

WNT/PCP SIGNALING AND MYELINATION IN THE PNS

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ABSTRACT OF THE DISSERTATION

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Myelination in Peripheral Nervous System (PNS) is established through Schwann cell (SC) migration, proliferation and radial sorting. After a phase of axonal association and SC radial sorting, SCs start to wrap around an axon at regular intervals before myelin compaction. A translational and transcriptional program elicits morphogenetic changes to SCs, which drives cytoskeleton rearrangement. Rho GTPases are the main effectors of dynamic cytoskeletal modification. Several studies implicate Rho GTPases, such as Rac1, Cdc42, and RhoA (and its downstream effector, ROCK) in SC proliferation, migration, radial sorting and compaction of myelin sheaths. Cdc42 is known to regulate SC proliferation, whereas Rac1 and RhoA show critical roles in myelination process such as radial sorting and formation of paranode compartment. Of many highly conserved signaling pathways by which morphogenetic changes can be established in SCs, the Wnt/Planar Cell Polarity (PCP) signaling pathway provides a versatile platform for understanding morphogenetic changes in SCs. Wnt/PCP pathway is known to promote cytoskeletal modifications through the activation of Rho GTPases, especially RhoA and Rac1, as effector modules to regulate actin polymerization. However, there

have been no studies to date investigating Wnt/PCP pathway in relation to PNS myelination.

In this study, I have characterized the presence of components for Wnt/PCP in SCs and their regulation during PNS myelination. Furthermore, I show that Wnt5a, a known ligand of the Wnt/PCP pathway, can modulate myelination in a culture model of myelination via a β -catenin-independent manner. In the context of purified SC culture, different concentrations of Wnt5a can elicit different responses of activation of Rho GTPases, especially RhoA and Rac1. Notably, 2.5 ng/ml of exogenous Wnt5a treatment showed hypermyelination compared to control, whereas > 200 ng/ml of Wnt5a had an inhibitory effect on myelination. Further investigations using two inhibitory constructs for Daam1, a key upstream regulator for small GTPases in Wnt/PCP pathway, revealed that Daam1/RhoA axis is necessary for the hypermyelination effect of Wnt5a (2.5 ng/ml). This Wnt5a concentration for hypermyelination does not affect the differentiation of Schwann cells into myelinating stage. Additionally, deletion of endogenous Wnt5a from SC resulted in less migration with higher expression of Prx, a non-migrating SC marker. Given the following two things; 1) existence of Wnt5a and machinery for Wnt/PCP signaling during myelination, and 2) cytoskeletal modulating function for Wnt5a without proliferative effect, my data suggest that Wnt/PCP signaling via Wnt5a/Daam1/RhoA axis may be involved in migration-association transition and/or spiral wrapping phase of SC lineage in the PNS.

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GLOSSARY OF ABBREVIATIONS

ABP	apical basal polarity
APC	adenomatous polyposis coli
CNS	central nervous system
Daam1	dishevelled-associated activator of morphogenesis1
dbcAMP	dibutyl cyclic adenosine monophosphate
DRG	dorsal root ganglion
Dvl	dishevelled
EdU	5-ethynyl-2'-deoxyuridine
Fzd	frizzled
HPRT	hypoxanthine-guanine phosphoribosyltransferase
IP3R	Inositol 1,4,5-Triphosphate receptor
MAG	myelin associated glycoprotein
MBP	myelin basic protein
MPZ	myelin protein zero
NFil	neurofilaments
NRG-1	neuregulin-1
OLs	dorsal root ganglion
OPCs	oligodendrocyte progenitor cells
PCP	planar cell polarity
PI	propidium iodide
PIP2	phosphatidylinositol diphosphate
PLC	phospholipase C
PLP	myelin proteolipid protein
PMP22	peripheral myelin protein 22
PNS	peripheral nervous system
Prx	periaxin
qRT-PCR	quantitative reverse transcription PCR
SC	Schwann cell
TCF/LEF	T-cell factor/lymphoid enhancer-binding factor

CHAPTER I

Background and Introduction

Myelin refers to a lipid-rich structure that is found surrounding the axons of neurons, in the Central Nervous System (CNS) as well as in the Peripheral Nervous System (PNS) (see Fig. 1). The oligodendrocyte in the CNS and the Schwann cell in the PNS are the specialized glia cells involved in forming the myelin sheath. The most recognized function of the myelin sheath is to facilitate and increase the propagation of the action potential, compared to non-myelinated axons. To understand that statement, I will in the next sections present i) the morphogenetic movements associated with myelination, ii) the structural organization of the myelin sheath and iii) the composition of the myelin sheath. The main focus will be on PNS myelination, and therefore on the Schwann cell.

Morphogenetic movements associated with PNS myelination

Schwann cells are derived from multi-potent cells called the Neural Crest Cells, or NCCs (1). As the NCCs migrate along the growing axons (Fig. 2) during early embryonic stages (embryonic days E11.5 to E15.5; (2, 3), they transition to the Schwann cell precursor stage under the influence of multiple axon-derived factors such as type III neuregulin-1 (type III Nrg1), Notch and FGF2 (4-6). This is essentially a proliferative, survival and migratory stage that allows the Schwann cell precursors to populate the axons (7) as they extend towards their target. Schwann cell precursors are characterized by the expression of several molecular markers, in particular $\gamma 1$, $\beta 1$ and $\alpha 6$ integrins (8, 9) that allow them to interact with the surrounding extracellular matrix. Under

the combined influence of axon-derived factors (type III Nrg1) and extracellular matrix-derived signals (mostly $\alpha 2$ and $\alpha 4$ chain-based laminins), the Schwann cells extend processes within the bundles of axons (Fig. 2; (9-19)). These processes, which are plasma membrane extensions, initially surround large groups of axons (early radial sorting; E12.5 to post-natal day p2; (3)). These large bundles of axons are further infiltrated by the Schwann cell plasma membrane extensions (late radial sorting; E17.5 to post-natal day p10; (3)) until axons are either i) in a 1-1 association with Schwann cells (1 Schwann cell, 1 axon) or ii) multiple axons associate with one Schwann cell (3, 20, 21). The Schwann cells undergoing radial sorting (Figs. 2 and 3) are called the immature Schwann cells, the Schwann cells in a 1:1 association with axons are the pro-myelinating Schwann cells, and the Schwann cells engulfing multiple axons in single pockets (yet 1 axon per pocket) are the non-myelinating Schwann cells (3). Through a process that is not fully understood, one edge of the pro-myelinating Schwann cell then passes under its own opposite edge (Fig. 2), and starts spiral wrapping around the axon. These wraps eventually compact under the action of several proteins, most notably the myelin basic protein (MBP), the myelin protein zero (Mpz) and the peripheral myelin protein 22 (PMP22), thereby leading to the formation of the compact myelin sheath (Figs 2 and 3).

Structural and molecular organization of the myelin sheath/axon unit

The myelin sheath is not a continuous structure all along an axon. It is interrupted at regular intervals by gaps that are called the nodes of Ranvier (Figs. 1 and 5). The myelin sheath, in between nodes, is referred to as a myelinated segment, and is made by one Schwann cell in the PNS.

As Schwann cells migrate and elongate along axons until they form a 1:1 association with the underlying axon, the longitudinal ends extend microvilli that make contact with the axon. These microvilli are enriched in cell adhesion molecule NrCAM that associates with extracellular matrix protein gliomedin to form a high-affinity ligand for axon-expressed cell adhesion molecule Neurofascin 186 (NF186; (22-24)). This interaction recruits and confines axonal AnkyrinG (AnkG) which then recruits voltage-gated sodium channels (Nav1.6; (25)). As adjacent Schwann cells elongates, their microvilli “drag” along the NF186/AnkG/Nav1.6 complexes. When adjacent tips meet, they fuse and form the node of Ranvier (24). The complex is further stabilized by the recruitment of scaffolding protein β IV spectrin on the axonal side (26). While the Nav1.6 channels are responsible for the axonal membrane depolarization and regeneration of the action potential, the axonal membrane at the node is also enriched in voltage-gated potassium channels (Kv3.1b) that are crucial to ensure directionality to the action potential, down along the axon (27, 28).

Next to the nodes are the paranodal loops and paranodal junctions (Fig. 5). These loops are formed when the Schwann cell plasma membrane spiral-wraps of around the axon. When the multi-lamellar structure compacts to form the myelin sheath, the longitudinal extremity of every wrap is not compacted, thereby leaving the presence of an un-compacted cytoplasm-filled loop for every turn of the plasma membrane around the axon. The paranodal loops are in tight association with the underlying axon, through septate-like junctions mediated by cell adhesion molecule Neurofascin 155 (NF155) on the paranodal loops and cell adhesion molecules Contactin and Contactin-associated protein 1 (Caspr1) on the axon (29). The paranodal loops are further stabilized by the

recruitment of the scaffolding molecule 4.1B under the axonal membrane (29). The tight junctions between the paranodal loops and the axonal plasma membrane electrically insulate the space between the axon and the myelin sheath from the extracellular environment, thereby contributing to the insulating properties of the myelin sheath.

The formation of the paranodal loops also contributes to the proper localization of juxta-paranodal components (30, 31). The juxta-paranodal is next to the paranode, and just at the beginning of the compact myelin sheath (Fig. 5). The juxta-paranodal domain is not identifiable by any ultra-structural features, but is defined by the very localized enrichment in specific sets of molecules: Caspr2, Kv1.1 and Kv1.2 on the axon, 4.1B under the axonal membrane, and cell adhesion molecule TAG-1 on the Schwann cell plasma membrane (29, 32, 33).

The myelin sheath itself represents about 99% of the overall myelinated segment length. As alluded to in the previous section, the myelin sheath is made by a plasma membrane extension of the Schwann cell, which first surrounds and then spiral-wraps around an axon, creating a multi-lamellar structure (Figs. 2 and 3). That multi-lamellar structure then compacts through the action of specific myelin proteins: MBP, Mpz and PMP22 (34-43).

This repeating organization, node-paranode-juxtaparanode-myelin sheath-juxtaparanode-paranode-node represents what is referred to as longitudinal polarity. It is critical to the major function of the myelinated axon unit, i.e. efficient and fast propagation of the action potential

Composition of the myelin sheath

Although MBP, Mpz and PMP22 represent the bulk (57-90%) of the myelin proteins (MBP: 5-15%; Mpz: 50-70%; PMP22: 2-5%; (44-47)), myelin proteins account for only about 15 to 30%, by dry mass, on the myelin sheath molecular composition. The major defining characteristic of the myelin sheath is its high, 70 to 85% lipid content, by dry mass (48-50). The major lipids of the PNS myelin sheath are cholesterol, galactocerebrosides, sphingomyelins and phosphatidylcholines (about 27%, 16%, 17% and 19% by lipid dry weight, respectively, in rat PNS; (46, 49, 51)). The myelin sheath is also characterized by the nature of the fatty acid chains that are a part of the myelin lipids. About 75% of the fatty acids are composed of very long carbon chains (\geq C18; 18 or more carbons) and are mostly unsaturated (52). Myelin lipids carry several functions, the major ones being: i) compaction of, and maintenance of the myelin sheath and ii) electric insulation of the underlying axon.

i) For example, mice that are deficient in cholesterol synthesis in the PNS are hypo-myelinated, and present many features of un-compacted myelin (53, 54). These features correlate with an associated decrease in myelin proteins that are implicated in myelin compaction. It is interesting that cholesterol, along with sphingomyelins interact with membrane-bound myelin proteins while self-assembling in the trans-Golgi network to form lipid rafts (55, 56). Both sphingomyelins and cholesterol have an impact on the sorting and trafficking of myelin proteins that are involved in compaction (53, 57). Myelin sheath abnormalities are also prominent in various lipid metabolism disorders (and their animal models) associated with galactocerebrosides synthesis dysfunction (48, 49, 51).

ii) A plasma membrane's main function is to establish a selective barrier between the inside of a cell and its extracellular environment (58). Specialized carrier proteins are involved in the regulated transport of molecules in and out of a cell (59, 60). Although small, uncharged molecules such as water can, very slowly, passively diffuse through the lipid bilayer of a plasma membrane, charged molecules such as Na^+ , K^+ , Ca^{2+} , although small, cannot. The hydrophobic nature of the core of the plasma membrane, formed by the hydrophobic fatty acid chains of phospholipids, prevents any significant amount of passive diffusion of charged molecules. The myelin sheath, which results of the compaction of several wraps (up to 40) of a plasma membrane that is inordinately rich in lipids (70 to 85%) is therefore a particularly inadequate structure to allow ionic exchanges between the axon cytoplasm and the extracellular environment, along most of the myelinated axon length. The myelin sheath acts therefore as an electric insulating material that isolates the axon from the conductive extracellular environment. This is further reinforced by the electric isolation of the extracellular space between the myelin sheath and the axon from the general extracellular environment by the tight association of the paranodal loops to the axons through septate-like junctions (Fig. 5; (30, 31)).

Myelinated axon structure and saltatory conduction

When an action potential is initiated, voltage-gated Nav1.6 channels open, which allows for an influx in Na^+ inside the action (61). The Na^+ ions then quickly diffuse away from the initial site of depolarization. As they do, they depolarize adjacent sections of the axonal plasma membrane (Fig. 4), generating an action potential that open the voltage-gated sodium channels in

these adjacent sections. More Na^+ ions enter, further spreading the action potential. The ability of the influx of Na^+ ions to elicit an action potential at a distance is limited by 2 factors: i) as the Na^+ ions diffuse, their concentration decreases, which decreases their ability to maintain a net positive charge on the cytoplasmic side of the axonal plasma membrane; ii) in addition to gated channels, the axonal plasma membrane contains non-gated channels that are involved in maintaining the resting plasma membrane potential. These channels “leak cations outside the cell to re-establish the resting membrane potential and affect how far an influx of Na^+ can propagate the action potential. In an un-myelinating axon, the spread of the Na^+ ions inside the axon is limited and new Na^+ influx need to be generated at short distances. The constant need to open voltage-gated sodium channels in a slow process. By insulating 99% of the axon from the conductive extracellular environment with the myelin sheath and paranodal junctions to reduce “leakage” from non-gated resting channels, a myelinated axon takes advantage of the passive spread of the Na^+ ions inside the axon, whose levels remains sufficiently high to elicit an action potential at a longer distance from the initial site of depolarization. As passive spread of ions is a fast process, myelinated axons propagate action potential at a faster speed than un-myelinated axons.

Molecular control of PNS myelination

The molecular control of myelination is best understood in the PNS. PNS myelination is the end result of a very complex crosstalk between: i) the Schwann cell and the underlying axon, and ii) the Schwann cell and its surrounding extracellular matrix. In effect a myelinating Schwann cell is “sandwiched” between the axon and the extracellular matrix (Fig. 6) and need

to coordinate and integrate signals from both sides in order to radial sort out axons and myelinate.

i) The transition between a non-myelinating and a myelinating Schwann cell is driven by the abundance of axon-bound type III neuregulin-1 (type III Nrg-1; (62, 63)). There are four neuregulin genes, Nrg-1 to Nrg- 4 (64-67), but Nrg-1 has to date been the most studied. The Nrg-1 gene encode for several splice variants that are classified in 6 main types, I to VI (68). All of them have an EGF-like domain that is necessary and sufficient to bind to their receptors (69), which belong to the ErbB family of tyrosine kinase receptors. Schwann cells express ErbB2 and ErbB3. All Nrg-1 isoforms are synthesized as trans-membrane proteins. They however undergo a cleavage processing that is mediated by the neuron-derived β -secretase 1 (BACE1; (70)). All types, except type III, are then released as soluble growth factors that function as paracrine signaling molecules. Type III is anchored to the plasma membrane through 2 trans-membrane domains and therefore remains tethered to the axon after BACE1 cleavage to function as a juxtacrine signal. Several isoforms of Nrg-1 have long been known to regulate many aspect of Schwann cell biology, such as proliferation, survival and migration (71-75), except for the process of myelination. It was not until 2004 and 2005 that 2 seminal research articles (62, 63), analyzing the phenotypes of mice heterozygotic for type III Nrg1, demonstrated that type III Nrg1 was the key instructive signal that determined Schwann cell fate, i.e. i) a non-myelinating Schwann cell associated with multiple axons in a Remak bundle, or ii) a myelinating Schwann cell associated with an axon in a 1:1 relationship (Fig. 2). The phenotypic choice is driven by the abundance of axon-bound type III Nrg-1. Small axons, expressing lower

levels of type III Nrg-1 compared to larger axons, are ensheathed by Schwann cells in Reamk bundles. Larger axons, expressing larger amounts of type III Nrg-1 induce the Schwann cells to form a 1:1 association, to spiral wrap and to form a compact myelin sheath. Furthermore the levels of type III Nrg-1 on axons regulate the extent of myelination by determining the number of lamellae that myelinating Schwann cells will form around axons, and therefore the thickness of the myelin sheath (62, 63). Nrg-1 activates 3 major signaling pathways within the Schwann cells: i) the PI3 kinase/Akt pathway, ii) the Ras/Raf/Erk pathway and iii) the PLC γ /Ca²⁺/calcineurin pathway. While all three signaling cascades are positively involved in PNS myelination (76-84), the activation of the PI3 kinase/Akt pathway is key to the Schwann cell response to the type III Nrg-1, from proliferation to survival, from myelination to myelin thickness (76-78, 81).

ii) While the axon-bound type III Nrg-1 signal has been shown to be the on/off switch to PNS myelination, regulation of the actin cytoskeleton by the extracellular matrix and small GTPases of the Rho family has been shown to be critical to the radial sorting of axons. Radial sorting is dependent on the formation of lamellipodia by Schwann cells. As Schwann cells closely associate with and elongate along axons, the lamellipodia are classified in 2 categories: i) lamellipodia localized at both extremities of the long axis area called axial lamellipodia; ii) lamellipodia that are localized >20 degree off the long axis are classified as lateral lamellipodia (85). Rac1 activity in immature Schwann cells is critical to radial sorting, which is severally impaired in Rac1-deficient mice (85, 86). A similar phenotype is observed in β 1 integrin-null mice (85), in which Rac1 activity is reduced (85, 86). Interestingly while the formation of peripheral lamellipodia was impaired, β 1 integrin-null Schwann cells still formed axial

lamellipodia, which led Nodari et al (85) to hypothesized that $\beta 1$ integrin activates Rac1 to allow immature Schwann cells to switch from a migration/elongation mode (low Rac1 activity, axial lamellipodia, directional cell migration (87)) to a radial sorting mode (high Rac1 activity, peripheral lamellipodia extension into axon bundles). Small GTPase Cdc42, along with Rac1, has also been shown to promote Schwann cell migration (88).

It is interesting to note that while the molecular bases that regulate radial sorting and Schwann cells fate are starting to be well understood, the mechanisms that drive and control spiral wrapping are still a mystery. Recent findings in CNS myelination by oligodendrocytes however clearly indicate that a dynamic regulation of the actin cytoskeleton is required (42).

Radial polarization of the Schwann cell

In the section “Structural and molecular organization of the myelin sheath/axon unit”, I concluded by noting that the organization of the myelin/axon unit is polarized along the long axis of the axon, i.e longitudinal polarity. In a similar fashion, the Schwann cell, sandwiched between the axon and the basal lamina, exhibit a polarity along the radial axis of the myelin/axon unit (Fig. 6), hence the expression of radial polarization. The Schwann cell membrane in close contact to the axon is called the adaxonal membrane. It is characterized by the presence of molecules that are, among others, necessary to mediate axo-glial interactions (Nectin-like cell adhesion molecules Nectin2 and Nectin4; (89)) and to receive the pro-myelinating type III Nrg1 signal (ErbB2 and ErbB3 tyrosine kinase receptors; (10, 14, 62, 72)). The Schwann cell membrane in close contact to the basal lamina is called the abaxonal membrane. It is devoid of the molecules expressed at the adaxonal membrane, and is enriched

in molecular components dedicated to transducing extracellular matrix signals, in particular such as $\gamma 1$ (16, 19), $\beta 1$ (11) and $\alpha 6$ (9) integrins, dystroglycan (90) and Lgi4 (18).

Apical-basal cell polarity and myelination

Cell polarity reflects the asymmetrical distribution of proteins within a cell. Most of our understanding on cell polarity was initially derived from studies on *Drosophila* epithelia in which the apical-basal polarity (ABP) and the planar cell polarity (PCP) have been characterized (91-93).

Apical-basal polarity is established and maintained by 3 complexes of conserved proteins: the Par3/Par6/aPKC complex, the Pals1/Patj/Crumbs complex, and the Dlg1/Scrib/Lgl complex (94-96). As the structural and molecular organization of the myelin/axon unit strongly indicates that the Schwann cell is a polarized cell, the requirement of the 3 apical-basal polarity complexes in Schwann cell myelination has been explored. A recent study has demonstrated the importance of Par-3 in Schwann cell myelination (97). The authors showed that Par-3 becomes asymmetrically localized to the adaxonal Schwann cell membrane upon axonal contact. They also established that Par-3 has a key role in promoting myelination by recruiting the p75 neurotrophin receptor (p75^{NTR}) to this site at the onset of myelination. These results were the first to directly implicate cell polarity as a key event in the initiation of myelination. Further studies then showed that Pals1 and Dlg1 play a role in regulating the longitudinal extension and thickness of the myelin sheath, respectively (76, 98, 99). More recently Pals1 has also been shown to be involved in the process of radial sorting by Schwann cells in the PNS (100).

Planar cell polarity

The studies on *Drosophila* epithelia further identified a second type of polarity, perpendicular from the apical-basal polarity, and extending within the plane of the epithelia (Fig. 7). This polarity was coined “planar cell polarity”, or PCP. PCP is essential for a variety of developmental events that involve cell-fate decisions, morphogenesis, and organized cell movements (101). A number of non-epithelial morphological processes in vertebrates are controlled by vertebrate homologs of PCP genes, and appear to involve cell polarization (102).

The pathways required to establish molecular asymmetry within and between cells in PCP rest on three main protein modules: 1) a global module consisting of non-classical cadherins Fat and Dachsous (103) and the Golgi-resident protein Four-jointed (104-106); 2) a core module which includes the multi-pass transmembrane proteins Vangl, Celsr (107, 108), and Frizzled (Fzd; (109, 110), and the cytosolic proteins Prickle (111-113) and Disheveled (114-116); and 3) tissue specific effector module proteins) that function downstream of the global and core modules to generate cell-specific cytoskeletal modulation (117).

It is interesting to note that the effector module proteins of PCP are small GTPases (118), in particular Rac1, RhoA and Cdc42, all of which are important components of the PNS myelination process, radial sorting in particular (85, 86, 119). Yet the involvement of PCP in regulating small GTPases and PNS myelination has not yet been investigated. It is however interesting to speculate on the potential for PCP to regulate several aspects of Schwann cell development, from its origin in neural crest cells (NCCs), to radial sorting and

spiral wrapping.

NCCs are a transient, migratory cell population that make a cell-fate decision and differentiate upon reaching their final destination. In the case of the dorsal root ganglia (DRG) PNS formation, NCCs give rise to both Schwann cell precursors and DRG sensory neurons (6). This cell fate decision appears to be the result of the asymmetrical localization of the Notch antagonist protein NUMB upon neural crest cell division (120). NUMB-positive cells do not respond to Notch1 signaling and differentiate into neurons, while Notch1 signaling in the NUMB-negative cells direct them to differentiate into Schwann cell precursors (6, 120). Asymmetric cell division results from the asymmetric orientation and position of the mitotic spindle, which depends on apical-basal polarity (121). In the absence of a clear axis of apical-basal polarity (NCCs are mesenchymal cells), it is however unclear how NCCs establish asymmetry and localize NUMB in only one of the daughter cell after mitosis when they have reached the DRG position. Interestingly the PCP pathway has also been involved in the positioning of the centrosome and regulates asymmetric cell division (122-124).

Prior to myelination, immature Schwann cells send lamellipodial extensions radially into the axon bundles. This process, referred to as radial sorting, is a polarized phenomenon as the immature Schwann cells are wrapped around the axon bundles and send the lamellipodia within it. While the structural organization of the Schwann cell, sandwiched between axons and basal lamina may provide polarity clues, the role of PCP, and in particular of Wnt/PCP signaling (see next section) in this process has not been investigated. Yet Wnt molecules, signaling to small GTPases through the PCP machinery,

are ideally suited to provide a spatially localized regulation of the formation of the lamellipodia needed for radial sorting.

Finally, once a Schwann cell has made a 1:1 association with an axon, both side of the Schwann cell come in contact with each other, edge to edge. One edge will then move inward, between the Schwann cell and the axon, and start the process of spiral wrapping. While the molecular mechanisms are still unknown, this suggests that both edges are different. In an epithelium, this is the core function of PCP: to provide cell positioning information to individual cells within an epithelium in relation to all others, by asymmetrically localizing factors between the two lateral sides of the cells. An interesting question: could we extrapolate this principle to a unique Schwann cell making contact with itself, one lateral edge with the other one, once having wrapped around an axon?

Wnt signaling and myelination

Wnt proteins (19 in mammals) are secreted lipid-modified signaling glycoproteins, which function in development by regulating cell fate, proliferation, polarity, and migration. Wnt molecules signal through Fzd receptors and co-receptors such as Ror2 and LRP5/6 (125, 126). Upon Wnt binding, Fzd receptors recruit and form a complex with Dishevelled (Dvl), and is then internalized (127-130). Wnt/Fzd binding elicits several pathways that are classified in 3 main groups: i) the canonical Wnt/ β -catenin pathway, ii) the non-canonical Wnt-PCP pathway, and iii) the non-canonical Wnt-calcium pathway.

The activation of canonical Wnt/ β -catenin pathway leads to the inactivation of GSK3, which results in the accumulation of β -catenin in the cytoplasm and subsequent translocation to the nucleus where it acts as a transcriptional co-activator by binding with members of the T-cell

factor/lymphocyte enhancer factor family (TCF/LEF) of transcription factors (131, 132). In the absence of Wnt ligands, β -catenin is targeted for proteasomal degradation (133). The best characterized direct target gene for Wnt/ β -catenin is Axin2, which is typically used regarded as one of the best indicator of the activity of Wnt/ β -catenin signaling pathway (134). Although several Wnts can activate several of the main Wnt pathways, the actual pathway activated within a cell is determined by cellular context (135). However Wnt3a has been reported to be a specific ligand to the canonical Wnt/ β -catenin pathway in many cell types (136-138).

In contrast, the non-canonical pathways do not utilize β -catenin. As a representative ligand for the non-canonical Wnt/PCP pathway, Wnt5a has been shown to regulate small GTPases (effector module of PCP) by binding to the core module protein Fzd (isoforms 2, 3, 5) and to the orphan receptor tyrosine kinase Ror2 co-receptor (139, 140). This interaction results in the recruitment of Dvl. In turn, Dvl recruits Daam1 through interactions with PDZ and DEP domains. While Dvl serves as a platform to regulate small GTPase Rac1 activity (116), DAAM1 is involved in the regulation of small GTPase RhoA (141). Interestingly, in some instances, the Wnt5a/PCP can intersect with the canonical Wnt/ β -catenin pathway, blocking the accumulation of β -catenin (Fig. 8) (142). The molecular mechanism is not clear but appears to involve the co-receptor ROR2 (126).

Finally, the non-canonical Wnt/calcium pathway causes increased calcium concentration that activates calcineurin and Ca^{2+} /calmodulin-dependent kinase II (CaMKII). Unlike the other two Wnt pathways, the non-canonical Wnt/ Ca^{2+} pathway utilizes Fzd receptors (isoforms 2, 3, 4 and 6) to

interface with a G-protein directly. This interaction leads to the activation of phospholipase C (PLC; (143)), and the subsequent production of DAG and IP₃ from the plasma membrane component phosphatidylinositol diphosphate (PIP₂). IP₃ releases calcium from the ER by binding to its receptor IP₃R on the ER (144). It is interesting to note that this pathway regulates the activity of transcription factors of the NFat family (145), one of which, NFatc4 is a positive regulator of PNS myelination (83). While type III Nrg1 has been shown to activate NFatc4 (83), the intersection with the Wnt5a/calcium pathway provides an intriguing possible point for regulatory control that will be interesting to investigate in future studies on PNS myelination.

To date, studies on Wnt signaling relating to myelination have focused on key components of the canonical Wnt/ β -catenin signaling pathway. There is a growing consensus that in the CNS the Wnt/ β -catenin pathway provides a negative control to myelination by delaying differentiation of oligodendrocytes (146-149). Indeed, experiments using genetic and pharmacological activation of the canonical Wnt pathway result in delayed myelination by inhibiting oligodendrocyte differentiation (146-149). For example, conditional deletion of APC, a negative regulator of canonical Wnt signaling, in oligodendrocyte progenitor cells (OPCs) prevents their differentiation (150). In the PNS, GSK3- β inhibition, which leads to the accumulation of, and translocation of β -catenin into the nucleus, promotes Schwann cell differentiation and enhances myelination (149). Recent results from Grigoryan et al (151) suggest that in the PNS the canonical Wnt pathway is involved in promoting progression of the Schwann cells to the pro-myelinating stage. On the other hand however, conditional deletion of APC in Schwann cells results in hindlimb weakness and

impaired nerve conduction in sciatic nerves (152), suggesting that the canonical Wnt pathway is inhibitory to myelination as well in the PNS. The potential discrepancy regarding the function of the Wnt/ β -catenin pathway on myelination could reflect differences between PNS and CNS in terms of cellular context. It is clear also from several studies, for example on type III Nrg1 (62) and Pals1 (100), that oligodendrocytes and Schwann cells have developed different strategies to achieve the same goal, i.e. the formation of a myelin sheath. Indeed while type III Nrg1 is the on/off signal to PNS myelination, and Pals1 is necessary to radial sorting, neither one of them is required or necessary to CNS myelination (62, 100).

Scope of this thesis work

Recent studies on the role of the canonical Wnt pathway in PNS myelination is providing burgeoning evidence that at least some Wnt molecules (153) are present in the developing PNS. However, despite the strong evidence implicating small GTPases Rac1, RhoA and Cdc42 (85, 86, 154) in the radial sorting process by Schwann cells, that these small GTPases are the *bona fide* downstream effectors of Wnt/PCP (118) and that myelination is a highly polarized process, the involvement of PCP, and Wnt/PCP signaling in the process of myelination by Schwann cells has yet to be investigated.

The scope of my thesis work has been to identify the major components of the PCP pathway expressed by Schwann cells. I then investigated the function of the Wnt5a/Dam1/RhoA PCP pathway in Schwann cell myelination, using the long-standing and well-established culture model of PNS myelination (155) that has been central to many key insights in the molecular mechanisms involving axo-glial interactions, domain formations and

myelination (29, 30, 81, 89, 156-159).

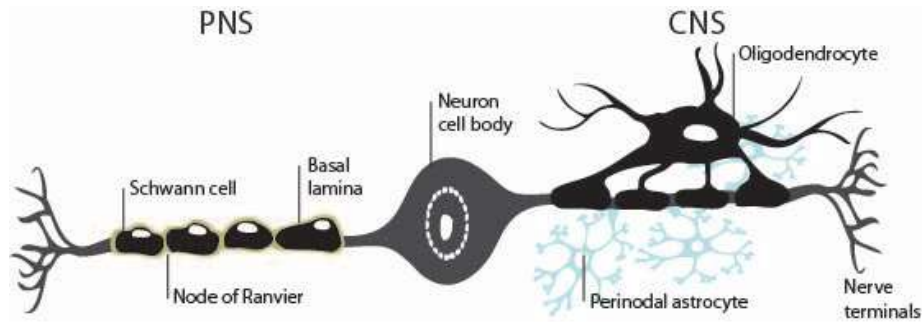


Fig. 1 Comparison of the myelinated axon in PNS and CNS.

Myelinating glial cells (Schwann cells in the PNS, at left; and oligodendrocytes in the CNS, at right) form myelin sheaths by extending their plasma membrane around the axon. This can be in the form of initial 'radial sorting' or 'spiral wrapping' during myelination. Schwann cells cover an axon from node to node by extending microvilli and basal lamina, while perinodal astrocytes contact the exposed node in CNS.

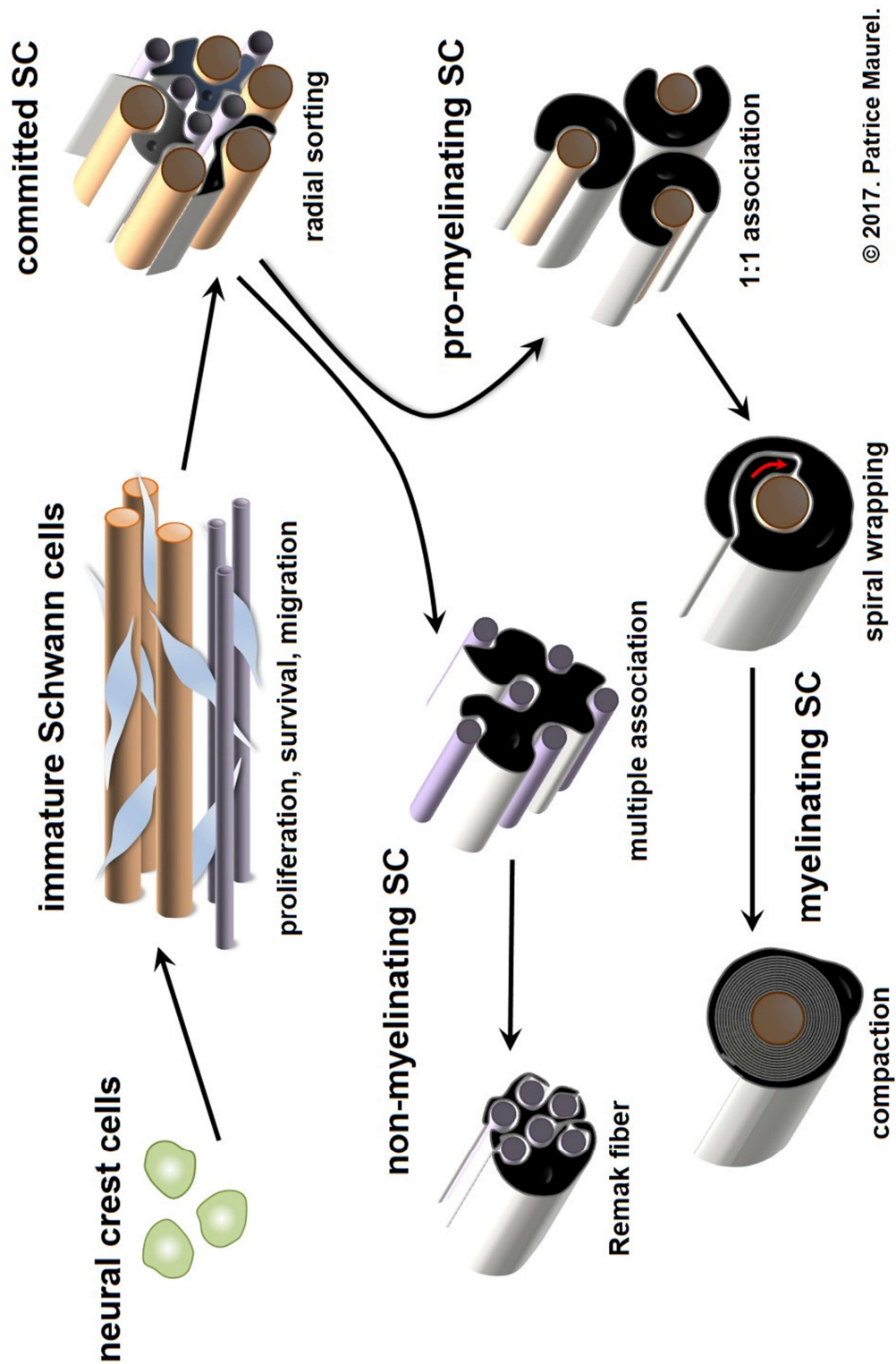


Fig. 2 Steps of Schwann cell differentiation. Myelinating and non-myelinating Schwann cells in the PNS are derived from neural crest cells. Prior to the association period (usually before E18 in rodent), the Schwann cell undergoes migration (< E16.5 in rodents) from the DRG's to peripheral sites around the body. Myelinating Schwann cells begin associating with a large caliber axon and segregate them from axon bundles of small caliber, a process called "radial sorting". Non-myelinating Schwann cells segregate small caliber axons from an axon bundle by multiple association, storing it into Remak bundle.

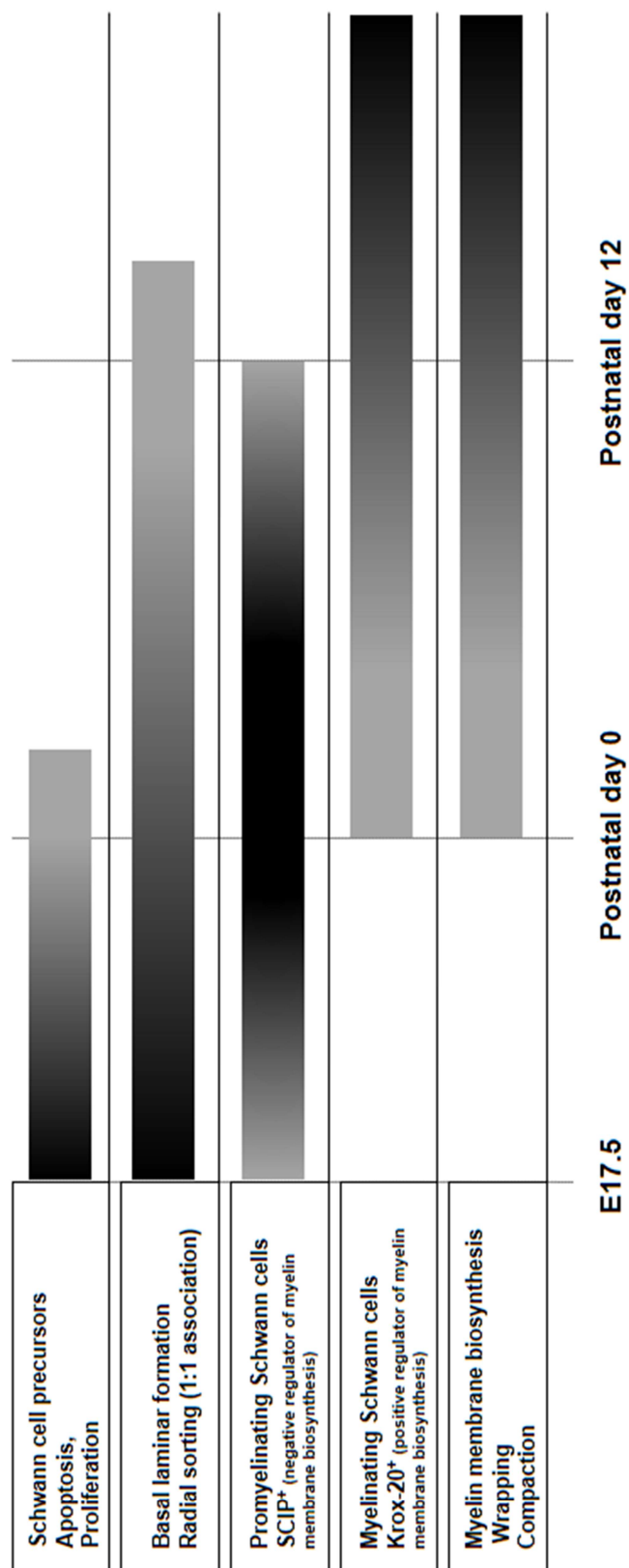


Fig. 3 Chronology of normal PNS myelination of the rodent model system.

From embryonic day (E) 12-13 to the time of birth, Schwann cells actively proliferate and undergo apoptosis. The transition from the migrating Schwann cell to the associated Schwann cell with an axon occurs from E18.5 to postnatal day 4. Normal myelination of the PNS of rodent is a process that begins at birth and culminates at about 3 months after birth.

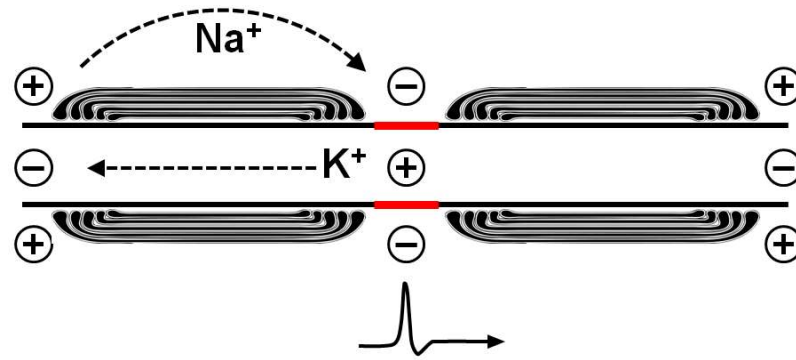


Fig. 4 Saltatory conduction in a myelinated axon. When the action potential initiates from soma site, the influx of Na^+ ions then quickly diffused away from the initial site of depolarization. As they do, they depolarize adjacent sections of the axonal plasma membrane, generating an action potential that open the voltage-gated sodium channels in these adjacent sections. More Na^+ ions enter, further spreading the action potential. Node of Ranvier was coded with red.

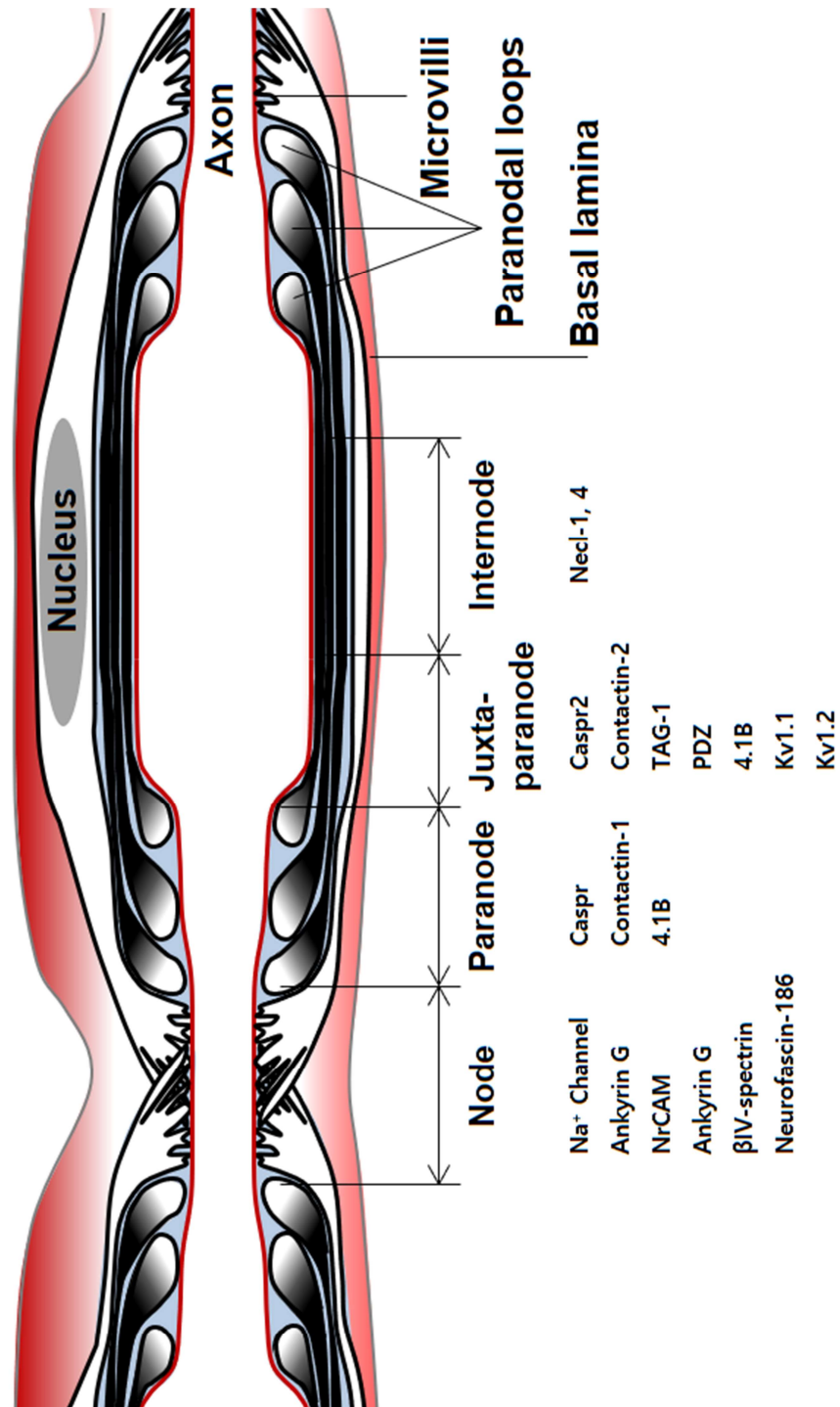


Fig. 5 Longitudinal compartmentalization of axon by myelination.

Molecules of the node include Na⁺ channel, NrCAM, Neurofascin-186, Ankyrin G, β IV-spectrin. The axonal membrane in the paranode contains Contactin and Caspr, which bind to Neurofascin-155 on the paranodal side of myelin. The juxtaparanode can be distinguished by distribution of a multiprotein complex containing TAG-1, Caspr2, PDZ, 4.1B, Kv1.1, and Kv1.2. Necl-4 is expressed in Schwann cell along the internode and juxtaparanode to bind Necl-1 on axonal membrane.

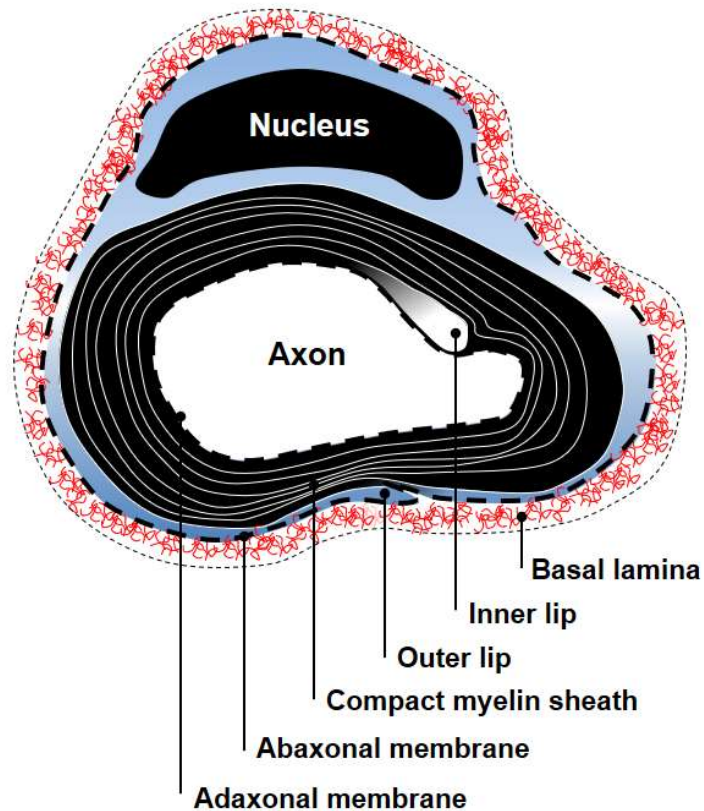


Fig. 6 Structural illustration of transverse section of myelinated axon at the internode. Inner lip of the myelin membrane continuously moves underneath the compact myelin sheath, whereas outer lip is stationary. The Schwann cell membrane in close contact to the axon is called the adaxonal membrane, whereas Schwann cell membrane in contact to the basal lamina is called abaxonal membrane.

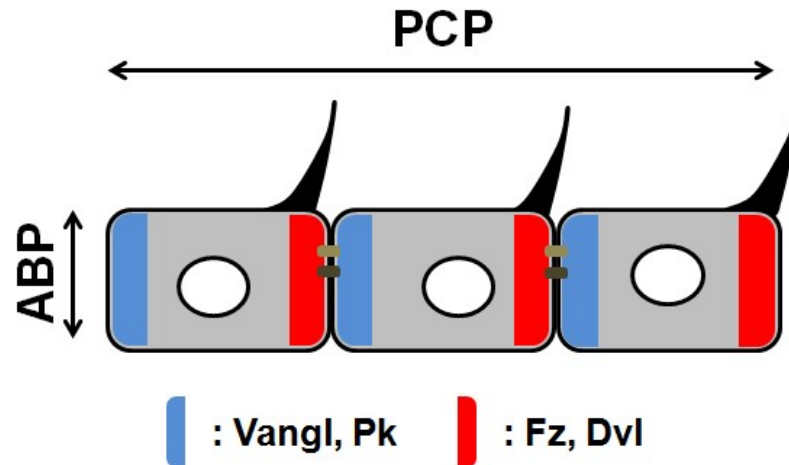


Fig. 7 Reciprocal subcellular localization of planar cell polarity (PCP) protein in the epithelium. The studies on epithelia identified two types of polarity, apical-basal polarity (ABP) and planar cell polarity (PCP), which is perpendicular to ABP. Three epithelial cells that line longitudinally are shown, with PCP components at proximal or distal face. Proximal face and distal face are coded with blue or red, respectively. Pk, Prickle; Strabismus (STB)/Vangl-like, Vangl; Fz, Frizzled; Dvl, Dishvelled.

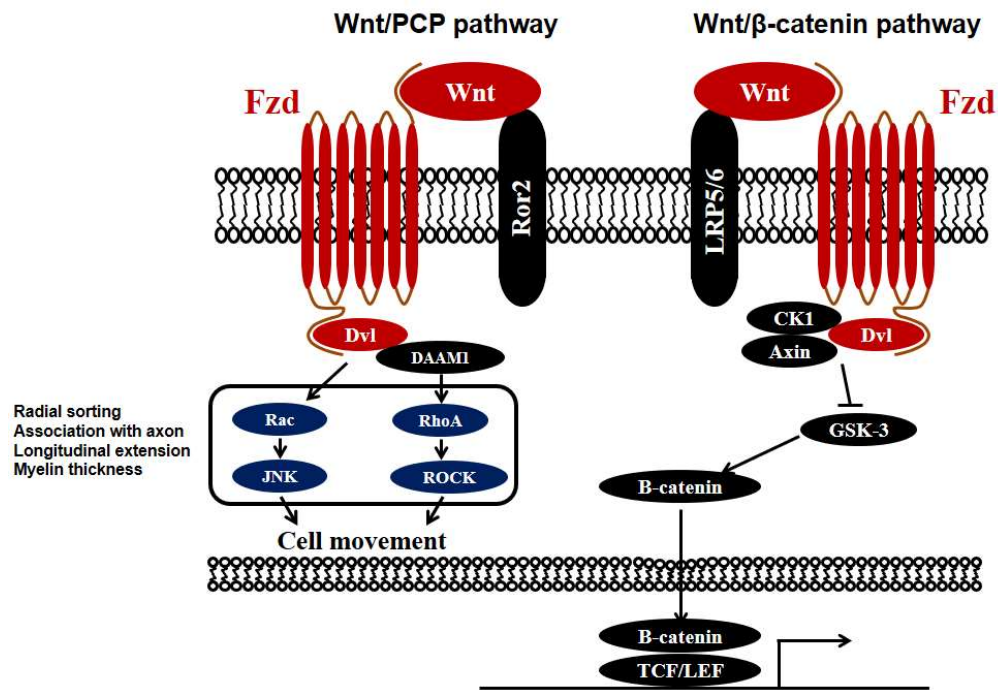


Fig. 8 Relationship between non-canonical Wnt/PCP pathway and canonical Wnt/β-catenin pathway. Wnt/PCP signaling is initiated by Wnt5a-Ror2 binding via small GTPases, such as RhoA and Rac activation. These induce cytoskeletal rearrangements that lead to the regulation of cell movement. The illustration also shows that Wnt5a/PCP signaling suppresses canonical Wnt3a/β-catenin pathway by blocking translocation of Wnt3a into nucleus, subsequently leading TCF/LEF-driven transcription.

CHAPTER II

Schwann cells and the developing sciatic nerves express the necessary components for Wnt/PCP signaling

RATIONALE

Decades of ultrastructural analyses have underscored the fact that the myelin sheath and the myelin/axon unit are highly polarized structures. Over the past 15 to 20 years, the biochemical characterization of these structures has reinforced the notion that myelination is a polarized process (160-162). Experiments manipulating the expression of the molecular components involved in apical-basal (AB) polarity have clearly shown that AB-like polarity is implicated in Schwann cell myelination (76, 99). Small GTPases Rac1, RhoA and Cdc42 are crucial to radial sorting, a necessary prelude to Schwann cell myelination (85, 86, 154). Numerous efforts to understand their regulation have focused on their activation by axonal signals (86, 163) and basal lamina signals (85, 164). Despite the fact that Rac1, RhoA and Cdc42 are the downstream effectors of PCP and Wnt/PCP signaling, and the fact that myelination is a highly polarized process, whether PCP components are expressed in the developing sciatic nerve, in Schwann cells and in DRG neurons, and whether their expression pattern is developmentally regulated has not yet been explored.

This chapter focuses on the characterization, in developing sciatic nerves, Schwann cells and DRG neurons, of components that have been determined to be involved in mediating Wnt/PCP signaling (165-167).

RESULTS

Schwann cells and developing sciatic nerves express components that are necessary for Wnt/PCP signaling

I first determined the expression of components that have been characterized as being key regulators of the Wnt/PCP pathway. Using RT-PCR (reverse transcription polymerase chain reaction) to amplify mRNA isolated from purified Schwann cells at postnatal day 2 (p2), I found that all Fzd receptors (Fzd1-10) were expressed in primary Schwann cells purified from p2 sciatic nerves (Fig. 9). It is noteworthy that non-canonical Fzd receptors, such as Fzd3, Fzd4, Fzd5, and Fzd7 (168-171), were all detected. qRT-PCR analyses of developing sciatic nerves demonstrated the expression of Wnt5a and Wnt11 mRNAs (Fig. 10), throughout the entire period analyzed (embryonic day E18.5 to postnatal day p21). Wnt7a and Wnt7b (172, 173), which are also considered non-canonical Wnt family members, were not detected from developing sciatic nerves. The detected mRNA for non-canonical Wnt molecules were developmentally regulated, being high at E18.5, and dropping dramatically (3-fold for Wnt5a, 9-fold for Wnt11) at birth (p0). Expression remained relatively constant until p14 (Wnt5a) and p21 (Wnt11). Polyacrylamide gel electrophoresis of qRT-PCR end-products (inserts, Fig. 10) revealed a unique band of the expected size for Wnt5a (96 bp) and Wnt11 (125 bp), underscoring the specificity of the reaction.

Quantitative RT-PCR (qRT-PCR) and ELISA analyses indicated that compared to DRG neurons, Schwann cells were the predominant cells expressing Wnt5a at both the mRNA and protein levels (Fig. 11), at least in the context of purified cultures. Western blot analyses of developing sciatic nerves

(Fig. 12; left panel) showed a consistent expression of Wnt5a receptors Frizzled 3 and 5 (Fzd3, Fzd5) from birth (p0) till postnatal day p30, while Frizzled 7 (Fzd7) was not detected (data not shown). Two major downstream effectors of the Wnt/PCP pathway, disheveled 2 (Dvl-2; (174)) and Disheveled-Associated Activator of Morphogenesis 1 (Daam1; (141)) were expressed during the first week postnatal (p0 - p7) and were not detected thereafter (p10 - p30). The transition in Dvl-2 expression between p7 and p10 appeared abrupt, whereas the expression of Daam1 decreased more gradually between p2 to p10. I also observed that Fzd3, Fz5 and Dvl-2 were expressed exclusively in Schwann cells while Daam1 was expressed in both Schwann cells and DRG neurons (Fig. 12, right panel). Taken together, these results suggest that Schwann cells may utilize the Wnt/PCP pathway during the early stages of myelination.

DISCUSSION

In this chapter, I showed that non-canonical Wnt family members and key components for Wnt/PCP pathway are expressed in developing sciatic nerve. It is interesting to note that these components are almost exclusively expressed by Schwann cells. As the expression and role of the small GTPases of the effector module has been studied (77, 85, 154, 175, 176), my findings complete these observations by demonstrating that the upstream global and core modules are also present. It is therefore possible that PCP and Wnt/PCP signaling are involved in regulating Schwann cell myelination in the developing sciatic nerve.

Of the Wnt/PCP-related ligands (172, 173, 177, 178), Wnt5a and Wnt11 were easily detected over a time course that included late embryonic stage E18.5 to post-natal day p30, whereas Wnt7a and Wnt7b were not detected.

Although Wnt ligands appear to be able to use several Fzd receptors (179), to date Wnt5a has been only associated with Fzd3 and Fzd5 receptors, both of which are expressed specifically by Schwann cell (Fig. 12, right panel). This result suggests the possibility that one or more aspects of Schwann cell biology, in particular the morphogenic events associated with myelination may be regulated by Wnt5a during PNS development. The mRNA levels of Wnt5a and Wnt11 are developmentally regulated, being expressed at higher levels during the late embryo stage (E18.5) compared to the post-natal period. It is interesting to note that this regulation corresponds in time with the period of Schwann cells transitioning from an immature, radial sorting phenotype to a myelinating phenotype, when Schwann cells initiate the expression of myelin genes such as MBP, PMP22 and Mpz. This time correlation raised the question of a possible inverse relationship between the levels of Wnt5a and Wnt11 and the ability of Schwann cells to express myelin genes. This suggests a possible scenario in which different amounts of Wnt molecules may control different developmental steps of sciatic nerves. The Wnt molecules are actually morphogens, i.e they do elicit different cell and tissue responses in a dose-dependent manner; the morphogen gradient also encodes positional information (180), which may be relevant to the differential association of non-myelinating and myelinating Schwann cells with axons.

Given that all Fzd receptors (1-10) are present in Schwann cells (Fig. 9), the activation of specific branches of Wnt signaling (canonical versus non-canonical) may be under the control of the type of ligand, and not receptor composition. However Fzd7, whose mRNA was detected in purified Schwann cells, was not detected at the protein level in the developing sciatic nerve (Fig.

9 and 12).

In conclusion, all necessary components for Wnt/PCP signaling are expressed in the developing PNS, particularly by Schwann cells. The expression pattern suggests that some of the processes involved in the formation of the myelin sheath may be regulated by the Wnt/PCP pathway.

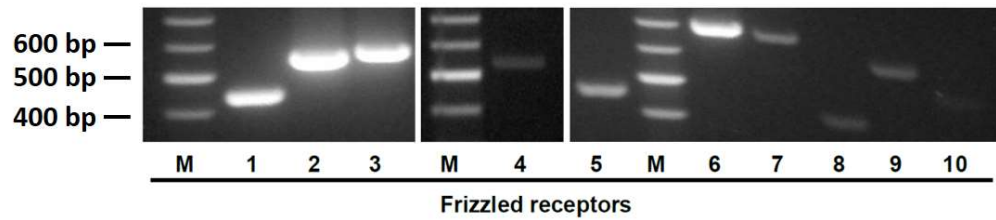


Fig. 9 mRNA expression of Wnt receptors, Fzd 1-10. Polyacrylamide gel electrophoresis of RT-PCR (reverse-transcription PCR) end products revealed a unique band of the expected size for Fzd 1-10.

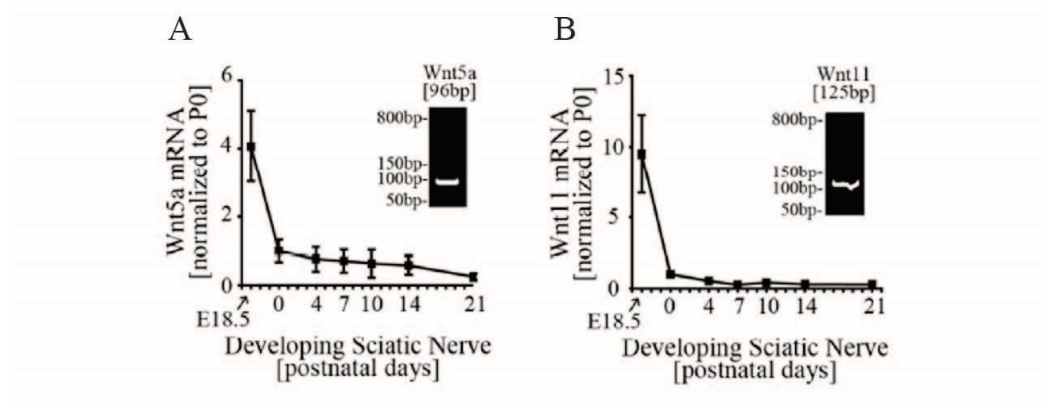


Fig. 10 Expression of ligands for Wnt/PCP signaling in developing sciatic nerve. Results for quantitative RT-PCR (qPCR) for Wnt5a (A) and Wnt11 (B) in developing sciatic nerve (embryonic day E18.5 to postnatal day p21), normalized to internal control, Hypoxanthine-guanine phosphoribosyl-transferase (HPRT) was presented by normalizing to stage p0. Polyacrylamide gel electrophoresis of qPCR end products revealed a unique band of the expected size for Wnt5a (96 bp) and Wnt11 (125 bp; see insets). Error bars present SEM. All results are from n=3-4 experiments.

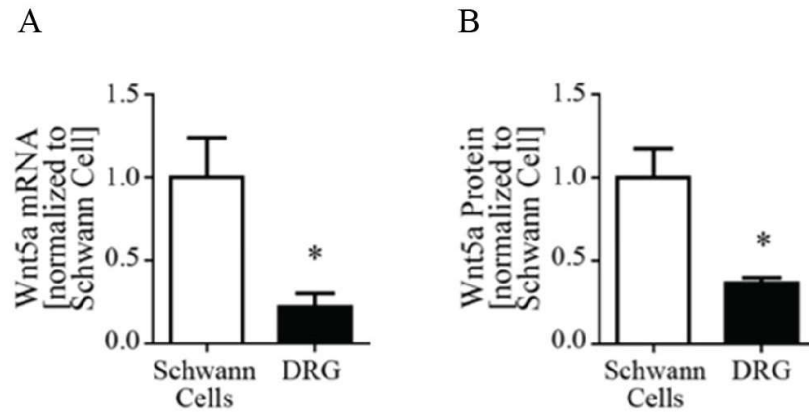


Fig. 11 Cell-specific expression of ligands for Wnt/PCP in the PNS. (A) qRT-PCR analyses for Wnt5a on cultured Schwann cells and Dorsal Root Ganglion (DRG). (B) ELISA analyses for Wnt5a on cultured SCs and DRG neurons. Results were presented by normalizing to SCs. All results are from n=3-4 experiments, T-test mean \pm SEM, *p<0.05.

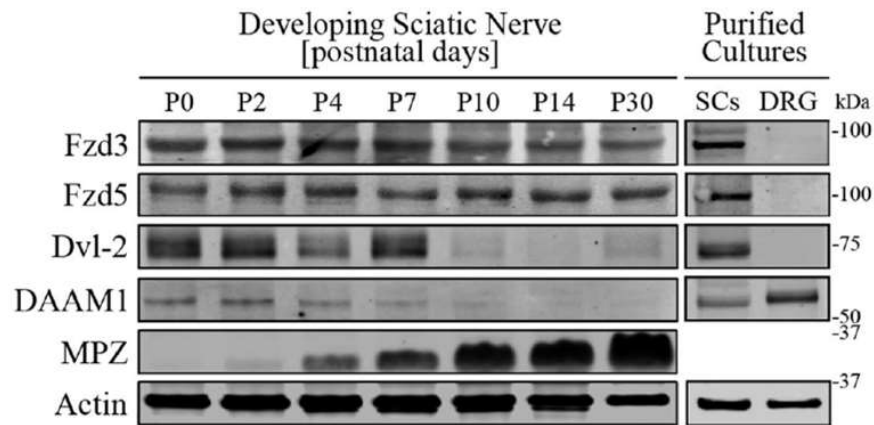


Fig. 12 Expression of key components for Wnt/PCP signaling in developing sciatic nerve. Western blot analyses of developing sciatic nerves (Birth p0 to postnatal day P30; left panels) and purified cultures of SCs and DRG neurons for expression of Wnt5a receptors Frizzled 3 and 5 (Fzd3, Fzd5), Disheveled 2 (Dvl-2) and Disheveled-Associated Activator of Morphogenesis 1 (Daam1). Data are a representative from 3 experiments. 10-400 sciatic nerves were collected for each time window. MPZ served as reference for development of myelin. β -actin served as internal reference.

CHAPTER III

Wnt5a regulates Schwann cell myelination in a biphasic, dose-dependent manner via Daam1 *in vitro*

RATIONALE

Ten 'Frizzled' receptors, and 19 of their possible 'Wnt' ligands, have been identified in the mammalian genome (179). With the numerous possible combinations of ligand and receptor, the molecular mechanisms underpinning the distinct activation of canonical Wnt/ β -catenin and non-canonical Wnt pathways are not clearly understood. They likely rely on different binding affinities, different pattern of expression of receptors and co-receptors (181, 182), and Dishevelled scaffold variants (183). However, Wnt ligands such as Wnt5a (139), Wnt7a (173) and Wnt11 (184), and scaffold adaptors Dishevelled 2 (Dvl-2) and Daam1 (141, 185), appear to preferentially activate the Wnt/PCP pathway. Non-canonical Wnt molecules have been shown to signal through Frizzled receptors Fzd3 (186, 187), Fzd5 (188-190), and Fzd7 (191, 192).

In Chapter II, I presented strong evidence that components necessary for the activation of the non-canonical Wnt pathway were detected in the developing sciatic nerves, and mostly expressed by Schwann cells. These included Wnt5a and Wnt11, the Frizzled receptors Fzd3 and Fzd5, Dishevelled 2 and Daam1.

In the present chapter, I tested the ability of Wnt5a and Wnt11 to affect Schwann cell myelination in an *in vitro* model of PNS myelination. Using loss-of-function approaches, I further determined the importance of Daam1

expression in Schwann cells for normal myelination, as well as to transduce potential Wnt effects.

RESULTS

Wnt5a affects *in vitro* myelination in a biphasic, concentration-dependent manner

I used the DRG neuron / Schwann cell myelinating co-cultures system to test *in vitro* the capacity of Wnt5a and Wnt11 to regulate myelination (Fig. 13 and 14). The cultures were first established in non-myelinating conditions for 3 days, at which time ascorbic acid was added (day 0) to initiate myelination (193). Wnt5a or Wnt11 were also added (experimental points) or not (controls) at final concentrations ranging from 2.5 to 200 ng/ml or 2.5 to 500 ng/ml, respectively. Myelination was assessed after 10 days of culture by the detection of MBP-positive compact myelin sheathes.

Myelinating co-cultures exhibited a biphasic response when treated with Wnt5a (Fig. 13). “Low” doses (2.5 to 10 ng/ml) induced a significant increase (\approx 2- and 1.5-fold at 2.5 and 10 ng/ml, respectively) in the number of myelin segments whereas higher doses (50 to 200 ng/ml) caused a concentration-dependent decrease in the number of myelin segments, with an almost complete inhibition at 200 ng/ml. Wnt11 did not affect myelination in any significant way (Fig. 14A, B) at any of the concentrations tested (from 2.5 to 500 ng/ml). The absence of effect of Wnt11, as well as that of the mock buffer that is common to both Wnts (Fig. 13B) further underscored the specific effect of Wnt5a. The bi-functional effects of Wnt5a discovered at day 10 in myelinating condition were sustained up to day 16 (Fig. 13C).

These results suggest a potential function for Wnt5a among other Wnts related to PCP in PNS myelination.

Wnt5a has no effect on axon-mediated cell proliferation and survival

I then analyzed Schwann cells survival and proliferation to determine whether the effect of Wnt5a could be a simple consequence of a decrease or increase in Schwann cell numbers that would indirectly translate into changes in the number of myelin segments.

To investigate whether Wnt5a had an impact on myelination by affecting axon-mediated Schwann cell proliferation, Schwann cells were allowed to populate axons for 3 days in culture, in non-myelinating conditions. The co-cultures were then switched to myelinating conditions, in absence or presence of Wnt5a (at 2.5 or 200 ng/ml) for 24hrs. Proliferation was assessed by the nuclear incorporation of EdU, which was added during the last 4hrs of culture. As shown in Fig. 15A and B, the incorporation of EdU in Schwann cell nuclei was on average 15% (ratio of Edu-positive nuclei to DAPI), and was not affected by Wnt5a, at either concentration.

Less than 1% of Schwann cells were undergoing cell death in the co-culture system as confirmed by propidium iodide incorporation, and no significant changes were observed in Wnt5a-treated co-cultures (Fig. 15C).

While Schwann cell density increased about 5-fold (Fig. 12D) during the 10 days of culture in myelinating condition, Wnt5a treatment at both myelin-promoting (2.5 ng/ml) and myelin-inhibiting (200 ng/ml) concentrations had no significant effects on that increase when compared to control (Fig. 12D).

These results strongly indicate that Wnt5a does not regulate the proliferation and survival of Schwann cell mediated by axonal contact, and that

the effect on myelination is not due to a secondary effect from changes in cell density.

Daam1 is necessary to promote Schwann cell myelination *in vitro*

In chapter II, I showed that two major downstream effectors of the Wnt/PCP pathway, disheveled 2 (Dvl-2; (174)) and Disheveled-Associated Activator of Morphogenesis 1 (Daam1; (141)) were expressed during the first week postnatal (p0 – p7) and not detected thereafter (p10 – p30; Fig. 12). As a scaffolding adapter protein required for the Wnt-induced assembly of the Disheveled-RhoA complex, Daam1 is a crucial component of the Wnt/PCP signaling cascade. To further investigate a role of the Wnt/PCP pathway in Schwann cell myelination, I used two distinct loss-of-function approaches to perturb Daam1 activity.

In a first approach, I used lentiviral constructs to drive the expression in Schwann cells of two separate short hairpin RNAs specific to Daam1 (shDaam1 #1 and #2). Controls were either non-infected Schwann cells (Ctrl), or cells infected with a construct expressing a non-specific scramble shRNA (shScr). The expression of GFP, also encoded by the shRNA lentiviral constructs, in > 90% of the infected Schwann cells demonstrated efficient lentiviral transduction (Fig. 16A). Both shDaam1 constructs effectively suppressed Daam1 expression in Schwann cells (down by > 90%) whereas the non-specific shScr construct did not affect Daam1 expression (Fig. 16B). These modified Schwann cells were then co-cultured with purified DRG neurons and maintained in myelinating conditions for 10 days. Consistent with a role for Wnt/PCP signaling in regulating myelination, Daam1-deficient Schwann cells were impaired in their ability to generate myelin segments (Fig. 16C) in comparison to shScr controls,

the cultures showing a significant 40% decrease in the number of MBP-positive segments (Fig. 16D).

In a complementary and independent set of experiments (Fig. 17), I used lentiviral transduction to express a cDNA encoding for a dominant-negative form of Daam1 (nDaam1, amino acids 1-233, (141)). Controls were either non-infected Schwann cells, or cells infected with a GFP-expressing lentiviral construct (vector). Western blot analyses (Fig. 17A) of Schwann cell lysates with an antibody against Damm1 (epitope aa 1-111) demonstrated the expression of a band at the expected molecular weight (\approx 25 kDa) for nDaam1. Endogenous levels of Daam1 (\approx 110 kDa) were not affected by lentiviral transduction and ectopic expression of the nDaam1 variant. Schwann cells were then added to purified DRG neurons and maintained in myelinating conditions for 10 days. The expression of nDaam1 in Schwann cells resulted in a strong inhibition in myelin segment formation compared to vector controls (Fig. 17B). The quantitation of the number of MBP-positive myelin segments demonstrated a significant 50% decrease with the nDaam1 construct (Fig. 17C).

Daam1 is necessary for Wnt5a-(low dose)-mediated increase in myelination

These results highlighted a potential role for a Wnt5a/PCP/Damm1 cascade in signaling the effects of Wnt5a on Schwann cell myelination. Therefore, I disrupted Damm1 activity in Schwann cells using the shDamm1 #1 and nDaam1 lentiviral constructs (Figs. 16B and 17A), and determined how Daam1-impaired Schwann cells responded to Wnt5a in the context of myelination (Figs. 18 and 19). As previously showed in Fig. 16C-D and 17B-C, impairing Daam1 activity either by knockdown (shDaam1 #1; Fig. 16) or

competition with a negative-dominant form (nDaam1; Fig. 17) in Schwann cells dramatically decreased their ability to form myelin segments (Figs. 18B and 19B; white columns), about 45% on average. Wnt5a at 200 ng/ml inhibited myelin segment formation by another 50%, bringing overall inhibition to about 70% (compare white versus black columns for shDaam1 (Fig. 18B) and nDaam1 (Fig. 19B). Since knockdown is at least > 90% efficient (Fig. 16B), this result suggests that the inhibitory effect of 200 ng/ml of Wnt5a may be mediated by a mechanism not dependent on Daam1. The pro-myelinating effect of Wnt5a at 2.5 ng/ml (Fig. 18B, shSCR; Fig. 19B, vector; white versus grey columns) was however completely abolished (Fig. 18B, shDaam1; Fig. 19B, nDaam1; white versus grey columns), a finding that supports the hypothesis that Daam1 function is necessary for Wnt5a pro-myelinating effect.

DISCUSSION

One of the major finding of this set of experiments is the demonstration that Wnt5a, a characterized PCP ligand (139, 142, 185, 194, 195), does influence the formation of myelin segments by Schwann cells, at least in an *in vitro* model of PNS myelination. Furthermore, the biphasic response, promoting or inhibiting myelination in a concentration-dependence manner, is consistent with the nature of Wnt5a as a morphogen. A cell exposed to a morphogen exhibit a different response that depends on the concentration of said morphogen (196-198). Recent studies have suggested that the N-terminal sequence of Wnt ligands could directly be the key to differential cell responses, such as activating different sets of target genes (199). Thus, it is possible that the N-terminal of Wnt5a may be regulating Wnt5a bi-phasic effect on myelination *in vitro*.

The finding that Wnt5a pro-myelinating effect requires Daam1, a known element of the Wnt/PCP signaling platform, further strongly support a role for Wnt/PCP signaling in regulating Schwann cell myelination. As only Schwann cells were Daam1-deficient, this result also suggests that the effect of Wnt5a on myelination was mediated by affecting Schwann cells response to Wnt5a, and possibly not by affecting DRG neurons. More importantly, myelination was deficient when Daam1 expression was ablated from Schwann cells, without the need to add Wnt5a. This suggests that the endogenous Wnt/PCP is involved in normal Schwann cell myelination. Interestingly, the inhibitory effect of high-dose of Wnt5a appears to be Daam1-independent, and suggests that a different pathway may be involved, possibly the Wnt/calcium pathway (200-202). Wnt5a has also been shown to activate c-Jun through the Wnt/PCP/Rac1 pathway (203); c-Jun is an inhibitor of PNS myelination (204) and is activated in de-differentiating demyelinating Schwann cells (204).

Although mRNA levels of Wnt11 were high in embryonic sciatic nerves just before birth, Wnt11 had no effect on myelination in the *in vitro* myelinating culture system, at any of the concentrations tested. Although some studies have reported that the function of Wnt5a and Wnt11 may be interchangeable (205-207), it does not appear so, at least in *in vitro* conditions. However this lack of effect does not preclude a possible role for Wnt11 *in vivo*.

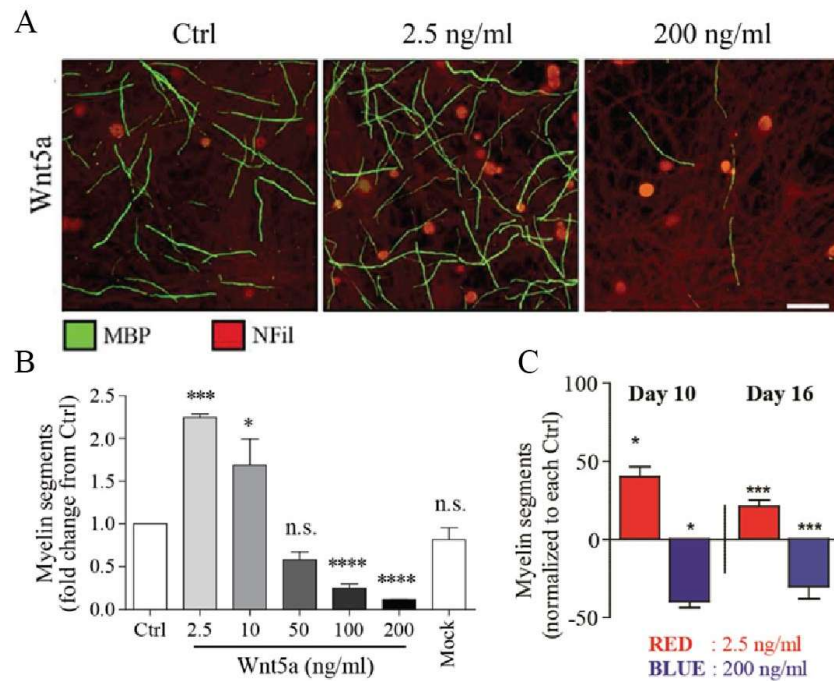


Fig. 13 The bi-functional effect of Wnt5a on myelination culture in concentration dependent manner. (A) representative culture immunostained with MBP (green) and NF (red). (B) Quantification of *in vitro* myelination treated with Wnt5a is in bar graph. (C) Effect of Wnt5a on myelination at different time windows (at day 10 and 16). Bars represent the normalized average number of MBP-positive segments per coverslip \pm SEM. * $P < 0.05$, *** $P < 0.001$, **** $P < 0.0001$, one-way ANOVA with Bonferroni's Multiple Comparison Test.

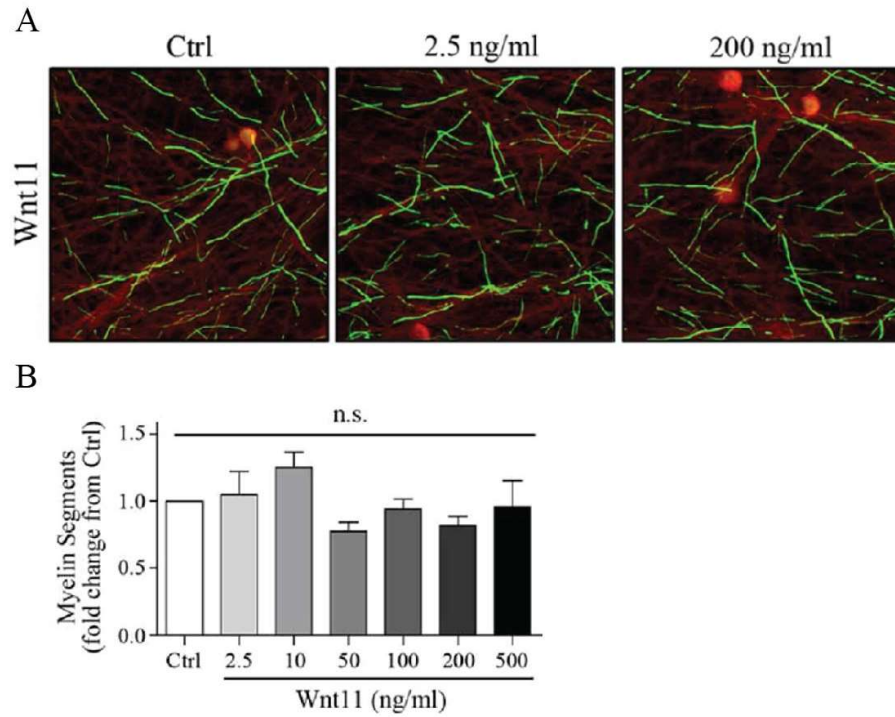


Fig. 14 The effect of Wnt11 on *In vitro* myelination culture in concentration dependent manner. (A) Cultures were immunostained with MBP (green) and NF (red). (B) Quantification of invitro myelination treated with Wnt11 is in bar graph. Bars represent the normalized average number of MBP-positive segments per coverslip \pm SEM. n.s. means no significance.

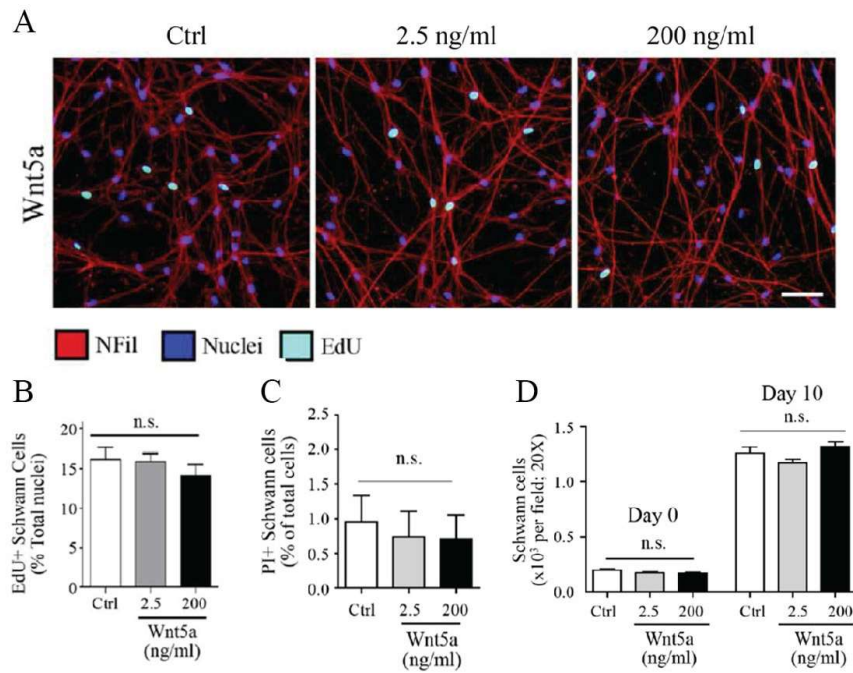


Fig. 15 Effect of Wnt5a on Schwann cell cycle. (A) Representative co-cultures with NFil (red) and Edu (green). (B) Graph showing Edu incorporation results from *in vitro* co-cultures with DRG neuron with average percentage of Edu-positive SCs to total cell number measured by DAPI. (C) Cytotoxic effect of Wnt5a on Schwann cell. Quantification of propidium iodide (PI) staining with Wnt5a is represented as the average percentage of PI-positive SCs to total cell number measured by DAPI. (D) Quantification of total Schwann cell numbers treated with Wnt5a. Total cell numbers were measured by DAPI at day 0 and 10 after treatment of ascorbic acid with Wnt5a. n.s. means no significance. Data are presented as mean \pm SEM.

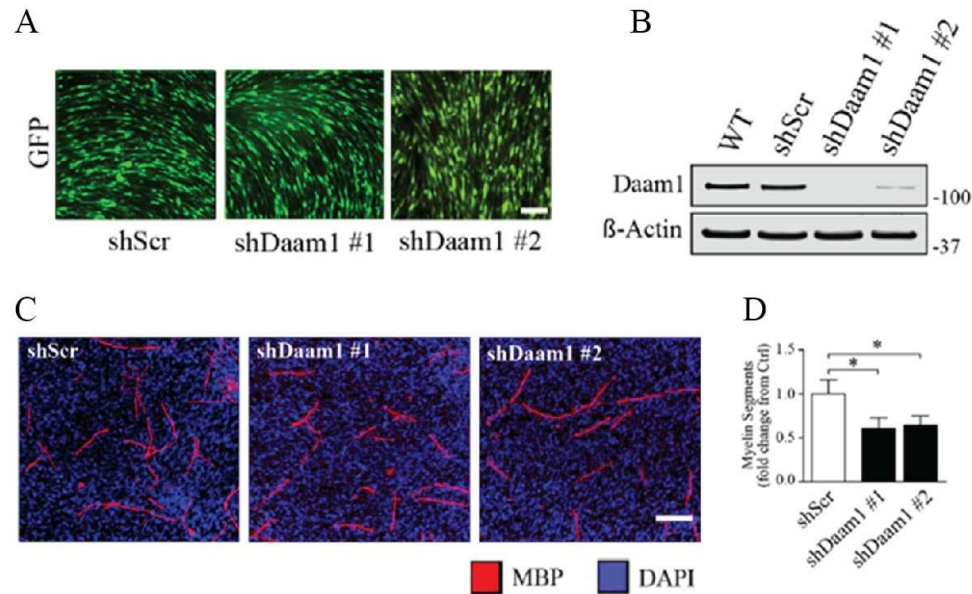


Fig. 16 Daam1 is necessary for myelination. (A) Representative pictures showing SCs infected GFP expressing Lenti-viral vectors against Daam1. (B) Western blot analysis of Daam1. SCs were infected with control or two different Daam1 shRNA-containing Lenti-viral vector. (C) The effect of Daam1 knockdown on myelination culture in concentration dependent manner. Cultures were immunostained with MBP (red) and DAPI (blue). Quantification of *in vitro* myelination is in (D). Bars represent the normalized average number of MBP-positive segments per coverslip \pm SEM. *P<0.05, 1way ANOVA with Bonferroni's Multiple Comparison Test.

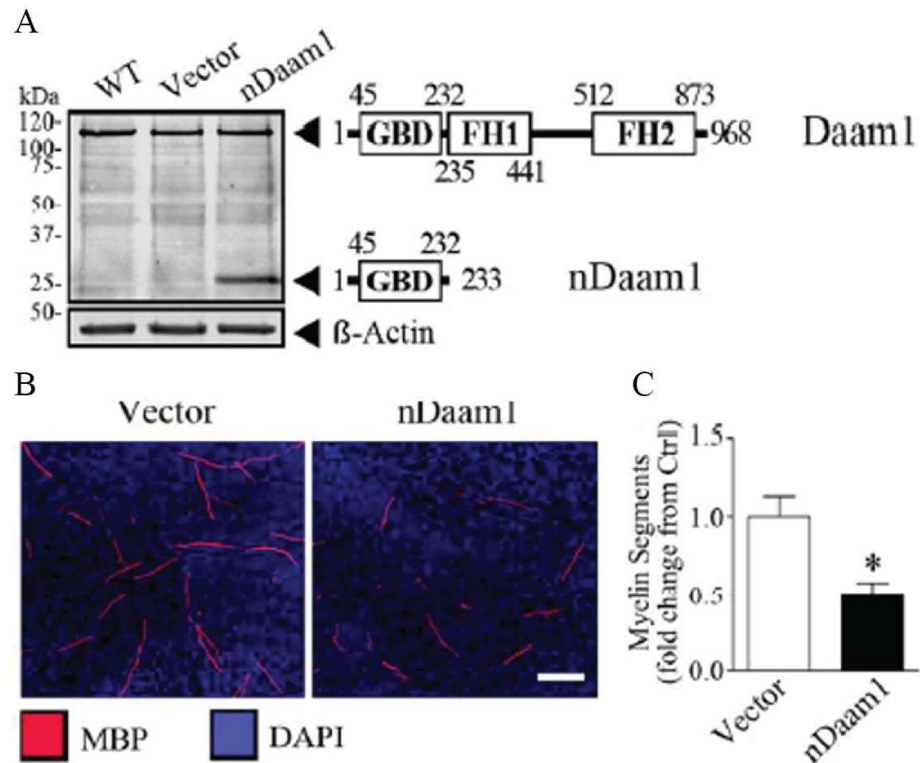


Fig. 17 Myelination with ectopic expression of nDaam1 Schwann cell *in vitro* (A) Western blot analysis of ectopic expression of n-terminal of Daam1 (nDaam1). (B) The effect of Daam1 knockdown on myelination culture in concentration dependent manner. Cultures were immunostained with MBP (red) and DAPI (blue). Quantification of *in vitro* myelination is in (C) Bars represent the normalized average number of MBP-positive segments per coverslip \pm SEM. * $P < 0.05$, 1way ANOVA with Bonferroni's Multiple Comparison Test.

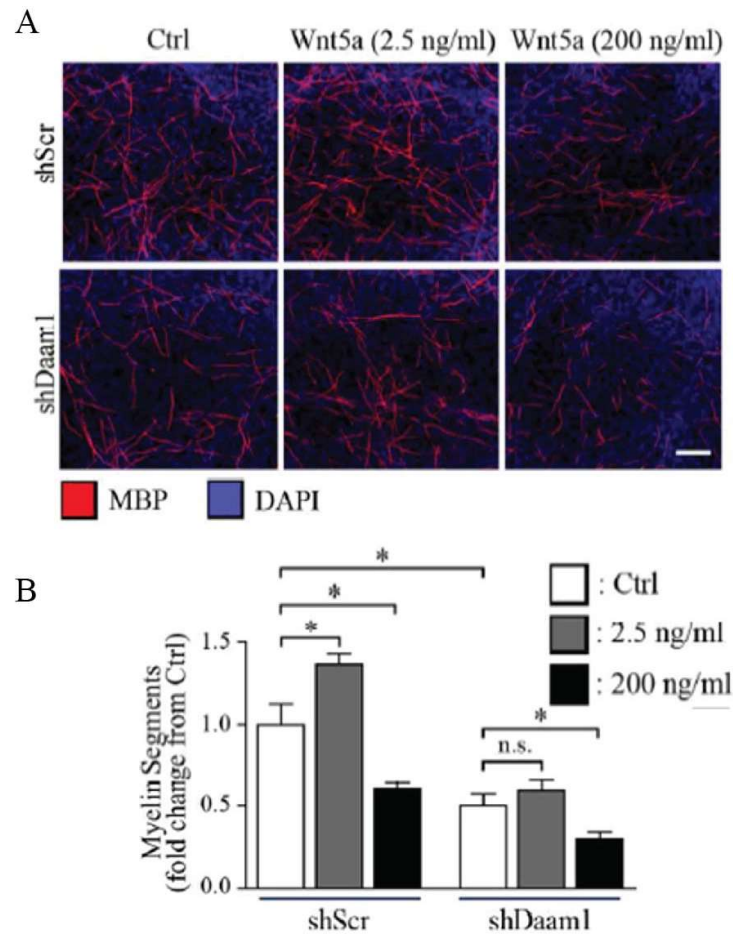


Fig. 18 Low dose of Wnt5a modulates *in vitro* myelination through Daam1 in Schwann cells (A) Representative pictures and quantification of myelination showing the effect of Daam1-deficient SCs on promyelinating effect of Wnt5a (2.5 ng/ml). Cultures were immunostained with MBP (red) and DAPI (blue). Quantification is in (B). Bars represent the normalized average number of MBP-positive segments per coverslip \pm SEM. * $P < 0.05$, 1way ANOVA with Bonferroni's Multiple Comparison Test. n.s. means no significance.

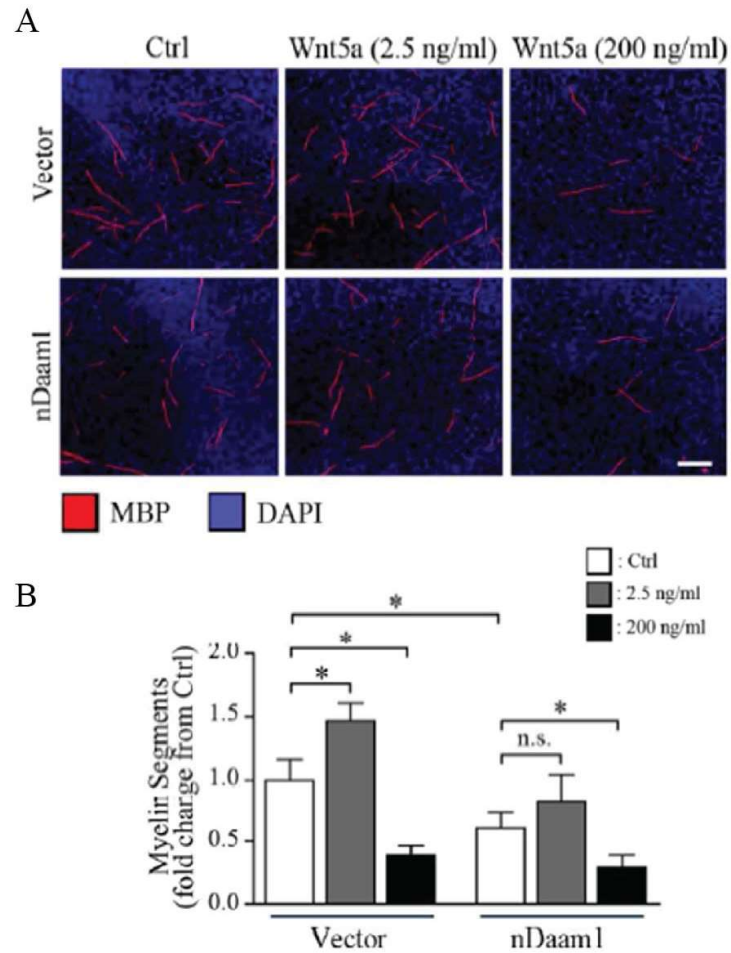


Fig. 19 Effect of Wnt5a on nDaam1-expressing Schwann cell myelination *in vitro*. (A) Representative pictures and quantification of myelination showing the effect of ectopically expressed nDaam1 in SCs on promyelinating effect of Wnt5a (2.5 ng/ml). Cultures were immunostained with MBP (red) and DAPI (blue). Quantification is in (B). Bars represent the normalized average number of MBP-positive segments per coverslip \pm SEM. * $P < 0.05$, 1way ANOVA with Bonferroni's Multiple Comparison Test. n.s. means no significance.

CHAPTER IV

Molecular aspects of Wnt5a effect in Schwann cell

RATIONALE

Several studies have established that the dynamic regulation of the actin cytoskeleton and myosin motor proteins in Schwann cells is important for the morphogenetic changes associated with the process of PNS myelination. For example cytochalasin D, a potent inhibitor of actin polymerization, prevents ensheathment and myelination in the Schwann cell / DRG neuron myelinating co-culture system (208). Schwann cells that are deficient in N-WASP, a factor involved in actin filament nucleation, are stopped at the pro-myelinating stage, failing to spiral-wrap and myelinate PNS axons *in vivo* (209). Furthermore, the pharmacological inhibition of key regulators of the cytoskeleton dynamics such as myosin II (210) and myosin light chain kinase (211) also impair the segregation of axons, ensheathment and myelination *in vitro*.

Rac1, RhoA and cdc42, small GTPases of the Rho family, are well-established regulators of actin assembly (212), thereby contributing to changes in cell morphology and movements by integrating multiple external stimuli. In the context of PNS myelination, Schwann cells coordinate Rac1, RhoA and cdc42 activity by integrating signals from both the extracellular matrix. Schwann cell-specific mouse knockouts for $\beta 1$ integrin (85) and integrin-linked kinase (213) have decreased Rac1 and RhoA activity, and radial sorting of peripheral nerve axons is severely impaired in these mice. Delayed radial sorting is also observed in Schwann cell-specific Rac1 and cdc42 knockout animals (85, 86), and Rac1-defective Schwann cells do not form myelin sheathes *in vitro* (85).

The abnormal myelin segments that are formed *in vitro* after the disruption of RhoA activity by inhibiting the downstream Rho kinase effector (ROCK) (119) also implied a functional role for the RhoA/ROCK pathway in coordinating Schwann cell branching and wrapping around axons. Finally, hypo-myelination was observed *in vivo* from Schwann cells that were deficient in RhoA expression (154), which suggests a role for RhoA in regulating Schwann cell wrapping. Altogether these findings strongly underscore the requirement of regulated morphogenetic events during PNS myelination.

In chapters II and III, I showed that Wnt5a, a defined ligand for the Wnt/PCP pathway, regulates myelination. The pro-myelinating effect of Wnt5a at “low dose” (2.5 ng/ml) is mediated via Daam1. Interestingly over the years, small GTPases of the Rho family have emerged as the key mediators of the Wnt/PCP pathway (118), and Daam1 is the scaffolding protein that is recruited upon Wnt/PCP activation to establish the signaling platform that regulates Rac1 and RhoA (214).

In this Chapter, I therefore investigated the activity of small GTPases Rac1, RhoA and cdc42 in Schwann cells upon treatment with different levels of Wnt5a.

RESULTS

Biphasic regulation of small GTPases Rac1 and RhoA by Wnt5a in Schwann cells *in vitro*

I used G-LISA assays to assess Rac1, RhoA and cdc42 activity in Schwann cells at various time points (1.5 to 20 minutes) after treatment with Wnt5a at concentrations that either promoted (2.5 ng/ml; red line in Fig. 20) or inhibited (200 ng/ml; blue line in Fig. 20) myelination. Both treatments elicited

changes (increase or decrease) within 5 minutes from the basal level of RhoA (Fig. 20A) and Rac1 (Fig. 20B) activity. Changes in RhoA (Fig. 20A) activity persisted for about 20 minutes whereas the changes in Rac1 activity (Fig. 20B) returned to basal level within 10 minutes. Interestingly, Wnt5a affected Rac1 and RhoA activity in an opposite, dose-dependent manner. While 2.5 ng/ml Wnt5a (red line) increased RhoA activity (Fig. 20A), Rac1 activity decreased (Fig. 20B). Conversely 200 ng/ml (blue line) of Wnt5a decreased RhoA activity (Fig. 20A) while increasing Rac1 activity. Wnt5a did not affect the activity of small GTPase cdc42 at either concentration (data not shown).

These results suggest that Wnt5a has a biphasic effect on the activity of small GTPases Rac1 and RhoA, either activating or inhibiting depending on the concentration of Wnt5a. Although purely speculative, it is tempting to draw a parallel between this biphasic regulation and the biphasic effect of Wnt5a on Schwann cell myelination *in vitro* (see Chapter III).

Effect of Wnt5a on small GTPases activity in Daam1-deficient Schwann cells

I then determined the activity of RhoA and Rac1 in Daam1-deficient Schwann cells (shDaam1) in response to Wnt5a treatment (Fig. 21). The analyses are done at time point 5 minutes at which time the maximal differences in Rac1 and RhoA induced by Wnt5a were observed (Fig. 20).

The expression of the non-specific shScramble (shScr) construct did not affect the response previously observed from Schwann cells in Fig. 21A and B: up-regulation of RhoA and down-regulation of Rac1 at 2.5 ng/ml (Fig. 21A and B; shScr; red columns), down-regulation of RhoA and up-regulation of Rac1 at 200 ng/ml (Fig. 21A and B; shScr; blue columns). When treated with Wnt5a at

2.5 ng/ml, shDaam1-expressing Schwann cells failed to respond and activate RhoA compared to shScr-expressing Schwann cell controls (Fig. 21A, red columns). The decrease of RhoA activity at 200 ng/ml was not significantly affected (Fig. 21A, blue columns) by the expression of shDaam1. Although shDaam1-expressing Schwann cells did not respond as efficiently to Wnt5a at 200 ng/ml to increase Rac1 activity, the “high dose” of Wnt5a in Daam1-suppressed condition resulted in 10% activation compared to non-treated (Fig. 21B, blue columns). The trend for a further decrease in Rac1 activation by Wnt5a at 2.5 ng/ml is not significant (Fig. 21B, red columns).

These results suggest that the increase in RhoA activity by “low dose” of Wnt5a (2.5 ng/ml) and the increase in Rac1 activity by “high dose” of Wnt5a (200 ng/ml) in Schwann cells upon treatment with Wnt5a are mediated through the PCP/Daam1 pathway.

Wnt5a does not activate the Wnt/ β -catenin canonical pathway in Schwann cells

Although Wnt5a is mainly characterized as a Wnt/PCP/small GTPase activator, it has been shown to activate the canonical pathway in certain conditions (135, 215). To determine whether Wnt5a activates the canonical pathway in Schwann cells, I treated purified Schwann cells at the two concentrations that had opposite effect on myelination, i.e., 2.5 and 200 ng/ml. I performed qPCR for the induction of Axin2 gene expression, a downstream target of the Wnt/canonical pathway (216). Wnt3a, a well-established ligand for the Wnt/ β -catenin pathway (217), was used as a positive control.

As expected, Wnt3a increased Axin2 mRNA levels, by about 3.5-fold in a concentration-dependent manner (Fig. 22). A unique band of the expected

size (93 bp) was observed after running the qRT-PCR reaction on a polyacrylamide gel (inset, Fig. 22). These results underscore the effectiveness of our qRT-PCR conditions to detect Axin2. Wnt5a did not induce changes in the basal level of Axin2 gene expression (Fig. 22).

One of the best-described markers of the Wnt/canonical pathway is β -catenin (218), whose cellular protein levels increase after stimulation of the Wnt/canonical pathway. Another response to the Wnt/canonical activation is the translocation of β -catenin to the nucleus. I therefore tested for nuclear translocation (Fig. 23A) and cellular accumulation (Fig. 23B) of β -catenin in Schwann cells treated with either 2.5 or 200 ng/ml of Wnt5a.

As for the qRT-PCR analysis, Wnt3a was used as a positive control, and Schwann cells treated with Wnt3a showed a translocation of β -catenin to the nucleus (Fig. 23A, second row) as well as an increase in protein levels within 2hrs post-treatment (Fig. 23B). Wnt5a did not induce translocation of β -catenin to the nucleus (Fig. 23A, two bottom rows), and there was no significant change in the basal level of β -catenin compared to non-treated cells, up to 4hrs after Wnt5a treatment (Fig. 23B).

Altogether these results suggest that Schwann cells do not respond to Wnt5a through the Wnt/canonical pathway.

DISCUSSION

The demonstration that Wnt5a, at low doses, promoted myelination in the *in vitro* culture system in a Daam1-dependent manner, strongly suggested that the Wnt5a/PCP pathway was involved. The findings that Wnt5a does regulate downstream effectors small GTPases RhoA and Rac1 in Schwann

cells, in a Daam1-dependent manner, without affecting the canonical Wnt/ β -catenin pathway, further strengthen the case for the involvement of the Wnt/PCP pathway in regulating PSN myelination.

One of the most striking finding, yet only correlative is that the regulation of Rac1 and RhoA by Wnt5a in Schwann cells also follows a biphasic response, reminiscent of the biphasic response of the myelinating Schwann cell /DRG neurons co-cultures (Fig. 20). The current inferred correlation is that “low dose” of Wnt5a would promote myelination by decreasing Rac1 activity and increasing RhoA activity, whereas “high dose” of Wnt5a would inhibit myelination by increasing Rac1 activity and decreasing RhoA activity.

At face value, these results strongly suggest that a high RhoA / low Rac1 activity ratio is important for the formation of compact myelinated segments *in vitro*. This could be the result of simply increasing the number of Schwann cells associated with axons in a 1:1 relationship, and therefore more Schwann cells able to undergo the spiral-wrapping that leads to the formation of the myelin sheath. This would imply a regulation of the process of radial sorting. However decreased Rac1 activity *in vivo* results in deficits in radial sorting (85, 86). On the other hand, the inhibition of the ROCK, a downstream target of RhoA, prevents Schwann cells to extend only one lamella around an axon, which leads to a Schwann cell to form multiple myelinated segments instead of one (119). This suggests that RhoA activity might be more important for steps that are after the radial sorting process. Indeed very recent *in vivo* data (154) have described hypomyelination (i.e. defects in spiral-wrapping) without affecting Schwann cell/axon association in the PNS of mouse whose Schwann cell' RhoA activity was depressed by lentiviral-mediated shRNA knockdown. This may therefore

suggest that Wnt5a at low dose may promote myelination not by increasing the number of Schwann cells that associate in a 1:1 relationship with axons, but by inducing Schwann cells that are in a 1:1 relationship with axons to undergo spiral-wrapping. This is a hypothesis that I will test in the next Chapter V.

On the other hand, the results also suggest that a low RhoA / high Rac1 activity ratio is inhibitory to the formation of compact myelinated segments *in vitro*. As mentioned earlier, high Rac1 activity has been proposed to mediate radial sorting by allowing Schwann cells to form lateral lamellipodia, while a low Rac1 activity would promote migration and elongation along axons (77, 85, 86, 176, 219). Could a low RhoA / high Rac1 activity ratio maintained artificially by “high dose” of Wnt5a prevent Schwann cells from exiting the radial sorting stage and move on with the formation of the myelin sheath? What the readout for this hypothesis could be is unclear. Indeed cells could form a 1:1 association but fail to spiral wrap, or cells may never be able to properly form a 1:1 association with axons.

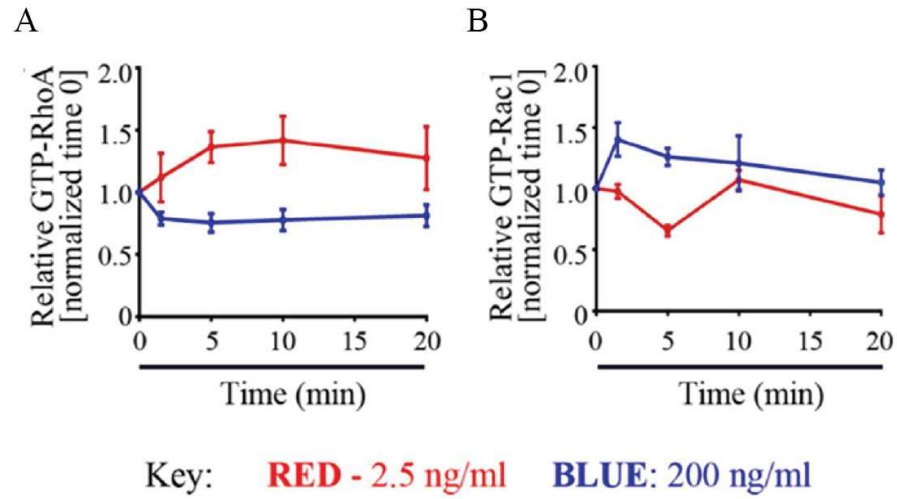


Fig. 20 Effect of Wnt5a on small GTPases activity in Schwann cells. Graph representing the active form of RhoA (A) and Rac1 (B) in SCs treated with low (2.5 ng/ml, red) and high (200 ng/ml, blue) concentration of Wnt5a. Data are mean \pm SEM (n=4).

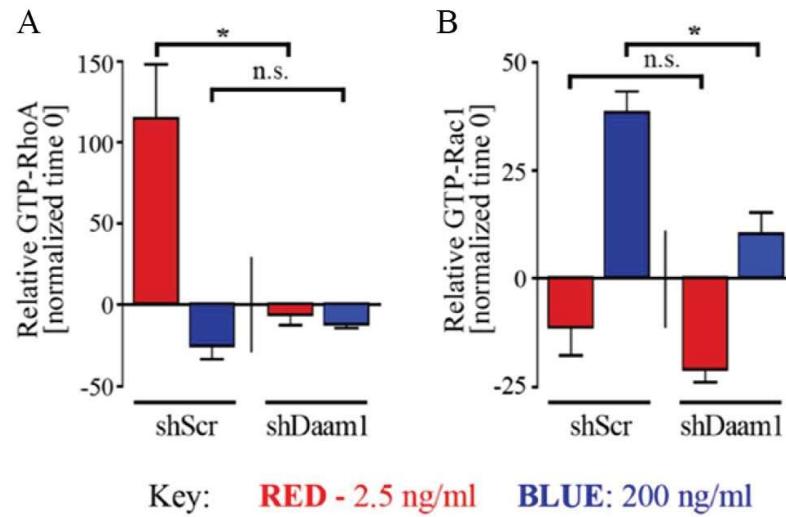


Fig. 21 Effect of Wnt5a on small GTPases activity in Daam1-deficient Schwann cells. (A) Graph representing the active form of RhoA in Daam1-deficient SCs treated with low (2.5 ng/ml, red) and high (200 ng/ml, blue) concentration of Wnt5a. Data are mean \pm SEM (n=4). *P<0.05, Two-tailed t-test. n.s. means no significance. (B) Graph representing the active form of Rac1 in Daam1-deficient SCs treated with low (2.5 ng/ml, red) and high (200 ng/ml, blue) concentration of Wnt5a. Data are mean \pm SEM (n=4). *P<0.05, Two-tailed t-test. n.s. means no significance.

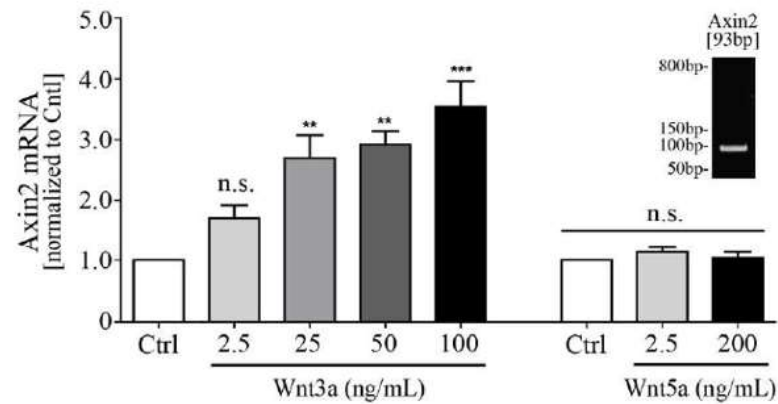


Fig. 22 Effect of Wnt5a on Axin2 mRNA expression. qRT-PCR for Axin2 gene expression in Schwann cells treated with Wnt3a (2.5, 25, 50, 100 ng/ml) or Wnt5a (2.5 or 200 ng/ml). A unique band of the expected size (93 bp) was observed after running the qRT-PCR reaction on a polyacrylamide gel electrophoresis (inset).

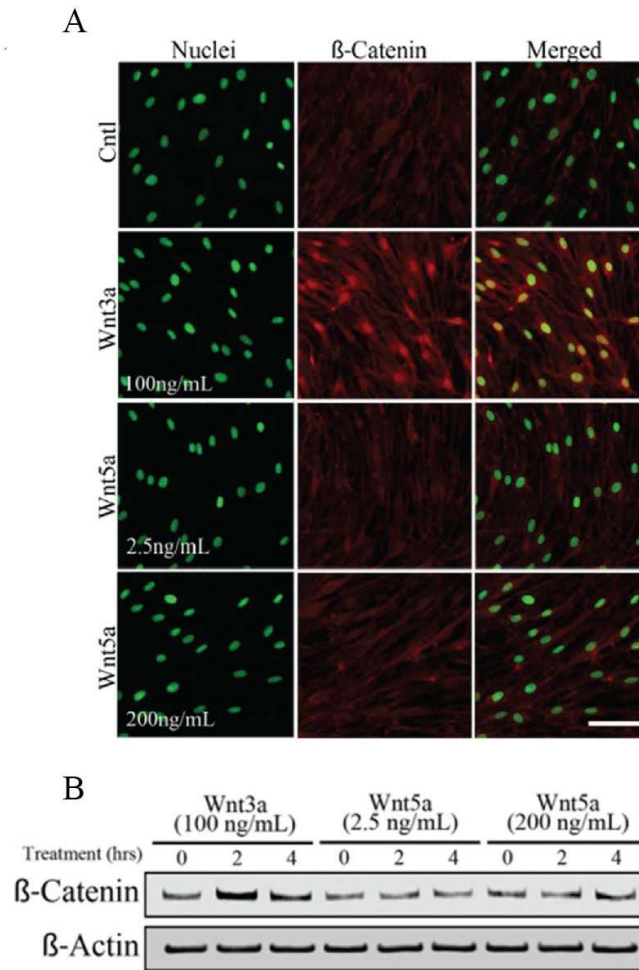


Fig. 23 Wnt5a does not activate canonical Wnt/ β -catenin pathway. (A) Effect of Wnt5a on translocation of β -catenin. Immunofluorescent analysis for nuclear translocation of β -catenin in Schwann cells treated with either Wnt3a (100ng/ml; positive control) or Wnt5a (2.5 or 200 ng/ml). Scale bar = 100 μ m. (B) Effect of Wnt5a on accumulation of β -catenin. Western Blot analysis of cellular accumulation of β -catenin in Schwann cells treated with either Wnt3a (100 ng/ml; positive control) or Wnt5a (2.5 or 200 ng/ml).

CHAPTER V

Morphogenetic effects of Wnt5a in Schwann cell

RATIONALE

The previous chapters have identified the developmentally regulated expression of components that can potentially elicit a Wnt/PCP response in Schwann cells. In the *in vitro* Schwann cell culture system, I showed that low levels of Wnt5 increased the numbers of compact myelin sheathes that are formed, whereas higher doses lead to an inhibition in a concentration-dependent manner. Daam1, a major component of the Wnt/PCP signaling platform is required to mediate the Wnt5a, low dose effects. Interestingly I also showed that the activity of the Wnt/PCP downstream effectors Rac1 and RhoA follow the same biphasic response as the myelination response. Low dose of Wnt5a increased the RhoA/Rac1 ratio, whereas high dose of Wnt5a decreased the RhoA/Rac1 ratio.

The small GTPases RhoA and Rac1 having been shown to play a major role in radial sorting of axons by Schwann cells (85, 86), and potentially in spiral-wrapping as well (119, 154), the next question that needs addressing is to determine whether, and how the morphogenetic movements of Schwann cells are affected by Wnt5a treatment, either in purified cultures, or in co-culture with axons.

RESULTS

Wnt5a has a biphasic effect on Schwann cell elongation, and low doses increase the number of lateral lamellipodia

The first assay was to determine changes in the overall morphology of the Schwann cells when plated onto a laminin-1 substrate, after Wnt5a treatment (Fig. 24) at low (2.5 ng/ml) or high (200 ng/ml) doses.

i) One of the features that appeared to change upon Wnt5a treatment was cell elongation. Cells baseline (i.e. un-treated Schwann cells) elongation was $167 \pm 11 \mu\text{m}$. Treating the Schwann cells with low and high doses of Wnt5a lead to opposite changes in the baseline elongation. Wnt5a at 2.5 ng/ml promoted Schwann cell elongation by $\approx 14\%$ ($190 \pm 5 \mu\text{m}$ at 10 min.) whereas 200 ng/ml of Wnt5a had an opposite effect and reduced elongation by $\approx 20\%$ ($134 \pm 7 \mu\text{m}$ at 10 min.) (Fig. 24A). The response was fast (within 1.5 min. for Wnt5a at 2.5 ng/ml, and 5 min. for Wnt5a at 200 ng/ml), and the extent of these changes in length increased gradually overtime (Fig. 24A).

ii) Another feature that presented an obvious change was the total number of, and position of lamellipodia. As determined after 10 minutes of treatment, the total number of lamellipodia was increased by $\approx 30\%$ on Schwann cells treated with 2.5 ng/ml of Wnt5a; this increase essentially reflected a 2-fold increase in the numbers of lateral lamellipodia. Wnt5a at 200 ng/ml had no effect on the total number and distribution of the lamellipodia (Fig. 24B) compared to control (un-treated) Schwann cells.

I then examined the morphology of Schwann cells in contact with axons once co-cultured for 24hrs with purified DRG neuron cultures, in non-myelinating conditions. Schwann cells were transduced with a construct

expressing a form of green fluorescent protein (GFP) form that does not express a nuclear localization signal and, therefore remained in the cytosol and allowed to visualize the morphology of the Schwann cells. The analysis was done after treatment the co-cultures in non-myelinating conditions with Wnt5a for 10 minutes. Schwann cells remained bipolar and elongated when co-cultured with axons, with no particular lateral branching (Fig. 24C). However, as was observed with isolated Schwann cells cultured on laminin-1, a similar effect on elongation was noted. Wnt5a at 2.5 ng/ml induced an increase of $\approx 10\%$ in length over the average $226 \pm 3.6 \mu\text{m}$ length measured in un-treated cultures, whereas Wnt5a at 200 ng/ml induced a decrease of $\approx 10\%$ in length.

Altogether, these results show that Schwann cells alter their morphology when responding to external Wnt5a stimulation. Furthermore, instead of responding with an increasing, more accentuated phenotype (i.e more elongation OR less elongation) with increasing Wnt5a concentrations, Schwann cells respond in an opposite manner: more elongation with low-dose of Wnt5a and less elongation with high-dose of Wnt5a.

“High dose” of Wnt5a increases Schwann cell motility

The effect of Wnt5a on cell migration was analyzed with Schwann cell cultured in two different conditions: i) purified isolated Schwann cells and ii) Schwann cells in contact with axons.

i) The Boyden chamber assay was used to determine the effect of Wnt5a on purified Schwann cells. Schwann cells were seeded in the upper chamber and incubated for 5hrs before the addition of Wnt5a, either at low (2.5 ng/ml) or high (200 ng/ml) dose in the bottom chamber. Migration of Schwann cells through the filter of the Boyden chamber was assessed 8hrs later by detecting and counting

DAPI-stained Schwann cells present at the bottom surface of the membrane. While the low dose of Wnt5a had no significant effect, the number of Schwann cells migrating through the membrane of the Boyden chamber with high dose of Wnt5a (200 ng/ml) was increased about 2.5 fold (Fig. 25).

ii) The pattern of migration was then analyzed when Schwann cells were in contact with a neurite network established with purified DRG neuron cultures. The Schwann cell nucleus was stained with Hoechst 33342, which allowed for live-cell image tracking of cell movements, and therefore allowed for measuring the extent of Schwann cell migration from their start point established at time point 0 (start of image capture). While 2.5 ng/ml of Wnt5a had no effect of Schwann cell motility, Wnt5a at 200 ng/ml increased cell mobility, Schwann cells migrating on average 10% farther from their starting point than Schwann cells in un-treated co-cultures (Fig. 26).

Wnt5a does not affect Schwann cell 1:1 association with axons *in vitro*

One critical aspect to myelination in the PNS is the necessity by Schwann cells to sort-out bundles of axons until a 1:1 association is established with the axon to be myelinated. It is a process highly dependent on the regulated activity of small GTPases such as Rac1 and RhoA (85, 86, 119). In view of the regulation of small GTPases Rac1 and RhoA in Schwann cells, and of their morphology, all of which in a biphasic manner similar to the biphasic effect on myelination, I used two different approaches to determine whether the effect on myelination by Wnt5a could reflect differences in the ability of Schwann cells to establish a 1:1 association with axons in the myelinating co-culture system.

Krox-20 is a transcription factor that is required for the myelinating

phenotype of Schwann cells (220). It is usually associated with Schwann cells at the pro-myelinating stage, i.e Schwann cells that are in a 1:1 association with axons (20). To determine whether Wnt5a biphasic effects on myelination was by promoting (2.5 ng/ml) or inhibiting (200 ng/ml) the progression of Schwann cells to the pro-myelinating stage, and therefore their 1:1 association, I assessed the number of Schwann cells positive for Krox-20 in myelinating co-cultures that were treated with Wnt5a at concentrations of either 2.5 or 200 ng/ml. Neither concentration of Wnt5a significantly affected the proportion of Schwann cells that stained positive for nuclear Krox-20 (Fig. 27).

An interesting feature of Schwann cells is that they deposit a basal lamina. This basal lamina organizes itself as a tubular structure that surrounds the Schwann cell / axon unit that is easily identifiable in the *in vitro* myelinating co-culture system. In Fig. 28, immunostaining for the $\gamma 1$ chain of laminin detected the basal lamina along Schwann cell / axon units of 10-day old myelinated co-cultures. Schwann cells were detected by DAPI nuclear staining, and the percentage of nuclei within the basal lamina tube, i.e Schwann cells in 1:1 association, was determined as about 5.5% on average. Neither Wnt5a at “low dose” (2.5 ng/ml) or “high dose” (200 ng/ml) had a significant impact in the number of Schwann cell nuclei detected within the tubular-shaped basal lamina (Fig. 28).

This result along with the Krox-20 data strongly suggests that Wnt5a did not affect Schwann cells until they achieved a 1:1 association stage with axons.

DISCUSSION

Small GTPases Rac1 and RhoA are well-established regulators of the actin cytoskeleton assembly (212), and changes in their activity has been

shown to affect the morphology and movements of many different type of cells (221-223). This is certainly true of the Schwann cells, whose morphogenetic behavior (morphology, migration and lamellar extensions) is regulated by Rac1, RhoA, cdc42 and Rock (85, 86, 119, 154). In its simplest interpretation, the data regarding the effects of Wnt5a on Schwann cell elongation and migration are therefore a direct corroboration of the previous findings regarding Rac1 and RhoA modulation by Wnt5a.

Interestingly some of the findings are different from what could have been expected. For example, the analysis of the radial sorting defects of the $\beta 1$ integrin and Rac1 Schwann cell conditional knockout mice, and of the ability of these Schwann cells to extend lamellipodia *in vitro* on a laminin-1 substrate (11, 85), have led to the conclusion that low Rac1 activity is associated with the extension (i.e. elongation) of the Schwann cells along axons. The increase in Rac1 activity, localized to the abaxonal plasma membrane of the Schwann cell (see Fig. 8) by $\beta 1$ integrin transducing ECM laminin signals was associated with the formation of multiple lateral lamellipodia *in vitro*, which *in vivo* would be infiltrating bundles of axons to affect the radial sorting process (85). We did detect increased lateral lamellipodia formation when Schwann cells were treated with low dose (2.5 ng/ml) of Wnt5a. This is however a condition that decreased Rac1 and increased RhoA activities in Schwann cells (Fig. 20). The increased Schwann cell elongation is however in line with Nodari et al (85) assessment that low Rac1 activity is associated with elongation of Schwann cells along axons. A major difference between our experimental setup and that of Nodari et al (85) is that ablating $\beta 1$ integrin or Rac1 expression may result in all-or-none kind of results. In our setup, Rac1 activation by $\beta 1$ integrin is still in

effect. Whether the decreased in Rac1 activity elicited by Wnt5a at 2.5 ng/ml reflects an interference with the $\beta 1$ integrin-mediated Rac1 activation is unknown. However if such were the case, a probable readout would then have been a decrease in the number of lateral lamellipodia, and not an increase. The observation of both elongation (low Rac1 activity as per (85)) and increased number of lateral lamellipodia (high Rac1 activity as per (85)) is intriguing and potentially suggests that laminin/ $\beta 1$ integrin and Wnt5a/PCP pathways provide differentially localized regulation of Rac1 activity within Schwann cells.

The increased numbers of lateral lamellipodia by Wnt5a at low dose could have provided an explanation as to why Wnt5a at low dose promoted myelination in the in vitro model of PNS myelination. More Schwann cells would have undergone radial sorting, ultimately leading to more pro-myelinating Schwann cells in a 1:1 association with an axon, a pre-requisite to spiral-wrapping and formation of the compact myelin sheath. However, I did not detect any increase in the number of Schwann cells expressing the pro-myelinating marker Krox20 (Fig. 27), nor did I detect an increase in the number of Schwann cells in 1:1 association with axons (Fig. 28). In fact, when co-cultured with axons, the formation of numerous lamellipodia is not elicited by treatment with Wnt5a at 2.5 ng/ml. Schwann cells elongated and associated with axons comparably to control, un-treated cultures.

Other than a slight reduction in elongation and a slight increase in motility, Schwann cells in co-cultures treated with 200 ng/ml (high dose) of Wnt5a associate as well as controls with axons.

Altogether these results suggest that wnt5a affects another aspect of myelination which might be spiral wrapping, thereby leading more (low dose) or

less (high dose) Schwann cells that are in a 1:1 association to extend their plasma membrane around the axon to form a myelin sheath upon compaction.

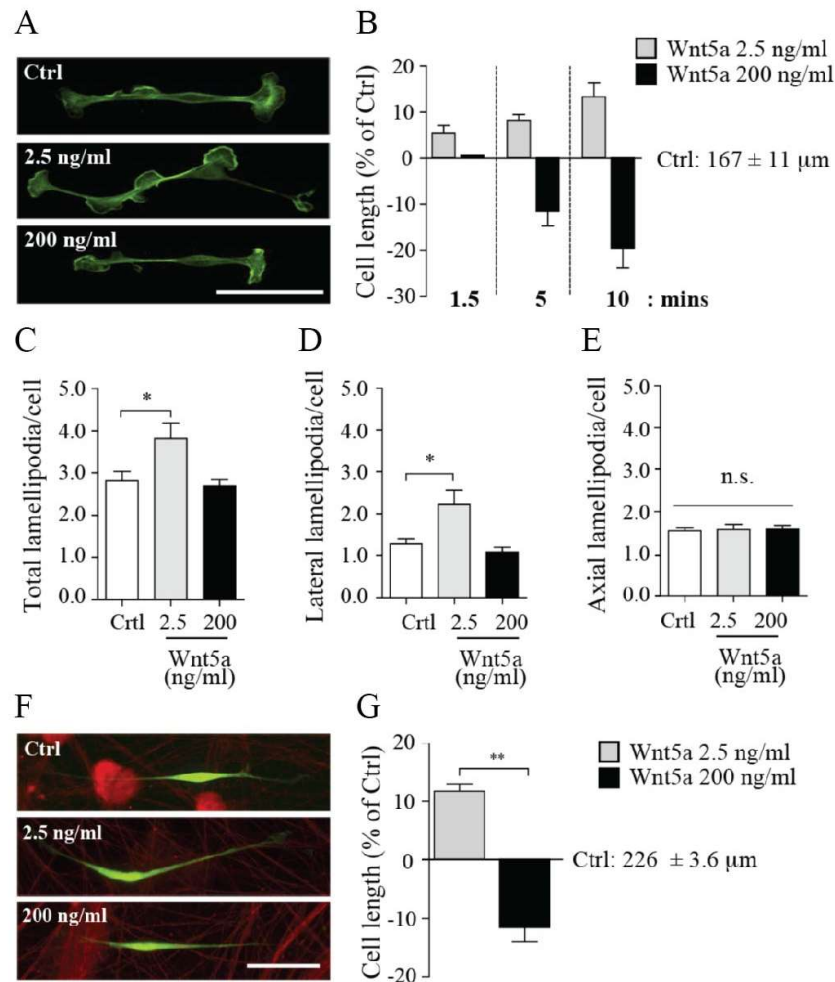


Fig. 24 Effect of Wnt5a on lamellipodia formation in Schwann cell. (A)

Representative pictures of SCs in presence or absence of Wnt5a. Two different doses of Wnt5a (2.5 ng/ml and 200 ng/ml, corresponding to hypermyelinating and hypomyelinating condition, respectively), were treated in low serum condition (3% of FBS). Alexa Fluor 488 Phalloidin was used to follow their morphology. (B) In the context of purified Schwann cell culture, average Schwann cell length was significantly affected by different concentrations of Wnt5a compared with non-treated. Quantification of total numbers lamellipodia per cell (C) were detailed into lateral lamellipodia per cell (D) and axial lamellipodia per cell (E). Quantitation shows results from more than 200 cells

of $n=3$ experiments, mean \pm SEM one-way ANOVA, n.s. = not significant. (F) In the axon contact condition, the effect of Wnt5a on cell length was measured with GFP expressing Schwann cells. (G) Quantification of cell length shows the bi-functional effect of Wnt5a on Schwann cell in the axon contact condition. Graphs represent mean \pm SEM normalized to control with Student T-test. * $p < 0.05$. ** $p < 0.01$.

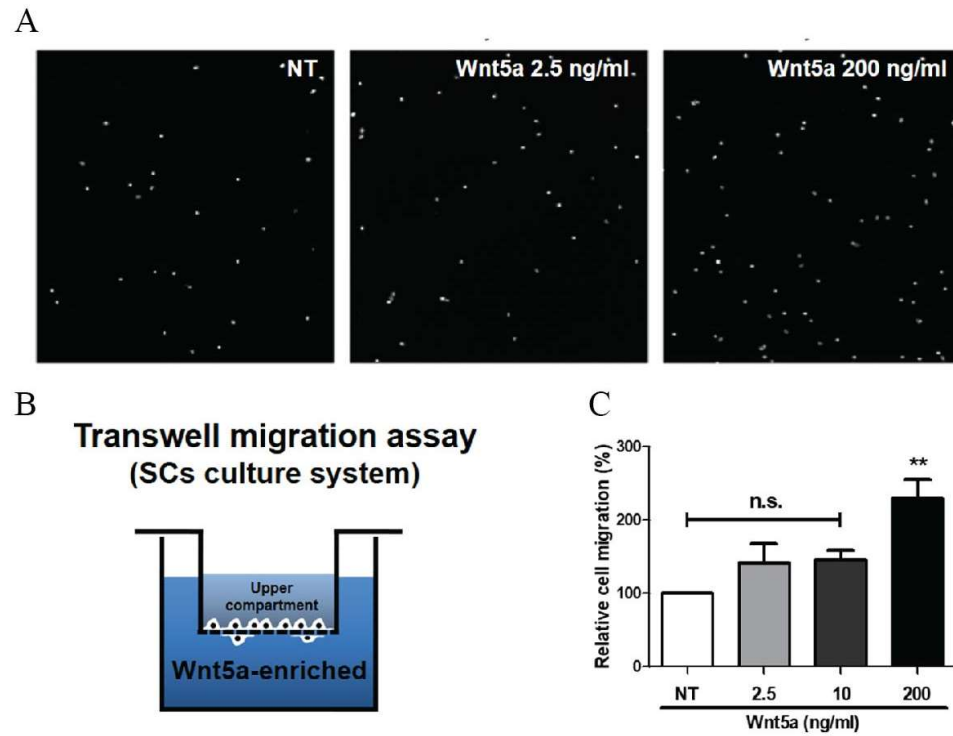


Fig. 25 Effect of Wnt5a on the migration of purified Schwann cells. (B) Migration assay was performed in a Boyden chamber, with Schwann cells seeded on the top of the filter and Wnt added in the bottom chamber. Schwann cells that migrated through the filter were detected by DAPI staining; representative images are shown in (A). (C) the quantitative analyses indicate an increased migration of Schwann cell when treated with Wnt5a at 200 ng/ml (average \pm SEM normalized to non-treated control; Student T-test $**p < 0.01$, $n=4$)

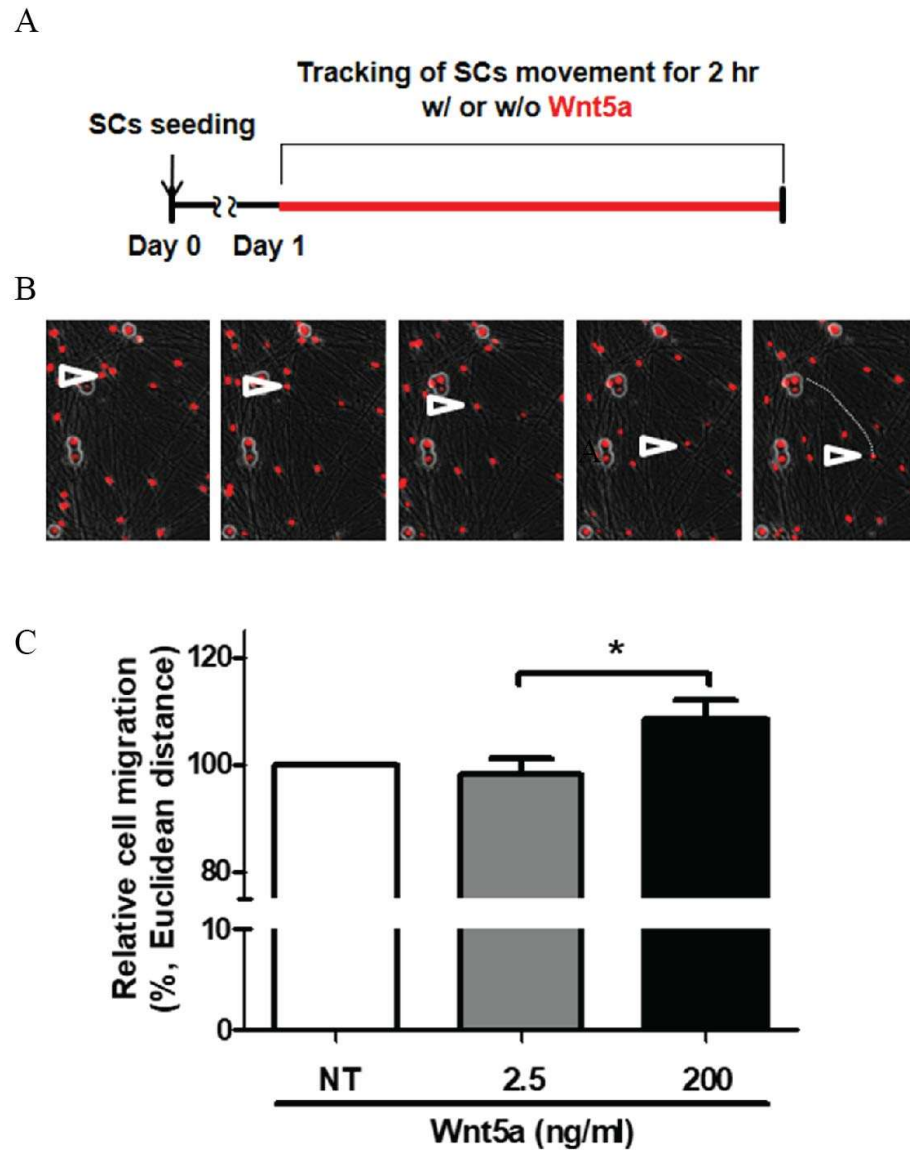


Fig. 26 Effect of Wnt5a on migration of SCs in contact with axons. (A) Experimental design of live Schwann cell tracking in SC/DRG co-cultures. (B) Representative pictures showing Schwann cell migration along an axon; migrating Schwann cells indicated at arrows. (C) Quantification of migrating Schwann cells; 400-500 cells were tracked for each condition. Graph represents mean \pm SEM normalized to control with Student T-test. * $p < 0.05$.

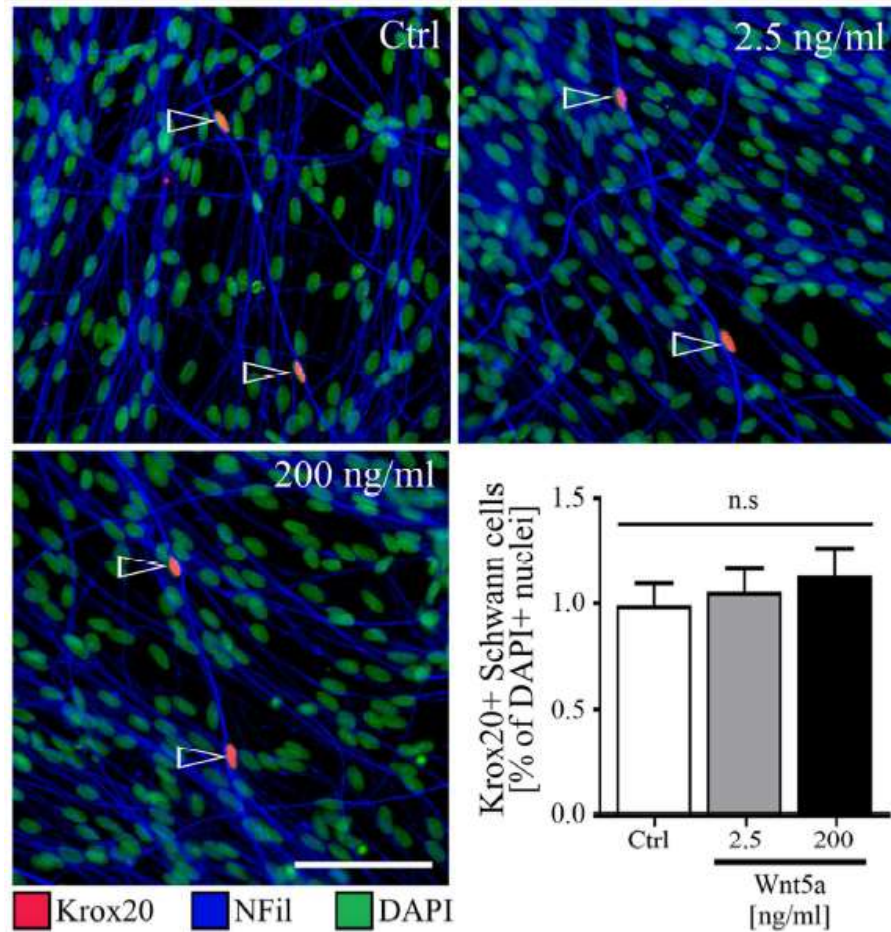


Fig. 27 Wnt5a does not promote or inhibit the progression of Schwann cells to the myelinating stage *in vitro*. Immunofluorescent staining (for Krox20, shown in red, arrowheads) of DRG neuron / Schwann cell myelinating co-cultures treated with Wnt5a (2.5 or 200 ng/ml) for 10 days. Quantitation shows results from n=3 experiments, mean \pm SEM one-way ANOVA, n.s. = not significant.

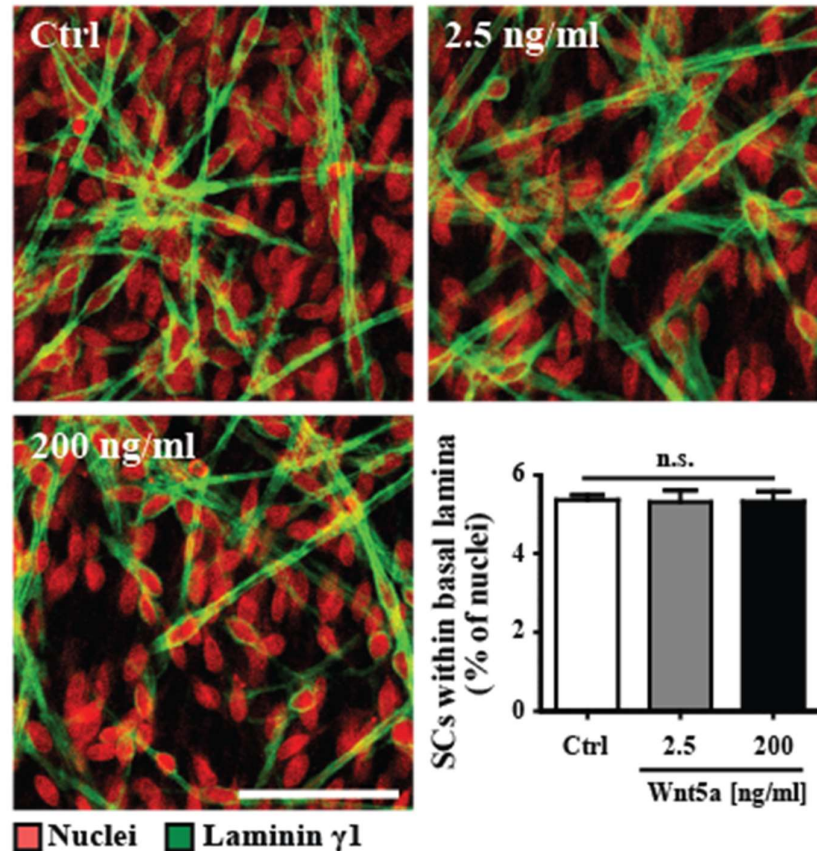


Fig. 28 Exogenous treatment of Wnt5a does not affect Schwann cells associations with DRG neurons. SCs co-cultured with DRG neuronal axons were conditioned with Ascorbic acid for 10 days to induce the association with or without two different doses of Wnt5a, which are corresponding to hypermyelinating dose (2.5 ng/ml) and hypomyelinating dose (200 ng/ml), respectively. Antibody against Laminin $\gamma 1$ was used for basal lamina, which is an indication of SCs/Axon association. Nuclei of SCs in the tubular structure stained with laminin $\gamma 1$ were plotted after normalization to total SCs number. Quantitation shows results from $n=3$ experiments, mean \pm SEM one-way ANOVA, n.s. = not significant.

CHAPTER VI

Regulation of Schwann cell differentiation and myelination by endogenous expression of Wnt5a

RATIONALE

In the previous chapters, I provided strong evidence in support for a function of Wnt/PCP signaling in regulating Schwann cell myelination. This evidence revolved around using Wnt5a, a well-accepted Wnt/PCP ligand, to promote or inhibit, in a concentration-dependent manner, the formation of myelin segments in the *in vitro* culture model of PNS myelination. Furthermore, loss-of-function of Daam1, a required component of the Wnt/PCP signaling platform abolished Wnt5a's effects at low dose.

Most interesting, Wnt5a is expressed in the developing sciatic nerve, from at least embryonic day E18 to post-natal day p30, and its levels of expression are developmentally regulated. While the *in vitro* experiments manipulated the culture system by adding exogenous Wnt5a, the *in vivo* data support the possibility for endogenous levels of Wnt5a, essentially expressed by Schwann cells (Fig. 12), to have a function in the *in vitro* system. This hypothesis is further supported by the facts that the loss of function of Daam1 alone does affect Schwann cell myelination *in vitro*, regardless of the addition or not of exogenous Wnt5a. Finally Wnt11, another Wnt/PCP ligand, had no effects on the formation of myelin segments by Schwann cells *in vitro*, further pointing to a specific function for Wnt5a.

In this chapter, I used a lentiviral-mediated loss-of-function approach to determine the role of Schwann cell-expressed Wnt5a in the process of myelination in the *in vitro* culture system.

RESULTS

Endogenous expression of Wnt5a is necessary for myelination

To characterize the function of the endogenous Wnt5a produced by Schwann cells, I designed constructs expressing short-hairpin RNAs specific against Wnt5a and delivered them into Schwann cells by lentiviral transduction. These constructs also express the green fluorescent protein GFP whose expression in $\geq 90\%$ of the cells demonstrates the efficient viral transduction of the Schwann cells (data not shown). Both Wnt5a shRNAs (shWnt5a #1 and #2) inhibited Wnt5a mRNA levels by 60 to 75% compared to control shScr-transduced cells, as determined by qRT-PCR (Fig. 29A). These modified Schwann cells were then co-cultured with purified DRG neurons and maintained in myelinating conditions for 10 days. Wnt5a-deficient Schwann cells were impaired in their ability to generate myelin segments in comparison to shScr controls, the cultures showing a significant 50% decrease in the number of MBP-positive segments (Fig. 29B).

Wnt5a increases its own production by Schwann cells

A positive-feedback mechanism has been shown to exist for Wnt5a in various cell types, such as ST2 bone marrow stromal cells, HEK293A cells, and 967 cancer cell lines (224). To determine whether Schwann cells also could be further induced to increase Wnt5a expression upon Wnt5a treatment, I performed qRT-PCR analyses on mRNA that was isolated from control Schwann cells (not treated) or Schwann cells that were treated with 200 ng/ml

of Wnt5a for 24 hrs. Wnt5a mRNA levels were increased by about 75% when Schwann cells were treated with exogenous Wnt5a (Fig. 30A). This positive feedback at the level of mRNA expression was confirmed at the protein level by Western blotting (Fig. 30B). Of note, Wnt5a protein levels are very low and not detectable in a “conventional way”, as can be seen in Fig. 30B (NT lane). Secretion of Wnt molecules however requires palmitoylation (225), a biochemical modification catalyzed by the palmitoyl acyltransferase porcupine (226). I therefore treated Schwann cells with the potent porcupine inhibitor C59 (Proffitt 2013) to increase the intracellular levels of non-secreted Wnts, and therefore Wnt5a as well. Although C59 alone did not bring Wnt5a in Schwann cell lysates to detectable levels (lane C59 in Fig. 30B), the association of Wnt5a with C59 did. A band at slightly less than 37 kDa was easily detected (Fig. 30B). The mature Wnt5a protein backbone being calculated at 35.6 kDa, this results strongly suggests that exogenous Wnt5a stimulates Schwann cells' own production of Wnt5a, and support the quantitative PCR data.

Cell density regulates Wnt5a production by Schwann cells

Since Schwann cells produce their own Wnt5a (Fig. 30), it stands to reason that Schwann cells could auto-stimulate their own production in a Wnt5a-dependent positive feedback loop. While there are several molecules that could be used to block Wnt signaling (227-229), none is specific to Wnt5a. I therefore resorted to determine whether cell density could have an impact on Wnt5a expression by Schwann cells.

Schwann cells were seeded at a constant number of cells (250,000) per plate. However, plate size was different: ø100mm dish for low density Schwann cell culture ($250,000 \text{ cells} / 60 \text{ cm}^2 = 4,167/\text{cm}^2$) and ø35mm dish for high

density Schwann cell culture ($250,000 \text{ cells} / 9 \text{ cm}^2 = 27,778/\text{cm}^2$). Cultures were kept in the same volume of media to ensure that the only variable was cell density. Changes in Wnt5a levels were determined by ELISA assay from: i) the culture media and ii) the Schwann cells lysate after collecting the culture media (Fig. 31A).

Wnt5a level detected in the culture media of high-density Schwann cell cultures was about double that of Wnt5a detected in the culture media of low-density Schwann cell cultures (93 pg/ml vs 48 pg/ml, respectively; Fig. 31B). Wnt5a levels from the cell lysates presented a similar trend, lysates from high cell density culture containing about 25% more Wnt5a than lysates from low cell density cultures (Fig. 31C).

These results are of course not a direct demonstration that the increased expression in Wnt5a by Schwann cells at high cell density is a result from Wnt5a secretion. However, combined with findings that exogenous Wnt5a stimulates its own expression (Fig. 30), it suggests that high cell density increases Wnt5a production in an autonomous manner.

Endogenous expression of Wnt5a enhances Schwann cell migration

I previously showed that exogenous Wnt5a was promoting Schwann cell migration (Figs. 25 and 26). I therefore wondered whether endogenously expressed Wnt5a could have a similar regulatory role on Schwann cell migration. As described earlier, Schwann cells were transduced either with control (shScr) or Wnt5a-specific shRNA-expressing lentiviruses to specifically abolish Wnt5a expression. 6 days post-transduction, Schwann cells were harvested and re-seeded onto well-defined small (2 mm^2) patches. These patches were delimited with a PDMS mask to ensure sharp edges from which

migration could be easily measured (see Chapter IX “Materials and Methods” for more details). The number of Schwann cells migrating out of the patches and the distance of migration were determined after 18hrs of culture (Fig. 32). Schwann cells deficient in Wnt5a expression (by about 60 to 75% on average; see Fig. 29) also presented a deficiency in their ability to migration. The number of cells exiting out of the culture patch was significantly decreased by about 20% (Fig. 32C).

These results, and those presented in Figs. 25 and 26 suggest that Wnt5a promotes Schwann cell migration.

Wnt5a prevents the differentiation of Schwann cells when cultured in a high cell-density context

It has long been known that cyclic AMP (cAMP) is a key factor regulating the differentiation of isolated Schwann cells into a “myelinating” phenotype (230, 231); Schwann cells treated with cAMP are induced to express typical myelin markers such as the pro-myelinating transcription factor Krox-20, the myelin-associated glycoprotein MAG, the myelin protein zero (Mpz) and periaxin (Prx). A similar effect is observed with several cAMP derivatives such as dibutyryl-cAMP (dbcAMP; (232)).

The expression of myelin markers indicates that Schwann cell are differentiating into a pro-myelinating phenotype and are no longer migrating. As the previous section showed that Wnt5a promoted Schwann cell migration, I then tested whether Wnt5a could then be inhibitory to dbcAMP-induced Schwann cell differentiation.

In a first series of experiments, Schwann cells were seeded at low or high density as previously described: a ø100mm dish for low density Schwann

cell cultures ($250,000 \text{ cells} / 60 \text{ cm}^2 = 4,167/\text{cm}^2$) and a $\varnothing 35\text{mm}$ dish for high density Schwann cell cultures ($250,000 \text{ cells} / 9 \text{ cm}^2 = 27,778/\text{cm}^2$). Cells that are not treated with dbcAMP do not stain for periaxin, regardless of cell density (Fig. 33A; panel NT). Interestingly, the response to dbcAMP is cell density-dependent (Fig. 33A; lower panels). 3 days after treatment, 35% of Schwann cells that are cultured at low densities had up-regulated the expression of periaxin, whereas only 5% of Schwann cells cultured at high density were positive for periaxin (Fig. 33B).

In a second series of experiments, lentivirus-transduced Schwann cells (shSCR control; shWnt5a knockdown) were seeded onto well-defined small (2 mm^2) patches delimited by a PDMS mask (Fig. 32; top panels). Cells were then treated with dbcAMP for 3 days and then assayed for periaxin expression. The PDMS mask was kept in place during the experiment to prevent Schwann cells from migrating out. While no to little periaxin-positive cells were expected in the control Schwann cell patch, a substantial amount (about 21%) of cells were actually expressing periaxin. The pattern of expression was however striking, being mostly restricted to the edge of the patch (Fig. 34A; ctrl panel). The number of periaxin-positive Schwann cells was increased by 57% when Wnt5a expression was abolished (Fig. 34A; right panel). Also in contrast to the control Schwann cell set-up, there was no particular pattern of expression and periaxin-positive Schwann cells were present throughout the culture spot (Fig. 34; right panel).

DISCUSSION

In this chapter, I expanded on my findings derived from adding exogenous Wnt5a to a myelinating Schwann cell / DRG neuron co-culture system. The facts that: i) Wnt5a is expressed by the developing PNS, ii) preferentially produced by Schwann cells, iii) affects Schwann cell myelination whereas Wnt11 does not, iv) that the effect is Daam1-dependent and, more importantly that v) Daam1 alone can impact Schwann cell myelination all pointed to a role for the Schwann cell endogenously-controlled Wnt/PCP pathway, and more particularly to endogenous Wnt5a in regulating myelination. A possible role that I addressed in this Chapter.

One of the findings is that Schwann cells produce very low amounts of Wnt5a. The amounts released in the culture media are in the pg/ml range (Fig. 31), varying from about 50 to 100 pg/ml depending on cell density. Yet ablating this low level of expression by Schwann cells resulted in a dramatic and significant inability of these cells to form myelin segments. This result further support that secreted Wnt5a, when at “low concentration”, are myelination in vitro, and that Schwann cells respond positively up to a concentration of 2.5 ng/ml (2,500 pg/ml) (Fig. 13).

Interestingly the amount of Wnt5a secreted by Schwann cells is dependent on their density when in culture. Furthermore, Wnt5a itself induces Schwann cells to express more Wnt5a. It is therefore likely that as Schwann cells are cultured at high density, they more easily respond to Wnt5a signaling through a paracrine-mediated positive feedback loop.

Surprisingly, the amounts of Wnt5a secreted by Schwann cells when cultured at high density are promoting Schwann cell migration (Fig. 32). It is

surprising as exogenous Wnt5a at 2.5 ng/ml was not promoting Schwann cell migration, but higher amounts (200 ng/ml) did (Figs. 25 and 26). This raises the question as to what must be considered a “low” or “high” dose of Wnt5a. The amounts that were calculated correspond to secreted Wnt5a, in solution. That does not provide information as to the amount of Wnt5a that is available in the pericellular space, whether initially added as an exogenous molecule, or secreted by the Schwann cells themselves. While the concentrations in 4 ml of culture media ranged from about 50 to a 100 ng/ml, the concentrations in the pericellular space could be higher even though the amount of Wnt5a detected may be lower.

While surprising, this result was interesting. Indeed, Schwann cells cultured at high density usually do not respond to differentiating agents such as dbcAMP. However, while Schwann cells that were within the culture patch did not, cells that were at the periphery substantially did (Fig. 34). This localized response to dcbAMP was lost once the expression of Wnt5a was abolished. Schwann cells at high density within the patch were also no longer prevented to differentiate, and up-regulated the expression of myelin marker periaxin (Fig. 34). An important parameter for Schwann cells response to dbcAMP appears to be context. Indeed cells at the periphery of the patch are not fully surrounded by other Schwann cells, whereas cells within the patch are. It is an intriguing possibility that Schwann cells received positional information that made them sensitive or not to dbcAMP. It is important to note that positional information is one of the major function of PCP, regardless of Wnt signaling or not (104-106, 233). However, as Wnt molecules do use the PCP platform to signal to cells, it is exciting to speculate that Wnt5a is using the positional cues providing by the

PCP platform to allow Schwann cells to differentiate or not. Also, as the ablation of Wnt5a impaired Schwann cells ability to migrate, I would like to propose that the Wnt5a/PCP pathway may provide a mean of inversely correlating the capacity of a Schwann cell to migrate or to differentiate.

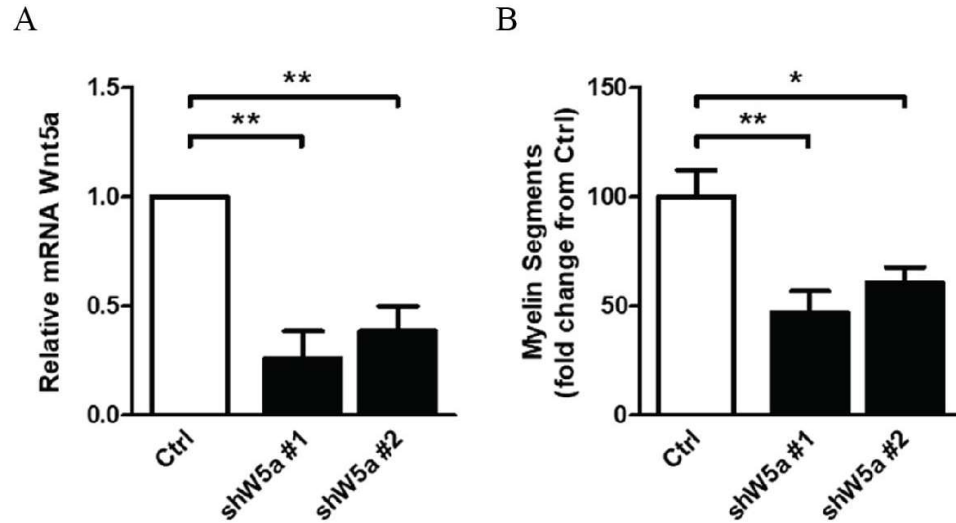


Fig. 29 Endogenous Wnt5a is necessary for myelination (A) qRT-PCR analysis for knockdown rate of Wnt5a. SCs were infected with control or one of two different lentiviruses harboring shRNA for Wnt5a. Results normalized to control. (B) The effect of Wnt5a knockdown on myelination in vitro culture. Myelination assessed by MBP+ segments. Bars represent the normalized average \pm SEM. * $P < 0.05$, ** $P < 0.01$, 1way ANOVA with Bonferroni's Multiple Comparison Test.

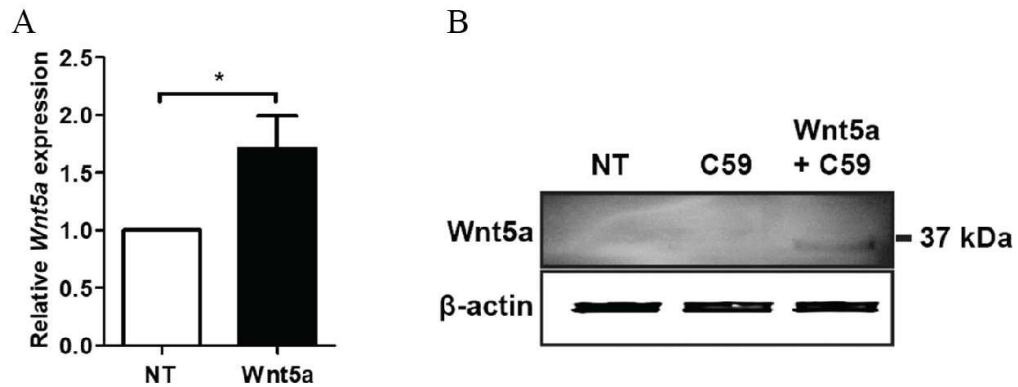


Fig. 30 Wnt5a stimulates its own expression by Schwann cells. (A) Normalized Wnt5a mRNA levels after exogenous Wnt5a treatment (200 ng/ml). Graphs represent mean \pm SEM normalized to control with Student T-test. * $p < 0.05$. (B) Western blot showing induction of endogenous Wnt5a expression after exogenous Wnt5a treatment (200 ng/ml). C59, an inhibitor of post-translational modification of Wnt molecules, was used to distinguish newly synthesized Wnt5a from exogenously treated-Wnt5a.

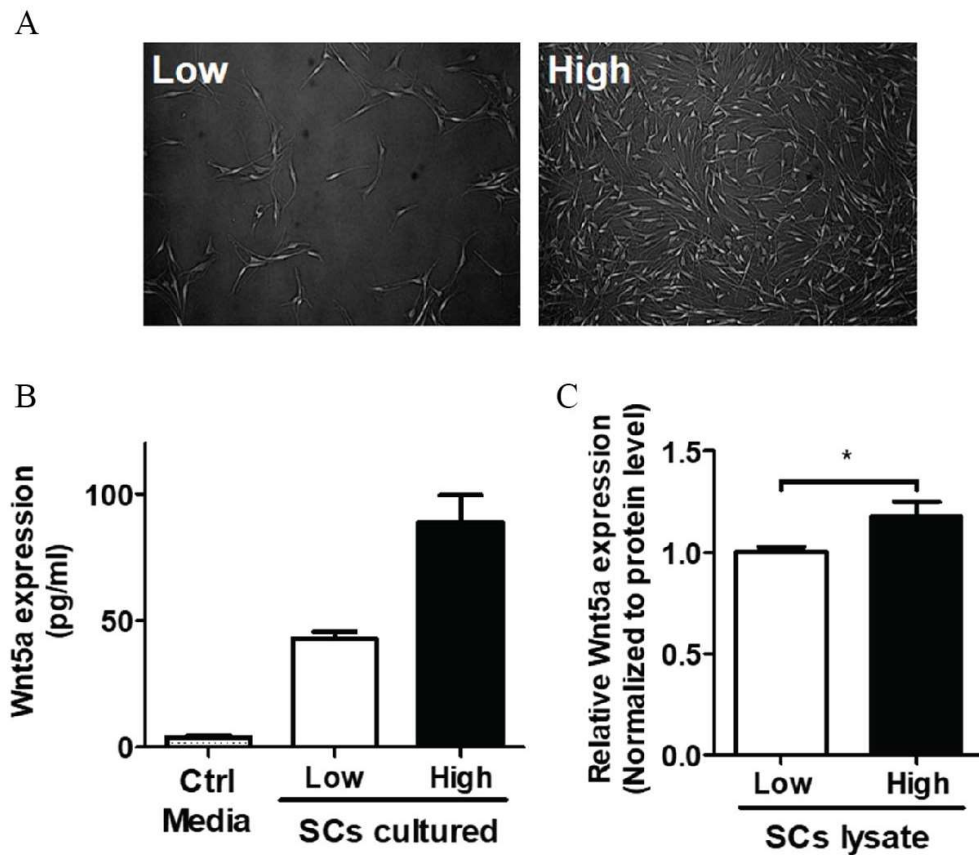


Fig. 31 High cell density increases Wnt5a expression in an autonomous manner. (A) Representative pictures for Low and High cell density. Level of Wnt5a in cultured media (B) or cell lysate (C) in Low and High cell density cultures were measured by ELISA. Graph represents mean \pm SEM, normalized to control with Student T-test. * $p < 0.05$.

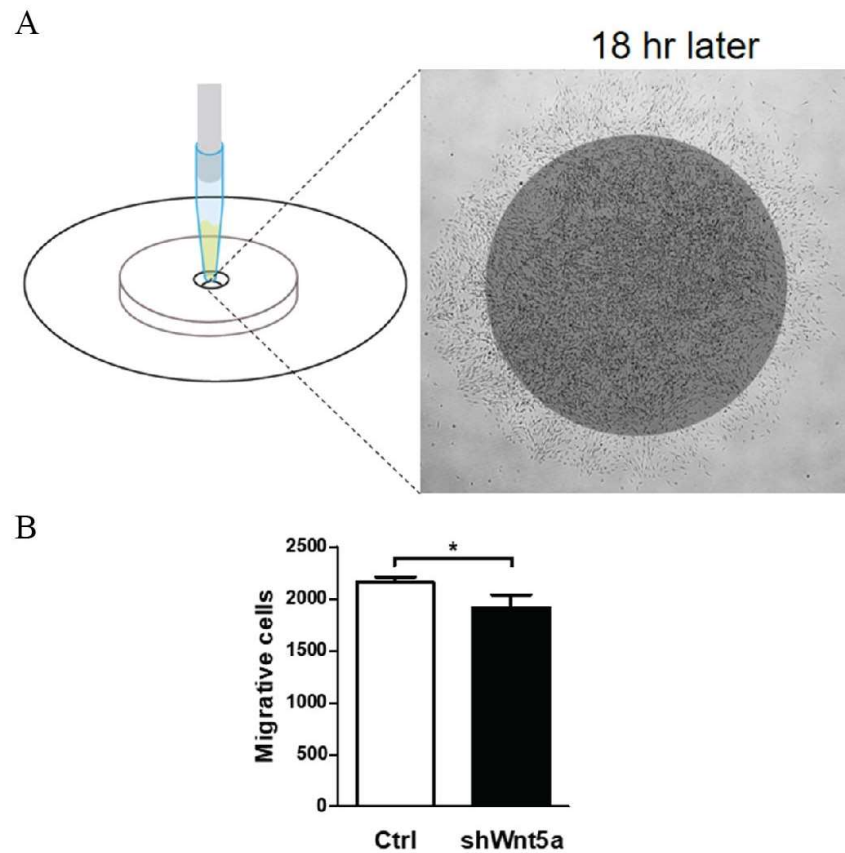


Fig. 32 Wnt5a is necessary for proper Schwann cell migration. (A) Stamp-PDMS migration assays were used to measure the effect of endogenous Wnt5a on Schwann cell migration. Endogenous Wnt5a expression was inhibited by shRNA knockdown. (B) Graph represents mean \pm SEM with Student T-test. * $p < 0.05$. $n=6$.

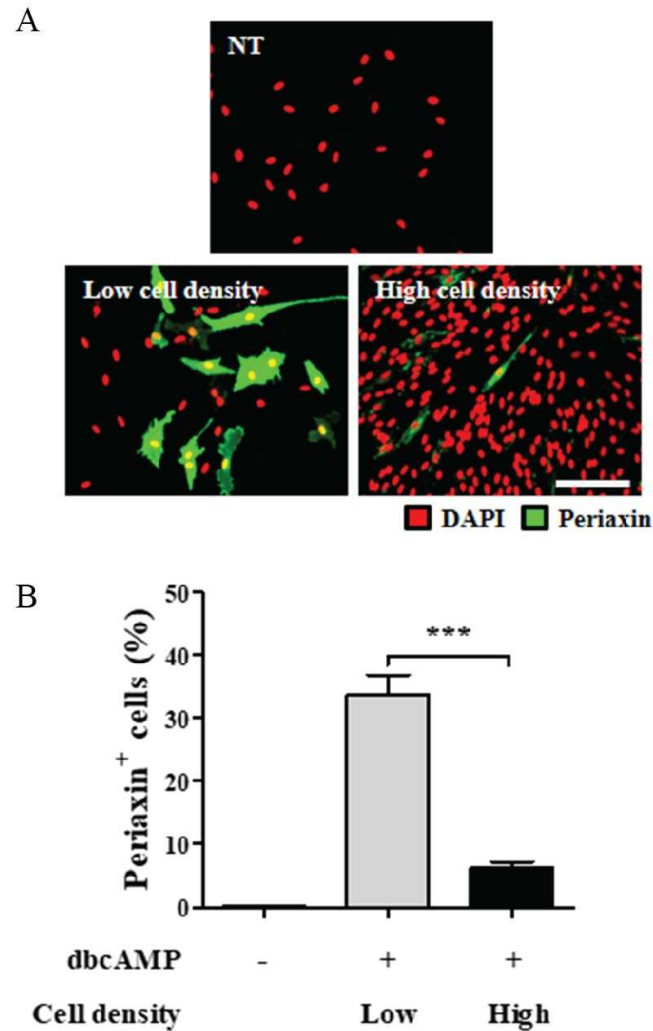
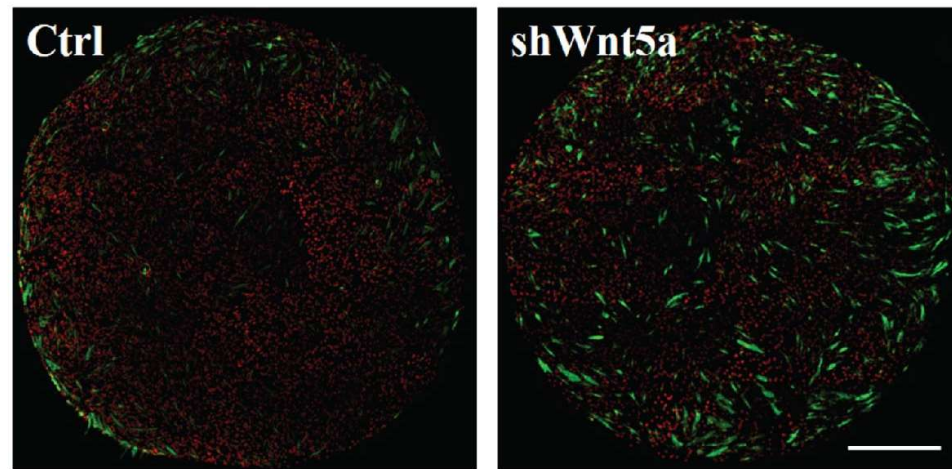


Fig. 33 Effect of cell density on induction of Periaxin. (A) Representative pictures showing Periaxin expression of Schwann cells at different cell densities. Schwann cells were treated with dbcAMP (1mM) for 3 days in low serum condition (3% of FBS). Periaxin is used as a marker for non-migrating Schwann cell. (B) Quantification of (A) presenting a suppressed expression of Periaxin by high cell density compared to cultures with low cell density. Periaxin⁺ Schwann cells in green were counted and plotted after normalization to total SCs number (by DAPI in red). Quantitation shows results from n=3 experiments, mean \pm SEM one-way ANOVA.

A



B

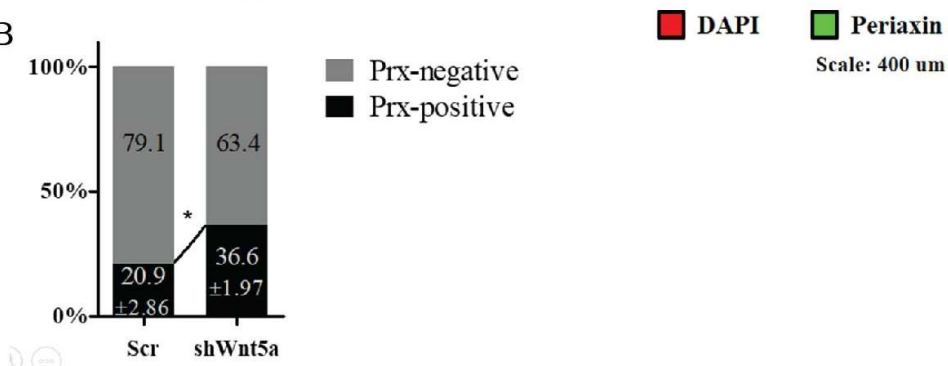


Fig. 34 Wnt5a prevents Periaxin expression in cell-dense area. (A) Ectopic Prx expression of Wnt5a-deficient SC in limited area compared to control. 20,000 of SCs extracted from post-natal 2 were restricted in PDMS 2 mm diameter-well with differentiating condition (1 mM of dbcAMP). Specification of differentiation into non-migrating condition of SCs was demonstrated by immunostaining of Prx with counterpart staining (DAPI). (B) Quantification of Prx⁺ expression by comparing the ratio between Prx⁺ and Prx⁻; with Student T-test. *p < 0.05.

CHAPTER VII

Wnt5a counteracts the inhibitory effect of the Wnt3a/canonical pathway on myelination

RATIONALE

Depending on the intracellular transducing signals elicited inside the cells, the Wnt pathways have been classified into 2 broad categories: i) the canonical pathway that is mediated by the stabilization of intracellular β -catenin, which then act as a co-transcription factor to transcription factors of the Tcf/Lef family to regulate gene expression (131, 132), and ii) the non-canonical pathway, a rather broad categorization that encompasses signaling through the PCP and small GTPases platform (116, 126, 139-141) or by increasing calcium levels and regulating gene expression mediated by transcription factors of the NFat family (83, 143-145).

Due to the complexity in Wnt-mediated signaling provided by the plethora of Wnt (19 molecules) and Wnt receptors (Frizzled; 10 molecules), and a certain level of ubiquity between which Wnt interact with what Frizzled receptor, most of the efforts in understanding the molecular mechanisms enabling Wnt signal transduction has been focused on the intracellular components. To date, the best-deciphered and understood pathway is the Wnt/canonical pathway (181), and is the pathway that so far as received most of the attention in the context of myelination, both in the CNS and PNS (146, 147, 149-153, 234).

In the CNS, activation of the canonical Wnt pathway by genetic or pharmacological means inhibits oligodendrocyte differentiation and leads to delayed myelination (146-149). The conditional deletion of APC, a negative regulator of canonical Wnt signaling, in oligodendrocyte progenitor cells (OPCs) also prevents their differentiation (150). Finally Wnt3a, a well-characterized ligand associated with the activation of the Wnt/ β -catenin pathway (136-138), is sufficient to perturb oligodendrocyte maturation (149) *in vitro*. These results support the hypothesis that in the CNS the Wnt/ β -catenin pathway provides a negative control to myelination by delaying differentiation of oligodendrocytes.

In the PNS, the assessment of the Wnt/catenin function in Schwann cell myelination is more confused. On one hand the inhibition of GSK3- β , which leads to the accumulation of, and translocation of β -catenin into the nucleus, promotes Schwann cell differentiation and enhances myelination (149). Tawk et al. have also provided some evidence that the Wnt/catenin can drive the expression of myelin genes in Schwann cells (153). While these results suggest a positive role for the Wnt/catenin in the PNS, other data suggest the opposite. For example, the conditional deletion of APC in Schwann cells results in hindlimb weakness and impaired nerve conduction in sciatic nerves (152), suggesting that the canonical Wnt pathway is inhibitory to myelination in the PNS. The analyses of mice carrying loss-of function and gain-of-function mutations in β -catenin also suggest that while the Wnt/ β -catenin pathway promote radial sorting and the progression of Schwann cells to the pro-myelinating stage, it is transiently inhibitory to the formation of the myelin sheath after birth (151).

Interestingly, the Wnt/ β -catenin and Wnt/PCP pathways have been shown to intersect. More specifically, the Wnt/PCP signaling pathway has been shown to be antagonistic to the canonical Wnt/ β -catenin (235-237).

In this Chapter, I therefore aimed i) to further investigate the function of the canonical pathway by treating the Schwann cell / DRG neuron myelinating culture system with Wnt3a and ii) determine a function for Wnt5a in interfering with Wnt3a effects.

RESULTS

Wnt3a inhibits myelination independently of Schwann cell proliferation.

The aim of this study was to assess the effect of exogenously added Wnt3a, an activator of the canonical Wnt/ β -catenin signaling, on the ability of Schwann cells to form myelinated segments in the well-established Schwann cell / DRG myelinating co-culture system. Three days after seeding Schwann cells onto the DRG neurite network, myelination was induced by the addition of ascorbic acid, without or with Wnt3a (0 to 100 ng/ml;). Myelination was assessed by immunostaining for MBP-positive compact myelin segments after 10 days of culture. Wnt3a induced a concentration-dependent decrease in the number of myelinated segments (Fig. 35A) compared to controls (Fig. 35A, NT lane), ranging from a $17 \pm 6\%$ (25 ng/ml) to a $91\% \pm 5\%$ (100 ng/ml) decrease. Schwann cell proliferation, as determined by Edu incorporation was not affected (Fig. 35B).

These results further comfort the notion that the Wnt/canonical pathway is inhibitory to the formation of compact myelin segments.

Low doses of Wnt5a counteract the inhibitory effect of Wnt3a on myelination *in vitro*

The non-canonical Wnt pathway, and in particular the Wnt/PCP pathway has been shown to counteract the activity of the canonical Wnt/ β -catenin pathway (142, 236). As I previously showed that Wnt5a, a prototypical non-canonical Wnt/PCP ligand, enhanced myelination at low doses (2.5 to 10 ng/ml), I designed experiments to determine whether Wnt5a could abolish the Wnt3a-mediated inhibitory effect on myelination. Schwann cells were seeded on DRG neurites and cultured for 3 days in non-myelinating conditions before the addition of ascorbic acid to initiate myelination. Two control groups were setup: i) cultures that did not receive any exogenous Wnts, and ii) cultures receiving an inhibitory dose (100 ng/ml) of Wnt3a. All other cultures received Wnt3a at 100 ng/ml along with varying amounts of Wnt5a (2.5 to 200 ng/ml) (Fig. 36). The number of MBP-positive myelinated segments were determined and normalized to the numbers obtained from the non Wnt-treated control cultures (set as a 100% myelination index). As was previously found (Fig. 35), Wnt3a at 100 ng/ml inhibited myelination by about 37 ± 7.9 %. Interestingly, this inhibitory effect of Wnt3a was countered by the addition of Wnt5a at low doses, in a concentration-dependent manner. The effect of Wnt5a at high doses (50 and 200 ng/ml) is not significant either way, i.e. neither promoting nor inhibiting the Wnt3a effect in a significant way. The possible interpretation of this result will be discussed later on.

Wnt3a inhibits SC differentiation

As mentioned earlier, cyclic AMP (cAMP) is a key factor regulating the differentiation of isolated Schwann cells into a “myelinating” phenotype (230,

231); Schwann cells treated with cAMP (20, 238)) and derivatives such as dibutyryl-cAMP (dbcAMP) (232) are induced to express typical myelin markers such as the pro-myelinating transcription factor Krox-20, the myelin-associated glycoprotein MAG, the myelin protein zero (Mpz) and periaxin (Prx).

I tested the effect of Wnt3a on the ability of Schwann cells to express the pro-myelinating marker Krox20 upon dbcAMP treatment. Total cell numbers were determined by counterstaining the nuclei with DAPI. As shown in Fig. 37, Wnt3a abolished in a concentration-dependent manner the expression and nuclear localization of Krox20 in Schwann cells that was induced by dbcAMP treatment. While $51 \pm 9\%$ of the control Schwann cells treated with 1mM dbcAMP were positive for nuclear Krox20 (compared to the $1.2 \pm 1\%$ of non-treated cells), only $6 \pm 2.8\%$ Schwann cells showed a nuclear Krox20 localization upon Wnt3a treatment at 100 ng/ml. As mentioned, the Wnt3a inhibitory effect was concentration-dependent (Fig. 35A).

These data suggest that Wnt3a inhibits the differentiation of Schwann cells into pro-myelinating cells, consequently leading to less myelination in the *in vitro* system.

Low-doses of Wnt5a counteracts the effect of Wnt3a on myelin gene expression

I further examined the effects of Wnt3a on the differentiation of Schwann cells. As I have shown previously, Schwann cells do respond to Wnt3a by increasing Axin2 expression, as determined by RT-qPCR, in a concentration-dependent manner (Fig. 38A, left panel). Interestingly, the basal mRNA levels of myelin markers Mpz and PMP22 were inversely related to Axin2 expression, showing a concentration-dependent decrease (Fig.38A, middle and right

panels, respectively). Wnt5a alone, at both low and high doses, did not activate the Wnt/ β -catenin pathway (lack of increase in Axin2 expression; Fig 38B, left panel), and also did not affect the basal levels of mRNA expression of both Mpz and PMP22 (Fig. 38B, middle and right panels, respectively).

Interestingly however, when cells were treated with both Wnt3a (at 25 ng/ml) and Wnt5a (2.5 and 200 ng/ml), the inhibitory effect of Wnt3a on Mpz and PMP22 expression was abolished (Fig. 38C). The activation of Axin2 by Wnt3a however was not inhibited by the low dose of Wnt5a, but by Wnt5a at 200 ng/ml (high dose) (Fig. 38C).

These results suggest that Wnt5a may modulate myelin genes expression by counteracting the Wnt3a-mediated Wnt/canonical pathway in Schwann cells, *in vitro*.

DISCUSSION

The current view is that the canonical Wnt/ β -catenin signaling pathway in the CNS is a negative regulator to myelination (147, 149). In the PNS, the role of the Wnt/canonical pathway has been more elusive, and has been proposed to either promote (151, 153) or inhibit myelination (152), on the bases of effects on radial sorting *in vivo*, expression of myelin markers *in vitro*, the progression to the pro-myelinating stage *in vitro* (151) and transiently delayed / hypo-myelinated *in vivo* phenotype (151). Surprisingly the effect of Wnt canonical ligands on Schwann cell myelination using the *in vitro* Schwann cell / DRG co-culture system has not been evaluated. Furthermore, the Wnt/PCP pathway having been shown to interact with the Wnt/canonical pathway in other cells (235-237), I wondered whether the pro-myelinating effect of Wnt5a at low dose could reflect, in part, a counteracting effect on the Wnt/canonical pathway.

The first interesting data is that the co-culture system responds to the treatment by Wnt3a, which suggest that Wnt3a *in vivo* may also have a regulatory function during the processes that lead to the formation of the myelin sheath. The finding is that Wnt3a was inhibitory to Schwann cell myelination. This is in line with Elbaz et al.'s findings (152) showing that the conditional ablation of APC expression in Schwann cell, which results in the activation of the Wnt/canonical pathway, is inhibitory to PNS myelination *in vivo*. It is also in agreement with the findings of Grigoryan et al. (2013), who showed that a different conditional activation of the Wnt/canonical pathway also leads to a transient delay in PNS myelination (151). It is however important to keep in mind that when creating conditional loss-of-function and gain-of-function mutants of β -catenin (151), one also perturbs cadherin-mediated cell-cell interactions (239) and that perturbing E-cadherin in Schwann cells also results in a transient delay in PNS myelination (240).

The second finding is that my data is at odds with some evidence provided by the work from Tawk et al. (153). While my data, both regarding Schwann cell myelination in the co-culture system as well as the analyses of myelin genes expression, outlined an inhibitory function for Wnt3a, Tawk et al.'s data suggested the opposite, that the Wnt/canonical is promoting to myelination. One of the major differences is that I used primary Schwann cells freshly purified from post-natal day p2 sciatic nerves, whereas the data from Tawk et al. was generated from the MSC80 immortalised Schwann cell line. Another major difference is that their work highlighted the pro-myelinating effect of Wnt1, another canonical Wnt, whereas my work focused on Wnt3a. It is possible for 2 different Wnts to be characterized as canonical ligands and yet elicit different

if not opposite responses from identical cells (241), even more likely so from different cells (primary Schwann cells versus immortalised Schwann cells). Nevertheless, the decrease in the basal levels of myelin genes such as *Mpz* and *PMP22* by Wnt3a (Fig. 38A) supports its inhibitory effect on the formation of compact myelin sheathes in the Schwann cell / DRG co-culture system (Fig. 35).

These data taken together suggest a possible negative role on PNS myelination for the Wnt/ β -catenin signaling when activated by the Wnt3a canonical ligand.

Finally I determined whether Wnt5a could achieve its promoting myelinating function by inhibiting Wnt3a. The preliminary data that I have obtained (Fig. 36) does strongly support such a possibility. Wnt5a at low doses (2.5 to 10 ng/ml) did counteract the inhibitory effect of 100 ng/ml of Wnt3a. It is interesting that in these conditions, 2.5 ng/ml is less promoting than 10 ng/ml, when compared to the effect of Wnt5a alone, where 2.5 ng/ml was more promoting than 10 ng/ml. This may indeed support the finding that the mechanism of Wnt5a to support myelination is, at least in part, by inhibiting an inhibitory pathway. Adding more of exogenous inhibiting molecules, such as Wnt3a, would lead to a shift in the effective amounts of Wnt5a needed to counteract the inhibition. The same reasoning can be applied to the results obtained with the high dose of Wnt5a, at 50 and 200 ng/ml. While the results are not significant, compared either to non-treated or to wnt3a-treated cultures, these concentrations appear to have a trend: not inhibiting Wnt3a as effectively as the concentration increases. There too higher concentrations than those

used with Wnt5a alone might be needed to fully recapitulate the results from Wnt5a alone.

When analyzing the effects of Wnt3a and Wnt5a on myelin gene expression, Wnt5a alone did not affect Mpz and PMP22 mRNA levels, as determined by RT-qPCR (Fig. 38A). As previously shown (Fig. 22), Axin2 was also not up-regulated. Interestingly, Wnt5a at low dose (2.5 ng/ml) was able to counteract the inhibitory effect of Wnt3a on both Mpz and PMP22 expression. However, although there appears to be an inverse correlation between increase in Axin2 with decrease in Mpz and PMP22 upon Wnt3a alone treatment, Axin2 activation by Wnt3a was not counteracted by Wnt5a at low dose. A counteracting response was observed only when using a high dose (200 ng/ml) of Wnt5a (Fig. 38C). Little is known about how the Wnt/PCP, and Wnt5a in particular interfere with the Wnt/canonical pathway. The PCP co-receptor ROR2 has been implicated (126), but the downstream molecular events have not been characterized. Our results suggest that there may be at least 2 different mechanisms at work that would be elicited in a concentration-dependent manner.

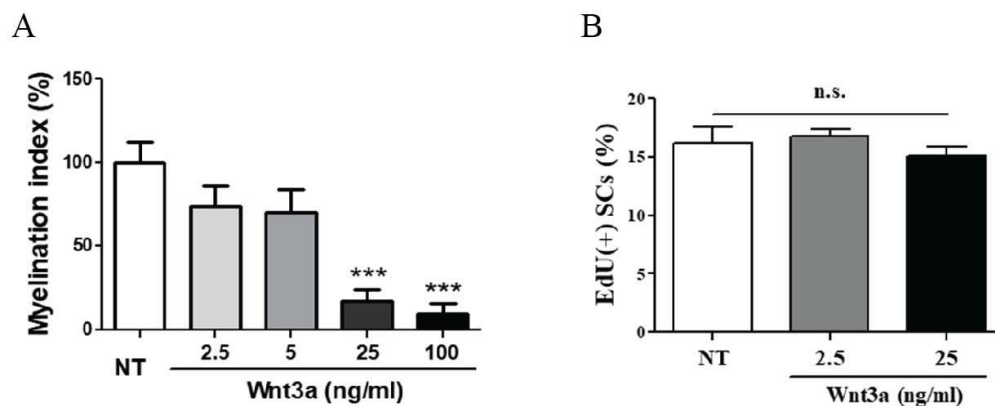


Fig. 35 Canonical Wnt signaling negatively regulates myelination. (A) The effects of Wnt3a, a prototype ligand of canonical Wnt signaling, on the expression of MBP segments. The number of MBP-positive segments was quantified by counting three independent experiment (cells of myelin segments normalized to non-treatment (NT) \pm SEM. ***p < 0.0001 versus NT) Bonferroni's post hoc comparison after one-way ANOVA). n.s. = no significance. (B) No effect of Wnt3a on SC proliferation in defined co-culture system DRG.

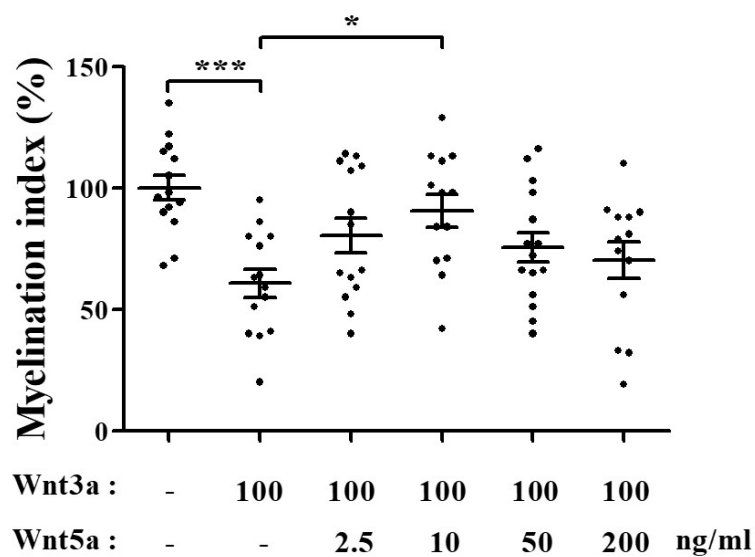


Fig. 36 Effect of Wnt5a on Wnt3a-treated myelinating culture. Comparison of average percentage of MBP segments in Wnt3a±Wnt5a treated cultures. Mean ± SEM, normalized to non-treated cultures. Individual experiments of triplicate are presented by block dot. *P<0.05, ***P<0.005, 1way ANOVA with Bonferroni's Multiple Comparison Test.

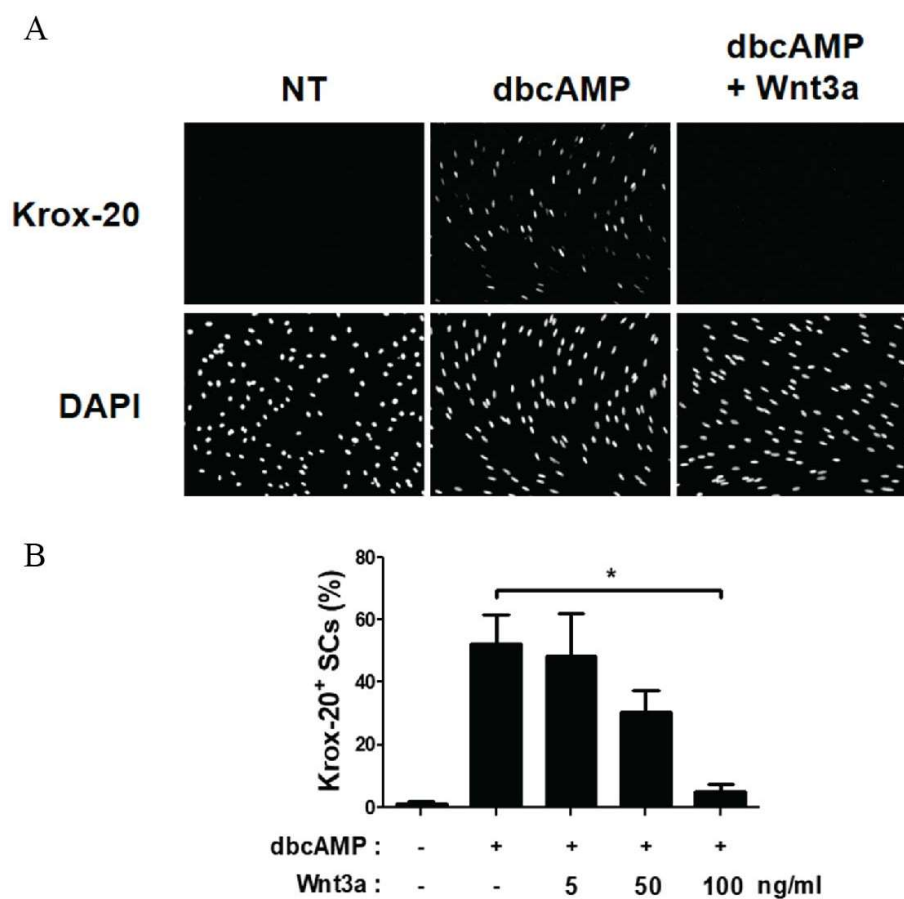


Fig. 37 Effect of canonical Wnt/ β -catenin signaling on SC differentiation *in vitro*. (A) Representative pictures showing the effect of Wnt3a on differentiating SCs. (B) Quantification of the differentiated SCs in presence of Wnt5a (% of total cell \pm SEM). Krox20⁺ SCs were quantified by counting three independent experiments and presented after normalization to total SC number. Bonferroni's post hoc comparison after one-way ANOVA. **p < 0.01. n.s. means no significance.

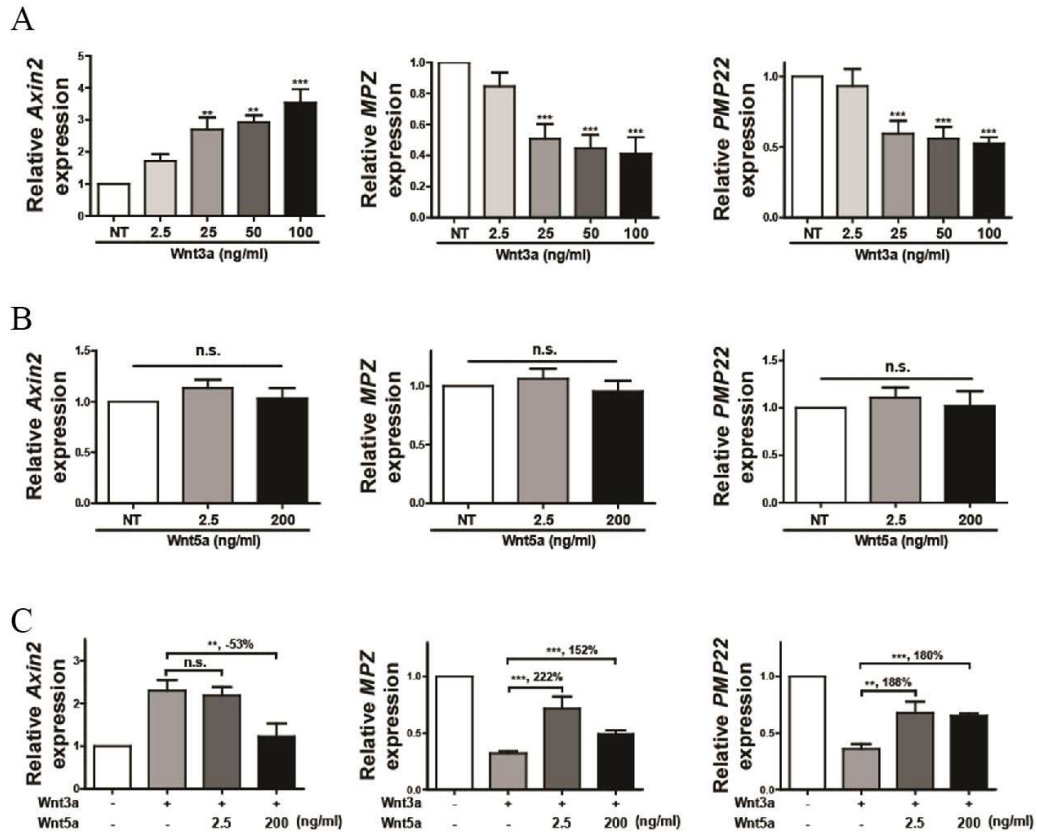


Fig. 38 Counteracting gene regulatory effect of Wnt5a against Wnt3a in Schwann cell. (A) The effects of Wnt3a on Axin2 (left), MPZ (middle), and PMP22 (right). Bars represent the normalized average values of mRNA expression from independent 5 experiments \pm SEM. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.005$, 1way ANOVA with Bonferroni's Multiple Comparison Test. (B) The effects of Wnt5a on axin2 (left), MPZ (middle), and PMP22 (right). Bars represents the normalized average values of mRNA expression from independent 5 experiments \pm SEM. n.s. means no significance. (C) The effects of Wnt5a on Schwann cell primed with Wnt3a. Data are presented as mean \pm SEM. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, 1way ANOVA with Bonferroni's Multiple Comparison Test.

CHAPTER VIII

Discussion and conclusion

The formation of a myelin sheath is a complex process that results from the integration by the myelinating glial cells of numerous signals provided by both the axons to be myelinated, and the extracellular matrix. Most of our current understanding of the molecular mechanisms and morphogenetic movements involved has been gained from numerous studies on the formation of the peripheral nervous system (PNS) myelin (161).

Indeed, these studies have identified key molecular events that regulate many aspects of the biology of the Schwann cells (the myelinating glial cell in the PNS), from proliferation to survival (81, 242), from migration (2, 243, 244) to radial sorting (11, 86, 209, 245), from a non-myelinating to a myelinating Schwann cell fate (62, 63, 246).

For example, among the many isoforms of the Neuregulin-1 family of growth factors, the membrane-bound type III Nrg1 was demonstrated to be the key instructive signal regulating the fate of the Schwann cell, from a non-myelinating to a myelinating phenotype (62, 63), as well as proliferation and survival (247). While soluble forms of Neuregulin-1 do promote survival and proliferation and can, as long as the Schwann cells have been “primed” by membrane-bound type III Nrg1, enhance Schwann cell myelination, soluble forms cannot instruct a Schwann cell to form a compact myelin sheath (248). In fact soluble forms of Neuregulin-1 are bi-functional and can also inhibit myelin formation (248, 249) as well as induce de-myelination (249). The key factor appears to reside in whether the MAP kinase pathway is activated or not

(248), an active MAP kinase pathway being conducive to the inhibition of myelin formation and to de-myelination (248).

Another major component that has been established as critical to PNS myelination is the extracellular matrix (ECM) that surrounds the Schwann cells that are in contact with axons (3). Indeed several mutant mice for the expression of ECM components such as laminin chains $\alpha 2$, $\alpha 4$ and $\gamma 1$ all show Schwann cells arrested at the early radial sorting stage, i.e the cells are around large bundles of axons but they fails to send lamellipodia within these bundles to sort-out axons within Remak bundles or in 1:1 pro-myelinating association (3, 12, 15, 17, 19). A similar phenotype is observed in mice mutated for the expression of ECM receptors, such as $\beta 1$, $\alpha 6$ and $\alpha 7$ integrins (9, 11) and dystroglycan (90), which further corroborates the importance of the ECM contribution to the process of myelination.

Interestingly, axo-glial interactions mediated through the type III Nrg1 ErbB2/ErbB3 axis seem also to be involved, as mice deficient for type III Nrg1 (on the axon) or ErbB2 receptor (on the Schwann cell) also present abnormalities in radial sorting (14, 62, 72). Other molecules involved in axo-glial interactions, such as Lgi4 (18) and L1 (250) also lead to radial sorting defects when mutated (Lgi4) or knocked out (L1). These results suggest that radial sorting, and therefore the formation of the lamellipodia that are needed for radial sorting, are polarized morphogenetic processes.

That Schwann cells are polarized, and that the formation of the compact myelin sheath is polarized event, has long been an accepted concept. Just the organization of the myelin/axon unit, in a repeating pattern of node of Ranvier-paranodal loops and domain-juxtaparanode-myelin sheath-juxtaparanode-

paranodal loops and domain- node of Ranvier demonstrates a polarized ultrastructural organization, later further confirmed and characterized at the molecular level (29-31). Furthermore, several studies have shown that all three complexes involved in apical-basal polarity (the Par3/Par6/aPKC complex, the Pals1/Patj/Crumbs complex, and the Dlg1/Scrib/Lgl complex) are regulating Schwann cell myelination in the PNS (76, 97-99). It is therefore not surprising that radial sorting, which requires a Schwann cell to send processes both radially (sorting) but longitudinally as well (elongation) would a polarized process.

Changes in cell morphology are the results of the regulation of that cell actin cytoskeleton (251, 252), which is achieved by integrating numerous external signals, in particular signals provided by interactions with the extracellular matrix (253, 254) and with adjacent cells (255). The intracellular molecular machinery that regulates the cytoskeleton has also been extensively characterized (252, 256, 257). The list is long, but mutants and conditional deletions / activations of regulators of the dynamics of the actin cytoskeleton such as small GTPases Rac1, cdc42 and RhoA (258-261), focal adhesion and integrin-linked kinases (262-265), scaffolding proteins such as Merlin (266) and actin-binding proteins such as Profilin and N-Wasp (267) all show abnormal radial sorting and myelination (85, 86, 176, 209, 245)

While the asymmetrical integration of axon- and ECM-derived signals can certainly provide a level of polarization to the process of radial sorting, the involvement of Planar Cell Polarity (PCP) has yet to be investigated. While PCP was initially identified as an in-plane epithelial polarity that provides positional information to cells within a tissue (101, 102), it also serves as a platform to

Wnt signaling to elicit the localized regulation of small GTPases of the Rho family, such as Rac1, RhoA and cdc42 (85, 86, 118, 119). PCP is not limited to epithelial tissues though, and regulates, among others, the collective and directional properties of mesenchymal cells migration by regulating small GTPases Rac1 and RhoA (109). Key to PCP is the asymmetrical repartition of cellular components (103) that locally affect the activation of downstream effectors. The Wnt/PCP pathway is therefore a platform that can provide spatial regulation of the actin cytoskeleton within cells, and may be of relevance to the morphogenetic processes that characterize myelination.

Wnt/PCP and the regulation of Schwann cell myelination

I therefore used the well-established, *in vitro*, Schwann cell DRG neuron myelinating co-culture system to lay out the bases demonstrating a role for non-canonical, PCP and Wnt/PCP signaling in Schwann cell myelination. Throughout the Chapters of my thesis work, I gradually established several facts and evidence that strongly support a role for Wnt/PCP signaling in regulating myelination.

Using both qRT-PCR and Western blot approaches, I showed that the developing sciatic nerves express Wnt ligands (Wnt5a, Wnt11), Wnt receptors (Fzd3, Fzd5) and scaffolding proteins (Dvl-2, Daam1) that have been shown to associate with the Wnt/PCP pathway (Fig. 10). In the context of purified cell cultures, Schwann cells express all of the molecules, whereas DRG neurons expressed only Daam1 and Wnt5a. Both Wnt5a and Wnt11 are developmentally regulated, being strongly expressed in embryonic stage E18.5. The mRNA levels dropped 3- and 9-fold (respectively) at birth and remained constant thereon. Similarly, Dvl-2 and Daam1 were essentially

detected from p0 to p7, which correspond in rodent to the period of radial sorting and the onset of myelination. These results expand the spectrum of Wnt signaling pathway molecules present in the developing sciatic nerve. Wnt1, Wnt2, Wnt6 and Wnt9b, Rspondin 1 through 4, Fzd1, Fzd3, Fzd7 and Fzd8 and Lgr4–6 have been detected in embryonic nerves (151). The developmental regulation of Wnt/PCP components suggests that there exist specific spatial and temporal requirements for Wnt/PCP signaling during peripheral nerve development and possibly myelination.

I used the well-established DRG neuron / Schwann cell culture system (81, 89, 159, 208, 268) to test that possibility. Myelinating cultures did not respond to Wnt11 treatment insofar as the number of myelinated segments was similar, irrespective of the concentrations of Wnt11 added to the cultures. Interestingly however, myelinating cultures showed a biphasic response to Wnt5a treatment (Fig. 13). At “low” concentrations ranging from 2.5 to 10 ng/ml, Wnt5a promoted myelination, whereas at “high” concentrations ranging from 50 to 200 ng/ml, Wnt5a inhibited myelination in a concentration-dependent manner. Biphasic and antagonistic effects of Wnt signaling on differentiation have previously been reported. For example, during embryogenesis the Wnt/canonical pathway promotes cardiomyocytes differentiation during the early stage of embryoid body formation, whereas Wnt/canonical pathway suppresses differentiation in late stages of embryoid body formation (269). A biphasic response in the levels of armadillo expression induced by Wnt has also been observed upon addition of soluble regulators of the Wnt pathway, such as Frizzled-related protein 1 (sFRP-1), at different concentrations (270). Therefore

it would appear that mediating biphasic responses is a normal mode of action of the Wnt pathway.

Schwann cells expressed Fzd5 (Fig. 9), which is a receptor to both Wnt5a and Wnt11 (189). It was therefore interesting to have a specific effect with only Wnt5a, and that Wnt11 did not affect myelination. While this result underscores the specificity of the biological response, it also suggests that additional regulatory molecules, such as co-receptors are present or absent. It will be interesting to extend the analysis of all identified component of the Wnt pathways, canonical and non-canonical, to the post-natal developing sciatic nerve.

It is intriguing that the inhibitory “high” concentration and promoting “low” concentration observed *in vitro* have an *in vivo* correlative at the mRNA level in the developing nerves; mRNA for Wnt5a were 3-fold higher in the embryo (E18.5) compared to the post-natal period of p0 to p7, which corresponds to active radial sorting and onset of myelination. It will be interesting to determine if this correlation holds up *in vivo* at the functional level as well.

I further determined the involvement of the Wnt/PCP axis in myelination by performing experiments with Schwann cells that were either deficient in Daam1, or expressed a dominant form of Daam1 (Figs. 16 and 17). These Schwann cells were greatly impaired in their ability to myelinate. This result suggests that the Wnt/PCP pathway is active for the normal process of myelination *in vitro*, regardless of the addition of exogenous Wnt5a. These cells however became unresponsive to the promoting effect of Wnt5a (Figs. 18 and 19), which further support that the effect of Wnt5a is mediated through the PCP pathway. Further inhibition at high concentration of Wnt5a was observed with

both the knockdown approach and the dominant-negative approach. Since the shDaam1 approach was very efficient, with > 90% of cells transduced and knocked-down for the expression of Daam1, it is therefore possible that Wnt5a at higher concentrations signal through a pathway that is different from the PCP (116).

Wnt5a has been described as a poor activator of the canonical pathway (271). The canonical pathway was not up-regulated in Schwann cell upon treatment with Wnt5a at either myelin-promoting or myelin-inhibiting concentrations (Fig. 22). This was further supported by the lack of nuclear localization of β -catenin in the nuclei of Schwann cells, as well as a lack of cellular increase in β -catenin amounts (Fig. 23). On the other hand, Wnt5a had a strong effect on the activation of two small GTPases that are important for radial sorting and myelination: Rac1 and RhoA (Fig. 20). Interestingly the effect is also biphasic, with “low” dose” of Wnt5a increasing GTP-bound RhoA while “high” dose has the opposite effect. Similarly, but in reverse, “low” dose” of Wnt5a decreased GTP-bound Rac1 while “high” increased levels of active Rac1. The ratio of active Rac1/RhoA would therefore decrease as the concentration of Wnt5a decreases. The activation of RhoA by Wnt5a at 2.5 ng/ml was completely abolished in the absence of Daam1, whereas Rac1 was inhibited comparably to control. This therefore strongly suggests that the pro-myelinating effect of Wnt5a at “low” dose is mediated through the Wnt/PCP/Dam1/RhoA cascade.

There of course remains to understand how Wnt5a either promote or inhibit *in vitro* myelination. I did not detect any differences on the pool of Schwann cells positive for Krox-20 in the nuclei, upon treatment with either

promoting or inhibitory Wnt5a concentrations. Krox-20 is a pro-myelinating transcription factor associated with Schwann cells that are in a 1:1 relation with axons (220). This result therefore suggests that Wnt5a, at either promoting or inhibiting concentrations, did not affect Schwann cell progression to the myelinating stage. Pro-myelinating Schwann cells are in a 1:1 association with axons and are surrounded by a basal lamina. I did not detect any differences in the number of Schwann cell nuclei within the basal lamina, a result that also suggests that Wnt5a did not affect the number of Schwann cells that are in a 1:1 association with axons. As the formation of a 1:1 association is the end-result of radial sorting, these results would suggest that Wnt5a did not promote or inhibit myelination that affecting radial sorting, but by affecting the morphogenetic event that comes next, i.e. spiral wrapping.

Attempting a correlation between the above interpretation of the *in vitro* results and *in vivo* data is difficult. Indeed, *in vivo*, different stages of Schwann cells stages overlap as the PNS develop. Immature Schwann cells undergoing radial sorting can be found from embryonic day E12.5. Until postnatal day p10, while pro-myelinating Schwann cells are present as early as E17.5 (3). Myelin sheath however will not start forming until p1 onward. It is interesting that, at least at the mRNA levels, Wnt5a is highly expressed before birth, then quickly decline. Is there a correlation with Schwann cells switching from a radial sorting phenotype to a pro-myelinating phenotype? Indeed “high” Rac1 activity has been proposed to be necessary for radial lamellipodia formation and radial sorting. I have shown that “high” levels of Wnt5a induce a high Rac1 activity in Schwann cells that low” Wnt5a doses (Fig. 20). On the over hand, “low” Wnt5a treatment of Schwann cells leads to an increase in RhoA activity (Fig. 20). It

was recently shown *in vivo* that RhoA may be regulating myelin thickness, i.e. regulating spiral wrapping (154).

There are of course additional mechanisms by which Wnt5a could have affected myelination. One such mechanism is the regulation of transcription factors of that NFat family. There are 2 Wnt/non-canonical pathways: i) the Wnt/PCP and ii) the Wnt/calcium pathway (143, 145). While the Wnt/PCP pathway regulates small GTPases, the Wnt/calcium pathway regulation of transcription factors of that NFat family. One of the members, NFatc4 was recently linked to the regulation of the pro-myelinating transcription factor Krox20 (83). While a possibility that will need to be investigated, it appears unlikely as I have not observed any changes in the number of Krox20 positive Schwann cells (Fig. 27). Another possible mechanism is the activation of the JNK/c-jun pathway by the Wnt/PCP/Rac1 cascade (Fig. 41; (272-274)). c-jun has been shown to be inhibitory to Schwann cell differentiation (204), to be up-regulated during de-myelination (204) and in Schwann cells undergoing de-differentiation (204), and to be antagonistic to Krox20 expression (204). Interestingly, the inhibitory levels of Wnt5a do increase the activity of Rac1 (Fig. 20), the upstream regulator of JNK in the Wnt/PCP/Rac1 cascade. There again though, no changes in Krox20 were detected, which weakens the possibility that this molecular mechanism was involved. It will nevertheless require further investigations.

Finally, I have extended our understanding on the role of the Wnt/canonical pathway with regards to Schwann cell myelination. In the PNS, the role of the Wnt/canonical pathway has been elusive, being proposed to either promote (150, 151, 153) or inhibit myelination (149, 152). Using the

Schwann cell DRG neuron myelinating co-culture system, I provide strong evidence that Wnt3a, a prototypical Wnt canonical ligand (136-138), inhibits the formation of compact myelin segments. Wnt3a has however been shown to activate the Wnt/PCP pathway in some cells (275), and further controls will be needed to rule that pathway out in the effect of Wnt3a on myelination *in vitro*. As expected for a pathway known to regulate transcription, the basal levels of transcription of myelin genes PMP22 and Mpz in purified Schwann cells were downregulated by Wnt3a (Fig. 38), in line with its inhibitory role *in vitro*. Interestingly, wnt5a at “low” dose was able to revert Wnt3a inhibition on the expression levels of PMP22 and Mpz mRNA, as well as on the formation of myelin segments in the co-culture system. A counteracting effect of the Wnt/PCP pathway on the Wnt/canonical pathway has been noted before (235-237) in other cell systems. This provides therefore an additional mechanism by which Wnt5a may have impacted myelination *in vitro*.

Planar cell polarity and PNS myelination

To date, there is absolutely no information regarding any possible function for PCP in PNS myelination. Throughout these Chapters, I have presented evidence for a role of the PCP-mediated Wnt signaling in regulating Schwann cell myelination. This and the characterization of components of the core and global modules of PCP suggest that Planar Cell Polarity is likely involved in regulating Schwann cell myelination, beyond serving as a “mere” platform for localized signaling.

PCP is a mechanism that provides cells, whether in an epithelium or in a mesenchyme, with positional information (101). i.e where they are in relation to each other. This positional information further allows cells to undergo cell fate

determination (120) and, for mesenchymal cells to move as a cohesive group. There are a few aspects of Schwann cell development that I would like to outline, where PCP might have a function.

Schwann cells migrate along nascent axons as a group. They need to parse themselves out along these axons so that the appropriate number of Schwann cells is associated with axons. Indeed the action potential velocity is directly related to the length of the myelinated sheath, therefore for a fixed length of axon, a fast fiber will have longer internodes than a slow fiber, i.e, a fast fiber will have less Schwann cells than a slow fiber (276). The molecular mechanism(s) that determine how many Schwann cells associate with a given axon are unclear. It has been proposed that Schwann cells compete for the axon-derive type III Nrg1 signal for survival (7). There are however a couple of caveats with this hypothesis. i) Indeed axon-derive type III Nrg1 signal is needed for the survival of Schwann cell precursors (E11.5) (7). By the radial stage (E12.5), cell survival is supported by an autocrine loop (cells secrete Nrg1; (277)) and by the ECM (85). ii) Schwann cells in Remak bundles are associated with up to 40 small caliber ($\leq 1 \mu\text{m}$) axons. Even though smaller in diameter and expressing less of type III Nrg1 (62), they “integrate” sufficient amount of signal to survive, even if they do not myelinate.

It is tempting to speculate and propose a model involving PCP. The axon is a very polarized structure, with a cell body at one end and a synapse at the other end. It is interesting that the last Schwann cell that migrate on an axon never myelinate, but becomes a perisynaptic Schwann cell (278). Although the molecular mechanisms are unknown, it is clear that this last Schwann cell to migrate along the axon identified itself as the last one, and therefore was not to

become a myelinating Schwann cells. Could this signal send a signal back to all the Schwann cells along the axon to inform them they will be the myelinating Schwann cells? Could the nature of the interaction between the axon and its target and/or the Schwann cell and the innervated target provide information as to the nature of the fiber, slow or fast? Could this information travel back to the other Schwann cells and therefore participate in the parsing of the proper numbers along any particular fiber? Could this information help Schwann cell in their cell fate determination, non-myelinating versus myelinating phenotype? This type of information would be positional in nature and could be relayed by PCP.

Indeed some of my data regarding the impact of Wnt5a on Schwann cell migration and differentiation is intriguing in that respect. I have shown that Schwann cells when cultured at high density cannot differentiate, unless when at the periphery of the culture (Fig. 34). This inability to differentiate is abolished when they do not express Wnt5a (Fig. 34). This suggests that the Schwann cells are able to sense their position among themselves, and that Wnt5a uses that positional information to prevent Schwann cells to differentiate. Interestingly Wnt5a stimulates Schwann cells' Wnt5a production. Therefore at high density, Schwann cells may be stimulating themselves to produce their own inhibitor to differentiation. In an *in vivo* setting, as cells migrate and parse themselves away from each other, this auto-stimulation would decrease, thereby allowing cells to differentiate.

Another aspect of Schwann cell development where PCP could be involved is spiral wrapping. Indeed my data regarding Schwann cell myelination *in vitro* does suggest that spiral wrapping is enhanced at "low dose" of Wnt5a

and inhibited by “high” doses. What is interesting about this process of spiral wrapping is that once the pro-myelinating Schwann cell has done a full one turn around the axon, it comes in contact with itself. Since one edge will remain “put” while the other one will initiate spiral wrapping, it implies that a mechanism is at work to make these two edges different. From the perspective of cell-to-cell contact within an epithelium, PCP establishes the difference between each side of a cell. Could PCP, in a similar fashion, establish the difference between what will remain the non-spiraling outer edge and what will become the spiraling inner edge of a myelinating Schwann cell?

CONCLUSION

In conclusion, I believe that I have established Wnt/PCP non-canonical signaling as a pathway that has the potential to regulate Schwann cell myelination in the PNS. It raises intriguing questions as to how PCP itself might be involved in the process of myelination, in the contexts of spiral wrapping, migration versus differentiation and, even more speculative, parsing of the Schwann cells along axons.

Undoubtedly, more investigations are required, not only to confirm the *in vitro* findings *in vivo*, but to also develop new concepts when thinking about the processes involved in myelination.

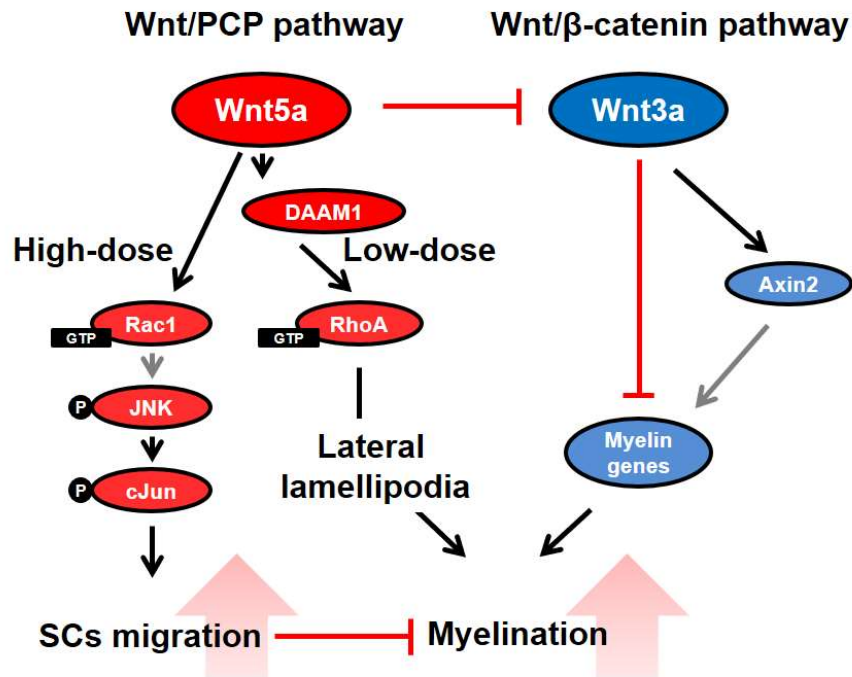


Fig. 39 Schematic representation of Wnt/PCP signaling on myelination.

“Low” dose of Wnt5a, a known ligand of the Wnt/PCP pathway, can promote myelination via Daam1/RhoA axis in Schwann cell, whereas “High” dose of Wnt5a inhibits myelination while promoting migration of Schwann cell. Additionally, Wnt5a alleviates the inhibitory effect of Wnt3a on myelination.

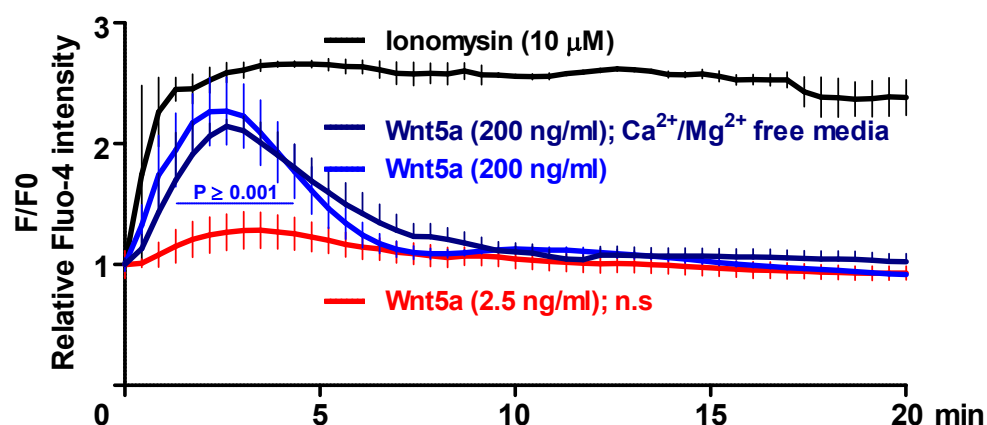


Fig. 40 Wnt5a-induced intracellular calcium induction in SCs. To analyze the intracellular calcium changes upon treatment Wnt5a, fluorescence recordings taken at 30 s intervals were corrected for dye photobleach and presented as relative values compared to initial intensity. 10 μ M ionomycin was used as a positive control. Changes in intracellular calcium as assessed by fluo-3 fluorescence from over 500 individual cells of 4 independent experiments with Wnt5a 2.5 ng/ml (red line) or 200 ng/ml (blue) in divalent ion-containing condition are shown. Line with dark blue means intracellular calcium induction upon 200 ng/ml Wnt5a treatment in $\text{Ca}^{2+}/\text{Mg}^{2+}$ free condition.

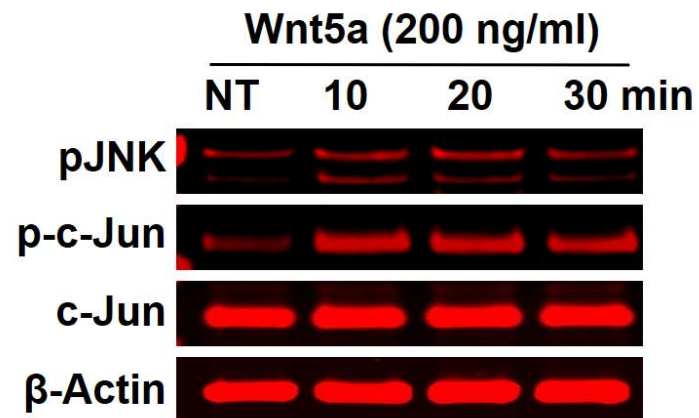


Fig. 41 Wnt5a treatment results in activation of the JNK pathway. Purified Schwann cells were treated with the 'high' dose of Wnt5a (200 ng/ml), which has a myelin-inhibitory effect. Wnt5a increased the activation level of JNK pathway (phosphorylation of JNK and c-Jun).

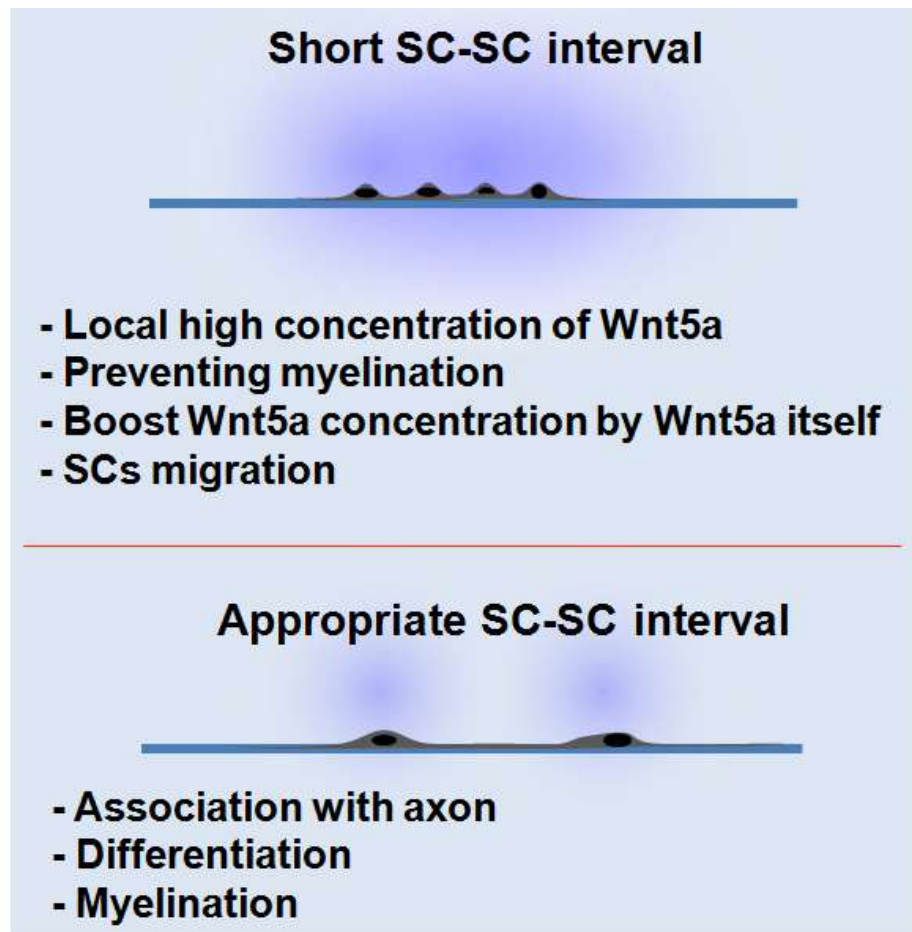


Fig. 42 Schematic model of the function of Wnt5a on SC-SC interval.

Wnt5a promotes SCs migration, supporting the observation of Wnt5a expression dominantly at E18.5 during which Schwann cells are actively migrating. In addition, Wnt5a inhibits Schwann cell differentiation into non-migrating stage in a 'high' cell density condition. This suggests Wnt5a from Schwann cells is activated by cell density dictates their distribution density along an axon. Additional *in vivo* studies will be important to clarify the role of Wnt5a as a key regulator of SCs distribution in the PNS.

CHAPTER IX

Materials and Methods

Animals

Sprague-Dawley rats, purchased from Hilltop Lab Animals Inc., were housed and cared for at the animal facility at Rutgers, The State University of New Jersey, Newark NJ, in accordance with an animal protocol approved by the Rutgers University Institutional Animal Care and Use Committee.

Antibodies

For immunofluorescence staining, primary antibodies included mouse monoclonal against myelin basic protein (MBP; SMI-94R at 1:500) and chicken monoclonal against neurofilament M (PCK-593P at 1:5000) from Covance, and mouse monoclonal against β -catenin (BD Biosciences 610153 at 1:1000). For Western blot analyses, primary antibodies included mouse monoclonals against β -actin (Sigma Aldrich A1978 at 1:5000), Dvl2 (Cell Signaling Technology 3224 at 1:1000), Daam1 (Abcam Ab56951 at 1:1000), β -catenin (BD Biosciences 610153 at 1:2000), chicken monoclonal to myelin protein zero (P0, Millipore AB9352 at 1:1000), goat polyclonal to Wnt5a (R&D Systems AF645 at 1:1000), rabbit polyclonals to Frizzled 3 (Ab102965 at 1:1000), Frizzled 5 (Ab14475 at 1:1000), Frizzled 7 (Ab64636 at 1:1000) from Abcam, and rabbit polyclonal to Krox-20 (1:200; kind gift from Dies Meijer, University of Edinburgh, UK).

Schwann cells, dorsal root ganglia (DRG) neuron primary culture

The isolation and purification of primary Schwann cells and DRG neurons has been previously described in detail (279). Briefly, Schwann cells were isolated from 2-day old rats and expanded in standard Schwann cell medium (DMEM, 1% GlutaMAX™-I, 10% heat-inactivated FBS) supplemented with 2 μ M Forskolin and 5 ng/ml of rhNrg1-EGFD. In all experiments Schwann cells were used at the 4th passage. DRGs were collected from embryonic day 15 rat embryos. After trypsinization cells were plated on \varnothing 10mm glass coverslips coated with Matrigel, in standard neuronal medium (neurobasal medium, 2% B27 supplement, 1% GlutaMAX™-I, 0.08% glucose, and 50 ng/ml 2.5S NGF). DRG cells were seeded at a density of 1.3 ganglia per coverslip. Feeding the cultures every 2 days with standard neuronal medium supplemented or not with 5-fluorodeoxyuridine and uridine (10 μ M each) for 8 days removed non-neuronal cells. Purified neurons were then kept in standard neuronal medium until use.

Myelinating co-culture

To establish myelinating co-cultures, purified neurons were repopulated with purified primary rat Schwann cells at a density of 100,000 cells per coverslip, in MEM supplemented with 10% heat inactivated FBS, 0.4% glucose and 50 ng/ml NGF. After 3 days, myelination was initiated by supplementing the media with 50 μ g/ml of ascorbic acid, and the formation of compact myelin segments was assessed 10 day later by immunostaining for MBP.

RNA interference of Daam1 expression in Schwann cells

A lentiviral construct expressing an shRNA targeting rat Daam1 mRNA at position 83-111 (ATCGGCTTAGGAATGATAGCAACTTTGCA; position relative to the start codon, GenBank NM_001108030.1) was purchased from Origene (TL706575). I used a non-effective scrambled shRNA cassette (Origene TR30021) as a control for non-specific effects. Transcription of the shRNA was under the control of the U6 promoter while the GFP marker was expressed under a CMV promoter (pGFP-C-shLenti vector; Origene TR30023). The lentiviral construct was transfected into 293FT cells (Invitrogen R70007) together with packaging plasmids pLP1, pLP2, and pLP/VSVG (Invitrogen K497500) using the calcium phosphate method (Invitrogen K278001). Viral supernatants were collected 48 hrs after transfection and centrifuged at 1,600xg for 20 min to pellet cell debris. For transduction, 400,000 Schwann cells (passage 2; plated for 24hrs) were treated overnight (16hrs) with 2 ml of viral supernatants (supplemented with Forskolin and rhNrg1-EGFD). The cells were then maintained in standard Schwann cell medium supplemented with Forskolin and rhNrg1-EGFD until passage 4, at which time they were tested for effective knockdown of Daam1 by Western blotting and used in experiments.

Expression of dominant-negative nDaam1 in Schwann cells

The rat Schwann cell cDNA was used to amplify cDNA sequence coding for the N-terminus (aa 1-233) of Daam1 cloned in place of the eGFP sequence of the pLL3.7 lentiviral vector (280). Expression was under the control of the CMV promoter. The original pLL3.7 served as control. Transfection of 293FT cells, viral supernatants preparation and infection of Schwann cells were as

described in the RNA interference section. Expression of nDaam1 was confirmed by Western blotting.

Western blot

Cell lysates were prepared in 25 mM Tris buffer pH 7.4, containing 95 mM NaCl, 1% SDS, protease inhibitors cocktail (Pierce 88266). Sciatic nerves, collected from rats at age ranging from birth (p0) to p21, were snap frozen and pulverized in a liquid nitrogen-cooled ceramic mortar. The powder was recovered in lysis buffer (25 mM Tris buffer, pH 7.4, containing 150 mM NaCl, 1% SDS and protease inhibitors cocktail. Lysates were boiled for 10 min and cleared by centrifugation at 16,000xg for 20 min at 14°C. Protein concentrations were determined by the BCA method (Pierce 23225). Twenty micrograms of proteins were fractionated on 10% Bis/Tris polyacrylamide gels and transferred onto 0.2µm nitrocellulose membranes (Biorad 162-0252). Appropriate regions of the blots were cut out and incubated with specific primary antibodies to probe for Frizzled 3, Frizzled 5, Frizzled 7, Daam1, Wnt5a, Dvl2, β -catenin and β -actin. After incubating with appropriate infrared-conjugated secondary antibodies (LI-COR), protein bands were visualized with the Odyssey imaging system (LI-COR).

Immunostaining

DRG neuron / Schwann cell myelinating co-cultures were rinsed in phosphate buffered saline (PBS) and fixed in 4% paraformaldehyde for 20 minutes. After washing with PBS, cultures were permeabilized in ice-cold methanol for 20 minutes and incubated in blocking solution (5% IgG-free BSA, 1% normal donkey-serum, 0.3% Triton X100) for 1hr at room temperature. This was followed by an overnight incubation at 4°C with primary antibodies against

MBP, Krox-20 and neurofilament M prepared in blocking solution. After washing with PBS, samples were incubated with secondary antibodies for 1hr. Nuclei of cells were visualized by staining with DAPI. Cultures were mounted in antifading agent Citifluor (Ted Pella). Preparations were examined by epifluorescence on a Nikon Eclipse TE2000-U (20x/0.75 objective) microscope. To quantify the extent of myelination, 15 to 20 random field images were acquired per culture using the MetaMorph software package (Molecular Devices). For each condition, the average number of MBP-positive segments per field of view was calculated and normalized to that of the control cultures (100%). Krox-20 nuclear localization was expressed as a percentage of Krox-20 versus DAPI labeled nuclei. Counting was done with the ImageJ software.

Proliferation assay

50,000 Schwann cells were plated onto dissociated DRG neuron cultures, in MEM supplemented with 10% heat inactivated FBS, 0.4% glucose and 50 ng/ml NGF. 24 hours later, cultures were fed with or without Wnt for an additional 24 hrs. Schwann cell proliferation was assessed using the EdU (5-ethynyl-2'-deoxyuridine) nuclear labeling assay. EdU (10 μ M final concentration; Invitrogen A10044) was added during the last 4 hrs of culture, and was detected using the Click-iT Alexa Fluor 488 imaging kit (Molecular Probes C10337) according to the manufacturer's instructions. Neurites were detected by immunostaining for neurofilament M and nuclei by DAPI. All cultures were mounted on glass slides in Citifluor, examined by epifluorescence microscopy (Nikon Eclipse TE2000-U, 20x/0.75 objective), and images acquired using the MetaMorph software package. EdU- and DAPI-labeled nuclei from 20 to 30 random fields per culture were counted. Three cultures per condition in three

separate experiments were analyzed. Typically, over a thousand cells were counted per condition. EdU incorporation was expressed as a percentage of EdU-labeled versus DAPI labeled nuclei.

Quantitative RT-PCR

TRIzol reagent (Invitrogen 15596) was used for the extraction of total RNA from Schwann cell cultures (1×10^6 cells) and from rat sciatic nerves at different stages of development. Total RNA was then used as template with the SuperScript III First-Strand Synthesis kit (Invitrogen 18080051) to produce cDNA from which specific targets were amplified by qRT-PCR performed on a LightCycler 480 II (Roche) using the Maxima SYBR green/ROX qRT-PCR master mix (Thermo Scientific K0221). Primers were designed using the web version of the Primer3 software (281) and the rat genome assembly version Rnor_6.0. Forward and reverse primer pairs were selected to amplify targets overlapping splice junctions at the position of long introns to minimize amplifications from unspliced mRNA-derived cDNAs. The list of primers is provided in Table 1. Melting point (T_m) curve analysis was performed on all qRT-PCRs to verify the detection of a unique peak for each reaction, at the expected T_m . All endpoint reactions were separated on a 15% DNA polyacrylamide gel to confirm the detection of a unique amplicon of the expected size.

Wnt5a ELISA assay

The Wnt5a Sandwich ELISA kit was from LSBio (LS-F15714). Isolated Schwann cells and DRG neurons were extracted from Sprague-Dawley rats. Cell lysates were obtained by ultrasonication 3 times for 10 seconds on ice. Proteins in supernatants after centrifugation at 1500xg for 10 minutes at 4 °C

were normalized and used immediately for the assay. The optical density (OD value) was determined using a microplate reader set to 450 nm.

Small GTPase activity assays

The activation of small GTPases upon Wnt5a treatment was assayed with G-LISA kits specific for Rac1, RhoA and cdc42 (Cytoskeleton, BK128, BK124 and BK127, respectively). 100,000 Schwann cells were seeded per well of 6-well plates (coated with PLL) in normal Schwann cell medium. 24 hrs later, cells were placed in 1% FBS containing DMEM medium (Corning, 10-017-CM) for 48 hrs with changing media every day. Cells were then treated or not with Wnt5a at 2.5 or 200 ng/ml. Cell lysates were prepared at various time points (1.5 to 20 minutes) and analyzed with the G-LISA kits following the manufacturer's instructions. Signal for GTP-bound small GTPases was detected using the GloMax multi-detection system fitted with the absorbance module (Promega).

Migration assay

For Boyden chamber, Schwann cells (2×10^4 cells) suspended in 400 μ l Schwann cell media containing 3 % heat-inactivated FBS were loaded into the upper chamber of the Boyden chamber (8 μ m pore size; BD Biosciences Falcon) pre-coated with poly-L-lysine for overnight. Bottom chamber was filled with the Schwann cell media containing "Low" or "High" concentration of Wnt5a (2.5 or 200 ng/ml, respectively). After incubation for 8 hrs, the pore membrane was fixed in 4% PFA, and stained with DAPI to count the migrating Schwann cell number. Schwann cells on the top of the membrane were removed before counting the migrating Schwann cell number on the bottom of the membrane. For Schwann cell migration assay in the condition of axonal contact, Schwann

cells (2×10^4 cells) suspended in 500 μ l co-culture media were loaded into DRG culture. Live cell images were acquired every 2 min for 2 hrs by a Zeiss Cell Observer Spinning Disk confocal microscope. Location of individual Schwann cell were marked by Hoechst 33342 (Thermo Fisher Scientific). The software ImageJ-Live Cell Tracker was used for cell tracking to measure the migration distance. A lentiviral construct expressing an shRNA targeting rat Wnt5a mRNA and a non-effective scrambled shRNA cassette as a control for non-specific effects were used for migration assay. Schwann cells (2×10^5 cells) suspended in 10 μ l Schwann cell media with 3% of FBS were seeded onto well-defined small (2 mm²) patches. These patches were defined by using a PDMS mask to ensure a sharp edge from which migration could be measured. The number of Schwann cells migrating out of the patches was measured after 18 hrs of culture.

Quantifications and statistical analyses

Statistical analyses (tests used are indicated in figure legends) were performed with the Prism software package (version 5.0d) from GraphPad.

Primers for PCR

Gene	Sequences	Amplicon size (bp)
<i>FZD1</i>	Se: ATCTGTTTCATCGGCACCTCTTTCC As: GTCTCCCCTTGTTTGCTGTTGGTA	442
<i>FZD2</i>	Se: AAGGTGCCGTCCTATCTCAGCTAT As: TGGCCAGGATGGTGATGGTTTT	553
<i>FZD3</i>	Se: AAGTGGGGCAGTGAAGCTATTGAG As: AGCTGGCCCATTGAAAGCATGT	577
<i>FZD4</i>	Se: GGTCACGAAGCCATTGAAATGCAC As: ACCTTCCCAGAATTGAGGAATCGG	545
<i>FZD5</i>	Se: CGAGAGCACAGCCACATTCACTAT As: TGCCAATGCGGATCATGAGCTT	457
<i>FZD6</i>	Se: ACTCCGTCTGTTACAGCATCGTCT As: GCTTTTGGGTTTGCCTGGTAAGGA	697
<i>FZD7</i>	Se: GGCCACGGAGTAACAGGTGATTCTT As: GGTTGGCCCTGGAACATTTTCGATA	679
<i>FZD8</i>	Se: CGTTGTGCGCTGCCTTTTCTATGA As: GGGACTTTGGCATTGCTTAGGGT	419
<i>FZD9</i>	Se: TACTCCTTGGCCTTCCTGATCAGA As: AAGTCCATGTTGAGGCGTTTCGAT	596
<i>FZD10</i>	Se: ATCGGCACTTCCTTCATCCTGTCT As: TGAAGGCTGAGCAGGAAGCTCATA	513
<i>W5a</i>	Se: CACAACAATGAAGCAGGTCG As: GCATGTCTTGAGGCTACAGGA	96
<i>W7a</i>	Se: GCCCGAACCCTCATGAACTT As: GTGGTCCAGCACGTCTTAGT	118
<i>W7b</i>	Se: ATGGCATTGACTTTTCCCGC As: AGTTTCATGCGGTCCTCCAG	103
<i>W11</i>	Se: GGATATCCGGCCCGTGAAG As: CGTTGGAGGTTTGTTCAC	125
<i>Axin2</i>	Se: GCTCCACAGGCAAATTCGTC As: GACTCTGGGCCACTTCAAGG	108
<i>MPZ</i>	Se: CCATGGAGAAGGGGAGATTT As: CCCAGCCCTTTTGATTTCTT	136
<i>PMP22</i>	Se: TCTTGTTGGGGATCCTGTTT As: GGCGGATGTGGTACAGTTCT	128
<i>HPRT</i>	Se: TGATAGATCCATTCTATGACTGTAGA As: TTTCCAGTTAAAGTTGAGAGATCA	117

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