Master's Thesis

The effects of Wnt5a and Wnt3a and PCP signaling on Schwann cell biology and myelination

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Abstract

Planar cell polarity (PCP) is known as the polarization of cells within the plane of the tissue layer. This form of polarization controls several epithelial and non-epithelial morphological processes, such as the orientation of primary cilia in the inner ear, convergent extension (CE) and directed migration. A three tiered model of PCP regulation has been proposed which consists of the global, core, and effector modules. However there is one addition level of modulation through non-canonical Wnt signaling pathway. Of the many Wnt proteins a few have been identified to signal primarily through this pathway. One such protein is Wnt5a, which has been shown to modulate PCP during directed cell migration. In this study we gather preliminary data for the presence of PCP signaling components in Schwann cells and investigate the effect of Wnt5a and its antagonist Wnt3a on Schwann cell proliferation, migration and myelination.

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INTRODUCTION

Chapter 1: Cell polarity

Cell polarity refers to the localized distribution of proteins and molecules within a cell. Our understanding of cell polarity derives from studies on invertebrate (Drospohila, C. elegans) epithelia from which two types of polarity have been characterized; Apical-basal polarity (Figure 1) and Planar Cell Polarity (PCP) (Figure 2). Subsequently cell polarity has been described in vertebrates and is a feature of epithelial cells, as well as cells of the mesenchyme (2, 18). Apical-basal polarity and PCP are essential for the development of organs and different tissues within the organism enabling cells to integrate multiple signals to initiate a proper biological response such as directed cell migration, asymmetric cell division, cell orientation, cell fate determination and differentiation, axon specification and dendrite formation (10, 19, 38) in neuronal cells. The formation of cell polarity is dependent on protein complexes that are conserved across species.

1.1 Apical- basal Polarity

There are three core regulatory complexes that control apical-basal polarity: the Partition-defective (Par3/Par6/aPKC) complex, the Crumbs (Crb/Pals1/Patj) complex and the SCRIB (Scrib/Dlg/Lgl) complex (Figure 1; 18). All proteins within these complexes (except aPKC, a serine/threonine kinase) are PDZ domain-containing proteins (72). PDZ is an acronym that stands for Post synaptic density protein, Drosophila disc large tumor suppressor protein, and Zonula occludens-1 protein, the three proteins that were first discovered to share this domain. PDZ domains are protein-protein interaction modules that coordinate the formation of molecular complexes and specialized domains such as cell junctions and synapses (87).

Of the PAR complex components, the Par6/aPKC complex is localized to the apical domain while Par3 segregates to the lateral/apical boundary. The interaction of

Par6 with aPKC suppresses aPKC basal kinase activity. Par-3, recruited to the initial site of cell-cell contact, recruits the Par6/aPCK complex. Par6 further recruits the small GTPase cdc42, which induces a conformational change that releases Par6 inhibition on aPKC. aPKC then binds to and phosphorylates Par3, which then dissociates from Par6/aPKC permitting aPKC to phosphorylate other target proteins and change their localization within the cell. For example aPKC phosphorylates the Lethal giant larvae (Lgl) protein of the SCRIB complex resulting in its basal lateral localization (72, 87). This complex series of events leads to the apical localization of Par6/aPKC.

Similar to the Par Complex, the Crumbs complex is also a regulator of apical polarity. Crumbs3, one of the three mammalian homologs of Crumbs protein, is localized to the apical membrane where it forms a complex with Pals1 (the mammalian homolog of Drosophila Stardust protein), and Patj (Pals-associated tight junction protein (76). Crumbs3 interacts directly to the N terminus of Pals1 (72), facilitating Pals1 binding to Patj (76). The newly formed Crumbs complex is able to interact with the Par complex by binding to the PDZ domain of Par6 (72), stabilizing the Par complex localization to the apical domain. Therefore, the Crumbs and Par complexes collectively regulate apical polarity.

In contrast to the previous complexes, the SCRIB complex coordinates the basal polarity of cells (87). The SCRIB complex comprises three proteins, Scribble (Scrib), Disc large (Dlg) and Lethal giant larvae (Lgl). Scrib co-localizes with Dlg at the basal lateral domain (Figure 1) while the localization of Lgl depends on its phosphorylation status, which is mediated by aPKC of the PAR complex (17). The SCRIB complex antagonizes the localization of the activated Par complex to promote basal membrane identity, facilitated by the competitive binding of Par complex between Lgl and Par3 (17, 20). The binding of Lgl sequesters the Par complex away from its apical lateral localization. aPKC phosphorylation of Lgl leads to inactivation of the SCRIB complex antagonism .

1.2 Planar cell Polarity (PCP)

Planar cell polarity (PCP) is a polarity axis perpendicular to the apical-basal polarity axis, i.e. parallel to the basement membrane, or luminal aspect, of adjacent epithelial cells (Figure 2). There are two aspects to PCP: The first is the cooperative organization of cells within an epithelium. The second is that this polarization occurs along an axis, for example the organization of cells along a proximal to distal axis (Figure 3). Vertebrate homologs of PCP genes also control several epithelial and non-epithelial morphological processes, such as the orientation of primary cilia in the inner ear (65), convergent extension (55, 83) and directed migration. (74). PCP has also been shown to initiate cytoskeletal rearrangement to via small GTPases activation. Mutations in components of PCP result in developmental anomalies such as cardiac abnormalities and neural tube defects through the disruption of gene transcription.

1.2.1 Modules of Planar Cell Polarity

Three modules coordinate PCP: 1) the Global module, 2) the Core module and 3) the Tissue Effector module (Table 1). The global module provides a global directional cue that links cell polarization to the tissue axis (20). Indeed mutations in global module components does not prevent cells from polarizing and coordinating their polarity with neighboring cells; however these cells do not do not organize themselves along the tissue axis. The most prominent proteins associated with this module are two atypical cadherins, Fat and Dachsous, and the Golgi-resident kinase Four jointed. The heterophillic interaction between Fat and Dachsous (18, 77) is regulated by Four jointed-mediated phosphorylation of Fat and Dachsous extracellular domains (Figure 4). Phosphorylation of Fat increases the interaction while phosphorylation of Dachsous decreases the interaction (77).Early in development, gradients of expression of Fat and Dachsous are observed in all polarized tissue. While the mechanisms of global PCP establishment are still poorly understood, it is proposed that the expression gradients of Fat and Dachsous in a tissue are translated into sub-cellular asymmetries of Fat-Dachsous heterodimers, which can orient in two directions: proximal to distal or distal to proximal (18, 77; Figure 4).

Proteins of the Core module coordinates the polarity of intracellular proteins (to the proximal or distal domains) within cells across a tissue, thus cells within a tissue display a polarized localization of proteins (20). Proteins associated with the core module are seven-pass transmembrane G protein-coupled receptor proteins (Frizzled 1 to 10), cadherin EGF LAG seven-pass G-type receptor (Celsr 1 to 3), four-pass transmembrane proteins Van Gogh-like (Vangl 1 to 3), cytoplasmic adaptors (Dishevelled 1 to 3, Ankyrin-D6), and transcription factor-binding proteins (Prickle 1 to 4). All Core proteins are localized uniformly around the apical-lateral cell membrane, once an appropriate PCP cue initiates their differential localization. Celsr facilitates contact-mediated cell communication (61) and is localized at both the proximal and distal ends (Figure 4) whilst Frizzled transduces polarity signals across the PCP axis by recruiting both Dishevelled and Diego (Figure4). This promotes activation of Dishevelled effectors to generate a polarized structure at the distal side of the cell. Vangl and Prickle are localized at the proximal end and work to limit Dishevelled activation (73; Figure 4, Figure 3 C). Vangl transmits directional cues of individual and groups of cells. Prickle regulates polarity at the proximal end through interactions with Vangl while competing with Diego for Dishevelled binding to modulate Frizzled/Dishevelled activity (73). The appropriate localization of core transmembrane proteins is essential for the proper localization of cellular core components (73). Without the presence of Vangl, Celsr and Frizzled there is reduced or even loss of localization of the remaining core PCP proteins.

The tissue effector module consists of small GTPases Rac, Rho, and cdc42, which organize the directional polarization of cells within a tissue through cytoskeleton rearrangement (Figure 4; 92).

A well-studied example of PCP is the orientation of hair in a parallel array on epidermal cells. In particular those present on the Drosophila wing (Figure3 A, B), and the stereocilia in the mammalian inner ear. In mutants of Core PCP components, hair adopt non-parallel orientations, displaying swirls and patterns of disoriented hair (Figure3 A). Another well-studied example of PCP is cell fate of the ommatidia cells in the Drosophila eye. In this system, core PCP module proteins Frizzled and Flamingo

induce cell fate determination by regulating the levels of Delta/Notch1 signaling (63, 66) and the unequal distribution of cell fate determinants such as NUMB, a Notch antagonist (70). PCP also regulates the collective migration of cells during vertebrate grastrulation by controlling acting cytoskeleton dynamics through the small GTPases RhoA (69) and Rac1 (1), and the serine/threonine Rho-associated kinase Rock that regulated by prickle and Vangl (69)

1.3 PCP signaling mediated through Wnt proteins

As discussed, PCP is mediated through three modules. Interestingly, Frizzled proteins are also known receptors for Wingless-type MMTV integration site family (Wnt) proteins, which bind to the cysteine rich domain (Frizzled domain) of Frizzled. The Wnt family represents a conserved group of secreted gylcoproteins. They regulate cell-cell interactions, cell polarity, development, and cell differentiation. Wnt proteins were first discovered in Drosophilia as effectors of body plan polarity (43). To date, nineteen Wnt protein homologs have been characterized in vertebrates (Table 2). Depending on the complement of Frizzled receptors (1-10) and co-receptors (LRP5/6, Ryk, Ror1, Ror2, and PTK7) that is present at the surface of a cell, as well as of Wnt proteins, different Wnt signaling pathways are activated, classified as canonical and non-canonical (Figure 5). For example Wnt1, Wnt3, Wnt3a, Wnt7a, and Wnt8a will preferentially activate the canonical pathway when co-bound to Frizzled and co-receptor LRP 5/6 (15). Wnt4, Wnt5a, and Wnt11 have been shown to activate the non-canonical pathway when bound to their Frizzled receptor ROR2 co-receptor (16).

The canonical pathway is also referred to as the Wnt/B-catenin pathway. In the absence of Wnt, β-catenin is part of a multi-protein degradation complex comprised of axin, adenomatosis polyposis coli (APC), diversin, and serine/threonine kinases casein kinase 1 (CK1) and glycogen synthase 38 (GSK-38). Upon phosphorylation by CK1 and

GSK-38, β-catenin is targeted for ubiquitination and is then degraded by proteasome. Therefore in the absence of Wnt signaling, β-catenin is maintained at low concentrations through degradation (Figure5; 76). Upon Wnt binding to Frizzled and LRP5/6, Dishevelled (Dsh) is recruited to the cytoplasmic membrane and activated by hyperphosphorylation. Dishevelled prevents the phosphorylation of β-catenin by displacing GSK-38 from the degradation complex. β-catenin then accumulates as a cytoplasmic pool of free molecules (43).β-catenin associates with T-cell factor (Tcf)/lymphoid enhancer factor (Lef)transcription factors and is translocated as a β-catenin/Tcf/Lef complex to promote the transcription of Wnt target genes. β-catenin also plays a role in cell adhesion through binding to the cytoplasmic domain of type 1 cadherins and linking them through a-catenin to the actin cytoskeleton.

There are two non-canonical pathways, which consist of: 1) the Wnt/planar cell polarity pathway and 2) the Wnt/Ca^{2+} pathway (Figure 5 b, c). Unlike the canonical pathway, the non-canonical pathway does not require transcriptional activity of β-catenin (43), and does not require LRP5/6, and has the ability to inhibit the canonical pathway. In the Wnt/PCP pathway (Figure5 B), ligand binding to its Frizzled receptor recruits disheveled, which forms a complex with disheveled –associated activator of morphogenesis 1 (Daam1). Daam1 activates the small G-protein Rho, which in turn activates Rho-associated kinase Rock. One of the major regulators of the cytoskeleton (35), Dishevelled, also directly activates Rac1 and mediates profilin binding to actin, which can result in restructuring of the cytoskeleton (). Rac1 also activates c-Jun N-terminal kinases (JNK). Vangl and Prickle, asymmetrically localized opposite to Frizzled and Dishevelled, are thought to limit disheveled activation (35)

In the Wnt/Ca^{2+} pathway (Figure5 C), Wnt binding to Frizzled induces the G-protein couple receptor to activate phospholipase-C (PLC). PLC cleaves the phospholipid phosphatidylinosotal 4, 5-biphosphate into Diacyl glycerol (DAG; membrane-bound)) an inositol 1, 4, 5-triphosphate (IP_3; cytosolic). When IP_3 binds to its receptor on the endoplasmic reticulum, intracellular calcium concentration increases. Calcium and DAG work together to activate protein kinase C which goes on to activate other molecules, in

particular cell division control protein 42 (cdc42). The increase in calcium also activates calcium/calmodulin-dependent protein kinase II (CamKII) and calcineurin. Calcineurin activates transcription factors such as NFATc and CREB (75).

1.3.1 Role of co-receptors in Wnt signaling

Despite their central role, Frizzled proteins are not the only receptors that modulate Wnt signaling. As previously described Wnt signaling is dependent on the complement of receptors and co-receptors (Figure 6) present at the cell surface. For example members of the LRP (low-density lipoprotein receptor-related protein) family are essential co-receptors for canonical signaling (16). LRP5 and LRP6 form a heterodimer that interacts with Frizzled receptors to form a complex upon Wnt binding.

Another group of co-receptors are single-pass transmembrane receptor tyrosine kinase orphan receptors, Ror1 and Ror2. Ror receptors are important for regulating skeletal and neuronal development, cell migration and cell polarity (Figure 6; 6). Knockdown of Ror2 expression results in PCP mutant phenotypes for convergent extension and migration. The expression of a mutant form of Ror2 in the organ of Corti during embryogenesis results in PCP abnormalities (6). Both Ror1 and Ror2 have a frizzled domain in their extracellular domain, suggesting that they could be receptors for Wnt proteins (89). Indeed Wnt5a has been shown to bind to the Frizzled domain of Ror2 (90) Furthermore Wnt5a mutant phenotype resembles that of a Ror2 knockout mutant (90). Wnt5a acts by activating the small GTPases Rac1 when binding to Frizzled 2 or Ror2 (6). One view is that the Ror2/Fz/Wnt complex triggers PCP signaling while another suggests that Wnt binds directly to Ror2 to activate PCP signaling (16). Interestingly Wnt5a can also suppress the canonical pathway activated by Wnt3a (), as well as activating it when binding to Frizzled 4 (58).

Another Co-receptor of Wnt is protein tyrosine kinase-7 (PTK7) (Figure 6). In vertebrates PTK7 has been shown to be deregulated in cancers and to be implicated in various morphological movements. Experiments conducted in Xenopus demonstrates that PTK7 binding to Wnt3a and Wnt8 (which activate canonical signaling in Xenopus

embryos) in the presence of Frizzled suggesting that PTK7 may inhibit canonical signaling by competing with LRP5/6 for the Wnt/Frizzled complex. This is similar to the proposed modulate of ROR2, which disrupts the canonical Wnt/ Frizzled/LRP signaling Wnt/Frizzled/ROR2 signaling complex.

Finally the atypical tyrosine kinase Ryk, which possesses a Wnt inhibitory factor (WIF) domain, was first discovered as a module present in secreted inhibitors of Wnt β–catenin signaling that sequestered Wnt proteins (16). The Ryk WIF domain binds to Wnt proteins with high affinity creating a complex independent of Frizzled (Figure6, 16). In Drosophila, the Ryk ortholog Derailed binds to Wnt5 to promote commissural axon guidance and proper salivary gland migration, possibly through the activation of members of the Src family of tyrosine kinases (16).

As demonstrated Wnt proteins have multiple signaling pathways and their signaling is intricately modulated by receptor availability. Their signaling has been extensively studied in epithelial tissues however studies in the nervous system are often limited to the canonical Wnt signaling pathways. Here I propose the possible role of non-canonical Wnt PCP signaling within the peripheral nervous system.

Chapter 2: Cell polarity and Myelination

2.1 Myelination

Myelination is the process by which the compact myelin sheath is formed around axons. This compact myelin sheath (Figure 7) is necessary for the proper function of the nervous system, facilitating optimal velocity of the action potential propagation and the synchronization of afferent inputs (5). A myelinated axon is a highly specialized structure that forms as the result of instructive contact-dependent signals between the axon and the myelinating glial cell; the Schwann cell in the peripheral nervous system (PNS), the oligodendrocyte in the central nervous system (CNS). In this thesis work, I will focus on the Schwann cell.

Schwann cells are derivatives of neural crest cells (Figure 8). When the neural folds come together to form the neural tube, the neural crest cells undergo an epithelial-mesenchymal transition (EMT), emigrate from the neural epithelium, and migrate laterally and ventrally. Ventrally travelling neural crest cells will give rise to dorsal root ganglia sensory neurons and glial cells, the latter forming Schwann cell precursors, that proliferate and migrate along growing axons and undergo sharp transitions into first immature Schwann cells then either mature myelinating or non myelinating Schwann cells.

Prior to differentiating into Schwann cell precursors Neural crests cells undergo two distinct cell processes that have been shown to be controlled by PCP. Once the neural tube closes neural cells undergo directed cell migration to reach their target locations. Direct cell migration is facilitated by protein gradient within the neural crest cells controlled by core PCP proteins and non-canonical Wnt signaling. For example Dishevelled is localized to the front of migration by PTK7 a co-receptor of Wnt signaling shown to promote Wnt/PCP signaling pathway while inhibiting the Canonical pathway (40). Wnt proteins (specifically, Wnt11, a non-canonical Wnt protein) are also shown to be localized at the front of migration (42). A form of cohesive directed cell migration is also exhibited by Schwann cells as they migrate along the growing axons. Classically it is thought that these Schwann cells migrate along the axon solely for the trophic support that they provide. However it is possible that PCP mechanisms are present in migrating Schwann cell precursors to facilitate directed migration.

These cells also undergo asymmetrical cell division, a process mediated by planar cell polarity, in order to give rise to DRG neurons and Schwann cell precursors. During asymmetrical cell division of neural crest cells there is unequal distribution of cell fate proteins. Particularly Notch and NUMB cell fate proteins. Notch signaling has been shown to promote gliogenesis while inhibiting neurogenesis (38, 39). The activity of Notch signaling is counteracted by NUMB signaling (38, 39) When neural crest cells undergo cell division at the location of the dorsal root there is asymmetrical localization of Notch to one side and NUMB to the other. The daughter cell that receives Notch

signaling under goes gliogenesis to give rise to Schwann cell precursors while NUMB signaling containing cell will undergo neurogenesis, giving rise to sensory neurons of the dorsal root ganglion. This is similar to Drosophila ommatidia cell division. Ommatidia cell division is dependent on asymmetrical localization of Notch and NUMB facilitated by PCP.

Furthermore the master effectors of the planar cell polarity, the small GTPases, are known to function in reorganization of the cytoskeleton. These Small GTPases drive the polarity of Schwann cells to allow Schwann cells to put out multiple processes into an axon bundle and sort the axons for myelination (4).

The generation of Schwann cells (i.e., proliferation) as well as their survival is highly dependent on axon-derived signals (36). These axon-derived signals are also critical to the Schwann cell lineage progression, from precursor Schwann cells (E14-15 in the rat), to the immature Schwann cells (E16-17), to mature myelinating and non-myelinating Schwann cells (ref). Neuregulin-1 (NRG-1) is a growth factor that has been characterized as one of the key axonal signals that regulate many aspects of Schwann cell development (21). Neuregulin- 1 has been classified into three major isoforms (I, II, and III) (22) are synthesized as trans-membrane proteins. Types I and II are cleaved and function as paracrine signaling molecules (21) whilst Type III remains bound to the cell surface and functions as a juxtacrine signal (22). Knockout studies have provided evidence that NRG-1-type III, on the axon surface, is the key isoform necessary for Schwann cell proliferation and survival (23, 24). NRG-1- type III has also been found to be the instructive signal that determines in the PNS whether a Schwann cell will ensheath multiple axons to form Remak bundles (Figure8) or associate in a 1:1 relationship with a single axon and form a compact myelin sheath (Figure8; 25).

2.2 Polarization of Schwann cells is essential for myelination

Prior to myelination Schwann cells separate axons through a process called radial sorting. During this process, Schwann cells interact with both axons and basal lamina components, and acquire what may be viewed as a radial polarity. Schwann cells

present two cytoplasmic membrane compartments: the inner membrane, which is in close contact with the axon, and the outer membrane, which is in close contact with the basal lamina (5). In myelinating Schwann cells the classical apical and basal domains are defined as such; the outer membrane is seen as the basolateral-like domain and the inner and the Schmidt-Lantermann incisures are considered the apical-like domain (2). One of the hallmarks of cell polarity is the localized distribution of proteins to membrane domains within cells. Consistent with this idea, the two Schwann cell membranes differ in their protein composition (2). They contain distinct protein groups that receive and transduce axonal- derived and basal lamina-derived signals essential for myelination. The protein distribution creates specialized regions for interactions between Schwann cells and axons or Schwann cells and basal lamina.

The distribution of these proteins is likely to be controlled by polarity proteins of classical apical basal polarity. Indeed specific proteins of cellular polarity have been shown to be important for myelination in Schwann cells (48). For example Par3 of the Par complex, protein associated with Lin seven 1 (PALS1) of the Crumbs complex, and Disc Large (Dlg) of the SCRIB complex, have recently been shown to play an important role during myelination. At the apical-like membrane of the Schwann cell membrane there is localization of Par3 upon axo-glial contact. Par3 plays a key role in promoting myelination by recruiting the neurotrophin receptor ($p75^{NTR}$) to the site at the start of myelination [41]. Knockdown of the Par3 protein has been shown to inhibit myelin formation (26). While Pals1 and Dlg1, appear to play a role in the regulation of radial extension and thickness of the myelin sheath [49, 50]. Pals knockdown resulted limited extension of myelin and Dlg1 knockdown limited the number of lamellae formed (25).

More abstract arguments can also be made for the presence of PCP in Schwann cell biology. Schwann cells make an initial one turn around the axon in order to for a 1:1 relationship, bringing the two edges of the Schwann cell membrane into contact with one another. One edge remains stationary while the opposite edge begins to spiral inward circumventing the axon (Figure 9, Figure11). This indicates that a difference in protein composition and differences in the molecular motors and/or cytoskeletal

structure may be present at the spiraling lip compared to stationary lip (Figure 9, Figure11).

Individual Schwann cells will wrap only one axonal segment yet single axons are myelinated by multiple Schwann cells. The number of Schwann cells myelinating a single axon is highly regulated as is the length of each segment made. In order for this level of organization to take place a mechanism must exist that allows neighboring Schwann cells to communicate with one another to regulate this process (Figure10, Figure 11).This communication would facilitate the signaling of Schwann cell number along the axon to form internodes of equal lengths. This form of cell communication is characteristic of PCP in tissues. In tissues PCP allows for arrangement of cells in relation to neighboring cells to produce a uniform tissue layer (Figure3).

In this thesis preliminary data was gathered for the presence of PCP signaling components in Schwann cells and investigate the effect of Wnt5a (a Wnt protein characterized to act primarily through Wnt/PCP) and its antagonist Wnt3a on Schwann cell proliferation, migration and myelination.

Results

Chapter 3

3.1 Components for each of the three PCP modules are present in Schwann cells.

To being the studies, first the presence of PCP components in Schwann cells was investigated. cDNA, prepared by PCR from total RNA isolated from rat Schwann cells, was later used as template to amplify transcripts for global and core PCP components with specific primers. Schwann cells appear to harbor mRNA transcripts for subsets of each module. In particular, of the global module (Fat-1, Fat-2, Fat3, Dashsous 1 and 2), we amplified Fat-1 at the expected length and mRNA transcripts for both Daschsous isoforms were present at expected lengths (Figure12 A, B). Of the core module (Celsr-1-3, Vangl 1-2, and Prickle 1-4.) proteins I have successfully amplified Celsr2, as well as Vangl-1 and Prickle-3 (Figure 13) as well as Ankryn D6.

I have also started to confirm the protein expression of factors identified in the PCR screen. Thus far proteins of the core module (Celsr2, Vangl-1 and Prickle-3) are expressed (Figure14) at the expect molecular weights (Vangl-1 60Kda, Celsr2 317Kda, Prickle-3 68Kda). However analysis of global module components has been more difficult due to the large size of atypical cadherins and availability of proper antibodies for mammalian cells. Upon acquisition of proper antibodies expression of all PCP components will be analyzed.

The presence of Wnt protein co- receptors within Schwann cells was also investigated. Again cDNA was amplified from total RNA isolated from rat Schwann cells. It was obsereved that Schwann cells express detectable mRNA for Ror proteins (Ror1 and Ror2). Both Ror 1 and Ror 2 were amplified by PCR of Schwann cell cDNA (Figure15). PCR analysis for the remainder of Wnt co-receptors is in progress at present.

3.2 Wnt5a and Wnt3a are expressed in the rat PNS

The next step to further the study is the investigation of whether Wnt proteins, specifically Wnt5a, a ligand of Ror 2 that signals primarily through the Wnt PCP pathway, and its antagonist Wnt3a, are present in the PNS. Rat sciatic nerves were collected from pups at birth (p0) until 30 days after birth. Nerve extracts were analyzed by Western blot for the expression of Wnt5a and Wnt3a. The Wnt5a antibody detected a doublet at 70 Kda and 65 Kda (Figure 16 A) at all ages. However the levels of expression sharply decrease from p4 onward. Interestingly, Wnt3a exhibit an opposite pattern of expression. A single band is detected at 35 Kda. It is below detectable levels at birth and increases with myelination (Figure16 C).

3.3 B Wnt5a and Wnt3a are present within the in vitro culture system

Proteins extracts prepared from purified primary Schwann cells and dorsal root ganglion neurons, were analyzed by Western blotting for the expression of Wnt5a and Wnt3a. It is observed that Wnt5a is present in both Schwann cells and DRG neurons (Figure17). However the expression pattern is different in both cell types when compared to sciatic nerve extracts. Schwann cells express a similar doublet as the sciatic nerve extracts as well as an additional band at 48 Kda. The DRG neurons exhibit expression of only a single band at 70Kda for Wnt5a (Figure17). It is known that Wnt proteins are highly modified post translationally by N-glycosylation. To confirm that the banding pattern seen is due to a difference in N-glycosylation, the extracts were digested using N-Glycosidase F (PNGase F) to remove all type of N-linked oligosaccharides (Figure 17 B, C). Following deglycosylation in vitro some bands show marked decrease (doublet at 70 and 65 Kda in Schwann cells and 70Kda in DRG neurons) while others disappear completely (48 Kda band in Schwann cells; Figure17 C). An enrichment of a band at 30 Kda the predicted protein backbone for Wnt5a, results from deglycosylation (Figure 17 A, C). Western blot analysis of Wnt3a expression in the two cells types revealed that Wnt3a is present at 35 Kda within Schwann cells but did not appear to be expressed in the DRG neurons (Figure 18).

3.4. Wnt5a and Wnt3a do not have an effect on SMDF mediated Schwann cell proliferation.

To highlight possible effects of Wnt3a and Wnt5a on Schwann cell Proliferation, confluent Schwann cells were cultured in the presence of SMDF were treated with low dose (Wnt5a 10ng/ml; Wnt3a 0.25ng/ml) or high dose (Wnt5a 10ng/ml; Wnt3a 25ng/ml), as per the ED 50 and incubated for 24hrs. Following with 4 hours of incubation with BrdU incorporation, was applied to all cultures. At 24hrs of treatment cells were fixed and subsequently stained for Dapi nuclear stain for comparison of DAPI +/-BrdU nuclei. It was observed that there was no significant effect on SMDF mediated Schwann cell proliferation with Wnt5a (Figure19) or Wnt3a treatment (Figure20) compared cultures treated with SMDF alone. To determine whether effects of Wnt proteins on Schwann cells are axonal contact dependent, the assay was re-performed in a co-culture system (Figure21). Again, Wnt treatment, when compared to the co-cultures cultures had no significant effect on Schwann cell proliferation (Figure22).

3.5. Wnt5a enhances SMDF mediated Schwann cell migration

To investigate the possible effects of Wnt5a and Wnt3a on neuregulin-mediated Schwann cell migration, we performed a scratch assay. A scratch was made with a 200 µl tip, through confluent, quiescent Schwann cell cultures maintained in serum free conditions for 24hrs. The media was immediately changed to serum free media (F12), with or without not SMDF (500pM), and with or without the Wnt5a proteins at 10ng/ml or 100ng/ml. Images were taken at the time of the scratch (T0) and 16hrs later. It was observed that at T0 and T16 there is no marked changes in migration for all conditions in the absence of SMDF (Figure23). However in the presence of SMDF after 16 hours it was observed that there is a marked increase in migration after treatment of Wnt5a protein at concentration (100ng/ml) (Figure24 F).

3.6 Wnt5a Inhibits Myelin formation

To test the effects of Wnt proteins on myelination, DRG and Schwann cell cocultures were treated with Wnt proteins as follows Wnt5a protein at 200ng/ml, 100ng/ml, 50ng/ml, and 10ng/ml and recombinant Wnt3a protein concentrations as follows; 10ng/ml, 1ng/ml, and 0.5ng/ml. DRG neuron and Schwann cells co-cultures were maintained for three days before myelination was initiated with the addition of ascorbic acid (50ug/ml final). At this time Wnt proteins were also applied to cultures. After fourteen days the cultures were fixed and immunostained for myelin basic protein (MBP).

Total myelin segments were assessed for each condition and percent myelin formed was determined. The result for mock treatment of the co-cultures revealed no significant effect on myelination at a concentration of mock (Figure25 Panel1; B) when compared to the control. For Wnt5a treatment it was observed that Wnt5a at low concentrations (10ng/ml or 50ng/ml) had no significant effect on myelination (Figure25 Panel; C, D,) when compared to the control (Figure25 Panel 1; A). While high concentrations (200ng/ml and 100ng/ml) of Wnt5a inhibited myelin formation (Figure25 Panel 1; E, F, Panel 2). Preliminary experimental results from Wnt3a treatment showed more variability in the results. Three set of experimental data were collected and all sets shoed variable results in myelin segment formation for each concentration of Wnt3a treatment. No treatment of mock has been applied in these cultures as there was no significant effect at 200ng/ml a factor of 1/500. Treatment of Wnt3a had no significant effect on myelination at high or low concentrations (Figure26).

Discussion

Chapter 4

We were able to demonstrate in this study that PCP components are present within Schwann cells and these components are expressed by PCR and Western blot analysis. Amplification of cDNA revealed that specific isoforms of global (fat and dachsous) and core (Prickle, Vangl, and Celsr) protein mRNA transcripts are present within Schwann cells. There proteins are necessary for the establishment of proximal and distal cell boundaries in typical epithelial tissue. However the present function of these proteins in the Schwann cells has not been studied. Therefore we propose the possibility of the role of global and core PCP components in protein localization to create molecularly distinct edges on a one: one associated Schwann cells with axon. Expression of these mRNA is still under progress due to the availability of proper antibodies for PCP components. However I have been able to show expression of some core components. In order to determine if the mRNA transcripts were transcribed into proteins western blot analysis of Schwann cell and DRG myelinating co-culture lysates was performed. Due to antibody availability I was only able to test core PCP proteins. Specifically thus far I have shown that specific isoforms of transmembrane and cytoplasmic core PCP components (Vangl 1, Celsr2, and Prickle3) are expressed in Schwann cell and DRG cocultures. Interestingly Prickle3 and Vangl 1 show an expression pattern in which levels of Vangl 1 and Prickle3 decreases with myelination. These expression levels would indicate the role of radial cell boundaries at the onset of myelination, when Schwann cells make a one: one association with axons bringing the two opposite edges of the Schwann cell membrane together. The core PCP proteins would enable the localization of the molecular motors and proteins necessary to stabilize one edge and allow the opposite edge to spiral inward. The expression of these proteins could then be decreased after the initiation of myelination. Upon obtaining proper mammalian antibodies myelinating cocultures will be stained for core PCP proteins to determine their localization. The changes in localization of these proteins

with myelination can also be monitored by immunostaining of time course of cocultures. If our hypothesis is correct then we would expect to observe two different immunostaining along the length of the Schwann cell membrane. This signal would then be localized at the paranode upon compact myelin formation.

I was also able to demonstrate, by cDNA amplification of Schwann cell mRNA, that Ror1 and Ror2 are also present in Schwann cells. Ror receptors particularly Ror2 have been shown to be key regulators of PCP signaling during development. Mutations in Ror2 exhibit defects in convergent extension, migration, deficiency in cellular polarization and cell morphology (16). Ror receptors are also known coreceptors for Wnt proteins and modulate their signaling through the non-canonical Wnt PCP signaling pathways. One particular Wnt protein, Wnt5a, has been identified to preferentially signal through Wnt/PCP pathway in the presence of Ror2 receptors (16). The interaction between Ror2 and Wnt5a has been shown to be essential for convergent extension and cell migration during development (7). Phenotypes similar to Ror2 mutants are exhibited in Wnt5a knockout studies (16). Therefore the presence of Ror2 receptor mRNA lead to the postulation that Wnt PCP signaling maybe involved in regulating some aspect of Schwann cell biology. However due to lack of proper antibodies the expression pattern of Ror receptors remains to still be determined.

However I was able to show that in fact Ror2 ligand, Wnt5a is present in the developing PNS (sciatic nerve) as well as both cells (Schwann cells and DRG neurons). I observed that Wnt5a is present at high levels early on postnatally in the sciatic nerve and drops off sharply at 4 days postnatally. During development this is the point at which Schwann cells will undergo a transition from immature Schwann cells to myelinating Schwann cells (13). The expression pattern there by indicates that Wnt5a may play a role in regulating the immature Schwann cell behavior such as proliferation and migration along the axon bundle. Both of these processes are initiated late prenatal period and ongoing postnatally, with an overlap with the transition period. Interestingly the pattern of expression of Wnt5a in Schwann cells and DRG neurons appears to be different from each other as well as expression in the sciatic nerve. Wnts are known to

be modified by multiple N-linked glycosylations, involved in regulating both secretion and signaling. For example, upon enzymatic de-glycosylation of secreted Wnt are able to still interact with Frizzled receptors, though signaling activity is strongly reduced (29). Mutation of modified residues or inhibition of glycosylation strongly affects Wnt secretion (29). The glycosylation is variable between the different Wnt proteins and cell types (30). Differences in glycosylation could explain the pattern of expression I have observed in the Sciatic nerve and the individual cells. In fact this idea is supported by the Western blot analysis of de-glycosylated extracts. I observed that after PNGase F digestion a band at ~31 Kda, the size of the mature core protein backbone. This data indicates that Wnt5a is differentially expressed by both cell types by N-glycosylation modulation. As we observe expression of Wnt5a in both cell types it is hard to determine from which cell type Wnt5a is being secreted. Potentially Wnt5a may act as an autocrine signal in which it acts upon the cells which produce it. The presence of both the Wnt5a protein and Ror coreceptors support the idea that Wnt5a may have a role in the Schwann cell biology by signaling through the noncanonical PCP pathway.

It is known that Wnt3a antagonizes Wnt5a by competing for Frizzled receptor binding and preferentially activates the canonical Wnt signaling pathway. Due to this antagonism, it was hypothesized that I would observe opposing effects on Schwann cell biology to those observed with Wnt5a. Consistent with this notion I observe that in the developing sciatic nerve, Wnt3a is not detectable early on postnatally and begins to increase at postnatal day 4 until adulthood. This pattern is opposite of the pattern seen for Wnt5a expression in the sciatic nerve. These data suggest that Wnt3a may be antagonizing the function of Wnt5a and potential provide the molecular switch necessary for Schwann cells to transition into mature myelinating Schwann cells. This notion is supported by the fact that Wnt3a signals primarily through the canonical Wnt pathway, which is known to regulate target gene transcription upon β-catenin translocation to the nucleus upon Wnt/ frizzled interactions. We hypothesize that Wnt3a may be acting to regulate gene transcription within the axon of molecular signals for myelination (Nrg 1 type3) and proteins for Schwann cell and axon association (Necl

1). This hypothesis is supported by the western blot data which has shown that Wnt3a is selectively produced by Schwann cells but not DRGs. This combined with the Wnt3a expression pattern suggests a potential role of Wnt3a regulation of gene transcription for myelination. Further studies have been proposed to validate our hypothesis, primarily testing the expression levels of Necl 1 and Nrg1 type 3 in coculture after treatment with Wnt3a.

With the observed expression patterns of Wnt proteins during development and the two cells type it was then necessary to test the effects of Wnt proteins on aspects of Schwann cell biology and behavior. First of which is a BrdU incorporation assay to test the effects on Schwann cell proliferation. It has been shown that some Wnt proteins (Wnt3a) can promote proliferation through EGF receptor interactions (31, 32, 34). In Schwann cells it has been shown that NRG-1 type 3 signal from the axon is necessary to promote Schwann cell proliferation (33). This proliferation is dependent the ErbB tyrosine kinase receptors (ErbB2 and ErbB3) that are members of the EGF receptor family and transduce the NRG signal (33). Based on this evidence it was proposed that Wnt proteins could promote Schwann cell proliferation in synergy with NRG. I tested the effects of Wnt5a or Wnt3a treatment on Schwann cell proliferation in the presence of SMDF, a soluble form of NRG and in contact with axons in cocultures. However to the results show that both Wnt5a and Wnt3a treatment of Schwann cells one and in coculture had no significant effects on neuregulin-mediated Schwann cell proliferation. Therefore we can conclude that Wnt3a and Wnt5a are not able to interact with ErbB2 and Erb3 receptors to enhance proliferation.

Next I tested the effects of Wnt5a treatment on SMDF mediated Schwann cell migration. During the development of the PNS, there are dynamic interactions between Schwann cells and axons. When Schwann cell precursors first come into contact with axons they are still proliferative cells whose survival is dependent on neuregulin signal provided by the axon (36). They subsequently differentiate into immature Schwann cells with polarized processes (37). At this stage, the migrating cells extend processes, which

penetrate between axons and segregate them. Thus, Schwann cell motility is a major aspect of development of the PNS may be regulated by Wnt signaling.

It has been shown that in serum free conditions at 10ng/ml of SMDF the effects on Schwann cell proliferation (a 2.5% increase) are negliable (35). It has also been shown that at this concentration of SMDF, Schwann cell migration is increased by 84% (35). Though in our results we did not observe this increase in Schwann cell migration with treatment of SMDF alone when compared to the control condition, a positive control study of SMDF mediated effect on Schwann cell migration is underway. Furthermore Wnt5a proteins have been implicated in modulation of cell migration through interactions with Ror2 receptors. Loss of Wnt5a activity has shown to present defects in cell migrations. With this evidence in mind it was proposed that Wnt5a may be able to enhance Schwann cell migration in synergy with SMDF mediate enhancement of migration. In this study I observed that in fact after 16hrs Wnt5a (100ng/ml) is able to enhance SMDF migration. However control studies for measurement of Schwann cell proliferation are underway to confirm these results are true. These results are indicative of the possible regulation of Schwann cell migration along the axonal bundle during development. Thus treatment of Wnt5a should promote migration and arrest the transition from immature to mature Schwann cells there by inhibiting myelination in coculture.

Thereby leading to my final studies, which tested the effects of exogenous Wnt protein treatment on myelination. I have thus far shown that Wnt5a and Wnt3a are present within the developing sciatic nerve and seem to be expressed in a pattern that may potentially regulate aspects of Schwann cell biology and myelination. Also Wnt5a treatment enhance SMDF mediated Schwann cell migration. With our coculture treatment of myelinating cultures it was observed that Wnt5a treatment of Schwann cell and DRG neuron co-cultures showed that there was no significant effect for lower (10ng/ml and 50ng/ml) concentrations of Wnt5a. However to our delight, at higher concentrations of Wnt5a treatment, there is a marked inhibition of myelin formation. The likely hood of myelination inhibition due to changes in Schwann cell density is low

as I have shown with the proliferation assay that there were no significant effects on neuregulin-mediated proliferation. However our scratch assay results are positive for Wnt5a treatment there by suggesting that Wnt5a enhances neuregulin-mediate Schwann cell migration. This result could be attributed to continued Schwann cell migration with increased levels of Wnt5a therefore preventing the transition from immature Schwann cells to myelinating Schwann cells.

As a known antagonist of Wnt5a, treatment of Wnt3a would be expected to promote more myelin formation. Also our western blot analysis of Wnt3a in the developing sciatic nerve exhibit a pattern that would suggests a promotion of myelination. However to our disappointment when co-cultures of were treated with Wnt3a I did not see the expected increase in myelination. Thereby leading me to postulate that the increase observed, in Wnt3a expression during development, maybe the essential switch necessary for the sharp transition into mature Schwann cells. The results from Wnt3a treated cultures still remain preliminary and must be repeated to confirm their significance. The Western blot analysis of our culture system for Wnt3a shows the presence of Wnt3a in Schwann cells. This indicates that the protein is involved in some aspect of Schwann cell biology. However with present studies the exact role of Wnt3a still remains unclear.

These effects of Wnt treatment on myelination are new to present research on myelination and Wnt proteins. Though here I have shown preliminary evidence for the presence of Wnt regulation in Schwann cell behavior, the question of the mechanism of action of Wnt proteins still remains unclear. Further Studies must be performed to confirm that PCP signaling is involved. Therefore simple experiments have been proposed such as Elisa small GTPase activation assays upon Wnt treatment. Knockdown studies of β-catenin must also be performed to confirm. If our Wnt induced effects are myelination are results of Wnt PCP signaling we expect to see increased activation of small GTPases with treatment of Wnt proteins and no changes in myelination inhibition with β-catenin knock down.

Material and Methods

Chapter 5:

Primary cell cultures

Primary cell cultures were obtained from purified rat Schwann cells and dorsal root ganglion neurons (DRG neurons). Neurons were isolated from embryonic day 16 rat DRGs and plated on a matrigel substrate in neurobasal medium, supplemented with 2% B27 supplement, 2 mM L-glutamine, 0.04% glucose, and 50 ng/ml 2.5S NGF. Schwann cells were prepared from postnatal day 2 rat sciatic nerves following the Brockes protocol (Brockes et al., 1979) and expanded in high glucose-DMEM with 10% FBS, 2 mM L-glutamine (Schwann cell media (SCM)) and supplemented with 2 µM Forskolin and 5 ng/ml of the EGF domain (EGF-D) of rhNRG-1-β1. Myelinating Schwann cell/neuron co-cultures were established by seeding purified DRG neuron cultures with 200,000 Schwann cells in MEM supplemented with 10% FBS, 2 mM L-glutamine, 0.4% glucose, and 50 ng/ml 2.5S NGF (C media).

Wnt treated co-cultures

Myelinating Cocultures were maintained for 3 days in C media. On the 4^{th} day myelination was initiated by addition of ascorbic acid and Wnt5a/Wnt3a (R&D systems) supplementation to C media. Wnt protein concentrations (Wnt5a; 200ng/ml, 100ng/ml, 50ng/ml, and 10ng/ml and Wnt3a; 10ng/ml, 1ng/ml, 0.5ng/ml) were determined using ED50 provided by data sheet Cultures were maintained for 14 days in defined medium.

Lysates

Rat sciatic nerves were extracted over a time course in the following order postnatal day 0.5, postnatal day 2.5, postnatal day 4.5, postnatal day 7.5, postnatal day 10.5, postnatal day 14.5. The extracts were lysated in appropriate amount of lysis buffer containing 2% SDS, protease inhibitors, and phosphatase inhibitors. Lysates were homogenized,

centrifuged, and transferred to remove excess tissues. Co culture, DRG neuron, and Schwann cell culture lysated in 2 % SDS buffer containing protease and phosphatase inhibitors.

Western Blot

Lysates were analyzed using western blot analysis performed using a 10% bis tris gel system (Invitrogen). Smaller molecular weight proteins were separated using MES running buffer while larger proteins were separated using MOPS buffer (20x stock diluted to 1x). Gels were run at 100v constant for 1.5 hrs. Standard SDS-page transfer buffer was used to transfer blots onto nitrocellulose membrane over night at constant 100 mA. Membranes were blocked using 2.5% milk solution in 1x TBST at room temperature for 30 minutes. Then blots were washed 3 times 5 minutes each with 1x TBST after which the blots were incubated in primary antibodies over night at + 4 degrees. Afterwards the blots are washed 3 times 5 minute each with 1x TBST. Then incubated in secondary antibody at 1:2000 for 2 hours at room temperature in 2.5% milk solution in 1 x TBST covered. Western blots were visualized using the licor system

Schwann cell proliferation assay

50,000 cells were seeded on poly-L-lysine pre-coated ø10mm glass coverslips in SCM supplemented with 2 µM Forskolin and 5 ng/ml of the EGF domain (EGF-D) of rhNRG-1-β1. After 24 hours, cells were place in SCM without Forskolin and the EGF domain for another 24 hours. Cultures were then pre-treated with either Wnt5a (10 and 100 ng/ml) or Wnt3a (0.5 and 25 ng/ml) prepared in SCM. One hour later, culture media was supplemented with type III NRG-1. After 20 hrs at 37°C, 10% CO_2, BrdU (final concentration: 20 µM) was added for the last 4 hours. In the co-culture system, 72 hours after seeding 50,000 Schwann cells, the culture media were changed to fresh C medium supplemented with either Wnt5a (10 and 100 ng/ml) or Wnt3a (0.5 and 25 ng/ml) and incubated for 24 hours. BrdU was added during the last 4 hours.

Migration assay

Schwann cells were plated on poly-L-lysine-coated plates and maintained until confluency. After this time, the medium was changed serum free medium (DM with or without 10% FCS) for 12hrs after which, three a cell-free areas were generated by gently scratching the cell monolayer with a sterile 200 uL pipette tip, thus resulting in the formation of an approximately 1-mm-wide gap. Immediately after scratching the medium was replaced and Schwann cells were exposed to NRG for the duration of the assay at the concentrations stated. Migration of Schwann cells within the gap was documented by imaging done with a Nikon Epi-Fluorescence microscope. Images were taken at 200X with a Hamamatsu camera (Hamamatsu, Shizuoka, Japan) controlled by the Metamorph software (Sunnyvale, CA). Cell counting was done using the NIH ImageJ software, and one way ANOVA statistical analysis done with the Prism software (GraphPad). Migration was assessed by marking off the scratch area and counting all cells with their bodies across the scratch boundary. Migration was monitored at T0 (right after scratch) and 16h after treatment with Wnt5a.

Immunofluorescence

Wnt treated myelinating cultures:

Cultures were washed with 1x PBS again. Cultures were permeabilized with methanol After 14 day co cultures on glass cover slips were washed with 300ul of 1x phosphate buffer solution (PBS) 3 times for 5 minutes and then fixed in 4 % paraformaldehyde (20 minutes at room temperature (RT)) and permeabilized with methanol (20 minutes at -20˚ C). Then cultures were washed again and then incubated in immunofluorescence blocking solution (5% donkey serum, 1% bovine serum albumin and .25% triton in 1 x PBS) for 1 hour at room temperature. Then the cultures were removed from the plate and placed on a light protected and parafilm covered plate. Then the coverslips were incubated in primary antibodies (anti-MBP mouse smi 94 1:1800, anti-neurofilament chk Covance 1:1500,) over night at 4˚C. The next day the cultures were washed 3 time for 5

minutes each with 1 x PBS. After washing the cultures are incubated in secondary antibodies (1:100) for 1 hour at room temperature. The coverslips were then washed 3 times with blocking solution, 5 minutes and once with PBS, 5 minutes, and milli-Q (Millipore Corporation, Billerica, MA) water was used to do the last wash. All cultures were mounted in Citifluor (Ted Pella, Redding, CA) with DAPI (1 µg/ml) on glass microscope slide. Nail polish was used to seal around the coverslips, and the samples were stored at 4°C until imaging. Imaging was done with a Nikon Epi-Fluorescence microscope. Images were taken at 10X with a Hamamatsu camera (Hamamatsu, Shizuoka, Japan) controlled by the Metamorph software (Sunnyvale, CA). Cell counting was done using the NIH ImageJ software.

Proliferation assay:

Schwann only culture were fixed and permeabilized as described previously. Anti-BrdU is added after permeabilization with methanol and incubation using HCl. The next day, the coverslips were washed by PBS, 3 times for 5 minutes at RT and incubated adequately each culture system with 2nd antibody prepared in blocking solution: DL488-conjugated Donkey anti-mouse (1/100). Next, the coverslips were washed 3 times with blocking solution, 5 minutes and once with PBS, 5 minutes, and milli-Q (Millipore Corporation, Billerica, MA) water was used to do the last wash. All cultures were mounted in Citifluor (Ted Pella, Redding, CA) with DAPI (1 µg/ml) on glass microscope slide. Nail polish was used to seal around the coverslips, and the samples were stored at 4°C until imaging. Imaging was done with a Nikon Epi-Fluorescence microscope. Images were taken at 200X with a Hamamatsu camera (Hamamatsu, Shizuoka, Japan) controlled by the Metamorph software (Sunnyvale, CA). Cell counting was done using the NIH ImageJ software, and one way ANOVA statistical analysis done with the Prism software (GraphPad).

Co-cultures were fixed and permeabilized as stated above with primary antibody incubation overnight. The following day, after washing 3 times 5 minutes each, with 400 ul PBS, the slides were incubated with 2N HCl for 15 min, 37°C and neutralized 2 times

with 400 µl of boric acid (0.1 M, pH 8.5) for 10 min at RT. The cultures were washed twice with PBS, and then with L15 medium at least 5 times. After incubation with blocking solution for 30 min at RT, we added the mouse monoclonal anti-BrdU at 1:200 at 4 °C, overnight. Slides were mounted and images were taken as described above.

Figure 1 Core complexes of Apical Basal polarity

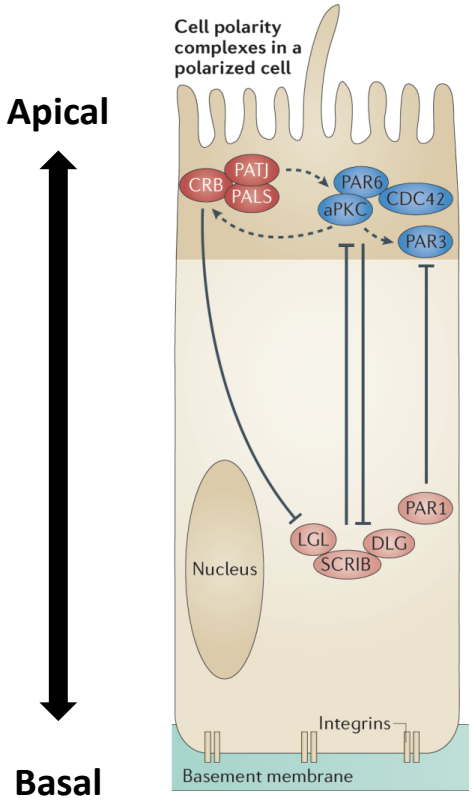
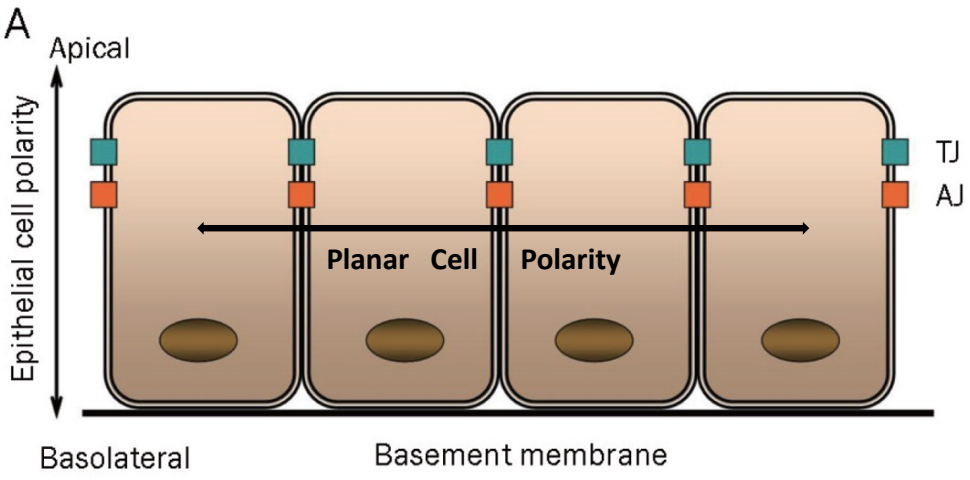


Diagram illustrating the localization and interaction of core regulatory complexes of apical-basal polarity in a polarized epithelial cell. Apical localized Crumbs complex proteins (red) Par complex (blue) and basal lateral localized SCRIB complex (pink). Par3 recruitment to apical lateral domain leads to recruitment of Par6/aPKC complex. aPKC kinase activity is activated and phosphorylated Par3 to stabilize it at the apical lateral domain. Crumb complex stabilizes Par6/ aPKC complex to apical domain. Also aPKC phosphorylates to localize it to the basal lateral domain (Image adapted from Nature Reviews Cancer 2012).

Figure 2 Planar cell polarity axis



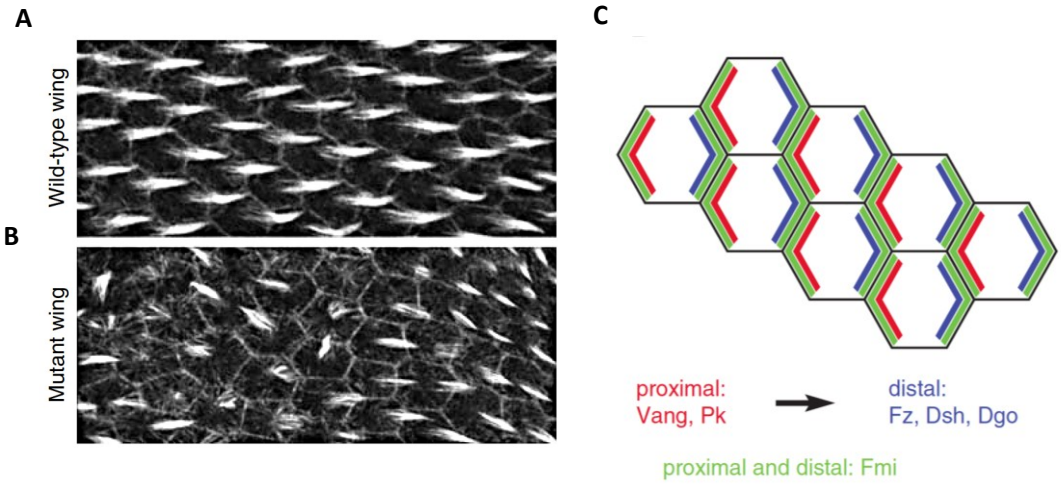
Schematic representation of planar cell polarity axis in relation of apical-basal polarity axis in polarized epithelial tissue. (Image adapted from Acta Pharmacologica Sinica (2011) review)

Table 1 Proteins associated with planar cell polarity

Global	Fat	Atypical Cadherin	Cell adhesion
Global	Dachsous	Atypical Cadherin	Cell adhesion
Global	Four-jointed	Golgi resident kinase	Phosphorylates Fat and Dachsous
Core	Frizzled	G-protein coupled receptor	Transduce proximal-distal polarity signal
Core	Dishevelled	Cytoplasmic phosphoprotein	downstream protein of frizzled
Core	Vangl	Membrane bound protein	Transmits directional signals of individual or groups of cells
Core	Prickle	Transcription factor binding protein	Regulates proximal polarity
Core	Ankryin repeat Diego	Tissue polarity gene	Competitor of prickle in regulating polarity
Core	Flamingo	Adhesion GPCR	Contact mediated cell communication
Effector	Rac	Small GTPases	Regulate intracellular actin dynamic
Effector	Rho A	Small GTPases	"
Effector	Cdc42	Small GTPases	"

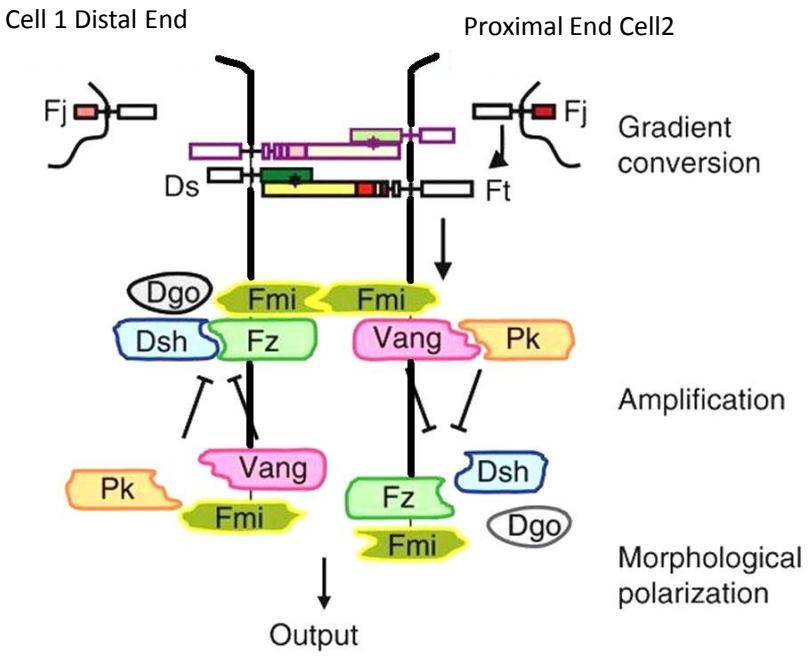
Global PCP components contain two atypical cadherins and a golgi resident kinase responsible for modulating activity of the atypical cadherins. The core module contains various transmembrane proteins (G protein coupled receptor, a four pass membrane protein, and adhesion G protein coupled receptor), and cytoplasmic proteins (phospho protein, and transcription factor binding proteins). The tissue effector module is composed of small GTPases

Figure 3 Localization of proteins at the proximal and distal end of cells



(A) PCP is important for orientation of hair in a proximal to distal growth pattern on the drosophila wing. (B)Mutations in the PCP proteins leads to dis orientation (swirly hair patterning). (C) Core PCP proteins localize at the proximal end (Vangl and Prickle) and distal end (Frizzled, Dachsous, and Diego) to coordinate the cell boundaries with the tissue axis. (image adapted from Vladar et al 2009)

Figure 4 PCP signaling



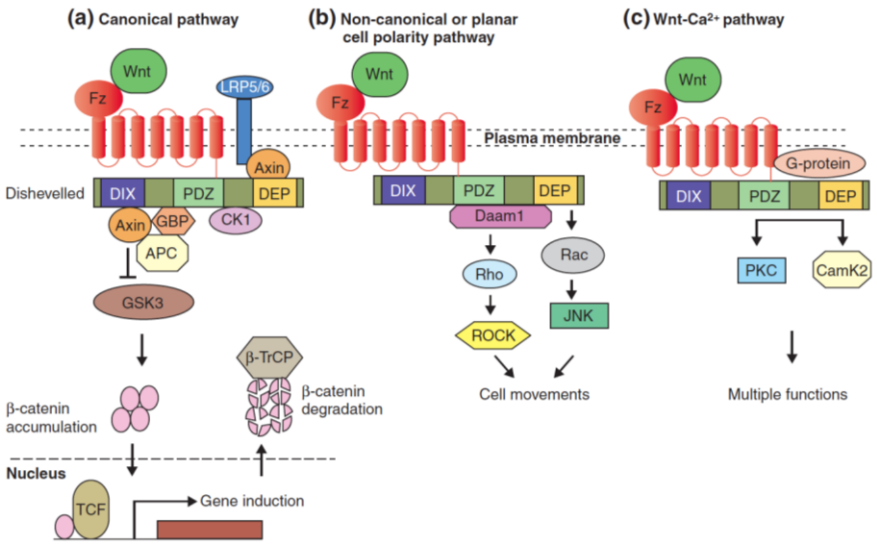
Schematic representation of a proposed PCP signaling model. Global module components (Fat (Ft) and Dachsous (Ds)) heterodimerize. Increase in heterodimerization of these proteins in one direction results in differential localization of core module components (Frizzled (Fz), Dishevelled (Dsh), Deigo (Dgo), Fmi(Flamingo), Vangl, Prickle (Pk)) to create in cell boundaries and activation of tissue effect module proteins (image adapted from Vladar et al 2009).

Table 2 Known Wnt proteins

Wnt Protein	Characterized As	Mouse	Human
Wnt 1	Canonical	X	X
Wnt2		X	X
Wnt2b/13		X	X
Wnt3	Canonical	X	X
Wnt3a	Canonical	X	X
Wnt4	Non canonical	X	X
Wnt5a	Non canonical	X	X
Wnt5b		X	X
Wnt6		X	X
Wnt7a	Canonical	X	X
Wnt7b		X	X
Wnt8a	Canonical	X	X
Wnt8b		X	X
Wnt9a		X	X
Wnt9b		X	X
Wnt10a		X	X
Wnt10b		X	X
Wnt11	Non Canonical	X	X
Wnt16		X	X

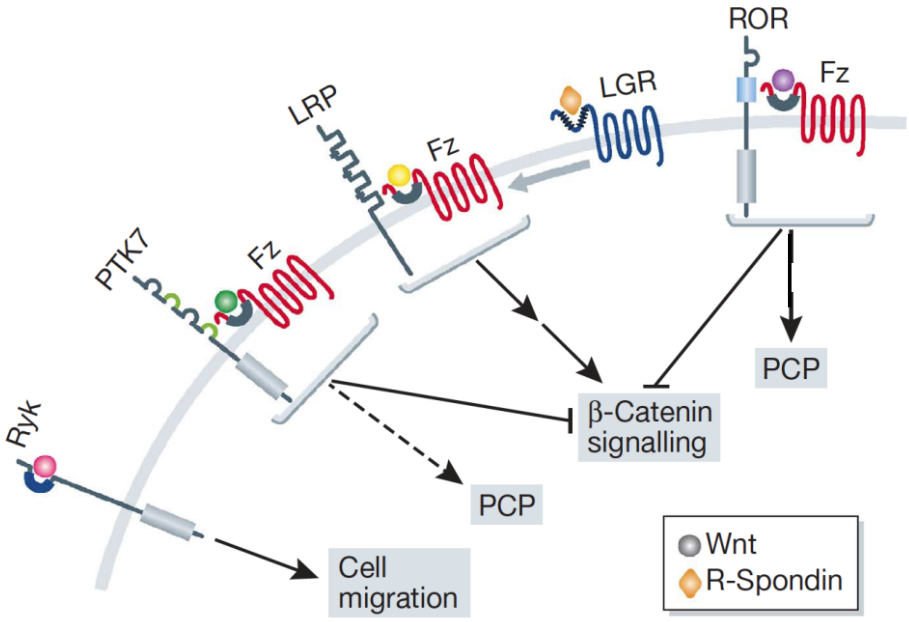
Table illustrates known Wnt proteins present in vertebrate. Some are proteins have been classified as canonical and noncanonical based on their primary signaling pathways.

Figure 5 Wnt signaling pathway



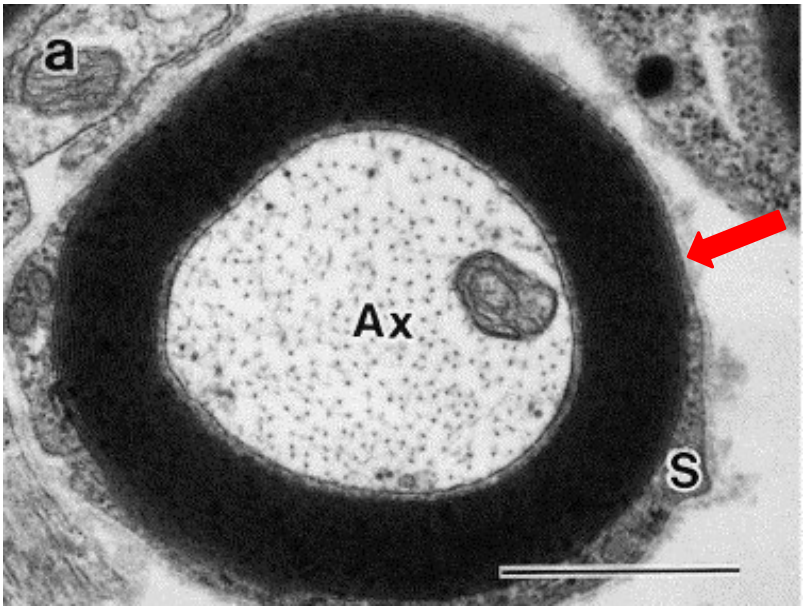
Schematic representation of the there Wnt signaling pathways. **(A)** Canonical Wnt signaling through the Frizzled (Fz) and LRP5/6 receptor complex prevents the degradation of β-catenin via Dishevelled (Dsh), Axin, glycogen synthase kinase 3 (GSK3) and casein kinase 1 (CK1). β-catenin accumulates in the cytoplasm and then translocates into the nucleus. In the nucleus it complexes with transcription factors to mediate transcriptional induction of target genes. **(B)** For Wnt/planar cell polarity (PCP) signaling, Wnt signaling is transduced through Frizzled independent of LPR5/6. Wnt Frizzled complex activates the PDZ and DEP domains of Dsh, inducing cytoskeletal rearrangement through activation of the small GTPases Rho and Rac. **(C)** For the Wnt-Ca^{2+} pathway, Wnt signaling via Frizzled mediates activation of heterotrimeric G-proteins, that in turn bind Dsh, calcium-calmodulin kinase 2 (CamK2) and protein kinase C (PKC). This pathway modulates cell adhesion and motility. (Image adapted from Wallingford, 2005)

Figure 6 Coreceptors of Wnt signaling



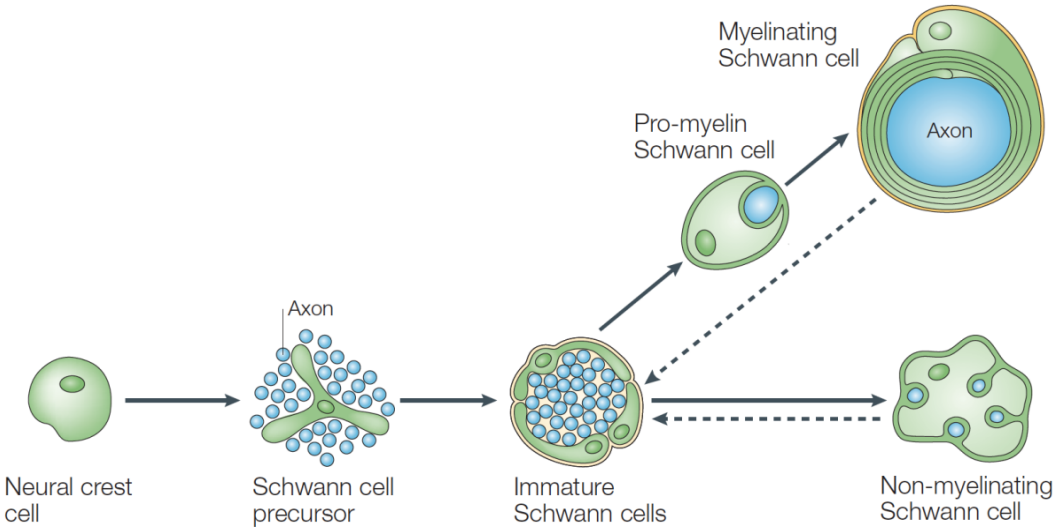
Schematic represent of coreceptor modulation of Wnt signaling. Though Frizzled proteins are the central receptors for Wnt signaling. The Wnt-Frizzled interaction is modulated by co-receptors. Wnt/Frizzled/LRP activates the canonical β-catenin/TCF signaling pathway. The presence of PTK7 or Ror inhibits β-catenin/TCF signaling and can activates Wnt/PCP signaling. Other receptors such as Ryk are thought to directly bind Wnt proteins independent of Frizzled receptors. (Adapted from Vincent and Beckett 2011)

Figure 7 The compact myelin sheath



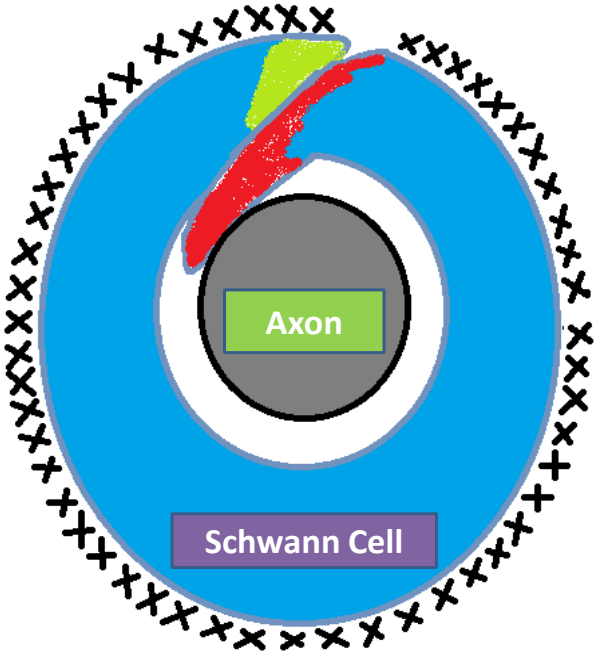
An electron micrograph of a cross section of a compact myelin sheath in the PNS. Red arrow indicates the compact myelin sheath, Axon (Ax), and Schwann cell (S). (Image adapted from Naito et al 2003)

Figure 8 Schwann cell lineage



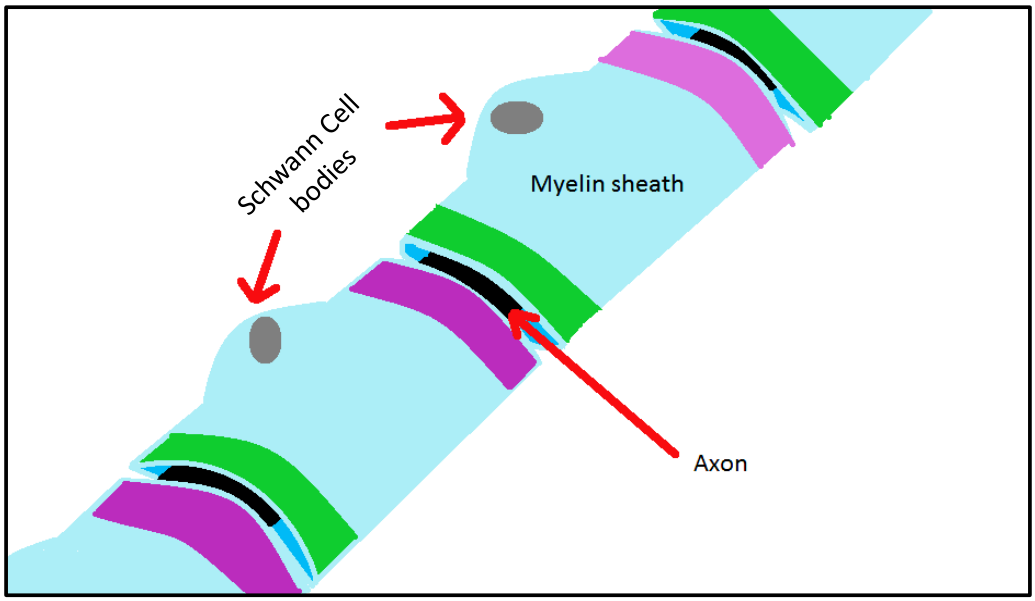
Schematic representation the developmental transitions in Schwann cell development. Migrating neural crest cells will travel ventral and upon reaching their location in the Dorsal root give rise to Schwann cell precursors. Schwann cell precursors will migrate and proliferated along growing axons. Immature Schwann cell fate is determined by the axons with which they associate. Myelination occurs only in Schwann cells that receive sufficient signal (Nrg1 type 3) from the axon. Schwann cells that do not make contact with axons with sufficient signal will ensheath multiple axons forming a Remark bundle and become mature non-myelinating cells. (Image adapted from Nature Review 2005)

Figure 9 Myelinating Schwann cells make a 1:1 association with axons



Schematic representation of cross section of Schwann cell axon 1:1 association. When Schwann cells make the initial wrap around the axon one edge of the schwann cell remains stationary (Green edge). While the opposite edge (Red edge) begins to spiral inward. This is indicative of a difference in molecular motors and protein composition at the spiraling edge compared to the stationary edge.

Figure 10 Number of Schwann cells myelinating an axon is regulated



Schematic presentation of Schwann cell organization along an axon. Schwann cell (light blue) number is highly regulated in order to maintain efficient nerve conduction and action potential velocity. Schwann cells exhibit contact inhibition at the nodes so that they do not overlap. Prevailing theories suggest that Nrg1 type 3 signal regulates Schwann cell number along the axon. However adjacent Schwann cell membranes due come into contact at the region of the node of Ranvier (region between two myelinating segments) which may allow for communication between Schwann cells to regulate number along the axon. This is reminiscent of communication between cells in epithelial tissue to coordinate the individual polarity with that of the tissue.

Figure 11 Schwann cells exhibit PCP-like cell boundaries

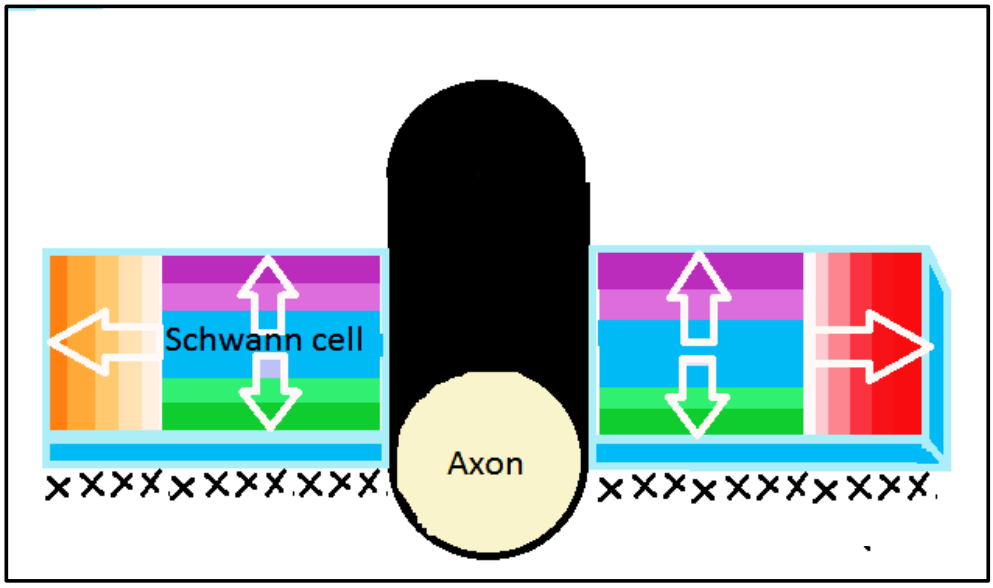
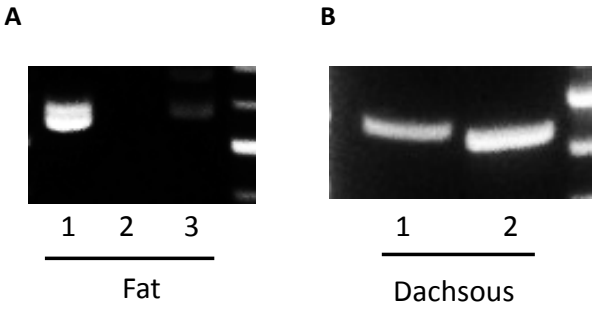


Figure 10 Schwann cells exhibit PCP-like cell boundaries

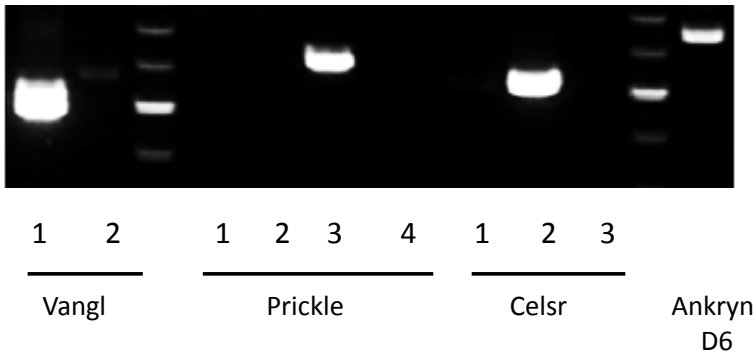
Schematic representation of a myelinating Schwann cell. The Schwann cell is unwrapped and flattened after the initial one turn. The opposite edges in contact with one another initially when unwrapped exhibit a similar protein distribution pattern as seen in epithelial tissue polarity by PCP core protein to create cell boundaries proximal and distally (Figure 1.3).

Figure 12 Global PCP components are present in Schwann cells



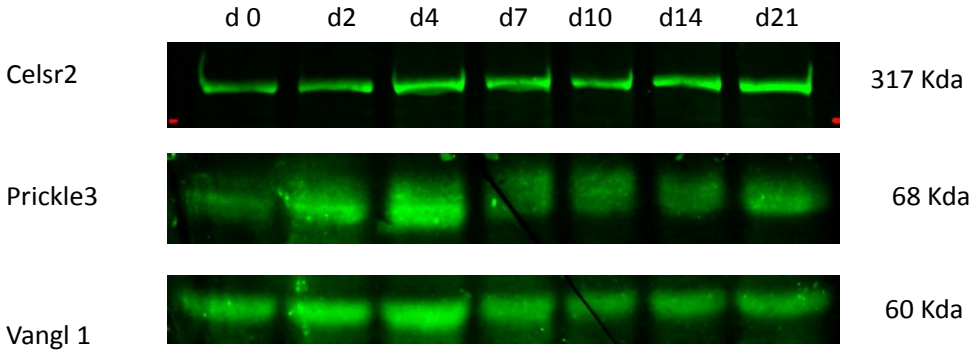
Gel electrophoresis image of PCR products for global module proteins in Schwann cells. **(A)** Atypical cadherin Fat has three known isoforms of which Fat1 mRNA is present in Schwann cells. **(B)** Atypical cadherin Dachsous (Dachsous 1 and Dachsous 2) has two known isoforms of mRNA of both is present in Schwann cells. All PCR products were made with specific primers for each protein.

Figure 13 Core PCP components are present in Schwann cells



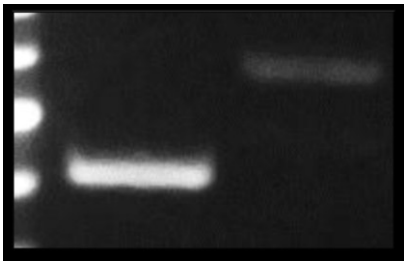
Gel electrophoresis image of PCR products of core module proteins in Schwann cells . There are two isoforms of transmembrane protein Vangl of which Vangl1 mRNA is present in Schwann cells. There are four isoforms of Prickle of which Prickle 3 mRNA is present in Schwann cells. There are three known isoforms of Celsr, Celsr 2 mRNA is present in Schwann cells. Ankryn D6 mRNA is present in Schwann cells. All PCR products were made with specific primers for each protein.

Figure 14 Core module proteins are expressed



Western Blot images of core module protein expression in myelinating Schwann cell and DRG neuron coculture extracts time course from d0 to d21. **(A)** Adhesion G protein couple receptor Celsr 2 is expressed at the correct molecular weight (317 Kda). **(B)** Transcription factor binding protein Prickle3 is expressed at the correct molecular weight (68 Kda). **(C)** Transmembrane protein Vangl1 is expressed at the correct molecular weight (60 Kda).

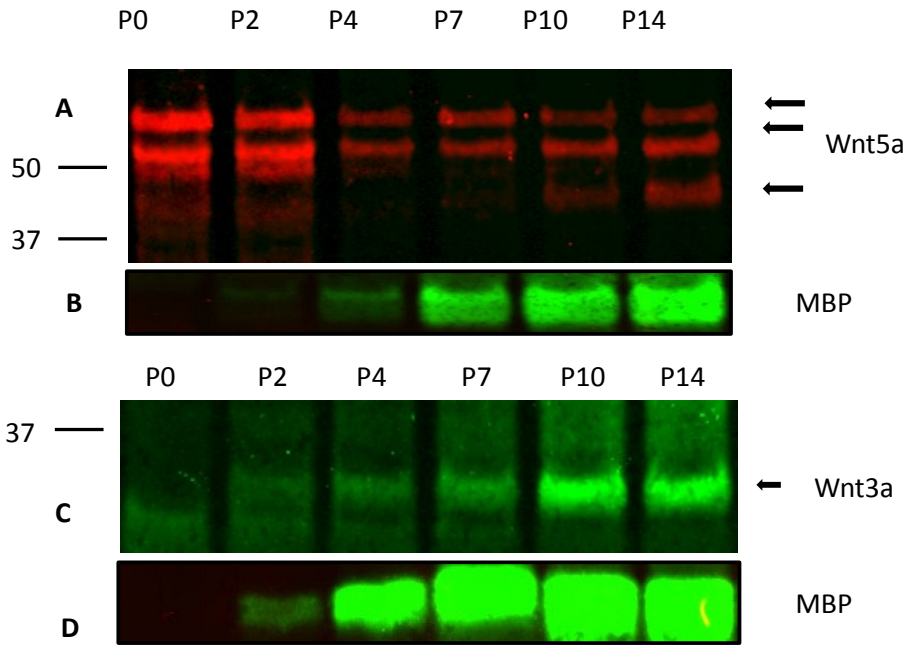
Figure 15 Wnt Co receptors Ror1 and Ror2 are present in Schwann cells



Ror1 Ror2

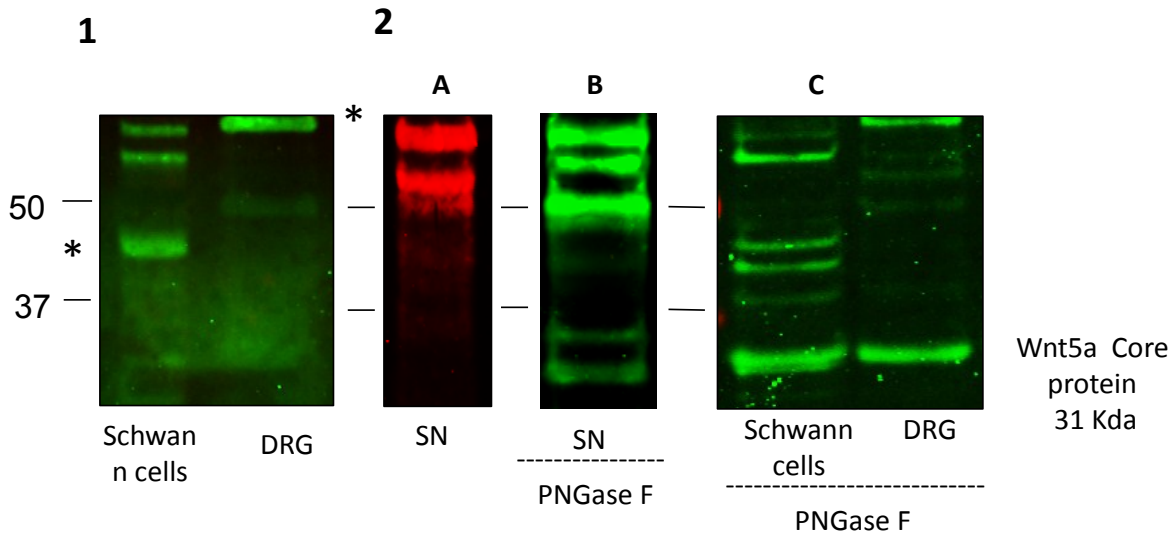
Gel electrophoresis image of PCR product of Wnt coreceptors in Schwann cells. mRNA of both Ror1 and Ror2 is present within Schwann cells. PCR was performed with specific primers for the Ror coreceptors.

Figure 16 Wnt5a and Wnt3a are present in the developing sciatic nerve



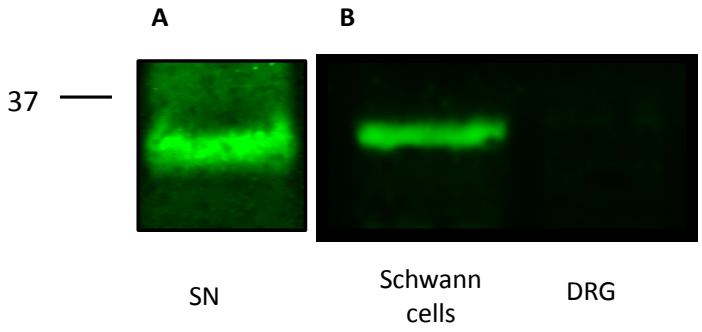
Panel 1: Western Blot image of sciatic nerve extracts analyzed for Wnt5a expression. Rat sciatic nerves were collected postnatally starting from right after birth (p0) until 14 days after (p14). Extracts were made in 2% SDS, analyzed by 10% Bis-tris gel and blotted for Wnt5a expression. **(A)** Wnt5a is expressed (doublet 65Kda and 70Kda) in the developing sciatic nerve at high levels right after birth and exhibits a sharp decrease in express at 4 days after birth and onward**. (B)** MBP (myelin basic protein) expression is consistent with developmental expression ensuring correct volumes were loaded and decrease in Wnt5a is due to expression levels and not sample loading error. Panel 2: Western blot image of Wnt3a expression in sciatic nerve extracts. Rat sciatic nerves were collected postnatally starting from right after birth (p0) until 14 days after (p14). Extracts were made in 2% SDS, analyzed by 10% Bis-tris gel and blotted for Wnt3a expression **(C)** Wnt3a is expressed in the developing sciatic nerve at ~ 35kda. Expression of Wnt3a is not detectable right after birth (p0) and increases with development. **(D)** MBP expression is consistent with development.

Figure 17 Wnt5a is a glycosylated protein and is present in the culture system



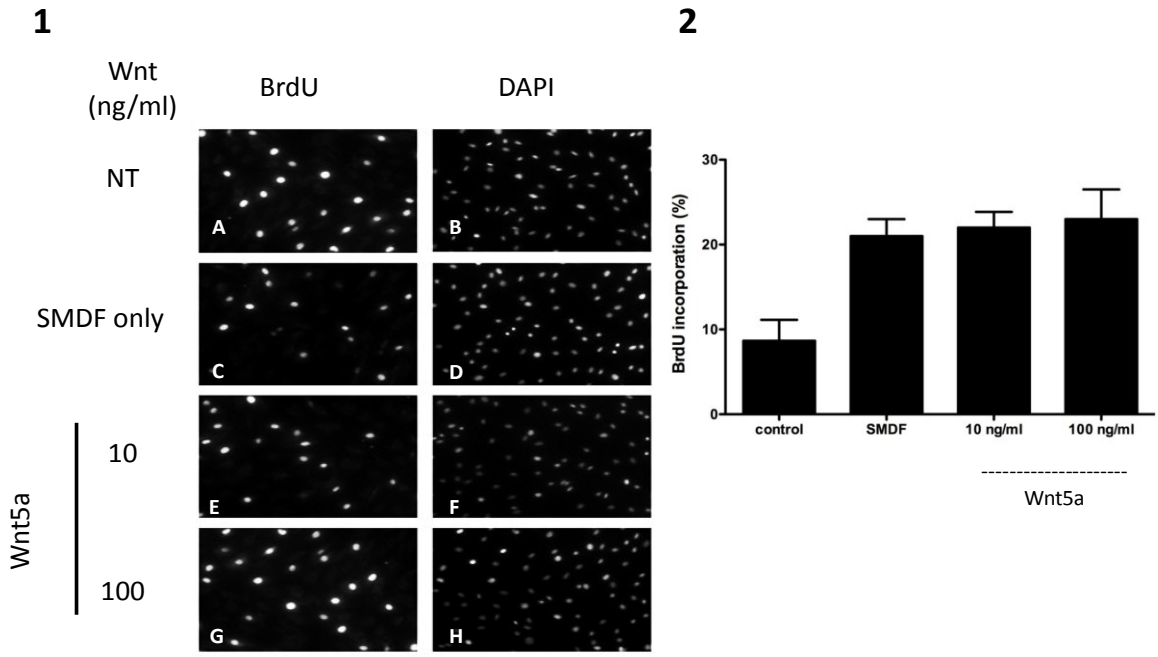
Western Blot image of Wnt5a expression in culture system . Lysates were prepared in 2 % SDS from cultures of primary rat Schwann cells only and cultures of embryonic DRG neurons only. Lysates were analysis by 10% bis-tris gel and blotted for Wnt5a expression. Panel 1: Wnt5a is expressed in both Schwann cells and DRGs. However both cells types exhibit a different expression pattern of Wnt5a in comparison to sciatic nerve expression of Wnt5a. (Prominent bands are denoted by the asterisk) Panel 2: Western blot analysis of glycosylation of Wnt5a in sciatic nerve, Schwann cells and DRGs. **(A)** Sciatic nerve expression of Wnt5a. **(B)** Wnt5a expression after deglycosylation of all N-glycosylated oligosaccharides by PNGase F. Appearance of 30 Kda band of core Wnt5a protein after deglycoslyation. Multiple banding pattern due to incomplete deglycosylation. **(C)** Wnt5a deglycosylated expression in Schwann cells and DRG neurons. Appearance of 30 Kda band and reduction in high molecular weight bands. Differences in banding pattern are indicative of varying levels of glycosylation of Wnt5a secretory regulation.

Figure 18 Wnt3a is present in Schwann cells of the culture system



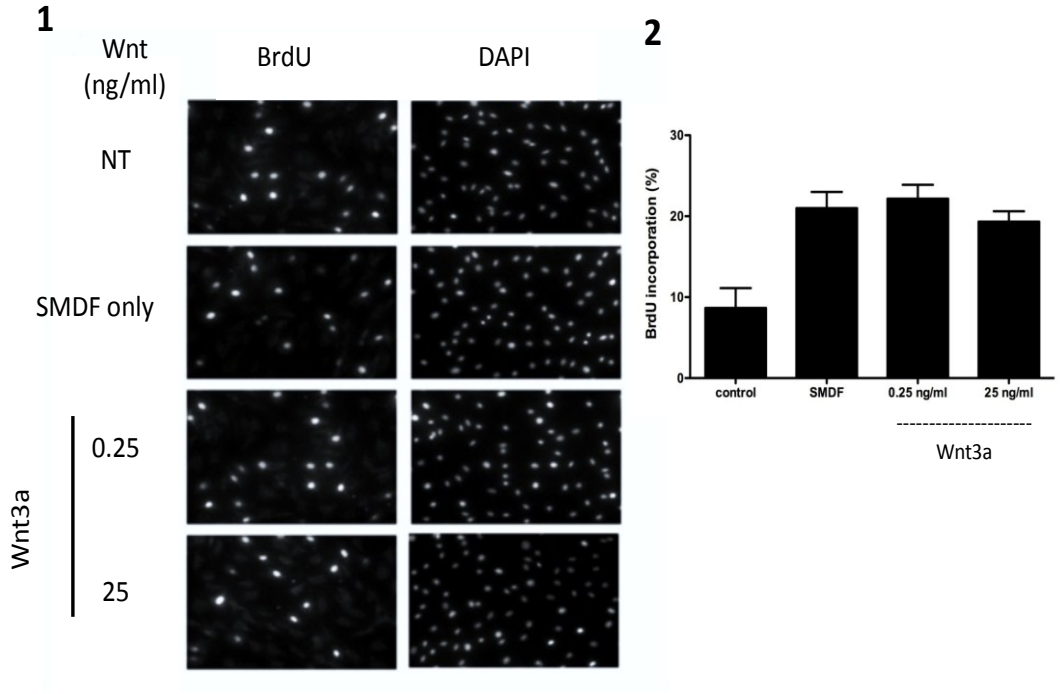
Western blot image of Wnt3a expression in culture system. Lysates were prepared in 2 % SDS from cultures of primary rat Schwann cells only and cultures of embryonic DRG neurons only . Lysates were analyzed by 10 % Bis- tris gel blotted and probed for Wnt3a **(A)** Wnt3a expression in sciatic nerve a band expressed at ~35 Kda**. (B)** Wnt3a is expressed in Schwann cells but is not expressed in DRG neurons.

Figure 19 Wnt5a does not effect SMDF mediated Schwann cell proliferation



Panel 1; Schwann cell cultures were maintained in DM for 24hrs with addition of SMDF and two different concentrations of Wnt5a protein. BrdU (left column) was incorporated at the last 4hrs of the 24hr period. Cells were fixed and stained for Dapi (right column). Panel 2; Quantitative analysis of SMDF mediated Schwann cell proliferation in the presence of Wnt5a treatment. Wnt5a treatment exhibited no significant effect on SMDF mediated Schwann cell proliferation.
Results are not significant $P > 0.05$.

Figure 20 Wnt3a does not effect SMDF mediated schwann cell proliferation



Panel 1; Schwann cell cultures were maintained in DM for 24hrs with addition of SMDF and two different concentrations of Wnt3a protein. BrdU (left column) was incorporated at the last 4hrs of the 24hr period. Cells were fixed and stained for Dapi (right column). Panel 2; Quantitative analysis of SMDF mediated Schwann cell proliferation in the presence of Wnt3a treatment. Wnt3a treatment exhibited no significant effect on SMDF mediated Schwann cell proliferation.
Results of Wnt treatment are not significant ($p > 0.05$) when compared to SMDF alone.

Figure 21 Wnt5a has no effect on Schwann cell proliferation in coculture

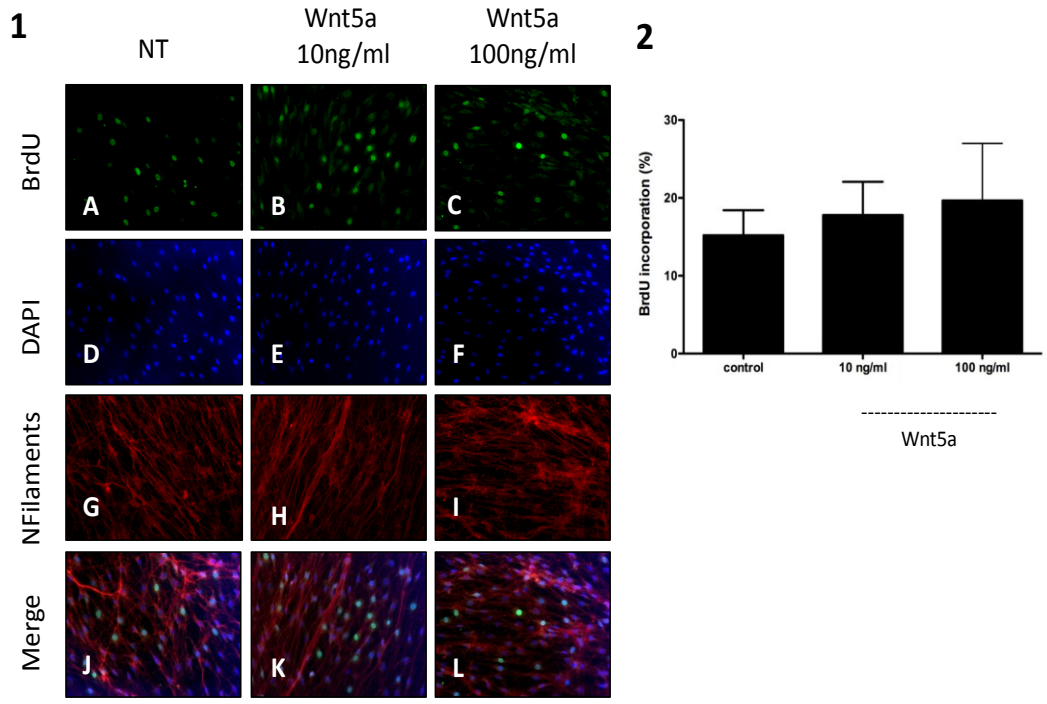
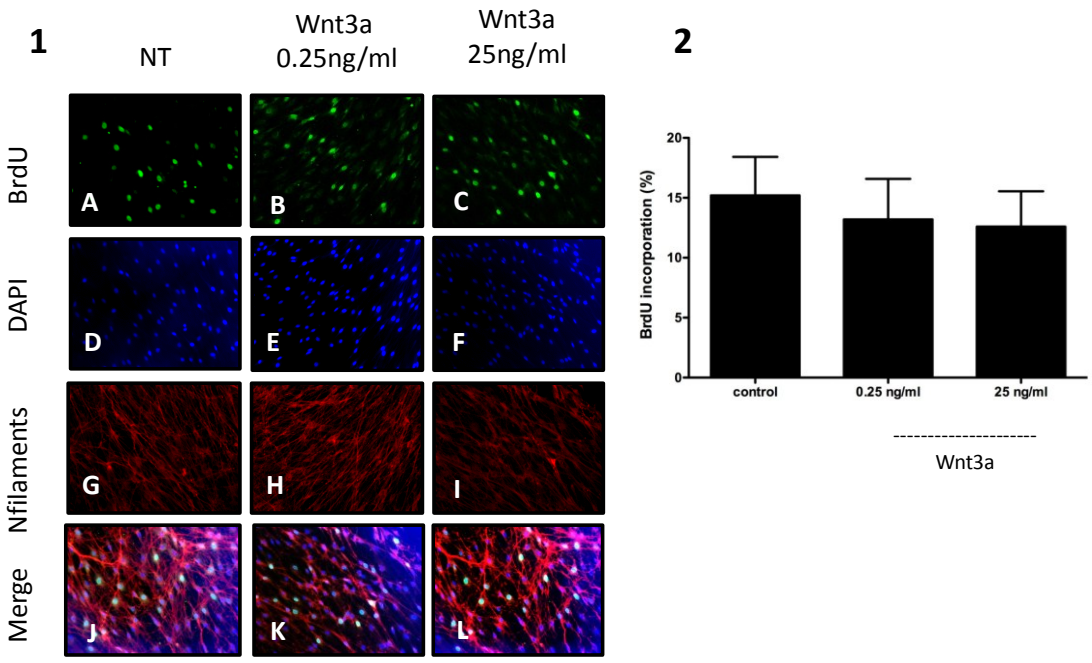


Figure 26 Wnt5a effect on Schwann cell proliferation in coculture

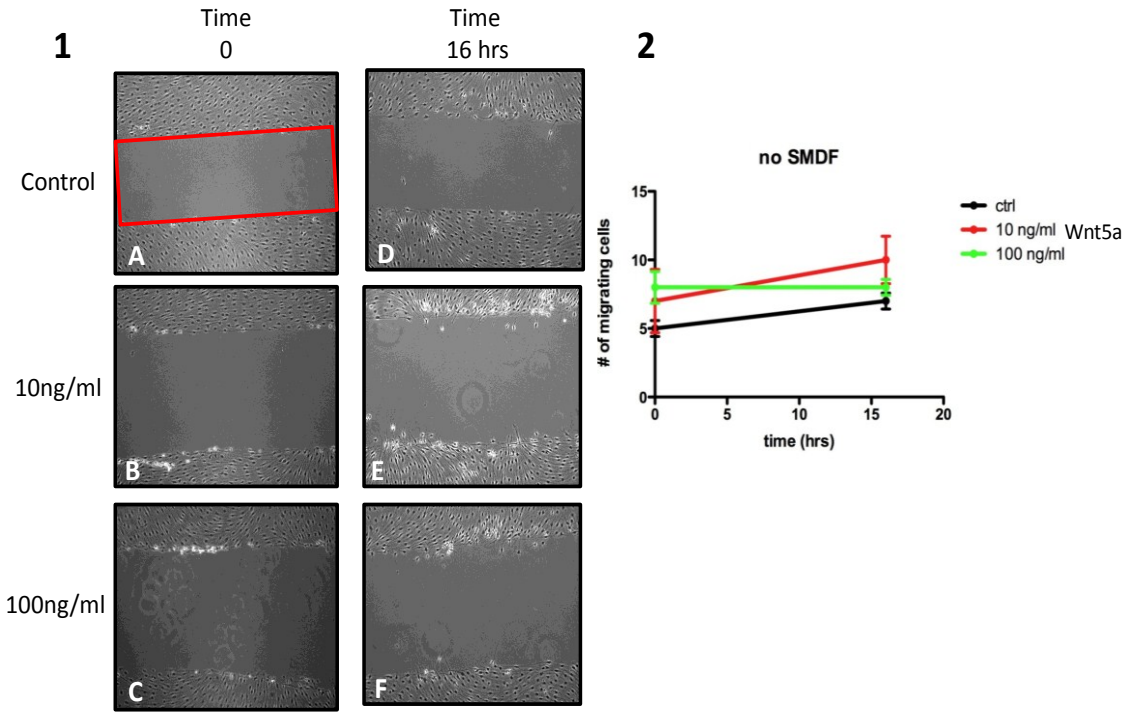
Panel 1; Schwann cell and DRG neuron cocultures were maintained in DM with Wnt5a protein at two different concentrations for 24 hrs. BrdU was incorporated during the last 4hrs . Cultures were fixed and stained for Dapi (Blue), neurofilaments (Red). All BrdU positive cells were counted. Panel 2; Quantitative analysis of Schwann cell proliferation in coculture, in the presence of Wnt5a treatment. Wnt5a treatment exhibited no significant effect on Schwann cell proliferation in coculture. Results are no significant ($p > 0.05$).

Figure 22 Wnt3a has no effect on Schwann cell proliferation in co culture



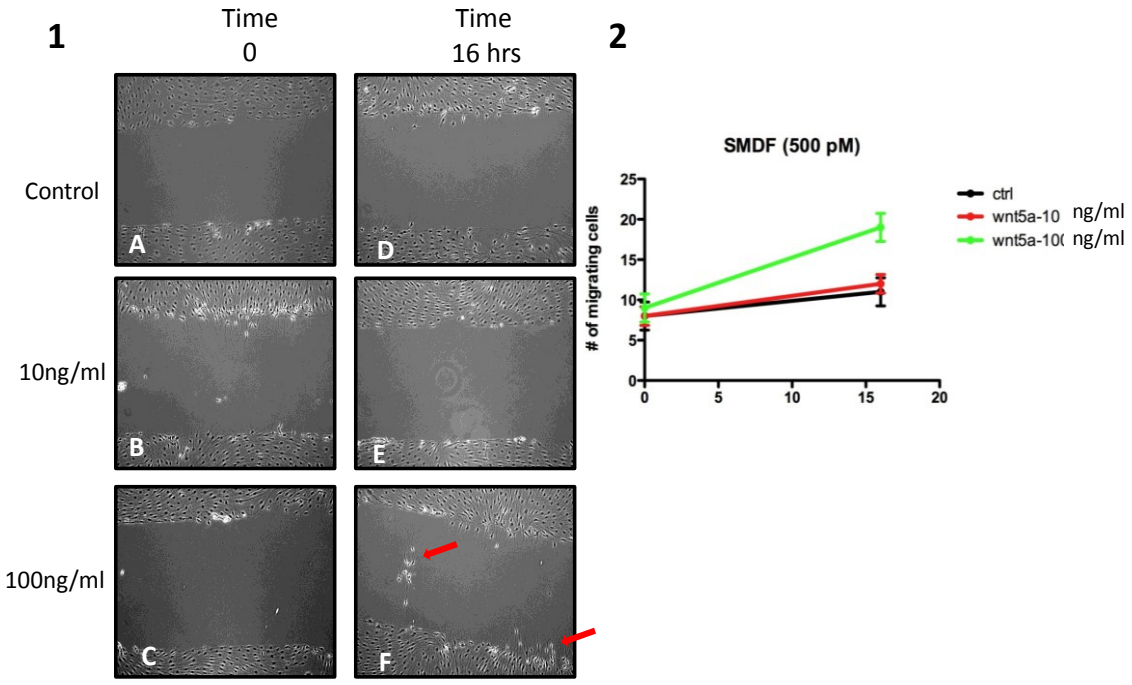
Panel 1: Schwann cell and DRG neuron cocultures were maintained in DM with Wnt3a protein at two different concentrations for 24 hrs. BrdU was incorporated during the last 4hrs. Cultures were fixed and stained for Dapi (Blue), neurofilaments (Red). All BrdU positive cells were counted. Panel 2: Quantitative analysis of Schwann cell proliferation in coculture, in the presence of Wnt3a treatment. Wnt3a treatment exhibited no significant effect on Schwann cell proliferation in coculture. Results are not significant ($p > 0.05$)

Figure 23 Wnt5a has no effect on Schwann cell migration in the absence of SMDF



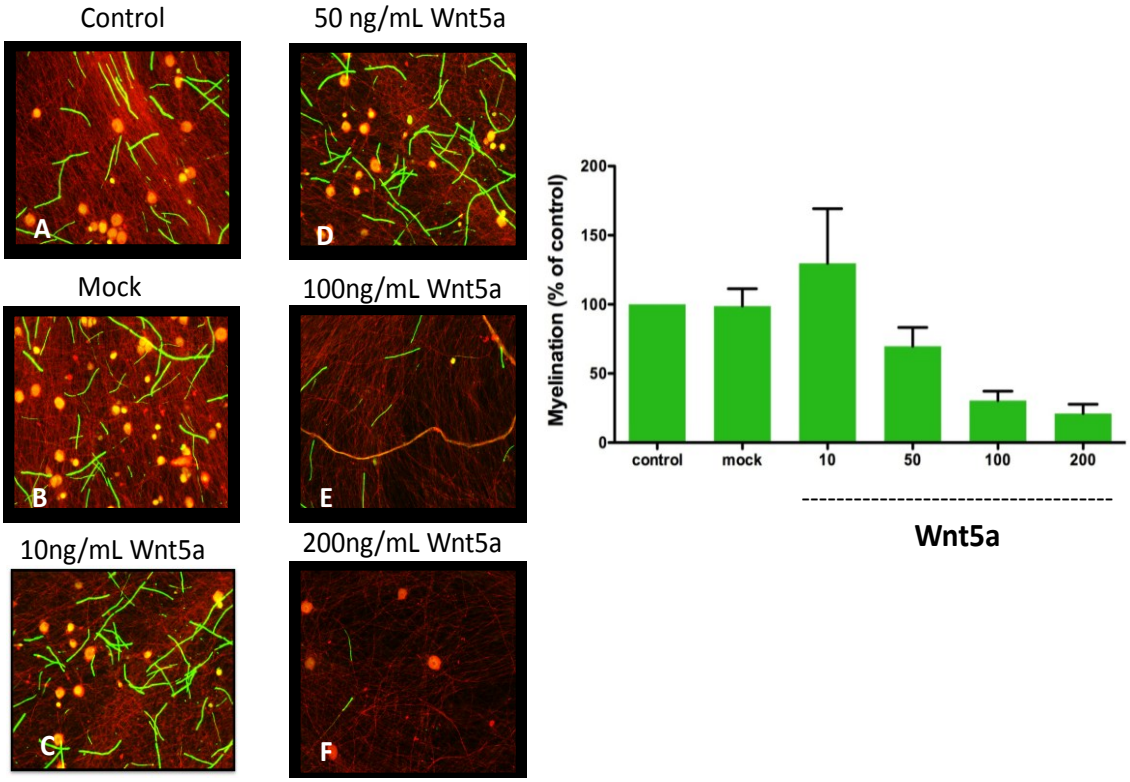
Panel 1: Schwann cell cultures were maintained in serum free conditions and the absence of SMDF. Wnt5a was added at two concentrations (10ng/ml and 100ng/ml). Phase contrast images were then taken at T0 (immediately after Wnt5s addition, Panels A to C) and again at T16 (16hrs after Wnt5a treatment, Panels D to F). Migrating cells were then counted for each time point. Migrating cells were defined as such, all cells that have cell bodies that moved past the initial scratch boundary (red box). Panel 2: Quantitative analysis of Wnt5a treatment effects on Schwann cell migration. No significant effect was seen on Schwann cell migration with Wnt5a at high (100ng/ml, green) or low (10ng/ml, red)treatment in the absence of SMDF. Results are not significant ($P > 0.05$).

Figure 24 Wnt5a enhances Schwann cell migration in the presence of SMDF



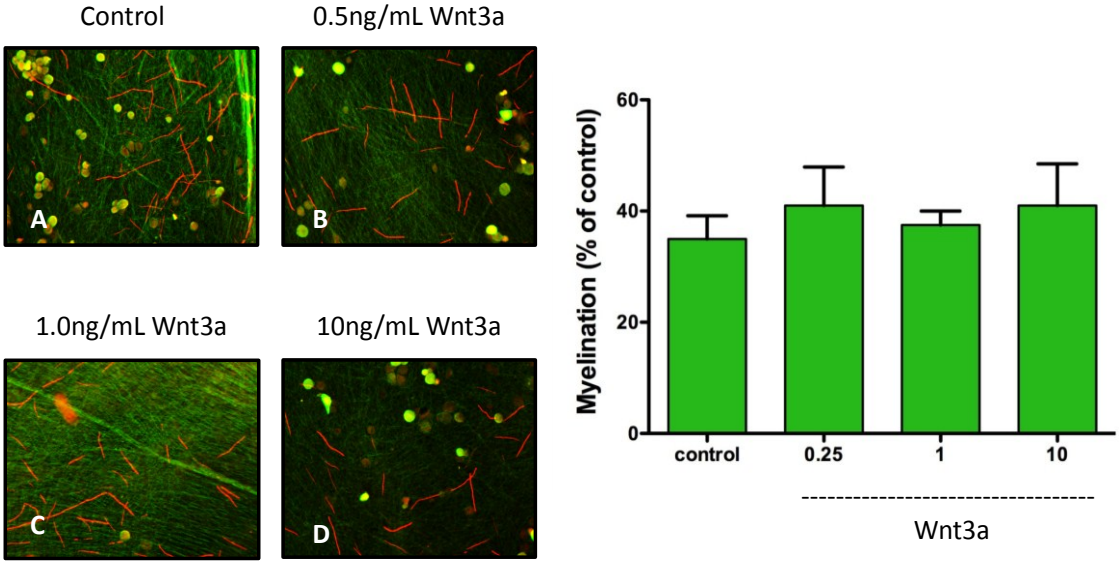
Panel 1: Schwann cell cultures were maintained in serum free conditions and the presence of SMDF. Wnt5a was added at two concentrations (10ng/ml and 100ng/ml). Phase contrast images were then taken at T0 (immediately after Wnt5s addition, Panels A to C) and again at T16 (16hrs after Wnt5a treatment, Panels D to F). Migrating cells were then counted for each time point. Increased number (Red arrows) in migrated cells after 16hrs of Wnt5a 100ng/ml treatment in the presence of SMDF was observed. Panel 2: Quantitative analysis of SMDF mediated Schwann cell migration with Wnt5a treatment. Low concentration treatment for (10ng/ml) Wnt5a (red) has no significant effect on Schwann cell migration. High Concentration (100ng/ml) of Wnt5a treatment enhances (green) SMDF mediated Schwann cell migration. Results for 100 ng/mL Wnt5a treatment are significant ($p < 0.05$).

Figure 25 Wnt5a treatment inhibits myelination in coculture



Panel 1: DRG neuron and Schwann cell cocultures were maintained for three days before myelination was initiated with the addition of asorbic acid. At this time DM was changed with the addition of Wnt5a (panel C- F) at increasing concentration (10ng/ml to 200ng/ml). Cultures were also treated with a mock solution (panel B). Cultures were fixed after fourteen days and were stained for MBP (green) and Neurofilaments (red). Images were taken and myelin segment number was counted. Panel 2: Quantitative analysis of myelin index. Wnt5a treatment did not have an effect on myelination at low concentrations (10ng/ml and 50ng/ml). Wnt5a treatment at high concentrations (100ng/ml and 200ng/ml) are inhibitory for myelination. Mock solution has no significant effect on myelination of cultures. Wnt5a concentrations >100 ng/ml are significant ($p < 0.0001$) for Wnt5a concentrations at 10, 50ng/ml and mock are not significant ($p > 0.05$) when compared to control for analysis.

Figure 26 Wnt3a has no effect on myelination in cocultures



Panel 1: DRG neuron and Schwann cell cocultures were maintained for three days before myelination was initiated with the addition of asorbic acid. At this time DM was changed with the addition of Wnt3a (panel B- D) at increasing concentration (0.5ng/ml to 10ng/ml) . Cultures were fixed after fourteen days and were stained for MBP (red) and Neurofilaments (green). Images were taken and myelin segment number was counted. Panel 2: Quantitative analysis of myelin index. Wnt3a treatment did not have an effect on myelination at low concentrations (0.5ng/ml and 1ng/ml) or high concentration (10ng/mL). Wnt3a treatment has no significant effect ($p > 0.05$).

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