cytoplasmic scaffolding proteins which, upon Wnt-stimulated phosphorylation, are capable of interacting with a large number of Wnt signaling effectors (Gao and Chen, 2010). This is critical for the different Wnt signaling pathways, since Dvl bridges the plasma membrane signals and the specific downstream intracellular signaling cascades (Boutros and Mlodzik, 1999; Gao and Chen, 2010). On the one hand, this ability seems to be possible due to several phosphorylation sites present in Dvl structure which, according to the received signal and intracellular context, are differently phosphorylated by several kinases (Gao and Chen, 2010; González-Sancho et al., 2013). On the other hand, the presence of distinct conserved domains (such as DIX, PDZ and DEP domains) allows Dvl to differently mediate protein-protein interactions and, consequently, trigger the different pathways. As an example, the DIX domain of Dvl is necessary for activation of the Wnt/β-catenin pathway; however, a mutant form of Dvl from which the DIX domain has been deleted is capable of activating both Wnt/PCP and Wnt/Ca^{2+} pathways (Gao and Chen, 2010; Heisenberg et al., 2000; Sheldahl et al., 2003; Tada and Smith, 2000).

2.1. THE CANONICAL WNT/β-CATENIN PATHWAY

As previously mentioned, the canonical Wnt/β-catenin pathway leads to the translocation of active β-catenin to the nucleus where it regulates the transcription of Wnt responding genes. The activation of this pathway has been involved in several biological processes, including cell proliferation, cell differentiation and embryonic patterning (Clevers, 2006; Logan and Nusse, 2004; MacDonald et al., 2009). In the absence of exogenous Wnt signaling, β-catenin is present in the cytoplasm where it is sequestered by the destruction complex which promotes its proteasome-mediated degradation, thus preventing β-catenin from reaching the nucleus **(Figure 1A)** (Logan and Nusse, 2004; MacDonald et al., 2009). At this complex, Axin and adenomatous polyposis coli (APC) promote phosphorylation of β-catenin by glycogen synthase kinase 3 (GSK3) and casein kinase 1α (CK1α) (Liu et al., 2002). Phosphorylated β-catenin is then recognized and ubiquitylated by the β-transducin repeat-containing homologue protein (β-TrCP), which targets β-catenin for degradation by the proteasome (Aberle et al., 1997; Kitagawa et al., 1999). The activation of the canonical Wnt/β-catenin pathway occurs when a Wnt ligand binds to Fz receptor, at its extracellular Cys-rich domain (CRD), and to co-receptor LRP5/6 **(Figure 1A)** (He et al., 2004). Upon formation of a putative Wnt-Fz-LRP5/6 complex, the cytoplasmic scaffolding protein Dvl is recruited to the Fz C-terminal domain. The Fz-Dvl interaction leads to subsequent recruitment of Axin-GSK3 complex, triggered by the interaction between Axin and Dvl. Once at the plasma membrane, Axin-bound GSK3 phosphorylates LRP5/6, thus activating it. On the other hand, phosphorylated LRP-5/6 further promotes Axin recruitment, suggesting a feed-forward loop (MacDonald et al., 2009). These events possibly cause the disassembly of the degradation complex, or at least the inhibition of its phosphorylation activity, preventing the degradation of β-catenin. Stable cytoplasmic β-catenin can then accumulate and translocate to the nucleus, where it functions as a transcriptional co-activator for TCF/LEF transcription factors, and thereby regulates Wnt target genes (MacDonald et al., 2009). However, recent evidence suggests that this model might be incorrect, arguing that relocalization of the destruction complex does not result in disassembling or inhibition of β-catenin phosphorylation, but instead in inhibition of ubiquitination which causes saturation of the complex (Li et al., 2012). Consequently, newly synthetized β-catenin can accumulate in the cytosol and be translocated to the nucleus.

A remarkable aspect of the Wnt/β-catenin pathway is that several genes which encode components of this signaling pathway, such as Fz, LRP6, Axin2, TCF/LEF, are amongst the β-catenin-TCF/LEF targeted genes (Logan and Nusse, 2004; MacDonald et al., 2009). This suggests that, at least in certain contexts, this signaling pathway has self-regulatory capacity to tightly control the magnitude and duration of the cell's response to the Wnt-triggered stimulus.

2.2. THE NON-CANONICAL WNT PATHWAYS

In contrast with the canonical Wnt/ β-catenin pathway, non-canonical Wnt signaling consists of those pathways which do not rely on β-catenin as a downstream effector. Like β-catenin-dependent signaling, these pathways may control transcriptional responses in the cell, but they may also control non-transcriptional responses **(Figure 1B,C)** (Niehrs, 2012). An intriguing, and still to be fully explained, aspect of non-canonical Wnt signaling is the often observed interaction and overlap between the apparently distinct pathways.

2.2.1. The non-canonical Wnt/PCP pathway

The planar cell polarity (PCP) pathway, the most extensively studied of the β-catenin-independent pathways, has been implicated in the establishment of cell polarity during morphogenetic processes, in particular for its role in the coordination of *Drosophila* epithelial tissue polarity (Gao, 2012; Wu and Mlodzik, 2009). Before activation of PCP pathway in the cells of the fly's wing epithelium, two core complexes of this pathway are evenly distributed around the plasma membrane (Gao, 2012). Upon PCP establishment, these complexes become asymmetrically distributed. Fz, Dsh/Dvl, Diego (Dgo) and Flamingo (Fmi)/Celsr preferentially localize at the distal side of the cell, while the other complex, composed of Strabismus/Van Gogh (Vang), Prickle (Pk) and Fmi/Celsr, localize proximally. Active Fz of the distal complex contacts with Vang from an adjacent cell, thus inducing polarization of the latter. This cell-cell interaction-based asymmetry is further propagated along the epithelium, resulting in tissue polarization (Gao, 2012). To date, it is unclear which upstream signal is involved in initiation and establishment of PCP in *Drosophila*. Since Fz is fundamental in PCP, it has been hypothesized that Wnts may be the ligands responsible for the initiation of this process, yet all studies intended to test this hypothesis have been so far unsuccessful in establishing a role for Wnts in *Drosophila* PCP (Gao, 2012; Wang and Nathans, 2007; Wu and Mlodzik, 2009). In vertebrates, however, Wnt ligands have been reported to be necessary for correct CE movements, an embryonic developmental process which is regulated by the PCP pathway (Gao, 2012; Simons and Mlodzik, 2008; Wang and Nathans, 2007). For instance, it has been reported that during mouse development, Wnt5a interacts genetically with Vangl2, one of the PCP core proteins (Qian et al., 2007). Moreover, a recent study showed that a Wnt5a gradient controls PCP in proximal-distal limb development by regulating, in a dose-dependent manner, the phosphorylation of Vangl2 and, consequently, its activity in each cell (Gao et al., 2011). Remarkably, it has been recurrently observed in vertebrate systems that asymmetrical localization of PCP core components is related to the direction of Wnt gradients. In mice and zebrafish, Dvl (and possibly Fz receptors) is frequently localized closer to the

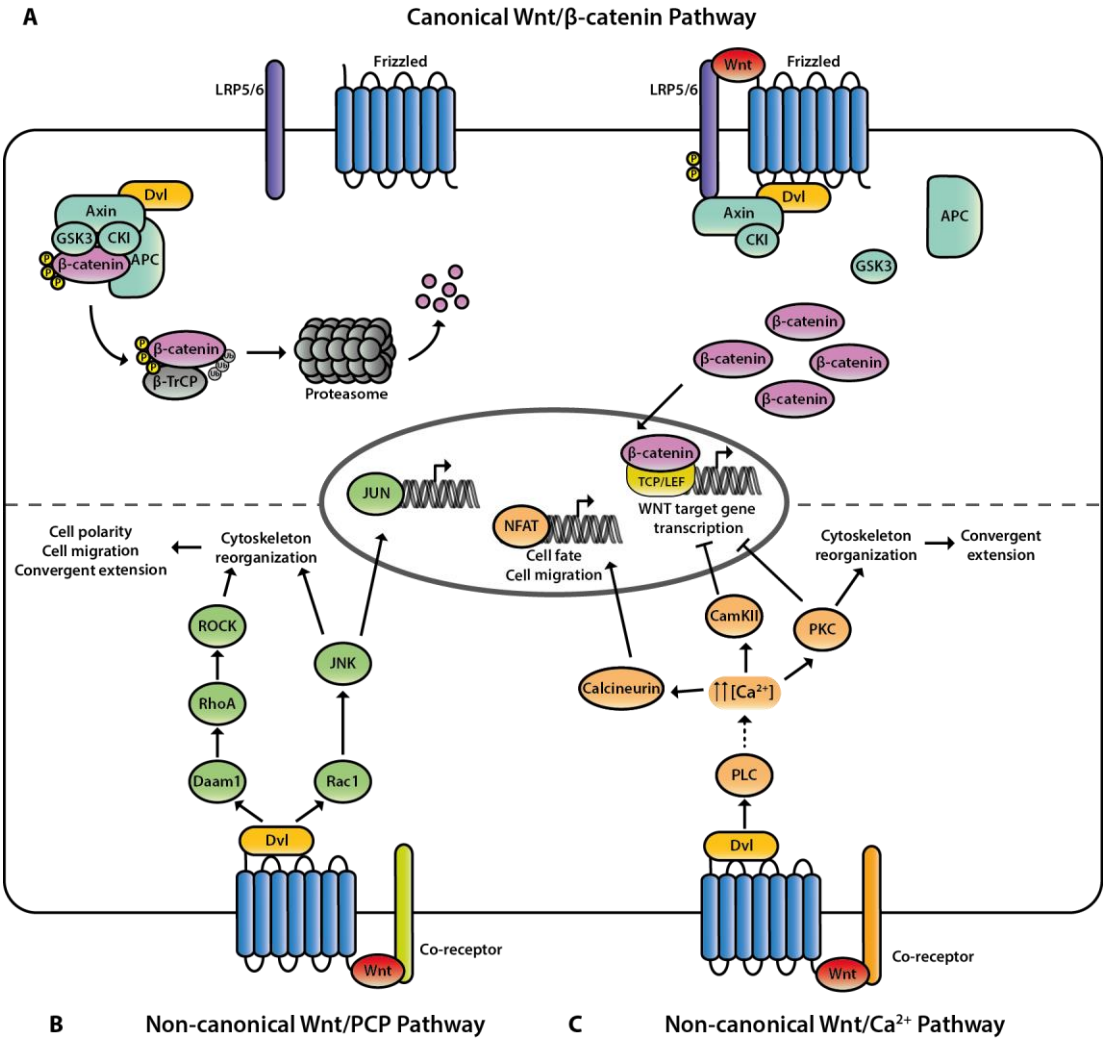

Figure 1 **The main pathways of Wnt signaling**. Simplified representation of the three main Wnt signaling transduction pathways. **(A)** Wnt/β-catenin pathway: in the absence of Wnt binding to Frizzled (Fz) receptors, β-catenin is targeted for degradation by the proteasome via the destruction complex (left); binding of Wnt to Fz and LRP5/6 inhibits the degradation of β-catenin which leads in its accumulation in the cytoplasm. Subsequent translocation of β-catenin to the nucleus results in the transcription of Wnt target genes (right). **(B,C)** Non-canonical Wnt pathways. Wnt binding to Fz and/or other co-receptors, such as Ror and Ryk, can activate the **(B)** Wnt/PCP pathway and the **(C)** Wnt/Ca^{2+} pathway. (Figure adapted from (Niehrs, 2012)).

Wnt source, while Vangl and Pk proteins are oriented away from this source. This supports the idea that Wnts can be upstream signals of this pathway (Gao, 2012).

Independently of how the pathway is initiated, Fz receptors are fundamental in the transduction of Wnt/PCP signaling. Similarly to the Wnt/β-catenin pathway, Fz-dependent initiation of the PCP pathway results in the recruitment and activation of Dvl **(Figure 1B)** (Gao and Chen, 2010). Activated Dvl mediates the activation of Rho family of GTPases, ultimately leading to cytoskeleton remodeling which is fundamental for cellular polarization and migration (Gao, 2012; Gao and Chen, 2010; Goodrich and Strutt, 2011). Dvl can associate with the small GTPase RhoA via Daam1, another scaffolding protein (Gao and Chen, 2010; Habas et al., 2001). In turn, RhoA activates ROCK kinase

which regulates the actin cytoskeleton. Dvl may also activate an alternative small GTPase, Rac1, by a still unclear mechanism (Gao and Chen, 2010; Habas et al., 2003; Rosso et al., 2005). Subsequently, Rac1 activates c-Jun N-terminal kinase (JNK), resulting in microtubule stabilization and activation of JNK-dependent transcription factors (Niehrs, 2012).

2.2.2. The non-canonical Wnt/Ca^{2+} pathway

The Wnt/Ca^{2+} pathway is another β-catenin-independent pathway involved in Wnt signaling **(Figure 1C)**. The discovery of this pathway was driven by the observation that Wnt5a signaling directly enhances an increase in intracellular Ca^{2+} both in *Xenopus* and zebrafish embryos (Slusarski et al., 1997a; Slusarski et al., 1997b). Since its identification, the Wnt/Ca^{2+} pathway has been linked to many developmental processes such as axon guidance, cell migration and fate determination, and CE movements during gastrulation (De, 2011). As in the signaling pathways previously described, the intracellular transduction of this Wnt-based pathway is also mediated by Fz receptors (De, 2011; Kuhl, 2000; Slusarski et al., 1997a; Slusarski et al., 1997b). Binding of Wnt to Fz stimulates the activation of membrane-bound phospholipase C (PLC). Subsequently, activated PLC induces the synthesis of diacylglycerol (DAG) and inositol trisphosphate (IP3). IP3 stimulates calcium release from the ER which in turn results in the activation of calcium dependent kinases such as calcium calmodulin-dependent protein kinase II (CamKII), protein kinase C (PKC) and calcineurin (De, 2011; Kuhl, 2000). Remarkably, the scaffold protein Disheveled is also involved in this Wnt pathway. In *Xenopus*, Dsh loss of function through injection of antisense morpholino oligonucleotide inhibits Fz7-mediated membrane translocation of PKC and affects cardiac development (Sheldahl et al., 2003). Additionally, overexpression of Dsh lacking the DIX domain is sufficient to cause the activation of PKC, CamKII and intracellular Ca^{2+} release.

2.2.3. The atypical Wnt receptors

As referred above, Fz and LRP5/6 are not the only receptors involved in mediating Wnt signaling. For instance, the receptor tyrosine kinase Ror has been broadly implicated in non-canonical Wnt signaling, with Wnt5a being recognized as the main ligand for vertebrate Ror receptors (Green et al., 2008; Hikasa et al., 2002; Oishi et al., 2003). In mice, loss-of-function of Ror2 results in phenotypes very similar to those observed in Wnt5a mutants. Remarkably, Wnt5a binding to Ror2 can induce both homodimerization of this receptor and formation of a complex with Fz, depending on the cellular context (Grumolato et al., 2010; Liu et al., 2008; Sato et al., 2010). This suggests a possible dual role for Ror-mediated signaling. On the one hand, these proteins may act autonomously as Wnt

receptors and directly mediate Wnt signaling (Hikasa et al., 2002; Oishi et al., 2003). On the other hand, they may act as Fz co-receptors and activate PCP signaling (Gao et al., 2011; Grumolato et al., 2010; Sato et al., 2010). However, the involvement of Wnt-Ror signaling in the PCP pathway is not completely clear, and may depend on the cellular process. For instance, mutant mice for Ror1 and Ror2 are phenotypically different from mutants of the core PCP protein Vangl2 (Niehrs, 2012). Wnt5a binding to Ror2 recruits Dvl, Axin and GSK3 to the membrane, which is followed by its activation through phosphorylation by GSK3 or CK1 (Ford et al., 2013; Grumolato et al., 2010; Niehrs, 2012). In fibroblasts, activation of Ror2 recruits filamin A, an actin-binding protein, inducing the activation of JNK which results in the reorganization of the actin cytoskeleton and consequent regulation of polarized cell migration (Nomachi et al., 2008). An interesting aspect of Wnt5a-Ror2 signaling is its role in the regulation of the Wnt/β-catenin pathway. Although the detailed molecular mechanisms are still unknown, several studies revealed that Wnt5a-Ror2 can inhibit the canonical pathway either by affecting β-catenin stabilization or by blocking TCF-mediated transcription (Mikels et al., 2009; Oishi et al., 2003; Yamamoto et al., 2007). Moreover, Wnt5a-Ror2 can also antagonize Wnt/β-catenin signaling by competing with canonical Wnt-LRP5/6 for the binding to Fz receptors (Grumolato et al., 2010; Sato et al., 2010). In addition, it was shown that formation of Wnt5a-Ror2-Fz2 ternary complexes leads to endocytosis of Fz2, which makes it unavailable for other Wnts. Remarkably, Wnt5a-Ror2 may also interfere with β-catenin-dependent signaling by activating the Ca^{2+} pathway. Ca^{2+}-dependent activation of CamKII inhibits β-catenin-induced transcriptional activation via mitogen-activated protein kinase (MAPK) (Ishitani et al., 2003). Another very intriguing role for Ror has been reported in *C. elegans.* Here, the Ror-homolog CAM-1 can function non-cell-autonomously to sequester Wnt ligands and regulate cell migration and cell fate-specification (see below) (Forrester et al., 2004; Green et al., 2007).

Like Ror receptors, Ryk is also a member of the receptor tyrosine kinase (RTK) superfamily which binds Wnt ligands. However, the tyrosine kinase domain of Ryk is inactive, making it 'kinase dead' (Hovens et al., 1992; Katso et al., 1999). The extracellular region of this receptor is also atypical, being relatively small when compared to other RTKs. *Drosophila* Derailed (Drl) receptor was the first Ryk ortholog to be discovered due to its role in the mediation of Wnt5 axon chemorepulsion in the fly's developing nervous system (Bonkowsky and Thomas, 1999; Yoshikawa et al., 2003). In vertebrates, Ryk is also involved in axon guidance, as it will be discussed later in this work, and in several other neurodevelopmental processes, such as neuronal migration and fate specification in mouse, and planar polarity establishment in *Xenopus* neural tube formation (Kamitori et al., 2005; Li et al., 2009; Liu et al., 2005; Zhong et al., 2011). In *Drosophila*, Drl/Ryk intracellular signal transduction is dependent on Src64B and Src42A, two members of the Src non-receptor tyrosine

kinase family (Wouda et al., 2008). Loss of each of these kinases results in phenotypes similar to those observed in *Wnt5* and *derailed* mutants. Moreover, Drl/Ryk and Src64B form a complex where the latter is catalytically active (Wouda et al., 2008). The stability of this complex relies on Src activation and Src-dependent phosphorylation of Drl/Ryk. Interestingly, mammalian homologs of Src and Drl/Ryk also form complexes, suggesting that this system might be conserved; however, *in vivo* evidence is still needed to confirm this hypothesis (Wouda et al., 2008). Although most studies have focused on Wnt5a-Ryk signaling, several other Wnt ligands (Wnt1, Wnt3a and Wnt11) have been reported to bind to this receptor (Kim et al., 2008; Lu et al., 2004; Macheda et al., 2012). Furthermore, Ryk has been implicated in both canonical and non-canonical pathways. For example, vertebrate axon guidance through Wnt5a-Ryk interaction was reported to implicate the Wnt/Ca^{2+} pathway (Hutchins et al., 2011; Hutchins et al., 2012; Li et al., 2009). Furthermore, Ryk has also been shown to interact with Wnt11 and Vangl2 during zebrafish CE movements and mouse neural tube closure, respectively, which involves this receptor in Wnt/PCP signaling (Macheda et al., 2012). Evidence also suggests that Ryk might activate the Wnt/β-catenin pathway by acting as a Fz co-receptor (Lu et al., 2004). Additionally, as in the case of all previous signaling systems described, Dvl can also act downstream of Ryk-mediated signal transduction (Kim et al., 2008; Lu et al., 2004).

Altogether, this evidence shows the important role of Ror and Ryk receptors in the transduction and regulation of the distinct Wnt pathways, and further demonstrates the dynamics and complexity of Wnt signaling.

3. WNT SIGNALING AND EARLY A-P NEURAL PATTERNING

3.1. WNTS AND NEURAL TUBE SPECIFICATION

During vertebrate early embryogenesis, the formation of the neural tube establishes the first three-dimensional structure of the central nervous system (CNS) which will give rise to the forebrain, midbrain, hindbrain and spinal cord in later stages. The neural tube expands along the A-P axis of the embryo, and is composed of a neuro-epithelium surrounding a hollow lumen. This neuro-epithelium arises from the neural plate, a flat sheet of neuro-ectoderm, which is formed during the final stages of gastrulation on the dorsal surface of the blastoderm (Clark et al., 2012). Classical experiments in amphibian models showed that Spemann's organizer is responsible for the induction of neural fate in these ectoderm cells (Kiecker and Lumsden, 2012). On the other hand, Nieuwkoop further proposed a double-gradient model which postulates that, after the acquisition of neural identity by these cells, neural tissues are induced to acquire anterior fate. After this induction, a gradient of posteriorizing signals (or factors) gradually induces posterior identity to the presumptive anterior neural tissues, resulting in the early A-P arrangement of the neural tube (Nieuwkoop, 1997). The molecular identity of these posteriorizing factors is still not completely understood; nevertheless, several candidates have been proposed, particularly FGFs, retinoic acid, Nodal and Wnts (Durston et al., 1989; Kiecker and Niehrs, 2001; Slack et al., 1996).

In a seminal study Kiecker and Niehrs demonstrated that an endogenous Wnt/β-catenin A-P signaling gradient in the presumptive neural plate of the *Xenopus* gastrula is both necessary and sufficient for A-P patterning of the neuraxis, making Wnts a prominent candidate as Nieuwkoop's posteriorizing signal (Kiecker and Niehrs, 2001). However, the posteriorizing role of Wnt signaling was first revealed in *Xenopus* animal caps, where ectopic expression of Xwnt3a or β-catenin resulted in induction of posterior neural markers expression (McGrew et al., 1995). Interestingly, this study showed that Wnt signaling components not only upregulate posterior neural markers, but also have a suppressor effect on the expression of anterior neural markers. This dual activator/suppressor role of Wnt signaling was further corroborated by several other studies in *Xenopus* where overexpression of Wnt ligands (Fredieu et al., 1997; Gamse and Sive, 2001; McGrew et al., 1997; McGrew et al., 1999), Wnt pathway components (Darken and Wilson, 2001; Hamilton et al., 2001; Kiecker and Niehrs, 2001; McGrew et al., 1995) and artificial induction of the Wnt pathway (Fredieu et al., 1997), lead to similar results. In agreement with these results, loss of Xwnt8 function results in the inhibition of posterior neural markers (Bang et al., 1999; McGrew et al., 1997) and expansion of the forebrain markers with

consequent defects in neuraxis posteriorization (Erter et al., 2001; Glinka et al., 1997; Lekven et al., 2001).

Conversely, suppression of posteriorizing Wnt signaling seems to be necessary for proper anterior identity. In zebrafish, *headless* (*hls*) mutants show severe head defects, which consist in the lack of anterior neural structures including eyes, forebrain, and part of midbrain **(Figure 2A)** (Kim et al., 2000). This phenotype results from a null mutation in T-cell factor-3 (Tcf3) which disrupts the transcriptional repression activity of Wnt signaling normally performed by this member of the TCF/LEF family (Kim et al., 2000). Similarly, *masterblind* (*mbl*) mutants also display strong anterior phenotypic defects. In these mutants telencephalon and eyes, which are the forebrain's most anterior regions, are reduced or completely absent, whereas posterior structures are expanded anteriorly **(Figure 2B)** (Heisenberg et al., 2001; van de Water et al., 2001). A single amino acid substitution at a conserved site of Axin1 was identified in the *mbl* mutant (Heisenberg et al., 2001). As previously denoted, Axin is a negative regulator of the Wnt/β-catenin pathway through its role in the β-catenin destruction complex. The point mutation observed in the *mbl* allele results in the inability of Axin1 to bind GSK3β, causing a reduction in GSK3β activity and, consequently, a gain of function in Wnt/β-catenin signaling (Heisenberg et al., 2001). In accordance, the *mbl* mutant defects are phenocopied by the overexpression of a dominant negative form of GSK3β or by lithium-mediated inhibition of GSK3β, while overexpression of wild-type Axin1 or GSK3β can restore the wild-type phenotype (Heisenberg et al., 2001; van de Water et al., 2001). Therefore, the evidence from *hls* and *mbl* mutants suggest that Wnt signaling inhibition is required for correct formation of the CNS' anterior structures.

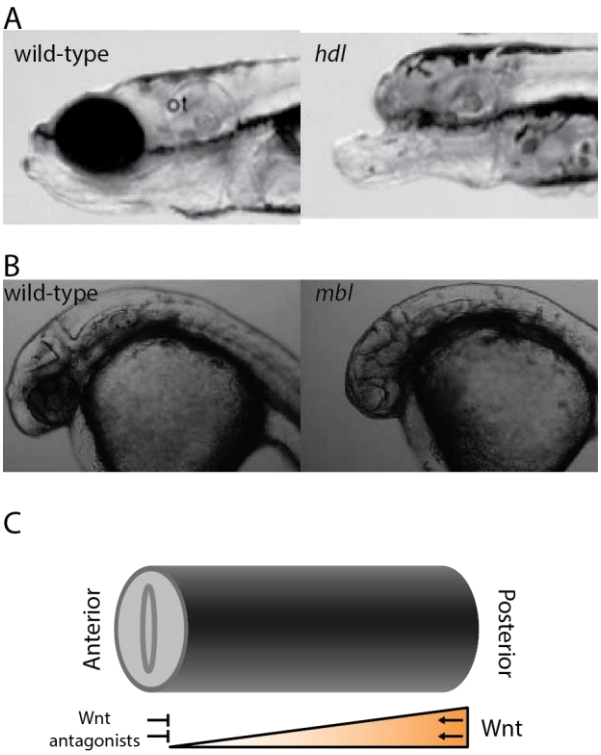

Figure 2 **Wnt signaling gradients in vertebrate early neural patterning.** Anterior inhibition of posteriorizing Wnt signaling is necessary for correct A-P establishment of early neural structures. In zebrafish **(A)** *headless* and *masterblind* **(B)** mutants, the disruption of anterior Wnt inhibition results in severe anterior defects. **(C)** A posterior-to-anterior gradient of Wnt combined with anterior expression of Wnt antagonists, creates an A-P gradient of Wnt signaling which is essential for neuraxis specification and neural tube formation. (Figures A and B adapted from (Kim et al., 2000) and (Heisenberg et al., 1996), respectively)

This idea is reinforced by the evidence that loss of the anteriorly secreted Wnt antagonist Dickkopf1 (Dkk1) function results in the posteriorization of anterior regions (Glinka et al., 1998; Kazanskaya et al., 2000); whereas overexpression leads to expansion of the head (Glinka et al., 1998). Moreover, posterior neural markers are not affected in *Dkk1* mutants, indicating that Dkk1 specifically affects anterior regions (Mukhopadhyay et al., 2001). Studies in mice also showed that Dkk1 specifically antagonizes Wnt3 to regulate anterior morphogenesis (Lewis et al., 2008). Other anteriorly expressed Wnt antagonists, such as the Secreted frizzled related proteins (Sfrps) have also been shown to be necessary for correct specification of the anterior neural plate in different model organisms (Esteve et al., 2000; Houart et al., 2002; Lopez-Rios et al., 2008)

In sum, the findings described above demonstrate that proper establishment of A-P neural tube patterning requires both activation and inhibition of Wnt signaling in the posterior and the anterior regions, respectively **(Figure 2C)**. This supports the hypothesis that polarized regulation of Wnt signaling along the anteroposterior axis is an evolutionarily conserved system in the patterning of this body axis across metazoans (Petersen and Reddien, 2009).

3.2. WNTS AND NEURAL TUBE CLOSURE

Neural tube closure is a fundamental step in the early development of the CNS. Moreover, several common human birth malformations such as anencephaly, craniorachischisis and spina bifida have been linked to defects in the closure of the neural tube (Greene and Copp, 2009). During neurulation, the neural groove is formed through the narrowing and elongation of the neural plate. Subsequent folding of the neural plate leads to the adhesion and fusion of its borders at the dorsal midline and consequent neural tube closure **(Figure 3)** (Gray et al., 2011).

Studies in *Xenopus* and zebrafish showed that the Wnt/PCP pathway has an essential role in this process through its mediation of convergent extension movements in the dorsal neural tube (Heisenberg et al., 2000; Wallingford and Harland, 2002). Inhibition of Xdsh translation revealed that its function is specifically required in the midline for normal neural tube closure (Wallingford and Harland, 2002). Accordingly, double mutant mice for *Dvl1;Dvl2* or *Dvl2;Dvl3* display craniorachischisis, the most severe neural tube closure defect (NTD), in which both brain and spinal cord remain open (Hamblet, 2002; Wang et al., 2006b). Several other components of the Wnt/PCP pathway have been implicated in correct neural tube closure. For instance, overexpression of the *Xenopus Strabismus/Van gogh* gene (*Xstbm*) affects posterior neural fold fusion and closure of the neural folds to form the neural tube (Goto and Keller, 2002), while mutations in its murine

homologue *Vangl2* also result in craniorachischisis (Kibar et al., 2001). In several other studies with mice, similar NTDs were obtained from genetic disruption of Wnt/PCP pathway components, including the *flamingo* homologue *Celsr1*, *Dact1*, *Fzd3*, *Fzd6, Scrb1*, Va*ngl1* and *Vangl2* (Curtin et al., 2003; Murdoch, 2003; Suriben et al., 2009; Torban et al., 2008; Wang et al., 2006a).

The importance of the Wnt/PCP pathway in correct neural tube closure has also been demonstrated in humans. Recently, several studies used DNA sequencing analysis to examine putative mutations in patients with NTDs (reviewed in Juriloff and Harris, 2012). These studies revealed statistically significant associations between NTDs and rare putative mutations in PCP-related genes, such as *VANGL1, VANGL2, FZD6, PRICKLE1, SCRIB* and *CELSR1* (Juriloff and Harris, 2012).

Despite the clear importance of the Wnt/PCP pathway in neural tube closure, it seems that the role of Wnt signaling in this process might not be restricted to this pathway. Some lines of evidence arising from phenotypic analysis of mice mutants also point towards a potential role for the canonical Wnt/β-catenin pathway in this process. For instance, it has been observed that several mutations in the murine co-receptor LRP6 lead to NTDs (Carter et al., 2005; Kokubu et al., 2004; Pinson et al., 2000). Moreover, defective neural tube closure was also observed in mice carrying a homozygous knock-in allele of Tcf3-ΔN, which prevents Tcf3-β-catenin interaction (Wu et al., 2012). Another example comes from Axin1 mutant mice where several neural tube malformations are observed (Perry et al., 1995).

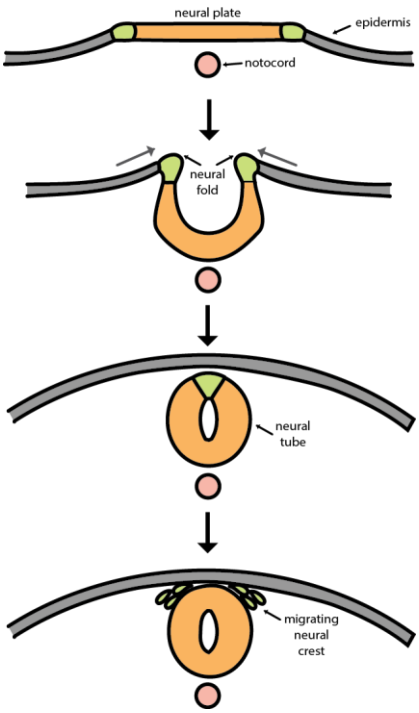

***Figure 3* The process of neural tube formation.** Schematic illustration of the main steps leading to vertebrate neural tube closure.

Altogether, these studies clearly show a relevant role for the Wnt/PCP pathway in the regulation of neural tube closure, and that defective signaling through this pathway seems to lead to NTDs in humans. Furthermore, despite the lack of a robust body of evidence, it seems plausible that the canonical Wnt/β-catenin pathway may also have a role in the normal course of this process. Thus, further investigation is needed in order to better understand the involvement of the canonical Wnt pathway in neural tube closure, and to explore potential interactions between these two pathways in this context.

4. WNT SIGNALING AND A-P NEURONAL CELL MIGRATION

Cell migration is a fundamental and decisive process during metazoan development and adult life. Impairments in this process lead to developmental abnormalities and are linked with several pathologies (Franz et al., 2002). Accordingly, cell migration is also a fundamental process in the correct establishment and maintenance of animal nervous systems. Neurons and/or neuronal precursors typically undergo considerable migration before arriving at their definite locations, in a highly directional manner which is regulated by several guidance mechanisms (Hatten, 2002). Wnt signaling has been implicated in the coordination of neuronal cell migration in general, and several lines of research have shown its role in directing neuronal cell migrations along the A-P body axis.

4.1. WNTS AND *C. ELEGANS* A-P NEURONAL CELL MIGRATION

Due to its relative simplicity, the nematode *Caenorhabditis elegans* (*C. elegans*) is a well-established model for the study of several metazoan developmental processes, including the development of the nervous system (Riddle et al., 1997). In fact, the study of nervous system's development and wiring was the initial driving force behind its implementation as a model organism (Brenner, 1974). Five Wnt ligands have been identified in *C. elegans*: LIN-44, EGL-20, CWN-1, CWN-2 and MOM-2 (Korswagen, 2002). Recently, using a single molecule mRNA FISH analyses, M. Harterink and colleagues demonstrated that the five *C. elegans* Wnt genes are expressed in partially overlapping domains along the A-P body axis in L1 larvae, with most prominent Wnt expression occurring in the worm's posterior body region **(Figure 4A)** (Harterink et al., 2011).

During the worm's embryogenesis several bilaterally symmetrical pairs of neuronal cells undergo A-P migration, in what has been shown to be Wnt signaling-dependent processes. One of the cases are the hermaphrodite-specific neurons (HSN), a pair of motor neurons which are born in the tail region and migrate to the mid-body, where they innervate the vulva muscles **(Figure 4B)** (Hedgecock et al., 1987; Sulston et al., 1983). Evidence suggests that HSN migration towards the anterior is largely dependent on a repulsive effect by EGL-20/Wnt possibly through the cell-autonomous function of the Wnt receptor MIG-1/Fz (Pan et al., 2006). In both *elg-20/Wnt* and *mig-1/Fz* mutants, the HSN terminate their migration prematurely, being found posteriorly to their wild-type positions (Desai et al., 1988; Pan et al., 2006). Furthermore, overexpression of *egl-20/Wnt* results in an anterior localization of these neurons, while ectopic expression of this gene in the head inhibits their anterior migration (Pan et al., 2006). On the other hand, HSN migration is not altered in single mutations of the other Wnt genes (*lin-44, cwn-1, cwn-2*, *mom-2*), of the Fz genes (*lin-17, cfz-2*, *mom-5*) nor of the

single Derailed/Ryk *lin-18* gene (Pan et al., 2006; Zinovyeva and Forrester, 2005). However, mutations in two or more Wnts resulted in synthetic or enhanced defects, suggesting that all the five Wnts contribute to HSN migration (Pan et al., 2006; Zinovyeva et al., 2008). Likewise, mutations in the *mom-5/*Fz, *cfz-2/*Fz or *lin-18/*Ryk significantly enhanced the HSN migratory defects observed in the *mig-1/Fz* mutant (Pan et al., 2006; Zinovyeva et al., 2008). Interestingly, mutation of *lin-17/Fz* significantly suppressed the HSN defects of *mig-1/Fz* and *mig-1/Fz; cfz-2/Fz* mutants, suggesting that LIN-17/Fz might antagonize signaling through the MIG-1/Fz receptor (Pan et al., 2006). Additionally, overexpression of the non-canonical CAM-1/Ror receptor results in a phenotype similar to what is observed in *egl-20/Wnt* mutants, while mutations in *cam-1/Ror* results in a more anterior migration of the HSN (Forrester and Garriga, 1997; Kim and Forrester, 2003). Based on these observations, together with the evidence that Ror kinases can bind Wnts both in *Xenopus* and mouse, it has been suggested that CAM-1/Ror inhibits EGL-20/Wnt signaling in this process (Forrester et al., 2004). Interestingly, after migration, the HSN cell bodies occupy a position adjacent to the CAN neurons, which express *cam-1/Ror*. Moreover, anterior misplacement or absence of CAN cell bodies results in HSN anterior overmigration (Forrester and Garriga, 1997; Kim and Forrester, 2003; Pan et al., 2006). Thus, it seems plausible that sequestration of EGL-20/Wnt by CAM-1/Ror arising from CAN neurons, regulates the correct positioning of HSN cells (Forrester et al., 2004; Kim and Forrester, 2003). Recently, it was also reported that overexpression of *sfrp-1* is capable of inhibiting the EGL-20/Wnt-dependent migration of HSN but, despite of the potential of SFRP-1 to inhibit EGL-20/Wnt, these observations do not have a biological implication since the migration of HSN was not affected in *sfrp-1* mutants (Harterink et al., 2011). The above evidences seem to suggest that, in HSN anterior migration, posterior-high to anterior-low EGL-20/Wnt gradient **(see Figure 4A)** functions as a repulsive factor mainly through MIG-1/Fz receptor. This effect is possibly fine-tuned with the help of the other Wnts ligands and the MOM-5/Fz, CFZ-2/Fz and LIN-18/Ryk receptors. On the other hand, LIN-17/Fz and CAM-1/Ror might intervene in these neuronal cells' movement by counteracting this repulsive effect, with CAM-1/Ror functioning as a sink to block anterior Wnt ligands spreading, in particular EGL-20/Wnt (Kim and Forrester, 2003).

Other interesting and extensively studied examples of neuronal migration are the Q neuroblast (QL and QR) and its descendants (QL.d and QR.d respectively). The Q neuroblasts originate at comparable positions on the left (QL) and right (QR) lateral sides of the worm, and migrate towards the anterior and posterior sides, respectively **(Figure 4B)** (Sulston and Horvitz, 1977). The observed migratory differences are specified by contrasting responses to EGL-20/Wnt which result in the expression of the homeotic gene *mab-5* in QL, but not in QR. In QL, *egl-20/Wnt* loss-of-function causes the inversion of its migration, with these cells moving anteriorly. It was demonstrated that MAB-5 directs

posterior migration of these neuroblasts. As observed in *egl-20/Wnt* mutants, disruption of *mab-5* results in anterior migration of the QL.d (Harris et al., 1996; Maloof et al., 1999; Salser and Kenyon, 1992). Furthermore, it was confirmed that this expression of *mab-5* is induced by EGL-20/Wnt through a canonical Wnt/β-catenin pathway which involves MIG-1/Fz and LIN-17/Fz receptors (reviewed in Korswagen, 2002). Interestingly, a recent study showed that *mig-1/Fz* and *lin-17/Fz* have different temporal patterns of expression, with *mig-1/Fz* being gradually downregulated during the course of migration while *lin-17/Fz* is upregulated (Ji et al., 2013). Moreover, this study also revealed that, after a period of initial variability, *mab-5* expression needs to be maintained above a certain high threshold in order to ensure the correct migration of QL cells. By combining mutant and expression analysis with theoretical modeling, the authors further suggest that a network of interlocked positive and negative feedbacks within the Wnt signaling pathway endogenously guarantees the stable levels of *mab*-5 necessary during this migratory process.

On the other hand, EGL-20/Wnt does not induce *mab-5* transcription in the QR and QR.d cells. However, in *egl-20/Wnt* mutants the migration of latter is also affected, resulting in QR.d undermigration (Whangbo and Kenyon, 1999; Zinovyeva et al., 2008); thus EGL-20/Wnt also regulates correct migration of the QR lineage. Interestingly, and in contrast with the QL.d, migration of the QR.d also requires the action of additional Wnts ligands and receptors. As in *egl-20/Wnt* mutants, disruption of *cwn-1/Wnt,* and to a lesser extent of *cwn-2/Wnt,* results in a more posterior localization of the QR.d (Zinovyeva and Forrester, 2005; Zinovyeva et al., 2008). Furthermore, these defects are enhanced in *cwn-1/Wnt; cwn-2/Wnt* mutants, and even more in *cwn-1/Wnt; egl-20/Wnt*. The role of these Wnt ligands is possibly mediated by MOM-5/Fz and CAM-1/Ror, as in mutants for these receptors undermigration of QR.d is also observed (Zinovyeva and Forrester, 2005; Zinovyeva et al., 2008). This phenotype is enhanced in *mom-5/Fz; cam-1/Ror* double mutants; thus indicating that they are involved in parallel pathways. Recent data from analysis of mutant combinations supports that in fact MOM-5/Fz and CAM-1/Ror act in parallel pathways, which control different aspects of QR.d long-range migration (R. Mentink *et al.*, unpublished). Moreover, the same authors showed that *cwn-2/Wnt* and *egl-20/Wnt* are part of the *mom-5/Fz* and *cam-1/Ror* genetic pathway, respectively. Remarkably, this work also demonstrated that termination of QR.d migration implicates a direct inhibition of these two pathways through the activation of Wnt/β-catenin signaling in a process which might involve *mig-1/Fz*. These authors further revealed the complex involvement of Wnt signaling in QR.d migratory process, by demonstrating that the PCP pathway components *vangl-1/Vangl* and *prkl-1/Prickle* control the final short-range migration of these cells. Inhibition of Wnt signaling is also necessary for correct migration of QR.d. In *sfrp-1* mutants, these cells are found to be positioned in more anterior locations similar to what is observed in *egl-20*/*Wnt* overexpression.

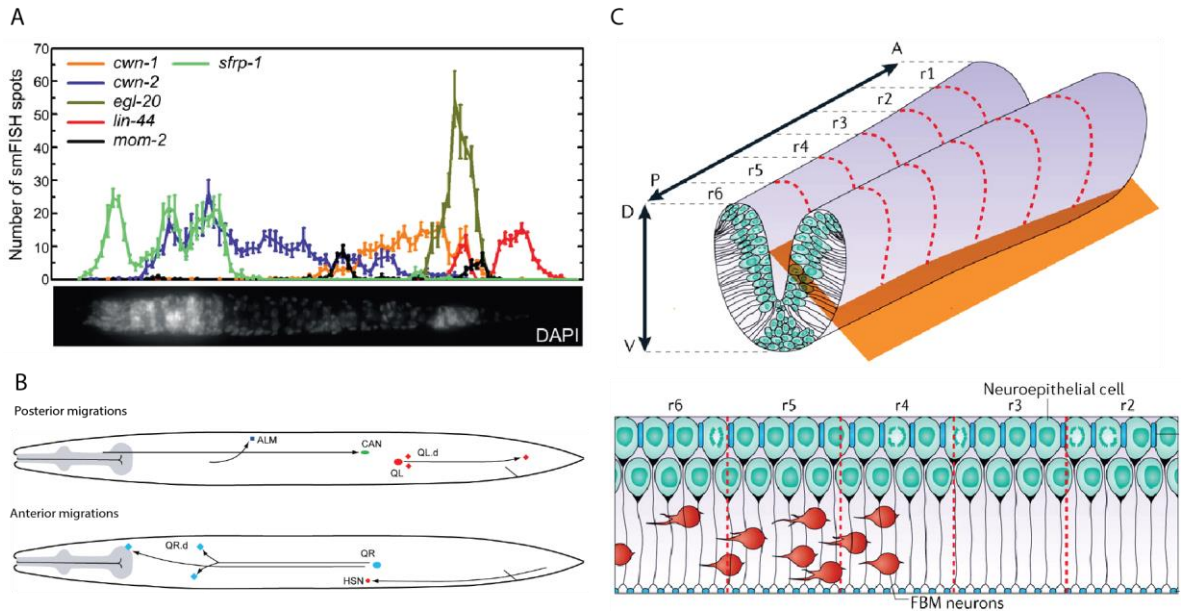

Figure 4 **Wnts determines A-P neuronal cell migration in invertebrate and vertebrate animals. (A)** Single molecule mRNA FISH analysis of *C. elegans* Wnt and *sfrp-1* genes in L1 larvae (top). Expression of the 5 Wnt genes is occurs mainly at the posterior, whereas Wnt inhibitor *sfrp-1* is expressed at the anterior side. Bottom panel depicts a DAPI stained L1 worm for orientation. **(B)** Embryonic and post-embryonic Wnt-dependent neuronal migrations towards the posterior (top) and anterior (bottom) sides. In A and B, anterior is to the left and posterior to the right. **(C)** Diagram of the developing vertebrate rhombencephalon (top). FBM neurons are born in the rhombomere 4 (r4) and migrate posteriorly to r6 in process dependent of Wnt/PCP pathway (bottom). Note that posterior is to the left and dorsal is up. (Figures A, B and C adapted from (Harterink et al., 2011), (Sawa and Korswagen, 2013) and (Tissir and Goffinet, 2013), respectively)

Likewise, overexpression of *sfrp-1* causes the inhibition of anterior migration by these cells (Harterink et al., 2011). Thus, a complex network of Wnt signaling regulates the migration of QR and its descendants. However, how the different Wnt pathways and regulatory mechanisms interact is still poorly understood and additional research is required.

The canal-associated neurons (CAN) and the anterior lateral microtubule cells (ALM) are two pairs of neurons which also migrate posteriorly, from the head to the mid-body, and from the anterior edge of the intestine to midway within the anterior body, respectively **(Figure 4B)** (Hedgecock et al., 1987; Sulston et al., 1983). Wnt signaling has also been shown to play an important role in the posterior migration of these neuronal cells. Although single mutations of the five *C. elegans* Wnts do not cause any significant impact on the migration of the ALM neurons, *cwn-1/Wnt; cwn-2/Wnt double* mutants show a strong anterior displacement, suggesting a partially redundant role for these two genes in this process (Zinovyeva and Forrester, 2005; Zinovyeva et al., 2008). This role is possibly mediated through the CFZ-2/Fz receptor. Disruption of the *cfz-2/Fz* gene causes a considerable undermigration of the ALMs as well, although not as strong as observed in the Wnt ligand double mutants (Zinovyeva and Forrester, 2005; Zinovyeva et al., 2008). Interestingly, simultaneous mutation of this gene and *mom-5/Fz*, mig-1/Fz or *lin-18/Ryk* resulted in the suppression of the observed phenotype, which can be an indication that these receptors have an antagonistic function (Zinovyeva et al., 2008). In the

case of the CAN cells, single mutations of Wnts only caused migration defects in *cwn-2/Wnt* mutants, where these cells where found at more anterior positions; however, double mutant combinations of this gene and *egl-20/Wnt* or *cwn-1/Wnt* result in an enhancement of this phenotype, pointing to a possible involvement of these two Wnts (Zinovyeva and Forrester, 2005; Zinovyeva et al., 2008). Single mutations in the *C. elegans* Frizzled receptors do not cause any significant misplacement of the CAN neurons. In contrast, *mom-5/Fz; cfz-2/Fz* double mutants lead to undermigration, thus suggesting that MOM-5/Fz and CFZ-2/Fz redundantly direct the migration of these neurons (Zinovyeva and Forrester, 2005; Zinovyeva et al., 2008). Recently, it was also shown that the Wnt inhibitory function of SFRP-1 is important for the migration of both ALM and CAN as well. Mutation of *sfrp-1* results in undermigration of both CAN and ALM (Harterink et al., 2011). Interestingly, inactivation of *cwn-1/Wnt* or *cwn-2/Wnt* in *sfrp-1* mutants rescued the defects of observed in these mutants, suggesting that anterior expression of *sfrp-1* modulates the activity of CWN-1/Wnt and CWN-2/Wnt in CAN and ALM migration (Harterink et al., 2011). Mutation of *cam-1/Ror* also produced a strong migratory defect in these cells, indicating that CAM-1/Ror receptor function is also necessary for correct migration of these cells (Kim and Forrester, 2003; Zinovyeva et al., 2008). Nevertheless, the mechanism behind its function is not understood and further investigation is needed.

It is clear that Wnt signaling is vital for correct migration of neuronal cells in *C. elegans*. Strikingly, with the exception of the QL lineage, all the described examples demonstrate that distinct, and sometimes overlapping, Wnt pathways are necessary for this complex process. Nevertheless little is known about how these different pathways interact, which demands further research into the details of these processes. In any case, it is clear that the low anterior to high posterior gradient of Wnts, specified not only by a differential expression along the A-P axis but also by the anterior inhibition of Wnt ligands by SFRP-1, is essential for patterning along this axis, further supporting the possible evolutionary conservation of this system.

4.2. WNTS AND VERTEBRATE A-P NEURONAL CELL MIGRATION

Wnt related signaling has also been implicated in vertebrate neuronal cell migration along the primary body axis. The facial branchiomotor (FBM) neurons are a group of neurons which form the motor component of the facial nerve. These neurons originate in the hindbrain rhombomere 4 (r4) and undergo caudal/posterior migration to r6, a process which is conserved from fish to mammals **(Figure 4C)** (Guthrie, 2007). It has been demonstrated that PCP signaling plays a key role in the regulation of this process. In zebrafish, transplantation experiments revealed that correct migration

of FBM neurons involves *vangl2/trilobite*, *fzd3a/off-limits*, *celsr-2/off-road* and *prickle1b*. Transplantation of *prickle1b*-deficient FBM neurons into wild-type embryos resulted in the lack of migration, while wild-type neurons failed to migrate caudally when transplanted into *vangl2*, *fzd3a* or *celsr-2* mutant embryos, evidencing cell-autonomous and non-cell-autonomous roles for the Wnt/PCP pathway (Jessen et al., 2002; Rohrschneider et al., 2007; Wada et al., 2006). Similarly, knockout of the murine *Scribble* homolog, *scrb1,* causes disruption of FBM neurons migration (Wada et al., 2005). Evidence form mice, demonstrated that mutations of *Vangl2, Frizzled3a, Celsr2* and *Scribble* also result in severe migratory defects of these neurons, confirming the evidence from zebrafish (Qu et al., 2010; Vivancos et al., 2009). The Wnt/PCP downstream components JNK and ROCK might also have a role in the migration of these neurons, since inhibition of these proteins in explants results in the disruption of migration (Vivancos et al., 2009). Intriguingly, loss of function of the PCP intracellular mediator Disheveled does not cause defects in FBM migration, both in zebrafish and mice (Glasco et al., 2012).

Experiments with murine hindbrain explants showed that Wnt5a and Wnt7a can act as chemoattractants for FBM neurons (Vivancos et al., 2009). Interestingly, these authors also revealed that Wnt5a is expressed in a posterior-to-anterior gradient across r4 to r6, which is in accordance with a possible attractive effect on FBM neurons. Additionally, Wnt5a knockout mice display partial defects in the migration of these neurons, agreeing with a possible role for this Wnt ligand in this process (Vivancos et al., 2009).

Taken together, the evidence from both zebrafish and mice demonstrates that PCP signaling is crucial for migration of FBM neurons along the primary body axis. Furthermore, there are good indications that an A-P gradient of Wnt is necessary for the precise course of this process. However, it is still obscure to which extent this migration is dependent on Wnt ligands.

5. WNTS AND A-P NEURITE DEVELOPMENT

The function of the nervous systems is grounded on the highly polarized organization of neuronal cells. The formation and development of axonal and dendritic processes, characterized for their morphological and functional asymmetry, is essential for correct nervous system wiring.

In *C. elegans*, Wnt signaling is deeply involved in the establishment of A-P neuronal cell polarity. The PLM neurons project two processes along the A-P axis: the presumptive axon is a long anterior process which forms gap junctions and synapses, and the shorter presumptive dendrite which extends posteriorly, and where no such features are observed **(Figure 5A)** (White et al., 1976). Correct polarization of these processes involves LIN-44/Wnt and LIN-17/Fz. Mutation of *lin-44/Wnt* or *lin-17/Fz* causes the inversion of PLM polarity, with the anterior process adopting the typical features of the posterior process, and vice-versa **(Figure 5A)** (Hilliard and Bargmann, 2006; Prasad and Clark, 2006). LIN-44/Wnt appears to control PLM polarity by determining the polarized localization of LIN-17/Fz to the dendritic process. This is suggested by the evidence that the asymmetric distribution of LIN-17/Fz is lost in *lin-44/Wnt* mutants (Hilliard and Bargmann, 2006). Intriguingly, a localized source of LIN-44/Wnt appears to not be essential for correct polarization of PLM neurons. Ubiquitous expression of LIN-44/Wnt from a heat-shock promoter partially rescues the *lin-17/Fz* mutant phenotype, suggesting that LIN-44 may have a permissive role in this process, rather than merely providing spatial information (Hilliard and Bargmann, 2006).

Contrarily to what is observed in PLM, mutation of *lin-44/Wnt* and *lin-17/Fz* does not affect polarity of the anteriorly localized ALM neurons, which have a single long process that extends towards the head **(Figure 5B)**. However, *cwn-1/Wnt; cwn-2/Wnt* and *cwn-1/Wnt; egl-20/Wnt* double mutants display bipolarity or reversed polarization in these cells, indicating that neuronal polarity in distinct body regions might be controlled by distinct Wnt signals **(Figure 5B)** (Hilliard and Bargmann, 2006; Prasad and Clark, 2006). Moreover, this phenotype is strongly enhanced in triple mutants, evidencing that this group of Wnts has partially overlapping roles in ALM polarity (Fleming et al., 2010). Interestingly, ectopic expression of *egl-20/Wnt* affects the normal development of ALM neurites, which suggests that EGL-20/Wnt provides spatial information to axon outgrowth of these neurons by acting as a repellent (Hilliard and Bargmann, 2006; Pan et al., 2006). It is still not completely clear how these Wnt ligands control ALM polarization. However, evidence from AVM and PVM neurons, where EGL-20/Wnt also acts as a repellent in axonal process growth, suggests that this EGL-20/Wnt function might be mediated by MIG-1/Fz and MOM-5/Fz (Pan et al., 2006). On the other hand, recent

evidence demonstrated that CAM-1/Ror and RIG-3, a cell surface Ig superfamily protein, regulate Wnt signaling to ALM cells (Babu et al., 2011).

Another interesting example of the Wnt signaling function in neurite development is the DD6 GABAergic neuron. This motoneuron is the most posterior of the DD neurons, which innervate the dorsal muscles of the worm. From the ventrally located cell body, this neuron extends a neural process which projects a dorsal posterior branch that terminates approximately at the same A-P position of its cell body **(Figure 5C)**. It has been reported that LIN-44/Wnt is involved in extension of this axonal process by acting as a repellent (Maro et al., 2009). In *lin-44/Wnt* mutants the axons extend posteriorly to their normal position, a phenotype which is enhanced in *lin-44/Wnt; egl-20/Wnt* double mutants **(Figure 5C)**. This suggests that these two Wnts cooperatively regulate axon guidance (Maro et al., 2009). Furthermore, defects in the extension of these processes are also observed in *lin-17/Fz* mutants. Interestingly, this phenotype is not enhanced in *lin-44/Wnt; lin-17/Fz* double mutants. On the other hand, expression of fluorescently tagged LIN-17::YFP revealed an enrichment at the posterior tip of DD6 axon, a sub-cellular localization that is not affected in *lin-44/Wnt; egl-20/Wnt*; thus giving good indications that LIN-17/Fz localization is Wnt-independent. Together, these observations indicate that LIN-44/Wnt and EGL-20/Wnt might control DD6 axon guidance by activating LIN-17/Fz at axon growth cones (Maro et al., 2009). Further analyses of potential downstream signaling revealed that this Wnt-Fz control of axon guidance acts through a canonical β-catenin-dependent pathway (Maro et al., 2009).

Besides their role in axon guidance, LIN-44/Wnt and LIN-17/Fz have also been involved in dendrite extension of the PQR neuron. PQR localizes at the posterior region of the worm, and has a long anterior axon and a short posterior dendrite **(Figure 5D)**. It was reported that in *lin-44/Wnt* and *lin-17/Fz* mutants, the dendritic process is absent or incorrectly projected towards the anterior **(Figure 5D)** (Kirszenblat et al., 2011). Given that these observations could be related with a role on cell polarity or on dendritic guidance, the authors decided to analyze the localization of presynaptic markers. YFP::RAB-3 fusion protein, specifically expressed in PQR neurons, revealed that these presynaptic markers were mainly found in the axons of these mutants, thus evidencing that these Wnt signaling components regulate dendritic guidance. Contrasting with the LIN-44/Wnt repellant effect on DD6 axonal guidance described above, PQR dendrites are attracted to the source of this Wnt. Anterior ectopic expression of *lin-44/Wnt* results in dendrite extension towards this anterior source (Kirszenblat et al., 2011). Curiously, *lin-17/Fz* is necessary quite prematurely in the development of PQR. When *lin-17/Fz* is expressed from a promoter that becomes active just before dendritic development (*Pgcy-36*), no rescue of *lin-17/Fz* mutant is observed. However, when the expression is driven by the *egl-17* promoter which is already active in PQR precursor (QL), the

phenotypic defects are rescued (Kirszenblat et al., 2011). In accordance with this evidence, when activated at the time of dendrite outgrowth, expression of *lin-44/Wnt* from a heat-shock promoter is insufficient to rescue the dendritic defects of *lin-44/Wnt* mutants, but rescue is observed when the construct is activated around the time of hatching (Kirszenblat et al., 2011). Additional mutant analyses also reveal that *mig-1/Fz* mutations enhanced the absent dendrite phenotype of *lin-17/Fz* mutants, indicating that these two act in parallel, with MIG-1/Fz possibly regulating the neuron's ability to form a dendrite, rather than direction of the growth (Kirszenblat et al., 2011). In addition, *lin-44/Wnt; cwn-1/Wnt* and cwn-1*/Wnt; cwn-2/Wnt* double mutants show enhanced misdirection defects and ectopic branching, respectively, suggesting that CWN-1 and CWN-2 may also have smaller roles in proper formation of PQR neurites (Kirszenblat et al., 2011).

The role of Wnt signaling on neurites goes beyond the level of individual neurons, as it is observed in the placement of the nerve ring. The nerve ring is a bundle of neuronal processes which surrounds the isthmus of the worm pharynx **(Figure 5E)**. In worms where *cnw-2/Wnt* gene is mutated, this structure is found at more anterior locations **(Figure 5E)** (Kennerdell et al., 2009). Additionally, cell bodies in the head region, and the amphid commissure, an axon bundle immediately posterior of the nerve ring, are also anteriorly misplaced, suggesting that CWN-2/Wnt might have a broad role in placement of anterior neuronal tissues. The observation that c*wn-2/Wnt* is strongly expressed in the pharynx bulb, a region that is posterior to the nerve ring, could indicate a repellant role for this Wnt ligand. Remarkably, anterior expression of CWN-2/Wnt is capable of rescuing the displacement defects observed in *cwn-2/Wnt* mutants, showing that the location of this source is not relevant for the nerve ring position which suggests that CWN-2/Wnt is a permissive signal in this process (Kennerdell et al., 2009). Three receptors are potentially involved in the correct placement of the nerve ring. Similar to what is observed in *cwn-2/Wnt* mutants, *cam-1/Ror* and *cfz-2/Fz* mutants have an anteriorly displaced nerve ring. When these mutations are independently combined with *cwn-2/Wnt* mutations, an enhancement of the defective phenotype is only observed in *cwn-2/Wnt; cfz-2/Fz* double mutants. Furthermore, the nerve ring placement defects were also enhanced in double mutants for these two receptors. This evidence suggests that CWN-2/Wnt and CAM-1/Ror are part of the same pathway, while CFZ-2/Fz could eventually be a receptor in an independent pathway which also functions in the nerve ring (Kennerdell et al., 2009). In addition, despite the fact that *mig-1/Fz* mutants do not show significant defects in the positioning of the nerve ring, in *mig-1/Fz; cfz-2/Fz* double mutants the defects are enhanced compared to *cfz-2/Fz* single mutants. On the other hand, no enhancement was observed in *mig-1/Fz; cam-1/Ror* double mutants. This suggests that MIG-1/Fz receptor functions in the same pathway as CAM-1/Ror and CWN-2/Wnt (Kennerdell et al., 2009). The function of CWN-2/Wnt in the position of the nerve ring seems to be achieved through its role on SIA

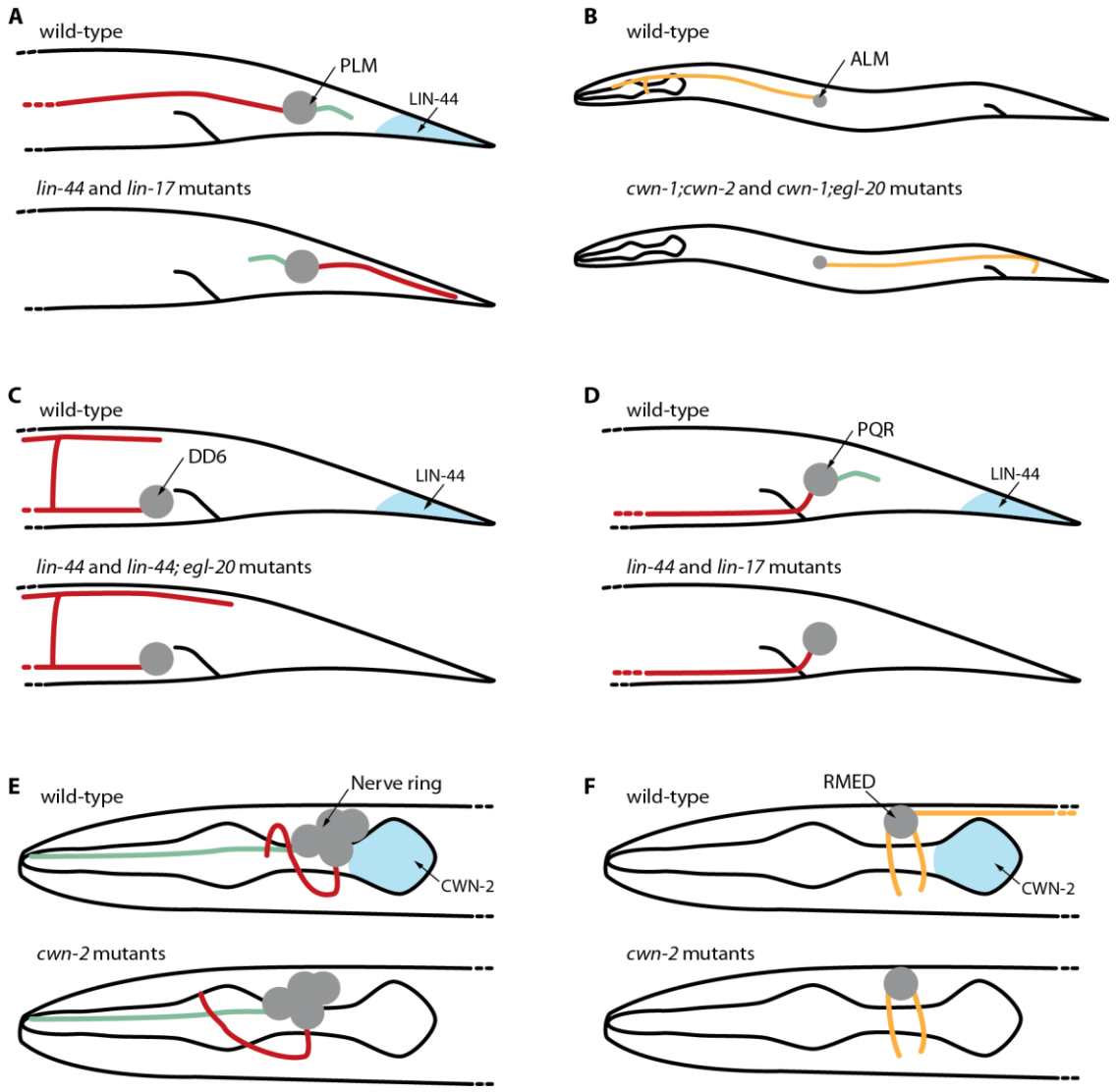

***Figure 5* Wnt signaling control of *C. elegans* neuronal processes polarity along the A-P axis.** Representative diagrams of the morphology of different neurons (top) and the observed defects in Wnt mutant backgrounds (bottom). **(A)** PLM; **(B)** ALM; **(C)** DD6; **(D)** PQR; **(E)** nerve ring; **(F)** RMED. In all panels anterior is to the left and dorsal is up. When pairs of neurons exist, only one is represented. Grey circles: cell bodies; red, green and yellow lines: axons, dendrites and neurites, respectively; blue: Wnt source. (Figure adapted from (Sawa and Korswagen, 2013))

and SIB neurons. Single mutations of *cwn-2/Wnt* and *cam-1/Ror* disrupt axonal trajectories of these two neurons, a phenotype considerably more complex than what is observed in other nerve ring neurons which only move to a more anterior position. Moreover, ablation of these two neurons causes nerve ring defects similar to what is observed in *cwn-2/Wnt* mutants (Kennerdell et al., 2009). Based on this evidence, Kennerdell *et al.* proposed an explanatory model where CWN-2/Wnt directly affects axon guidance of SIA and SIB neurons, which in turn instruct the positioning of the nerve ring (Kennerdell et al., 2009).

CWN-2/Wnt also guides the migration of neurites from the nerve ring. The RMEV and RMED motoneurons, whose cell bodies are located just posterior to the nerve ring, posteriorly project axonal

processes along the ventral and dorsal nerve cords, respectively **(Figure 5F)**. In *cwn-2/Wnt* mutants, the RMEV/D processes are completely absent or have a reduction in their length **(Figure 5F)** (Song et al., 2010). When *cwn-2/Wnt* is ectopically expressed in these mutants, proper neurite growth is rescued; however, when its source is localized anteriorly to the cell bodies, migration occurred towards the anterior, showing that CWN-2/Wnt has an attractive role in the navigation of RMEV/D axons (Song et al., 2010). At the receptor level, mutations in *cfz-2/Fz* and *mig-1/Fz* result in defects which are similar, although milder, to those of *cwn-2/Wnt* mutants. However, in double mutants the penetrance of the phenotype is comparable to what is observed in the Wnt mutants, suggesting that these two receptors are redundantly involved in this signaling process (Song et al., 2010). In addition, the CAM-1/Ror receptor also has a prominent role in the outgrowth of RMEV/D axons, possibly by acting as a receptor with CFZ-2/Fz and/or MIG-1/Fz in the transduction of CWN-2/Wnt signaling (Song et al., 2010). Worms carrying a *cam-1/Ror* null mutation display phenotypes similar to those observed in *cwn-2/Wnt* single and *cfz-2/Fz*; *mig-1/Fz* double mutants. Furthermore, a *cam-1/Ror* partial loss-of-function allele enhances the defects observed in *cfz-2/Fz* and *mig-1/Fz* single mutants. Intracellularly, DSH-1, one of the three *Disheveled* homologs present in the worm genome, plays a crucial role in axon extension (Song et al., 2010). In *dsh-1* mutants, the length of neurites is similar to what is seen in *cwn-2/Wnt* mutants. Furthermore, through yeast two hybrid assays, it was shown that the intracellular kinase region of CAM-1/Ror can physically interact with both the PDZ and the DEP domains of DSH-1. Conversely, no interactions were detected between DSH-1 or CAM-1/Ror and the intracellular domains of MIG-1/Fz or CFZ-2/Fz. This evidence points towards the idea that CAM-1/Ror, together with MIG-1/Fz and/or CFZ-2/Fz as co-receptors, mediates the activation of DSH-1 by CWN-2/Wnt in the outgrowth of RMEV/D axons (Song et al., 2010). However, this process might be more complex since the lack of CAM-1/Ror intracellular domain does not results in phenotypes as severe as those observed in *dsh-1* mutants.

The evidence for the importance of Wnt signaling in neurite development along the A-P axis is not restricted to *C. elegans.* Research in vertebrates has unraveled a crucial role of Wnt signaling in the guidance of axons along the anteroposterior axis of the spinal cord. In rodents, the dorsal spinal cord commissural neurons extend their axons to the ventral midline, crossing it into the contralateral side of the spinal cord, where they turn anteriorly towards the brain (Bovolenta and Dodd, 1990). *In situ* hybridization revealed that *Wnt4* has a decreasing anterior-to-posterior gradient during the stages when commissural axons turn anteriorly (Lyuksyutova et al., 2003). Applying an anterior ectopic source of Wnt4 to rat "open book" spinal cord explants where this migratory directionality was lost, is sufficient to rescue the A-P guidance defects (Lyuksyutova et al., 2003). Moreover, when this source is applied posteriorly, axons migrated towards the posterior, evidencing that Wnt4 is not only an intrusive cue, but also that it functions as a chemoattractant in the guidance of commissural axons (Lyuksyutova et al., 2003).

Furthermore, in *Frizzled3* mutant mice commissural axons are projected randomly along the A-P axis. Together, this evidence suggests that the attractant effect of Wnt4 in the guidance of these axons is probably mediated by Frizzled3 receptors (Lyuksyutova et al., 2003). Further research has demonstrated that atypical PKC (aPKC) signaling is necessary for this Wnt4-mediated guidance process, and that phosphatidylinositol-3-kinases (PI3Ks) act as positive switches for Wnt responsiveness after midline crossing (Wolf et al., 2008). Blocking of aPKC signaling results in random A-P turning by commissural axons. On the other hand, ectopic expression of PI3K catalytic subunit leads to precocious anterior turning of these axons (Wolf et al., 2008). A recent study reported that Wnt-dependent attraction of commissural axons is also mediated by the Wnt/PCP pathway (Shafer et al., 2011). Shafer *et al.* showed that, in spinal cord explants, exposure to Wnt5a causes phosphorylation of JNK which results in outgrowth of commissural axons. This phosphorylation process is mediated by Frizzled3 and Vangl. Vangl2, which is predominantly localized on the filapodia tips of the growth cone, reduces the phosphorylation and internalization of Frizzled3, thus promoting Wnt/PCP signaling. On the other hand, activated Disheveled acts antagonistically by promoting Frizzled3 phosphorylation and accumulation at the growth cone membrane, and consequent inhibition of Wnt/PCP signaling (Shafer et al., 2011).

Wnt1 and Wnt5a are also expressed in decreasing anterior-to-posterior gradients in the dorsal spinal cord of rodents. Contrarily to the function of Wnt4 in commissural neurons, these gradients function as a repellant for the posterior outgrowth of corticospinal track (CST) axons along the spinal cord (Liu et al., 2005). The posterior navigation of these axons occurs after crossing of the midline, the time at which these become Wnt-responsive. *In situ* hybridization experiments revealed that this is also the timing at which Ryk becomes expressed in these axons (Liu et al., 2005). Moreover, injection of anti-Ryk antibodies in the cervical spinal cord of mice blocks the posterior migration of CST axons (Liu et al., 2005), while knockdown of Ryk in developing hamster cortical slices results in arbitrary patterning of postcrossing axons (Hutchins et al., 2011). This evidence strongly supports that Wnt5a functions as a chemorepellant of CST axons during their postcrossing posterior migration. Evidence also suggests that the Wnt5a-Ryk interaction induces a Wnt/Ca^{2+} pathway. *In vitro* and *in vivo* analysis of cortical neurons and slices, respectively, revealed that Wnt5a-Ryk interactions cause changes in intracellular calcium activity (Hutchins et al., 2011; Hutchins et al., 2012; Li et al., 2009).

Based on the above evidence, it is irrefutable that Wnt signaling, in its various forms, plays a major role in the development of neurites along the primary body axis. In accordance to what was descried in the previous chapters, A-P Wnt gradients also seem to have recurrent and crucial roles as guiding cues for the correct establishment of neuronal cell processes in very different metazoans; thus reinforcing the idea of an evolutionary conservation of this signaling mechanism in the patterning of nervous systems along the A-P body axis.

6. CONCLUSION AND FUTURE PERSPECTIVES

Extensive research conducted during the last decades has revealed the critical importance for Wnt signaling in the correct development and function of the nervous system. In this review I summarized our current understanding of the roles for Wnt signaling in the formation and patterning of the nervous system along the anterior-posterior body axis.

In a recent publication, C. Petersen and P. Reddien proposed that Wnt signaling is the driving force behind the polarization of bilaterian anteroposterior body axis (Petersen & Reddien, 2009). Based on the review of a consistent body of evidence from both bilaterian and pre-bilaterian metazoans, these authors postulated that posterior activation and anterior inhibition of Wnt signaling is a highly conserved mechanism that drives primary body plan establishment. Interestingly, several lines of research reviewed here seem to corroborate and further extend this hypothesis. In vertebrate early embryogenesis, the A-P establishment of the neuraxis marks the first major developmental step in the emergence of the nervous system. As it was previously described (see section 3), posterior activation and anterior inhibition of the Wnt/β-catenin pathway generates an anteroposterior canonical signaling gradient which determines the correct formation and subsequent patterning of the neural tube **(Figure 2)**. Likewise, during *C. elegans* development a comparable Wnt signaling mechanism is also observed (see section 4) **(Figure 4)**. Here, the Wnt signaling gradient is profoundly involved in the correct migration of neuronal cells, such as the ALM, CAN and Q cells' descendants (Harterink et al., 2011). Despite the considerable evolutionary distance between vertebrate and invertebrate species, these observations evidence that the correct initial patterning of their nervous systems relies on a similar A-P Wnt signaling gradient. Thus, in the framework of C. Petersen and P. Reddien hypothesis, it is plausible to speculate that this, apparently, indispensable spatial orientation of the Wnt signaling gradient along the primary body axis might have had an essential role in the formation and organization of the nervous system of the ancient bilaterian common ancestor, which has been evolutionarily conserved.

An aspect that also becomes evident is the fact that Wnt gradients are determinant not only in the early A-P establishment of nervous systems but also in the delineation of their later architecture along the primary axis, as it is observed in the polarization and navigation of neuronal cell processes (see section 5). In *C. elegans,* Wnts may act as chemoattractants or chemorepellants during migration of axons and dendrites of several neurons. Similarly, guidance of some axonal processes along the vertebrate spinal cord has also been shown to depend on the attractive or repulsive effect exerted by gradients of Wnts, such as Wnt4 and Wnt5a.

But the function of Wnts in A-P neuronal cell polarity is not exclusively instructive. For instance, CWN-2/Wnt has a permissive role in the polarization of SIA and SIB axons which in turn instruct the positioning of the worm's nerve ring (Kennerdell et al., 2009). Here, this Wnt ligand is necessary for the correct trajectory of the axonal processes, yet the localization of its source is apparently not relevant. This contrasts with what is observed in the RMEV/D neurons, where CWN-2/Wnt has an attractive role in the navigation of their axons (Song et al., 2010). How these different responses to the same signal occur is not clear; however, different combinations of Wnt receptors are involved in these two distinct responses to CWN-2/Wnt. Different outcomes to similar Wnt signals are also observed in vertebrates. In axonal outgrowth of commissural neurons, the Wnt/PCP pathway is activated by a Wnt5a signal, possibly mediated by Frizzled (or other receptors) (Shafer et al., 2011). Conversely, Wnt5a-dependent guidance of CST axons, mediates the activation of the Wnt/Ca^{2+} pathway through the Ryk receptor (Hutchins et al., 2011; Hutchins et al., 2012; Li et al., 2009). Although further research is needed to understand the molecular mechanisms underlying the distinct observed outcomes in these specific cases, they seem to be in accordance with the idea that the aftermath of Wnt signaling is not dependent on the ligand, but rather in the nature of the receptor and/or receptor combination.

Another expanding paradigm of the Wnt signaling complexity that is also patent during A-P neuronal development is the intricate relationship between the different pathways. An interesting example of this is the stereotypical A-P migration of the QR cell and its descendants in *C. elegans*. The most recent evidence suggests a model where the different phases of this process are controlled by sequential activation of three different Wnt signaling mechanisms, including both β-catenin independent and dependent pathways (R. Mentink, unpublished). Intriguingly, the transitions from one mechanism to the other are not dependent on Wnt ligands, but arise from a still unknown cell intrinsic timing mechanism. Understanding how this uncommon cell autonomous process is controlled might give new insights into the complexity of Wnt signaling. Another case of the tangled involvement of different Wnt signaling pathways is the regulation of neural tube closure in vertebrates. A considerable body of evidence, ranging from zebrafish to humans, has revealed the critical role that the Wnt/PCP pathway has in this process, and evidenced its implications in severe human developmental defects (Juriloff and Harris, 2012). In addition to the Wnt/PCP pathway, phenotypic analysis of mouse mutants suggests that the Wnt/β-catenin pathway might also be involved in this process. However, this possible implication has not been significantly explored, despite its potential significance for our comprehension of Wnt signaling complexity and its relevance in human birth defects.

Altogether, the evidence summarized in this review reveals that Wnt signaling plays critical, often conserved, roles in the polarized development of nervous systems along the anteroposterior axis. However, and despite the considerable accumulated knowledge, the complexity of Wnt signaling challenges our comprehension of its detailed molecular and cellular mechanisms, not only in this context but also in animal development in general. Thus, questions like how the interplay between different receptors determines different outcomes, how different pathways crosstalk and influence each other, and to which extent cell context and history may have an impact on all this, will inevitably need to be in the center of future research which aspires to further elucidate the complex mechanisms of Wnt signaling.

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