Role of Cell Adhesion Molecules of the Nectin-like family in Peripheral Nerve Myelination:

Nectin-like 1 and Nectin-like 2 are negative regulators of myelination by Schwann cells

By

MING-SHUO CHEN

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ABSTRACT OF THE DISSERTATION

Role of Cell Adhesion Molecules of the Nectin-like family in Peripheral Nerve Myelination:

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Axo-glial interactions are critical for myelination and the domain organization of myelinated fibers. Cell adhesion molecules belonging to the Nectin like family, and in particular Necl-1 (axonal) and its heterophilic binding partner Necl-4 (Schwann cell), mediate these interactions along the internode. Using targeted shRNA-mediated knockdown, we show that the removal of axonal Necl-1 promotes Schwann cell myelination in the in vitro DRG neuron/Schwann cell myelinating system. Conversely, over-expressing Necl-1 on the surface of DRG neuron axons results in an almost complete inability by Schwann cells to form myelin segments. Axons of superior cervical ganglion (SCG) neurons, which do not normally support the formation of myelin segments by Schwann cells, express higher levels of Necl-1 compared to DRG neurons. Knocking down Necl-1 in SCG neurons promotes myelination. Finally, the extracellular domain of Necl-1 interferes in a dose-dependent manner with the activation of ErbB3 and of the pro-myelinating PI3K/Akt pathway, but does not interfere with the activation of the Mek/Erk1/2 pathway. While not in direct contradiction, these in vitro results shed
lights on the apparent lack of phenotype that was reported from in vivo studies of Necl-1-/- mice. Our results suggest that Necl-1 may act as a negative regulator of PNS myelination, potentially through the selective regulation of the signaling cascades activated in Schwann cells by axonal contact, and in particular by type III Nrg-1. Further analyses of peripheral nerves in the Necl-1-/- mice will be needed to determine the exact role of axonal Necl-1 in PNS myelination.

This opposite effect that Necl-1 in regulating myelination has led me to investigate the function of its another binding partner Necl-2. I have shown that in vitro Necl-2 expressed by Schwann cells is inhibitory to myelination. Strikingly, upon axonal contact, Necl-2 perturbs PDK1 and Akt activation (pro-myelinating signal) in direct correlation with the observed myelination results, suggesting the regulatory role of Necl-2 in axo-glial interaction. I have also observed that Necl-2 regulates actin cytoskeleton rearrangement in Schwann cells that is potentially due to regulating Rac1 activity. These findings provide another mechanism of internodal cell adhesion molecule in fine-tuning PNS myelination, both at signaling and cytoskeleton level.
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CHAPTER I

Background and Introduction

The myelin sheath

The myelin sheath is often loosely referred to as a “fatty substance” or “structure” that surrounds axons, both in the Central Nervous System (CNS) and in the Peripheral Nervous System (PNS) (Fig. 1). Specialized glial cells, the oligodendrocyte in the CNS and the Schwann cells in the PNS, generate the myelin sheath. Whether in the CNS or in the PNS, the myelin sheath is formed by an extension of the plasma membrane from this specialized glial cells, which surrounds and then spiral-wrap around axons, creating a multi-lamellar structure (Fig. 2). That multi-lamellar structure then compacts through the action of specific myelin proteins (see Molecular composition of the PNS myelin sheath) and the myelin sheath is formed. The myelin sheath is not however a continuous structure all along the axon. It is interrupted at regular intervals (Fig. 1) by gaps that are called the nodes of Ranvier [1] where the axonal plasma membrane is in communication with the extracellular medium. The myelin sheath between a pair of nodes of Ranvier is called a myelin segment or internode. There are a few structural differences between the CNS and PNS myelin, which are outlined in Fig. 1. For example, one Schwann cell forms only one myelin segment around one axon, whereas oligodendrocytes form multiple segments (up to about 40) around the same and multiple axons. In the PNS, myelin segments are also surrounded by a basal lamina that is not present around the myelin segments made by oligodendrocytes in the CNS. Finally the axonal plasma membrane at the nodes
of Ranvier is not fully “naked” and in direct contact with the extracellular medium. In the PNS, the axonal plasma membrane is covered by microvilli that are protruding from the edge of the outer layer of the myelin sheath. However in the CNS, oligodendrocytes do not extend microvilli and the node is surrounded by an extension from specialized astrocytes called perinodal astrocytes.

Regardless of these differences, whether in the CNS or the PNS, the function of the myelin sheath is to ensure the efficient and fast propagation of action potentials along the axons of the myelinated nerve fibers. This is achieved in part by the biophysical properties of the myelin sheath, and in part by the structural and molecular organization of the myelin/axon unit.

As mentioned earlier, structurally the myelin sheath is derived from the plasma membrane of specialized glial cells. The function of a plasma membrane is to provide a selective barrier between the extra- and the intra-cellular environment. While few small molecules such as water can slowly passively diffuse directly through the lipid bilayer of the plasma membrane, most small molecules undergo regulated passive or active transport through specialized carrier proteins [2]. This is particularly true for charged molecules, such as ions. Although ions such as sodium, calcium, chloride and potassium are physically sufficiently small to pass through, the hydrophobic nature of the core of the plasma membrane (formed by the hydrophobic tails of the phospholipids) prevents any significant amount of passive diffusion. In the myelin sheath, this plasma membrane surrounds the axons and wrap around into a multi-lamellar organization that ultimately tightly compacts (Fig. 2). In addition, compared to most mammalian cells,
the lipid content of the plasma membrane of these specialized glial cells is inordinately high and, in the myelin sheath is on average around 70 to 85% [3-5]. The combination of these two features (multi-lamellar plasma membrane-based structure, high lipid content) effectively transforms the myelin sheath into an insulating material that isolates the axon from the conductive extracellular environment.

On un-myelinated axons, the major ions channels that are implicated in action potential formation and propagation (voltage-gated sodium channels and voltage-gated potassium channels) as well as in maintaining the axon plasma membrane resting potential (resting potassium channels) are diffusely spread along the whole length of the axon [6]. In myelinated axons however, the channels are not present on the axonal plasma membrane that is directly under the compact myelin sheath [6]. They are concentrated in small domains, in particular the nodes of Ranvier (Fig. 4).

When an action potential is initiated by a neuron, there is an influx of Na$^+$ ion at the level of the initial segment (Fig. 4), through the opening of voltage-gated sodium channels [6]. Voltage-gated potassium channels ensure repolarization. These channels are less “responsive” to depolarization and open after the voltage-gated sodium channels, at the peak of Na$^+$ influx [7]. This delay allows for the Na$^+$ ions to travel in the axonal cytoplasm by passive diffusion (often referred to as passive spread) within the long axis of the axon, in both directions (see next paragraph). As the Na$^+$ ions move away from the initial depolarization site, they depolarize adjacent sections of the plasma membranes (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. However as the Na\(^+\) diffuse, the net positive charge on the cytoplasmic side of the plasma membrane decreases. In addition non-gated resting channels are “leaking” cations outside the cell to re-establish the resting membrane potential and affect how far an influx of Na\(^+\) can propagate the action potential. Although passive spread is a fast process, the need to constantly regenerate the action potential on an unmyelinated axon slows its propagation speed, as the action of opening voltage-gated Na\(^+\) channels is a “slow” process. On the other hand, the absence of channels on the axonal membrane under the myelin sheath, couple to the sheath insulating properties, Na\(^+\) ions diffuse quickly on a longer distance before the charge starts to dissipate. They are therefore able to depolarize the axonal membrane further away from the original site of depolarization compared to an unmyelinated axon. Since the action potential does not have to be regenerated as often, the speed of propagation of the action potential is dramatically increased, up to a 100-fold [8].

Of note, voltage-gated sodium channels undergo a refractory period during which they remain closed [9] and cannot be opened again until the membrane potential regains its resting potential. As a result only voltage-gated sodium channels downstream of the initial action potential can open, thereby assuring uni-directionality to the propagation of the action potential. This uni-directionality is further ensured by the action of voltage-gated potassium channels [10] that are implicated in the repolarization of the axonal plasma membrane. Repolarization by
voltage-gated potassium channels goes beyond the resting membrane potential and momentarily results in hyperpolarization. A higher action potential would then be required to open the nearby voltage-gated sodium channels.

**Molecular composition of the PNS myelin sheath**

As mentioned in the previous section, one of the major defining characteristics of the myelin sheath is its inordinately high lipid-to-protein ratio, 70 to 85% lipids for 15 to 30% protein, by dry mass [3-5] which relates to the insulating property of the myelin sheath. The major lipids of the PNS myelin sheath are cholesterol, galactocerebroside, sphingomyelin and phosphatidyl-choline (about 27%, 16%, 17% and 19% by lipid dry weight, respectively, in rat PNS; [4, 11-13]. The myelin sheath is also characterized by the nature of the fatty acid chains that are a part of the myelin lipids [11]. About 75% of the fatty acid have very long carbon chains (≥ C18; 18 or more carbons) and are mostly unsaturated or carry one unsaturation (i.e no double bonds or 1 double bond; for example C18:0 or C18:1, respectively). Oleic acid (C18:1) is the most abundant fatty acid chain in the PNS myelin sheath. Poly-unsaturation (>1 double bond) is essentially associated with the longest fatty acid chains of the myelin sheath, such as arachidonic acid (C20:4 n=6), docosatetraenoic acid (22:4 n=6) and docosahexaenoic acid (C22:6 n=3) [11].

Some of these lipids have an important function in the formation of the myelin sheath that extends beyond conferring an insulating property. For example, the fatty acid chains of sphingomyelin and cholesterol interact with membrane-bound myelin proteins PLP/DM2, MAL, CNP and MOG [14-16] while self-
assembling in the trans-Golgi network to form lipid rafts. Sphingomyelin and cholesterol therefore have an impact on the sorting and trafficking on myelin proteins [17-19]. Sphingomyelin is also the major source of ceramide [20, 21], itself the major building block to form galactocerebrosides, the most prominent sphingolipids in the myelinating glial cells [4, 13]. The studies of various lipid metabolism disorders and of their animal models associated with galactocerebrosides synthesis have underscored their importance in myelin formation and maintenance [3, 4, 13]. Mice that are deficient in cholesterol synthesis in the PNS are severally hypo-myelinated [19, 22] and present many features of un-compacted myelin [19], which correlates with an associated decrease in myelin proteins implicated in myelin compaction. Mostly affected is the transport of myelin protein zero (Mpz, or P0) from the endoplasmic reticulum to the plasma membrane [19]. It was recently reported that the qualitative and quantitative profiles of phosphatidylcholines were dramatically affected in in vitro-cultured Schwann cells that were deficient in the expression of the cell adhesion molecule Nectin-like 4 [23]. Although the lipid composition of the myelin sheathes isolated from mice that are knockout for Nectin-like 4 in Schwann cells has not yet been determined, the myelin in these animals exhibits numerous abnormalities such as focal hyper-myelination, tomacula and outfoldings [24], that are characteristics of the human Charcot-Marie-Tooth (CMT) neuropathology type 4 (CMT4). Analyses of Mtmr2 and Fig4 mutations, and their animal knockout models strongly point that a tight regulation of Pl_{(3,5)}P2 levels is needed for proper myelination in the PNS. Mutations in both genes are a cause of CMT neuropathy,
type 4J (Fig4) and type 4B (Mttr2) [25-27]. Mttr2+/− mice develop tomacula and focal hypermyelination [28]. Both are phosphatases, Mttr2 converting PI(3,5)P2 into PI(5)P, Fig4 converting PI(3,5)P2 back to PI(3)P. Phosphatidylcholine is an important source of phosphatidic acid [29] that is either converted to diacylglycerol or used for the synthesis of a class of important signaling and structural phospholipids, the phosphatidylinositols (PI) [30] and phosphorylated forms of PI, such as PI(3)P, PI(4)P, PI(4,5)P2, PI(3,4,5)P3, PI(5)P and PI(3,5)P2, all of which crucial for proper PNS myelination [31-34].

Not only are the PNS myelin proteins limited in total amount (15 to 30%; myelin sheath dry mass), the bulk (90%) is “limited” to five proteins [11]: Myelin protein zero (Mpz or P0; 50 to 70%; [12, 35, 36], Myelin basic protein (MBP; 5 to 15%;[37], Peripheral myelin protein 22 (PMP22; 2 to 5%; [38], Periaxin (5%;[39, 40] and Myelin associated glycoprotein (MAG; 1%). Each of the remaining 10% myelin proteins represents less than 1% of the total amount of myelin proteins. Mpz is an integral membrane glycoprotein whose extracellular domain contains a V-like immunoglobulin domain [41]. The C-terminal domain is cytoplasmic and rich in basic amino acid residues[42]. Several mutations in the Mpz gene have been characterized and linked to several types of peripheral neuropathies such as CMT1B [43, 44], Déjérine-Sottas syndrome [45] and congenital hypo-myelination [46]. The analyses of the structural abnormalities of the PNS myelin sheath associated with P0 mutations, along with crystallography studies of P0, strongly suggest that P0 is involved in the compaction of the myelin lamellae [47, 48] and maintenance of the myelin sheath once formed [49]. MBP is a surface membrane
protein associated with the inner leaflet of the plasma membrane of the myelinating glial cell. It is highly basic (25% of the amino acid residues are basic) and with an isoelectric point of 10 is highly positively charged in physiological conditions. MBP is involved in the compaction of the myelin sheath. The positively charged MBP interacts with the negatively charged PI(4,5)P2 to mediate a higher lipid order and condensation of the two apposing cytoplasmic inner leaflets [50-53].

MBP may also be involved in neutralizing the charges of other phospholipids, phosphatidylserine in particular. PMP22 is a small integral tetraspan glycoprotein present in the compact myelin sheath. Gene dosage is an important feature of PMP22 expression, as too much (gene duplication) and too little (gene deletion) of PMP22 expression have been linked to CMT1A and hereditary neuropathy with liability to pressure pals (HNPP), respectively [54]. This suggests that the stoichiometry of PMP22 is necessary to maintain the integrity of the myelin sheath, which in CMT1A patients presents abnormalities such as onion bulb formation and demyelination. The molecular bases underlying PMP22 importance in PNS myelin formation and maintenance are not completely understood. Biophysical studies with lipid vesicles showed that the addition of PMP22 results in the formation of “cylindrically-wrapped protein-lipid vesicles that share common organizational traits with the compact myelin of peripheral nerves in vivo” [55]. As in vivo, stoichiometry is important. The formation of these myelin-like structures is dependent on the PMP22-to lipid ratio, and requires the two extracellular loops (ECL1 and ECL2) of PMP22. It is interesting to note that the ECL1 domain has been proposed to mediate homophilic trans-interactions [56].
Finally these myelin-like structures also fail to form when mutant forms of PMP22 (associated with severe human dysmyelinating neuropathy) are substituted for wild-type PMP22.

While periaxin is associated with myelinating Schwann cells [39, 40], it is not an integral plasma membrane protein and it is not within the compact myelin sheath proper [57]. It is localized to the plasma membrane of the myelinating Schwann cells that is in close apposition with the axon (adaxonal membrane) (Fig. 3). The particular function of periaxin is still unclear. However, several mutations have now been linked to the type 4F of CMT [58-62] strongly suggesting that periaxin is somehow involved in myelin maintenance.

Although the remaining 10% of proteins associated with myelination individually account for less than 1%, a few of them have been well characterized and have been shown to be crucial to the proper development of the myelin sheath and the myelin/axon unit. Their nature and function will be addressed later on in greater details in the following two sections: i) “Axo-glial interaction through cell adhesion molecules is necessary for domain formation and myelination” and ii) “Nectin like molecules: intermodal candidates for regulating myelination”. Indeed, for a better comprehension of these sections, I would like to present first the morphogenetic movements that are associated with the formation of the PNS myelin sheath, and provide further relevant structural details of the myelin/axon unit.

**Schwann cells developmental stages and the morphogenetic movements associated with peripheral nerve myelination**
Myelination of axons in the PNS is a complex process that results from the spatial and temporal integration of numerous signals by the Schwann cell, the myelinating glial cell.

Schwann cells are derived from the neural crest cells [63]. As the neural crest cells (NCCs) destined to become Schwann cells migrate along the nascent axons during embryonic development, they undergo a series of transition that are characterized by the expression of particular sets of molecular markers, as well as by changes in their ability to respond to various factors [64]. The most usually characterized stages of Schwann cells development are: i) Schwann cell precursors, immature Schwann cells, pro-myelinating Schwann cells, myelinating Schwann cells and Remak (or non-myelinating) Schwann cells [64]. Beyond the molecular characterization, the criteria of the physical interaction of the developing Schwann cells with its surroundings (axon and basal lamina) must also be taken into consideration.

As NCCs migrate along the nascent axons, they are pushed into the glial lineage and become Schwann cell precursors under the influence of type III neuregulin-1 (type III Nrg1), Notch and FGF2 [65-67]. Under the influence of axon-derived type III Nrg1, Schwann cell precursors further proliferate and survive [68] to populate axons. As Schwann cell precursors progress into their glial fate, they develop a basal lamina and start expressing, among many markers, laminin chains α2, α4, and γ1, β1 and α6 integrins and Lgi4 [69]. The Schwann cells are now in the immature stage. At that stage, signals provided by the axon (type III Nrg1), the basal lamina and integrin receptors regulate the formation of plasma membrane
processes that expand and surround large groups of axons (Fig. 2) [69-79]. Ultimately these membrane processes infiltrate within these groups of axons, and segregate these axons into either: i) a 1-1 association, i.e. one Schwann cell around one axon; this Schwann cell is now called the pro-myelinating Schwann cell or ii) multiple axons associate with one Schwann cell; however each axon is within its own “pocket” of Schwann cell plasma membrane and isolated from all the other axons; this Schwann cell is now referred to as the non-myelinating Schwann cell (Fig. 2). Through a process that is yet 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 (Fig. 2).

The process of sorting axons into smaller bundles, until Schwann cells are either in a 1:1 association or in a Remak bundle, is called “radial sorting”. In the mouse, the model of choice to study myelination, and from which most of our understanding is derived, radial sorting starts embryonically (around E12.5) and continues after birth until post-natal day P10 [80]. Schwann cells in the pro-myelinating stage start appearing at E17.5 and can be observed until P15. These cells are also characterized by the expression of new molecular markers such as transcription factors Krox20, Oct6 and NF-κB [81]. Krox20 is particularly important and is considered a master regulator of PNS myelination. It activates the expression of several myelin protein genes [82] regulates the expression of genes
involved in the biosynthetic pathway of cholesterol and other lipids important to myelination [83] and suppress the expression of myelin inhibitors [82]. Mutations in the Krox20 gene have been linked to several types of CMT [84], Déjérine-Sottas syndrome [58] and Congenital Hypomyelinating Neuropathy [85]. Upon birth, pro-myelinating Schwann cells initiate spiral wrapping and compact myelin sheath can be observed as soon as 24 hrs later.

**Structural domains associated with the myelin/axon unit**

During the morphogenetic movements associated with the formation of the myelin sheath, the interaction between the myelinating glial cell and the underlying axon also results in the organization of domains that can be characterized both at the structural and molecular levels.

As described before, the spiral-wrapped plasma membrane compacts to form the compact myelin sheath. This represents about 99% of the zone of contact between the glial cell and the myelinated axon. The inner (adaxonal) and outer (abaxonal) layers are, however, not compacted (Fig. 3A). The cytoplasm between these two layers is in communication through cytoplasmic channels that are dispersed along the myelin sheath. These interruptions within the myelin sheath are called the Schmidt-Lanterman incisures. They appear after the formation of the compact myelin sheath [86], which suggests that they are likely not important for myelin formation, but that they may regulate myelin maintenance [39]. As these channels spiral around, from the outer to the inner cytoplasmic layers, the adjacent plasma membranes is enriched in gap junctions [87], further supporting the putative function of the Schmidt-Lanterman incisures in communication between
the inner and outer layers. Although direct evidence of such communication has yet to be provided, mutations in the connexin-32 that characterizes Schmidt-Lanterman incisures have been associated with the X-linked Charcot-Marie-Tooth disease [88], underscoring the importance of GAP junctions and Schmidt-Lanterman incisures in myelin maintenance.

The inner and outer membranes of the internode express different sets of molecules that allow the myelinating and myelinated Schwann cells to interact with the axon and the extracellular matrix. This localized repartition of various molecular components across the radius of the myelinating Schwann cell/axon unit is referred to as radial polarity (Fig. 3B)[89], and is particularly important to the process of myelination. For example, the localization of ß1 integrin to the outer membrane of the myelinating Schwann cell is necessary to the process of radial sorting [72, 90, 91]. Later on the localized expression of ß4 integrin to the outer membrane is crucial to the myelin sheath stability. ß4 integrin deficiency leads to abnormal myelin folding in old mice [92]. On the opposite side, the localization of the tyrosine kinase receptors ErbB2 and ErbB3 to the inner plasma membrane is necessary for the Schwann cell to receive the axonal type III Nrg1 whose levels control Schwann cell fate, i.e. myelinating phenotype or a non-myelinating phenotype (see section “Type III neuregulin-1 and peripheral nerve myelination”) [31, 93]. These examples do not represent an exhaustive list of asymmetrically, radially localized proteins and their function in myelination and myelin maintenance (see reviews [89, 94].

At both longitudinal ends of the myelin sheath, the plasma membrane of the
outer layer extends microvilli that come in close apposition with the axonal membrane at the node of Ranvier. These nodes are characterized by the localized presence of extracellular matrix molecule Gliomedin (microvilli) and cell adhesion molecules Neurofascin 186 (on the axon) [95]. The axonal membrane at the node is also enriched in the voltage-gated sodium channels and voltage-gated potassium channels that are crucial to salutatory conduction [96]. Additional markers such as AnkyrinG and βIV spectrin are also specifically enriched at the node of Ranvier, just below the axonal membrane [97]. On either side of the node are the paranodal domains, characterized at the ultra-structural level by Schwann cells loops (Fig. 4). These loops are formed by the spiral-wrapping of the Schwann cell plasma membrane around the axon. At the time of compaction to form the compact myelin sheath, the longitudinal extremities are not compacted, thereby leaving the presence of an un-compacted loop for every turn of the plasma membrane. As with the Schmidt-Lanterman incisures, the paranodal loops ensure continuity between the cytoplasm at the inner layer with the cytoplasm at the outer layer of the myelinated Schwann cell. The paranodal domains are biochemically characterized by the specific localization of various cell adhesion (Caspr and contactin on the axon; Neurofascin 155 on the Schwann cell loops) [98-100] and scaffolding molecules (4.1B under the axonal membrane) [98-100]. This domain is devoid of any ion channels. Finally, next to the paranode, and just at the beginning of the compact myelin sheath, are the juxta-paranodes (Fig. 4). Although not identifiable by any ultra-structural features, the juxta-paranodes are highly enriched in specific sets of molecules such as: i) Caspr2 on the axon, ii) TAG-1 on
the Schwann cell, iii) 4.1B under the axonal membrane [98-100]. This repeating organization, node-paranode-juxta-paranode-internode-juxta-paranode-paranode-node represents what is referred to as longitudinal polarity.

**Axo-glial interaction through cell adhesion molecules is necessary for domain formation and myelination**

As mentioned earlier, differentiated Schwann cells organize the myelinated axons into specialized domains such as node of Ranvier, paranode, juxta-paranode, and internode. The process of saltatory conduction is intimately linked to this structural organization. The formation of these domains is dependent on specific axo-glial interaction through cell adhesion molecules and extracellular matrix components that are present at the surface of axons and Schwann cells.

For example Gliomedin, which is secreted by Schwann cells [101] combines with glial cell adhesion molecule NrCAM to form a high-affinity binding partner for axonal Neurofascin 186 [102]. This interaction recruits and confines axonal Neurofascin 186 at the tips of the Schwann cells that are elongating along axons. AnkyrinG, binding with Neurofascin 186, also localizes to these tips and recruits voltage-gated sodium channels [103]. As adjacent Schwann cells elongates, they “drag” along the Neurofascin 186/ankyringG/sodium channel complexes. When adjacent tips meet, they fuse and form the node of Ranvier [102]. The complex is further stabilized by the recruitment of scaffolding protein βIV spectrin on the axon side [104]. Similar mechanisms are at play in the formation of the paranodal [105-107] and juxta-paranodal domains [108-110].

Although these domains are important to stabilize voltage-gated channels
for action potential propagation, these domains account for only 1% of the whole axon-glial contact length. Further, myelination is not impaired by the ablation of CAMs expression in node, paranode, and juxtaparanode. Only the formation of these domains will be affected [102, 105-110]. Compact myelin sheaths are still formed at the internode.

The cell adhesion molecules that mediate axo-glial interactions at the internode have recently been identified [111, 112]. To date, their function is not well understood (see section “Nectin like molecules: internodal candidates for regulating myelination”).

**Type III neuregulin-1 (type III Nrg-1) and peripheral nerve myelination**

One of the morphological characteristics associated with PNS myelination is that the compact myelin is essentially found around large caliber axons. On the other hand, small caliber axons are found within the pockets of the non-myelinating Remak Schwann cells. The axon diameter cut-off for myelination versus non-myelination is around 1 µm [113]. The molecular basis for this distinction was not understood for a long time, until research published by Michailov (2004) and Taveggia (2005) highlighted the key role of type III Nrg-1 in the process of myelination by Schwann cells.

Neuregulins are growth factors of the EGF family that are encoded by four distinct genes, Nrg-1 to Nrg-4. They are particularly implicated in several developmental processes such as Schwann cells [114] and oligodendrocytes [115] differentiation, cardiac development [116], and the formation of neuromuscular synapses [117]. Most of our understanding on neuregulins functions is mostly
derived from extensive studies of the Nrg-1 member of the family. Nrg-1 is declined in numerous isoforms that are derived from alternative splicing [118] of the Nrg-1 gene. All however have an EGF domain that is necessary and sufficient [117] to activate their receptors, which belong to the ErbB family of tyrosine kinase receptors, of which EGFR is the founding member (also referred to as ErbB1). Schwann cells express ErbB2 and ErbB3.

The different isoforms of Nrg-1 are organized in at least six major classes or types [119], I to VI. All isoforms are initially synthesized as transmembrane proteins. Type I, II and IV to VI are shed and function as soluble paracrine signaling molecules [120]. Type III however remains tethered to the cell surface and functions as a juxtacrine signal [118] (Fig. 5). Membrane-bound (type III Nrg-1) and soluble isoforms of Nrg-1 are produced by axons and glial cells [118].

Nrg-1 growth factors have long been the candidate axonal signals that regulate many aspects of Schwann cell development [114, 121]. Type II Nrg-1 is the glia growth factor 2 (GGF2) and has long been the Nrg-1 isoform associated with Schwann cell proliferation and survival [122]. The signal regulating Schwann cell myelination was however elusive. Indeed, GGF2 was initially shown to inhibit myelination in an in vitro system of PNS myelination [123], and to induce the demyelination of already formed compact myelin sheathes [123]. Furthermore in vitro studies using purified axonal plasma membrane strongly highlighted the dependence of Schwann cell to axonal contact for proliferation and survival [32]. Studies using knockout strategies to ablate the expression of the membrane-bound form of Nrg-1 were the first to provide compelling evidence that type III Nrg-
1 is the key isoform required for Schwann cell proliferation and survival [68, 124]. Furthermore, type III NRG-1 was also shown to be the instructive axonal signal that determines, in the PNS, whether a Schwann cell ensheathes multiple axons (Remak bundles) or associates in a 1:1 relationship with an axon and forms a compact myelin sheath [31]. The levels of type III Nrg-1 on axons further regulate the extent of myelination by determining the number of lamellae that myelinating Schwann cells will form around axons [31, 93]. Interestingly type III Nrg-1 is effective at promoting myelination only as a juxtacrine signal. Paracrine signals provided by a soluble form of type III Nrg-1, as well as other soluble isoforms, do not provide the necessary instructive myelinating signal [31, 125]. Interestingly however, it was also found that soluble Nrg-1, initially found inhibitory to myelin formation and maintenance [123], could actually enhance myelin formation once initiated by the type III Nrg-1 [125] The dual effect of soluble Nrg-1 on myelination (promoting or inhibiting) is concentration-dependent and the resulting signaling pathways (Akt or Erk1/2) that are activated [125]. The characterization of type III Nrg-1 as the key regulator of Schwann cell myelination provided the first idea as to the molecular bases for the observation that small caliber axons (< 1 µm) are not myelinated and large caliber axons are: small caliber axons express little amounts of type III Nrg-1 compared to large caliber axons [31].

As mentioned earlier, all Nrg-1 isoforms are initially generated as membrane-bound forms that, to the exception of type III Nrg-1, are released as soluble form by cleavage by β-secretases. One particular β-secretases of importance to PNS myelination is BACE-1, which cleaves type III Nrg-1 just C-
terminus of its EGF domain [126]. Type III Nrg-1 remains tethered to the axon through a cysteine-rich N-terminal domain [120] (Fig. 5). Ablating BACE-1 expression in mice results in a hypo-myelinating phenotype [127], which suggests that a change in the presentation of the EGF domain of type III Nrg-1 to the Schwann cell is necessary for its function. Interestingly type III Nrg-1 is also the target of an alpha-secretase, TACE that cleaves within the EGF domain. TACE knockout mice present a hyper-myelinating phenotype. These results strongly suggest that the regulated proteolysis of type III Nrg-1 likely regulates the amounts of active type III Nrg-1 [128], which may have an impact on the timing and extent of myelination by Schwann cell during the development of the PNS.

**Regulation of Schwann cell myelination by signaling pathways**

As mentioned earlier, all Nrg-1 isoforms have an EGF domain that is necessary and sufficient to activate their receptors. These receptors are ErBb1 (aka EGFR), ErbB2, ErbB3 and ErbB4 and belong to the family of receptor tyrosine kinases. Schwann cells express ErbB2 and ErbB3. Whereas the tyrosine kinase domain of ErbB2 is active, the domain of tyrosine kinase domain of ErbB3 is not functional [129]. On the other hand, while ErbB3 can bind Nrg-1 ligands, there are no known ligands for ErbB2 to date [130]. In order to form a signaling complex, Nrg-1 binds to ErbB3. This binding induces a conformational change to ErbB3 that allows it to recruit and for a heterodimer with ErbB2. Dimerization activates the tyrosine kinase domain of ErbB2, which now phosphorylates tyrosine residues on the cytoplasmic tail of both itself and ErbB3 [131]. The phosphorylated tyrosine residues act as docking sites to several adaptor proteins that serve as platforms
to recruits and activate three major signaling pathways: i) the PI3 kinase/Akt pathway, ii) the Ras/Raf/Erk pathway and iii) the PLC \( \gamma / \text{Ca}^{2+}/\text{calcineurin} \) pathway [132].

i) The PI3 kinase/Akt pathway is the major pathway involved in regulating all Schwann cell biological processes: survival, proliferation and myelination [32], including myelin sheath thickness [31]. Upon Nrg-1 stimulation, phosphorylated tyrosine residues (mostly on ErbB3) recruit the p85 subunit of class 1A PI3 kinase. p85 in turn recruits and activates the catalytic domain p110 that enables PI3 kinase to convert phosphatidylinositol-4,5-bisphosphate \((\text{PI}_{(4,5)}\text{P}_2)\) to phosphatidylinositol-3,4,5-trisphosphate \((\text{PI}_{(3,4,5)}\text{P}_3)\). Signaling effectors harboring a pleckstrin homology domain (PH), such as Arf6, PDK1 and Akt, get recruited to the plasma membrane by \((\text{PI}_{(3,4,5)}\text{P}_3)\). Once recruited, Akt is activated by phosphorylation on Threonine 308 \((T^{308})\) by PDK1 (phosphoinositide-depend kinase 1; also recruited by \((\text{PI}_{(3,4,5)}\text{P}_3)\) [133]. Akt is then further phosphorylated on Serine 473 \((S^{473})\) by the mTORC2 complex (mammalian target of rapamycin; also called PDK2) [133]. This second phosphorylation leads to Akt being fully active.

There are at least two mechanisms to terminate Akt signal. First, reducing the amount of activating phospholipid (PIP3) by PTEN, a phosphatase that dephosphorylates PIP3 to PIP2 [134]. Second, dephosphorylating Akt itself by the other two phosphatases – PP2A-type phosphatases [135] and PHLPP [136]. The analyses of the PTEN conditional knockout mice have confirmed the importance of this signaling pathway \textit{in vivo}. This mice not only show phenotypes such as multiple uncompact wraps of myelin around collagen fibers [33], but also
hypermyelination of small fibers and aberrant myelination of large diameter axons [34]. Treatments with the mTOR inhibitor rapamycin improved myelin abnormalities, indicating that mTORC signaling is important downstream of this signaling cascade. Schwann cell-specific inactivation of mTORC1 impaired PNS myelination and caused an arrest in longitudinal fiber growth [137], findings that are in line with the fact that mTORC1 is a downstream effector of the PI3K/Akt pathway. In addition, the conditional inactivation of Raptor in Schwann cell, an adaptor protein specific to the mTORC1 complex, impaired PNS myelination. This is due to perturbed lipid biosynthesis that depends on the mTORC1/RXRγ/SREBP-signaling axis [138]. The Schwann cell- and oligodendrocyte-specific expression of a dominant-active form of Akt in mice further strengthened the demonstration that the PI3 kinase/Akt axis controls the thickness of the myelin sheath, both in the PNS and in the CNS [139].

(ii) The case for the function of the MEK/Erk1/2 cascade in myelination is more complex. Indeed activation of the MEK/Erk1/2 cascade appears to be both pro-myelinating and inhibitory [125, 140] to PNS myelination. On one hand, *in vivo* studies have shown that the conditional knockout of Erk1/2 proteins in Schwann cells impaired PNS myelination [141], whereas the sustained activation of Erk signaling in myelinating Schwann cells caused hyper-myelination [142]. Furthermore a recent study has shown that a mild but sustained activation of the Erk signaling cascade could compensate for the deficient activation of the PI3K/AKT cascade in ErbB3 knockout mice [143]. These results all point for a pro-myelinating function of the Mek/Erk pathway and are in line with the demonstration
that this pathway can promote the transcription of the Krox20 gene [144]. On the other hand, *in vitro* studies have shown the inhibitory effect of soluble forms of neuregulins on myelin formation and myelin segments stability [123], a process that is dependent on the activation of the Erk pathway [125]. Furthermore, the expression of constitutively active Mek1 also abolishes the expression of myelin protein markers by Schwann cells when treated with forskolin [140], and the inhibitory effect of soluble neuregulin on myelin protein markers expression is abolished in Schwann cells harboring a dominant-negative form of Ras. These data underscore the opposite roles of the Mek/Erk pathway in PNS myelination. As the experimental approaches are varied, these apparently conflicting results probably reflects different role for Mek/Erk pathway at different time of PNS development, as has been shown in CNS development and oligodendrocyte myelination [142].

(iii) The PLCγ/Ca\(^{2+}\)/calcineurin signaling cascade is the third pathway activated by type III Nrg1 in Schwann cells. Its activations leads to the dephosphorylation of transcription factor NFATc4. Upon dephosphorylation, NFATc4 translocates to the nucleus and, in association with Sox10 induces the upregulation of transcription factor Krox20 [132].

**Nectin like molecules: internodal candidates for regulating myelination**

As previously mentioned, axons and Schwann cells interact through several cell adhesion molecules. These interactions are important as they participate in the organization of the specialized domains that are characteristic of myelinated axons (node of Ranvier, paranode, juxtaparanode) and whose structure and
molecular composition are crucial to the fast propagation of action potentials by saltatory conduction [98]. While the role of many of them in the formation of nodes, paranodes and juxtaparanodes has been extensively addressed, the role and mode of action of the cell adhesion molecules that characterize the internode, which accounts for 99% of longitudinal extent of axon, are not well understood. Recently, two independent groups [112, 145] identified the Nectin-like (Necl) proteins as the cell adhesion molecules that mediate axo-glial interactions along the internode.

The Necls (also referred to as Cadm [cell adhesion molecule] and SynCAM [synaptic cell adhesion molecule]) are type 1 transmembrane cell adhesion molecules [146, 147] of the immunoglobulin superfamily. They are about 400 amino acids in length, with molecular weights ranging from about 42 to 100 kDa due to varying extent of N-glycosylation [147], and are characterized by: i) three extra-cellular Ig-like domains, ii) one transmembrane domain, iii) a FERM- (4.1, ezrin, radixin, moesin)-binding domain and a class type II PDZ- (PSD-95, DLG, ZO1)-binding domain that are in the short cytoplasmic sequence. Biochemical analyses have shown that these domains can interact, respectively, with intracellular scaffolding proteins of the 4.1 family [148, 149] and PDZ domain-containing proteins [150-153].

Four different Necls have been identified: Necl-1, Necl-2, Necl-3 and Necl-4. Their structural organization (extracellular Ig loops, intracellular domains involved in scaffold organization and complex recruitments) strongly suggests that the Necls are implicated in the recruitment and stabilization of signaling complexes.
Several studies have shown that the Necls promote cell-cell adhesion through heterophilic and homophilic interactions [145, 146, 150, 152, 154, 155]. They also regulate various cellular activities such as cell polarization [156], differentiation [157], movement [158], proliferation [156, 159] and apoptosis [160]. Of particular interest to Schwann cell biology, Necl-1 and Necl-4 have the potential to directly interact in cis with the tyrosine kinase receptor ErbB3 [161, 162], and have been shown to regulate the tyrosine phosphorylation status of ErbB3 by recruiting PTPN13, a tyrosine phosphatase [161-163].

In the peripheral nervous system, Schwann cells mainly express Necl-4, while DRG neurons express Necl-1. Both cell types also express Necl-2. They promote axon-to-Schwann cell interaction along the internodal domain through heterophilic (Schwann cell Necl-4 and Necl-2 to axonal Necl-1) and homophilic (Necl-2 to Necl-2) binding. Schwann cell-specific Necl4 has been shown to be a key molecule in initiating PNS myelination [112, 145]. Indeed, shRNA-mediated knockdown of Necl4 blocked Schwann cell differentiation and myelination in a Schwann cell / DRG neuron myelinating co-culture system, as was indicated by the absence of myelin proteins and myelin segments [145]. The limited expression of transcription factors Oct6 and Krox20 indicated that Schwann cells were arrested before progressing to the promyelinating stage [145]. Experiments using the addition of the extracellular domains of Necl-4 or Necl-1 to perturb axo-glial interactions at the onset of myelination in the co-culture system similarly resulted in a drastic reduction in myelin segments formation [112], and suggest that proper axo-glial interactions are needed for proper myelination. Electron microscopy
analysis showed that Schwann cells ensheathed but failed to wrap axons [112]. In that respect it is interesting to note that, among the myelination abnormalities that were described in the recent analysis of a Necl-4 knockout mouse, there is a delay in the initiation of PNS myelination [24].

The Necl-4 knockout mouse also presents several abnormalities (outfoldings, tomaculae, focal hyper-myelination) along the axo-glial interface that are indicative that Necl4 is essential to promote a proper interaction between the axon and the myelinating Schwann cell [24]. Recent findings regarding lipid metabolism in Schwann cells lacking Necl-4 expression may provide a molecular basis to explain some of these abnormalities [23]. Necl-4 deficient Schwann cells exhibit an increase in intracellular choline, and the quantitative and qualitative patterns of expression of choline-dependent lipids are dramatically affected [23]. The primary choline-dependent lipid is phosphatidylcholine, a major lipid component of the myelin sheath. Phosphatidylcholine is also an important source of phosphatidic acid [29]. Phosphatidic acid is quickly converted to diacylglycerol or used for the synthesis of a class of important signaling and structural phospholipids, the phosphatidylinositols (PI) [30] and phosphorylated forms of PI, such as PI(3)P, PI(4)P, PI(4,5)P2, PI(3,4,5)P3, PI(5)P and PI(3,5)P2, all of which crucial for proper PNS myelination [25-28, 31-34, 50-52, 164] As mentioned in the section “Molecular composition of the PNS myelin sheath”, the tight regulation of PI(3,5)P2 levels by Mtmr2 and Fig4 is crucial to the proper folding and stability of the myelin sheath. Mutations in both genes are linked to Charcot-Marie-Tooth type 4 neuropathologies [25-28] that are characterized by outfoldings, tomaculae and
focal hyper-myelination myelin abnormalities, similar to the ones seen in the peripheral nerves of Necl-4 knockout mice [24].

**Scope of the thesis work**

1) **Characterization of the role and mechanism(s) of action of axonal Necl-1 in regulating Schwann cell myelination**

Necl-4 has two identified binding partners: Necl-1 and Necl-3 [145, 165]. While Necl-3 promotes axo-glial interaction in the central nervous system, its role in PNS is still not clear. Although it appears to be present in trigeminal neurons [166], Necl-3 is barely detectable in DRG neurons [112, 145] with limited amount in the neuron bodies and absent from the axons. While additional axonal binding partners for Necl-4, as yet uncharacterized, could be up regulated with myelination, the current published data strongly suggest that Necl-1, on the axon, is the obligate binding partner for Necl-4 present on the Schwann cell.

Surprisingly, the analyses of two independent Necl-1 knockout mice have shown no detectable defects in PNS myelination [24, 167]. This could potentially be attributed, in part, to the complexity of *in vivo* systems in which redundancy and functional overlap may obscure the detection of a phenotype. I therefore investigated *in vitro* a potential role for axonal Necl-1 in myelination, using the well-established Schwann cell/DRG neuron myelinating co-culture system that has been central to many key insights in the molecular mechanisms involving axo-glial interactions, domain formations and myelination.

The results are presented and discussed in Chapter III.
2) Characterization of the role and mechanism(s) of action of Schwann cell Necl2 in regulating Schwann cell myelination

Necl-2 can potentially mediate axo-glial interactions through two mechanisms: i) homophilic interaction between Necl-2 on the Schwann cell and the axon, and ii) heterophilic interaction between Schwann cell Necl-2 and axonal Necl-1 [145, 165]. More interestingly, the extracellular domain of Necl-2 has been shown, at least in biochemical conditions, to interact with the extracellular domain of ErbB3 [161]. This is a cis interaction, i.e. both components must be expressed at the plasma membrane by the same cell [161], a condition that is fulfilled by the Schwann cell. Furthermore Necl-2 can recruit PTPN13, a tyrosine phosphatase, through a PDZ/ PDZ binding domain interaction [161]. Finally overexpression of Necl-2, and several domain-deletion variants suggest that Necl-2 recruits PTPN13 to ErbB3, leading to ErbB3 inactivation by de-phosphorylation [161] with an associated inhibition of Akt, as well as Rac1 activity [161]. As presented earlier, the PNS myelination is dependent on the activation of the pro-myelinating PI3K/Akt signaling cascade in Schwann cells by binding of axonal type III Nrg1 to the tyrosine kinase receptors ErbB2/ErbB3 that are expressed by Schwann cells [31]. Necl-2 potential function in regulating ErbB3 and Akt activation highlight it as a potential candidate to regulate myelination by Schwann cells.

A prospect investigated in Chapter IV.
CHAPTER II

Materials and Methods

Animals

Sprague-Dawley rats were purchased from an accredited vendor (Hilltop Lab Animals Inc.) Upon delivery, animals were housed and cared for by the trained personnel of the animal facility at Rutgers, The State University of New Jersey, Newark NJ, in accordance with protocols approved by the Institutional Animal Care and Use Committee of Rutgers University.

Culture media, Antibodies, and Growth factors

DMEM and MEM were purchased from Mediatech, neurobasal media, Ham’s F1, B27 supplement, N2 supplement, GlutaMAX™-I and trypsin were from Invitrogen, glucose, Forskolin, 5-fluorodeoxyuridine, uridine, and ascorbic acid were purchased from Sigma-Aldrich, FBS was from Atlas Biological, and Matrigel was from Beckton Dickinson. For immunofluorescence staining, primary antibodies included mouse monoclonals to myelin basic protein (MBP; Covance SMI-94R at 1:250) and HA epitope (Covance HA.11 at 1:500) and Flag epitope (Sigma-Aldrich Anti-flag M2 at 1:200), and the chicken monoclonal to neurofilament M (Covance PCK-593P at 1:3000). For Western blot analyses, primary antibodies included mouse monoclonals to β-actin (Sigma-Aldrich A1978 at 1:5000), GFAP (Cell Signaling Technology 3670 at 1:1000), Cadm4 (Necl4, NeuroMab 75-247 at 1:1000), Erb2 (Thermo Scientific MS-730 at 1:500), ErbB3 (Thermo Scientific MS-
201 at 1:500), phospho-Akt (T308 and S473; Cell Signaling Technology 4051 at 1:500 and 1:1000), phospho-PDK1 (S241; Cell Signaling Technology 3061 at 1:1000), PDK1 (Cell Signaling Technology 3062 at 1:1000), mouse polyclonal against the Fc portion of human IgG (Jackson ImmunoResearch 209-005-098 at 1:1000), rabbit polyclonals against phospho-ErbB2 (Y1248; Santa Cruz sc-12352-R at 1:500), phospho-ErbB3 (Y1289; Cell Signaling Technology 4791 at 1:500), Akt (Cell Signaling Technology 9272 at 1:1000), phospho-Erk1/2 (T202/ Y204; Cell Signaling Technology 9101 at 1:1000), PTPN13 (Santa Cruz sc-15356 at 1:200), Nrg1-β1 (Santa Cruz sc-348 at 1:250), TrkA (168 at 1:500), Cadm2 (Necl3; [145]), guinea pig polyclonal against Cadm3 (Necl1; [145]), and chicken monoclonal against Cadm1 (Necl2, MBL CM004-3 at 1:1000). Recombinant EGF domain of human Nrg1-EGFD) was from R&D Systems (396-HB) and 2.5S NGF was from AbD Serotec (PMP04Z).

**Schwann Cells, Dorsal Root Ganglia (DRG) Neurons, SCG neurons, and myelinating Cocultures**

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 5 ng/ml of rhNRG1-EGFD and 2 µM Forskolin. In all experiments, Schwann cells were used at the fourth passage. DRGs and SCGs were collected from embryonic day 15 rat embryos and 2-day-old rats, respectively. After trypsinization cells were plated on ø10 mm 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 and SCG cells
were seeded at a density of 1.3 and 0.8 ganglia per coverslip, respectively. Alternately feeding the cultures every 2 days with standard neuronal medium supplemented or not with 5-fluorodeoxyuridine and uridine (10 µM each) for 10 days removed non-neuronal cells. Purified neurons were then kept in standard neuronal medium until use. To establish co-cultures, purified neurons were repopulated with purified primary rat Schwan 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 µg/mL of ascorbic acid. The formation of compact myelin segments was assessed by immunostaining for MBP, 10 days (DRG cocultures) or 60 days (SCG cocultures) later. To quantify the extent of myelination, 15-20 random fields were captured per culture (Nikon Eclipse TE2000-U, 20X/0.75 Plan Fluor objective). 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%). Counting was done with the ImageJ software [169].

**RNA interference of Necl-1 and Necl-2 Expression in DRG, SCG Neurons and Schwann cells**

Two 21-nucleotide long shRNAs (#1, GGCCAGAAGCTGTTGTTACAT and #2, GTGCCAAGTGAAAGACCATGA) targeting in the extracellular domain of Necl-1 at position 733-753 and 911-931, respectively (GenBank NM_001047103) and one 21-nucleotide (GCCGAATCTGTTCATCAATAA) targeting in the extracellular domain of Necl-2 at position 1057-1078 (GenBank NM_001012201.1), were designed using the following online tools: Easy siRNA (ProteinLounge), BLOCK-iT
RNAi designer (Invitrogen), and siRNA sequence selector (Clontech). The shRNA stem loops consisted of a sense shRNA sequence followed by a short non-specific loop sequence (TTCAAGAGA) and the reverse/complement antisense shRNA sequence, followed by six thymidines that serve as a stop signal for the RNA polymerase III. The oligonucleotides were cloned into the Hpal-XhoI sites of the pLentiLox pLL3.7 lentiviral vector (Addgene, plasmid 11795), in which the U6 promoter drives the expression of the shRNA while the GFP marker is expressed under a CMV promoter [170, 171]. The lentiviral constructs were transfected into 293FT cells together with packaging plasmids pMD2.G (Addgene plasmid 12259) and psPAX2 (Addgene plasmid 12260) using Calcium Phosphate Transfection kit (ThermoFisher Scientific). We used a pLL3.7 construct encoding a shRNA against luciferase as a control for non-specific effects. Viral supernatants were collected 48 h after transfection, centrifuged at 1,6000g for 20 min to pellet cell debris, and supernatants were aliquoted for one-time use and kept frozen at -80ºC. For the infection, DRG or SCG cultures were treated with viral supernatants (supplemented with NGF at 50 ng/mL) 24 h after plating the cells. The following day, alternate feeding to remove non-neuronal cells was initiated as described before. The knockdown of Necl-1 and Necl-2 expression were confirmed by Western blotting before using the cultures.

**Expression of Necl-1 in DRG neurons and Necl-2 in Schwann cells**

The sequence encoding the influenza hemagglutinin (HA) epitope was added by PCR to the amino-terminus of Necl-1 protein immediately after the signal peptide as predicted by the SignalP program [172]. The cDNA encoding HA-Necl-
1 was then inserted in the pLent6/V5-D vector by directional TOPO cloning (Invitrogen) and the construct was confirmed by sequencing. The cDNA encoding of Necl-2 fused with Flag tag at 363aa site was inserted in the pLL3.7 lentiviral vector, in which Necl-2 expression is expressed under CMV promoter. The lentiviral construct was transfected into 293FT cells together with packaging plasmids pMD2.G (Addgene plasmid 12259) and psPAX2 (Addgene plasmid 12260) using Calcium Phosphate Transfection kit (ThermoFisher Scientific). The pLenti6/V5 vector was used as control. Viral supernatant collection and infection of the DRG neurons was as described in the RNA interference section. Expression of HA-Necl1 at the surface of axons was confirmed by live immunostaining for the HA tag, 10 days post infection, before use. Expression of Flag-Necl2 at the surface of Schwann cell was confirmed by live immunostaining for the Flag tag, 6 days post infection, before use.

**Immunostaining**

DRG neurons, DRG/Schwann cell or SCG/Schwann cell cultures were rinsed in phosphate buffered saline (PBS) and fixed in 4% paraformaldehyde for 20 minutes. After washing with PBS, samples were permeabilized in ice-cold methanol for 20 min and incubated in blocking solution (5% normal donkey serum, 0.3% Triton X100) for 1 h at room temperature. This was followed by an overnight incubation at 4ºC with primary antibodies against MBP and neurofilament M prepared in blocking solution. After washing with PBS, samples were incubated with secondary antibodies for 1 h. 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 (203/0.75 objective) microscope; 15-20 random field images were acquired per culture using the MetaMorph software package (Molecular Devices). Quantitation was done in ImageJ.

**Proliferation Assay**

Five thousand Schwann cells were plated onto dissociated DRG neuron cultures, in MEM supplemented with 10% het inactivated FBS, 0.4% glucose, and 50 ng/mL NGF. After 24 h, cultures were fed with fresh media for another 24 h. Schwann cell proliferation was assessed using an EdU nuclear labeling assay. EdU (10 mM final concentration) was added during the last 4 h of culture, and was detected using the Clik-iT Alexa 488 or Alexa 555 kit according to the manufacturer’s instructions (Life Technologies). Neurites were detected by immunostaining for neurofilament M and nuclei by DAPI. All cultures were mounted on glass slides in Citifluor and examined by epifluorescence microscopy. Images were 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 1000 cells were counted per condition. EdU incorporation was expressed as a percentage of Edu-labeled versus DAPI labeled nuclei.

**Nec1-Fc production, signaling pathways experiment, and Western Blot Analysis**

The cloning of Nec1-Fc into the pcDNA3.1 TOPO vector (Invitrogen) and the production of the Nec1-Fc fusion protein were previously described [145].
Schwann cells were plated at a density of 300,000 cells per well of a 24-well plate (Techno Plastic Products) coated with poly-L-lysine. The following day, cells were incubated for 3 h in defined media [DMEM/Ham’s F12 (1/1), N2 supplement, 1% GlutaMAX™-I] to down regulate signaling pathways. Cells were then incubated for 30 min at room temperature with Necl1-Fc at concentrations ranging from 0 to 20 nM. rhNrg1-EGFD (2.5 and 10 nM) was then added and cultures were incubated at 37°C for 10 min, at which time cell lysates were prepared in 25 mM Tris buffer pH 7.4, containing 95 mM NaCl, 1% SDS, protease and phosphatase inhibitors cocktails (Pierce). Lysates were boiled for 10 min and cleared by centrifugation at 16,000g for 10 min. Protein concentrations were determined by the BCA method (Pierce). Ten micrograms of proteins were fractionated on 10% Bis/Tris polyacrylamide gels and transferred onto 0.2 mm nitrocellulose membranes (Biorad). Appropriate regions of the blots were cut out and incubated with specific primary antibodies to probe for ErbB2, ErbB3, PDK1, Akt, Erk1/2, Necl-3, β-actin, and β-tubulin. After incubating with appropriate infrared secondary antibodies (LI-COR), protein bands were visualized and quantified with the Odyssey imaging system (LI-COR). For each signaling molecule analyzed, the ratio of phosphorylated versus total was first determined. Then for each Necl1-Fc group, the background (0 nM of rhNrg1-EGFD) was subtracted. Finally each value was normalized to the activation of the control (no Necl-1-Fc) at 10 nM of rhNrg1-EGFD (100%).

Co-immunoprecipitation Analysis
Schwann cells (3 x 10^6) were plated per Ø100 mm plates coated with poly-L-lysine, in standard Schwann cell medium supplemented with rhNrg1-EGFD and Forskolin. After 24h, cells were incubated for 3 h in defined media to down regulate signaling pathways, and then were further incubated for 30 min at room temperature with either Necl1-Fc or human IgG (hu-IgG) at 20 nM. rhNrg-EGFD (10 nM) was then added and cultures incubated at 37°C for 10 min, at which time pathway stimulation was stopped by washing the cultures 3 times with ice-cold PBS. Proteins were extracted in 200 µL of a 1% NP-40 detergent solution prepared in 20 mM Tris, pH 7.4, 10% glycerol, 2.5 mM EGTA, 2.5 mM EDTA, protease and phosphatase inhibitors. Cell lysates were centrifuged (16,000 x g for 15 min at 4°C) and protein concentration of supernatants were determined. For each condition (hu-IgG or Necl-1-Fc treated, plus or minus rhNrg1-EGFD stimulation), 300 mg of protein lysate was precleared with 40 µL of agarose-conjugated goat anti-mouse beads (50% slurry; Sigma A6531) at 4°C for 30 min. Beads were then removed by a 15 sec centrifugation (16,000 x g, 4°C) and precleared lysates incubated overnight at 4°C with 1 µg of mouse anti ErbB3 antibody. ErbB3 was then immunoprecipitated with 40 mL of agarose-conjugated goat anti-mouse beads (50% slurry) for 90 min at 4°C. Beads were collected by a 15 sec centrifugation (16,000g at 4°C), washed 3 times in 500 µL of lysis buffer, and boiled in Laemmli sample buffer for 15 min. Immunoprecipitated proteins were fractionated as described in the previous section. Appropriate regions of the blots were cut out and incubated with specific primary antibodies to probe for ErbB2, ErbB3, Necl1-fc, Necl2, Necl-
4, and PTPN13. After incubating with appropriate infrared secondary antibodies, protein bands were visualized the Odyssey imaging system.

**Neurite Membrane Preparation**

Neurite membranes were prepared according to established protocols [31, 32, 173]. Briefly, purified DRG neuron cultures from 24-well plates (12 wells) were washed with ice-cold PBS, collected in a 1 mL Dounce homogenizer (Kontes, Vineland, NJ) in 1 ml of ice-cold PBS, and homogenized with 30 strokes. The homogenate was centrifuged (80g, 20 min, 4ºC) to remove debris and collagen. The supernatant, diluted up to 7 mL in PBS, was then centrifuged at 35,000g for 1 h, at 4ºC. The supernatant was discarded and the pellet was resuspended in ice-cold lysis buffer.

**PCR for Splice Variants of Necl-1**

Trizol reagent (Invitrogen) was used for the extraction of total RNA from purified DRG neuron cultures. Total RNA was then used as template with the SuperScript III First-Strand Synthesis kit (Invitrogen) 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 qPCR master mix (Thermo Scientific). Primers were designed using the online version 4.0.0 of Primer3web [174, 175]. Sense primers were designed so that the last five bases overlapped exons 1-2 (X1 variant; 5’-ATCTTTCCAGGACGGCTAC) or exons 1-3 (canonical variant, lacks exon 2; 5’-CAATCTTTCCAGGACGATAGC) boundaries. Antisense primers were specific to exon 2 (5’-AGGGCTGGCTATCTTGA) or to exon 3 (5’-AGACCACCTGCAGGATGAGT). Control primers were designed against
HPRT1 (sense 5’-TGACACTGGTAAAACAATGCAGA in exon 6, and antisense 5’-ACTTCGAGAGGTCCTTTTCACC in exon 7). Melting point (Tm) curve analysis was done on all qRT-PCRs to verify the detection of a unique peak for each reaction, at the expected Tm. All endpoint reactions were separated on a 15% DNA polyacrylamide gel to confirm the detection of a unique amplicon of the expected size (Nec1 canonical: 118bp; Nec1 X1 variant: 129bp and HPRT1: 105bp)

**Small GTPase activity assays**

The activation of small GTPases upon laminin matrix stimulation was assayed with G-LISA kits specific for Rac1, and RhoA (Cytoskeleton, BK128, and BK124, respectively). 150,000 Schwann cells (knock down or overexpression Nec1) were seeded per well of 6-well plates (coated with laminin I, Sigma, L2020) in normal Schwann cell medium. 4 hrs later, cells lysates were collected 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). Absorbance was read at 490 nm.

**Statistical Analysis**

Myelination and proliferation data obtained were analyzed by one way ANOVA followed by Bonferroni post-tests. Quantitative Western blots were analyzed by paired Student’s t-test. The effects of Nec1-fc on the stimulation of pathways by rhNrg1-EGFD were analyzed by two-way ANOVA. Tests were performed with the Prism software package from GraphPad.
CHAPTER III

Axonal Necl-1 is a negative regulator of Schwann cell myelination in vitro

INTRODUCTION

The Nectin-like (Necl) cell adhesion molecules belong to the immunoglobulin superfamily. Several independent groups have shown that they promote cell adhesion through homophilic and heretophilic interactions [145, 146, 150, 152, 154, 155, 165].

Most interesting to studies on the regulation of myelination by Schwann cells in the PNS, the Necls were shown to be the long-elusive cell adhesion molecules promoting axo-glial interactions along the myelinated internode [112, 145]. Three of the four characterized Necl-3 are expressed in the PNS: Necl-1 (on the axon), Necl-4 (on the Schwann cell) and Necl-2 (axon and Schwann cell) [112, 145]. These analyzes of the binding properties of the Necls [145, 146, 150, 152, 154, 155] suggest that the following three axo-glial interactions are present along the internode: i) heterophilic Necl-4 to Necl-1, ii) heterophilic Necl-2 to Necl-1 and iii) hemophilic Necl-2 to Necl-2. Semi-quantitavive analyses indicate that the heterophilic Necl-4 to Necl-1 is the strongest interaction among all three and suggest that it may therefore be the predominant axo-glial interaction [145].

The importance of Schwann cell Necl-4 has been demonstrated both in vitro and in vivo [112, 145] In vitro, the ablation of Necl-4 expression [145] or the perturbation of Necl-4 and Necl-1 mediated [112] led to an inhibition of myelin segment formation by Schwann cells. The in vivo results from the analyses of a
Schwann cell-specific Necl-4 knockdown mouse [24] indicate that Necl-4 is involved in the initial steps of myelination and myelin maintenance.

Although the *in vitro* studies indicate that the heterophilic Necl-4 to Necl-1 interaction is likely to be the major one along the internode, surprisingly the analysis of two independent Necl-1 knockout mice have shown no detectable defects in PNS myelination.

The *in vitro* myelinating culture system is ideally suited to analyze axo-glial interactions and identify putative role in Schwann cell myelination. It is a simplified system (only 2 cell types) that bypasses the complexities of *in vivo* system. The *in vitro* myelinating culture system was established about 35 years ago and mimics PNS myelination. It has been instrumental since its implementation in all the major discoveries pertaining to PNS myelination. The importance of such a culture system is so significant that when a CNS myelinating culture system was finally developed [176], Prof. Ben Barres, one of the most respected leading figure in the field of neurobiology and CNS myelination, wrote that the lack of an efficient CNS myelination culture system had been “a long-time limitation in studying the molecular basis of CNS myelination” [177].

In this chapter, I manipulated the expression of Necl-1 in DRG neurons using loss and gain of function mediated by lentiviral transduction. An expected outcome of suppressing the Necl-1 expression would have been a decrease in myelination by Schwann cells. However, I report that axonal Necl-1, the partner of Schwann cell Necl-4, has the opposite effect on myelination. Low level of Necl-1 increased the level of myelination, whereas high level of Necl-1 impaired
myelination. I show that superior cervical ganglion (SCG) neurons, which do not normally support (both in vitro and in vivo) the formation of myelin segments by Schwann cells, express higher levels of Necl-1 compared to DRG neurons. Knocking down Necl-1 expression in SCG neurons promoted myelination. Finally, I show that the binding of the extracellular domain of Necl-1 to Schwann cells inhibited the activation of the pro-myelinating ErbB3/PI3K/Akt signaling cascade by the EGF-like domain of Nrg1β1.

RESULTS

Axonal Necl-1 is not necessary to promote Schwann cell myelination in vitro

To characterize the function of axonal Necl-1 in Schwann cell myelination, we designed constructs harboring short-hairpin RNAs (shRNAs) specific to Necl-1, and delivered them into DRG neurons by lentiviral transduction.

These constructs also express the green fluorescent protein (GFP) whose extensive expression in the neurite network demonstrates the efficient viral transduction of the DRG neurons (Fig. 6A). Both Necl-1 shRNAs (shNecl-1 #1 and #2) that were tested effectively inhibited Necl-1 expression in DRG neurons (Fig. 6B). A control shRNA targeted against luciferase (not expressed in mammalian cells; shLuc) did not affect Necl-1 expression (Fig. 6C), underscoring the specificity of the Necl-1 shRNAs. Immunostaining for neurofilament did not indicate any obvious defects in the formation of a dense neurite network by DRG neurons deficient in Necl-1, when compared with control (Fig. 6A). We then tested the ability of Schwann cells to myelinate DRG neurons lacking Necl-1 at the surface of their neurites. Schwann cells myelinated equally well both control and shLuc-infected
DRG neurons (Fig. 6C,D). Necl-1 deficient neurites also efficiently supported the formation of compact myelin segments by Schwann cells. In fact a substantial and significant 3.5-fold increase in the number of myelin segments was observed.

At 65±1 µm, the average myelin segment length was not statistically different from that of control (69±3 µm) and shLuc-infected DRG neurons (65±2 µm; p=0.485; one-way ANOVA; n = 4 experiments (ctrl, shLuc and shNecl-1 #1); n = 3 experiments for shNecl-1 #2; 3 cultures per condition, per experiment).

These results suggest that Necl-1 expression on axons is not necessary to promote Schwann cell myelination. However they also suggest that axonal Necl-1 may be inhibitory to myelin formation.

**Overexpression of axonal Necl-1 impairs Schwann cell myelination in vitro**

To corroborate the hypothesis that axonal expression of Necl-1 may be inhibitory to myelination, we used lentiviruses to deliver into DRG neurons a cDNA encoding the full length Necl-1 tagged with the hemaglutinin epitope (HA) at the N-terminus [145]. Live immunodetection of the HA tag indicated that Necl-1 was properly targeted at the surface of neurites (Fig. 7A, top panels, red), and that the extent of the neurite network was comparable to that of control DRG neuron cultures (Fig. 7A, bottom panels, green). Numerous and comparable myelin segments were formed by Schwann cells co-cultured in myelinating conditions with either control DRG neurons (Ctrl; non-infected) or DRG neurons infected with the empty pLenti6 viral vector (Fig. 7B,C). This result indicates that the formation of myelin segments is not affected by lentiviral transduction. Increased expression of Necl-1 at the surface of axons however resulted in a dramatic decrease (75%) in
the numbers of myelin segments formed by Schwann cell in contact with DRG neurites (HA-Necl-1; Fig. 7B, C).

This result strongly suggests that indeed axonal Necl-1 may be inhibitory to myelin formation by Schwann cells.

**SCG neurons are myelinated when deficient in axonal Necl-1**

To further corroborate a possible inhibitory role of Necl-1 in myelination by Schwann cells, we compared by quantitative Western blotting the levels of Necl-1 expression between NGF-dependent peripheral neurons isolated from two different sources: the DRG and the SCG (superior cervical ganglia). SCG neurons are sympathetic neurons whose axons are only ensheathed and unmyelinated in vivo. In co-culture with Schwann cells, SCG axons are also poorly ensheathed [178] and remain largely unmyelinated.

Interestingly, we found that the level of expression of Necl-1 by SCG neurons is about 2-fold higher than that of DRG neurons (Fig. 8A, B). To account for differences in the diameter of neurites derived from SCG and DRG neurons, which would affect surface-to-volume ratio, the data was normalized to either β-actin (volume) or receptor tyrosine kinase TrkA (surface membrane) without any significant difference (Fig. 8A, B).

To test whether these high levels of Necl-1 expression may prevent Schwann cells to myelinate these normally unmyelinated axons, SCG neurons were infected with the lentiviral shRNA constructs that effectively inhibited expression of Necl-1. Similar to DRG neurons, neurite outgrowth was not impaired and an extensive network developed (Fig. 8C; right panels). Rat Schwann cell
were then co-cultured with control or Necl-1-deficient SCG neurons in myelinating media for 60 days. While few myelin segments were detected in control cultures as expected, a significant number of compact myelinated segments were detected, by staining for myelin basic protein (MBP), around axons deficient for Necl-1 (Fig. 8C, D).

This result strongly supports the hypothesis that axonal Necl-1 may be inhibitory to myelin formation by Schwann cells.

**Axonal Necl-1 inhibits axon-mediated Schwann cell proliferation**

To elucidate whether axonal Necl-1 was involved in regulating axon-mediated Schwann cell proliferation, we cultured Schwann cells onto the neurites of DRG neuron cultures that either normally express Necl-1, or that had been knocked down with shRNAs against Necl-1. Proliferation was assessed by the nuclear incorporation of Edu.

As shown in Fig. 9, the ablation of Necl-1 expression at the surface of DRG neurites resulted in a 2-fold increase in the incorporation of Edu by Schwann cells.

This result strongly indicates that contact-dependent stimulation of Schwann cell proliferation is regulated by the expression of Necl-1 at the surface of axons.

**Type III Nrg1 and Necl-3 expression is not affected by the presence or absence of Necl-1 in DRG neurons**

DRG neurons regulate Schwann cell proliferation and myelination through several axon-derived factors [94], in particular the type III isoform of Nrg1. We therefore performed quantitative Western blot analyses on lysates from neurite
membrane preparations to determine possible changes in the expression of type III Nrg1 by DRG neurons in the absence of, or overexpression of Necl-1.

As shown in Fig. 10, changes in Necl-1 expression did not affect the expression of type III Nrg1 significantly. We also analyzed for the expression of Necl-3, the only other identified cell adhesion molecule ligand for Necl-4 to date [145]. Similarly to type III Nrg1, Necl-3 expression was not affected in DRG neurons, whether they were over-expressing Necl-1 or deficient for Necl-1.

These results suggest that the effect of axonal Necl-1 on Schwann cell myelination and proliferation may not be due to compensatory mechanisms, such as i) the regulation of type III Nrg1, a known axon-bound Schwann cell mitogen as well as the key instructive signal to PNS myelination [31, 93] or ii) the expression of an axonal cell adhesion molecule that would promote the axo-to-glia interaction with Necl-4 that is normally provided by Necl-1.

**Comparative levels of expression of Necl-1 in vitro and in vivo**

Necl-1 levels are developmentally regulated in vivo (Fig. 11C; compare p0 and p30 time points), a finding that is in line with our previous in vitro data showing up-regulation of Necl-1 upon axo-glial contact, in non-myelinating and myelinating conditions. Since the levels of Necl-1 can potentially be regulated by context, we attempted to evaluate the relative amount of Necl-1 expression by DRG neurons in vivo and in vitro.

Quantitation was done for the band at 43 kDa (Fig. 11A; open triangle). The molecular weight corresponds to that calculated for the canonical form of Necl-1. It is DRG-specific (Fig. 11A) and both the shNecl-1 (Fig. 11B) and the PCR for
canonical Necl-1 (Fig. 11B) confirm that it is Necl-1. PCR analysis failed to confirm the expression of a putative splice variant (X1; NCBI XM_006250323.2) with an expected MW of 47 kDa that has been predicted by automated computational analysis (NCBI annotation; [179]).

Relative intensities of the 43 kDa band were measured by quantitative Western blots (Fig. 11C, top panel) from purified DRG neurons, p0 and p30 sciatic nerve extracts, and normalized to relative intensities of neurofilament M (DRG marker). In sciatic nerves, Necl-1 expression is increased almost 8 fold from p0 to p30 (Fig. 11C, bottom panel). The relative amount of Necl-1 in purified DRG neurons is about 50% that observed in p30 sciatic nerves (Fig. 11C, bottom panel).

Although this quantitative analysis between in vivo and in vitro samples is quite relative (see “Discussion”), the results suggest that the levels of Necl-1 in vitro are likely to be within the range of in vivo levels. The in vitro results on myelination may therefore be physiologically relevant.

**Necl-1 negatively regulate Nrg1-mediated activation of ErbB3 and Akt**

The activation of the PI3 kinase/Akt signaling cascade by Nrg1 is necessary to Schwann cell proliferation and myelination [32, 139, 140]. Another major pathway activated by Nrg1, with important implication to PNS myelination, is the Mek/Erk signaling cascade [32, 140, 141, 144, 164].

To determine whether the interaction of Necl-1 with Schwann cells could have an impact on the activation of these signaling pathways by Nrg-1, Schwann cells were first incubated for 30 minutes with Necl-1-Fc (0 to 20 nM), and then stimulated for 10 minutes with the EGF domain of the recombinant human Nrg1
(rhNrg1-EGFD), at 2.5 and 10 nM. We used quantitative Western blotting to determine the phosphorylated levels of Akt (S\(^{473}\)), Erk1/2 (Thr\(^{202}\)/Tyr\(^{204}\)), ErbB3 (Tyr\(^{1289}\)) and ErbB2 (Tyr\(^{1248}\)).

In all experimental conditions ErbB2, ErbB3, Akt and Erk1/2 were significantly activated (p < 0.0001) in a concentration-dependent manner by the rhNRG1-EGFD treatment (Fig. 12A-D; solid line). However, the extent of activation of Akt and ErbB3 in Schwann cells was dramatically and significantly (p < 0.0001) reduced (up to ≈ 50%) by the presence of Necl-1-Fc (Fig. 12A, B) in a concentration-dependent manner. There appears to be an inhibitory trend of the Erk1/2 phosphorylation (up to ≈ 30%), but it was not significant (Fig. 12C; p = 0.1012). ErbB2 phosphorylation on Tyr\(^{1248}\) was not affected by the presence of Necl-1-Fc (Fig. 12D, p = 0.8292).

These experiments were designed to detect and measure the effect mediated on Schwann cells solely by the extracellular domain of Necl-1, independent of possible effects of Necl-1 levels on DRG axons, and demonstrate concentration dependence. Therefore, we added Necl-1-Fc to Schwann cells instead of using the primary DRG cultures to perform these experiments. The Fc portion of the human IgG ensures that the extracellular domain of Necl-1 is presented as a cis-homodimer, which mimics the normal assembly of full-length Necl-1 [152]. One pitfall is that we cannot control the orientation of the Fc chimeric protein to the cells (in cis or in trans).

The results are nonetheless informative as to the role of an interaction of the extracellular domain of Necl-1 with Schwann cells on signaling pathways
activation, at concentrations (nM range) that are likely to be physiological.

As to the EGF-like domain of neuregulin, it is necessary and sufficient to activate ErbB receptors and downstream signaling cascades [117]. Finally, within the time frame used in our experiments (10 minutes of treatment), the EGF-like domain of Nrg1 activates the ErbB receptors with kinetics that are similar to that of full-length neuregulins [125, 180].

**ErbB3 co-immunoprecipitates with Necl-1, but not with Necl-4 and Necl-2**

The effect of the extracellular domain of Necl-1 on ErbB3 and Akt activation suggested a possible interaction, direct or indirect, between Necl-1 and the ErbB receptors. To determine whether exogenous Necl-1 could be part of a complex with the ErbB receptors on Schwann cells, cells were first incubated for 30 minutes with the Necl-1-Fc protein at 20 nM. The human IgG (hu-IgG) at a similar concentration was used as control; its MW is close to that of Necl-1-Fc, and it also accounts for the Fc portion. Schwann cells were then stimulated, or not, with 10 nM of rhNrg1-EGFD for 10 minutes. Schwann cells were then lysed, ErbB3 immunoprecipitated, and the immunoprecipitated material analyzed by Western blotting (Fig. 13).

As expected, ErbB2 was co-immunoprecipitated with ErbB3 upon Nrg-1 stimulation (Fig. 13), demonstrating the effective recruitment of ErbB2 by ErbB3 upon ligand binding to ErbB3. We did not notice any obvious differences in the amount of ErbB2 that was co pull-downed from Schwann cells that had been treated with Necl-1-Fc or hu-IgG. Interestingly, Necl-1-Fc was also enriched in the ErbB3 IP lanes, with or without rhNrg1-EGFD stimulation. The absence of hu-IgG in the ErbB3 IP lanes in the control experiment suggests that Necl-1-Fc co-
immunoprecipitated with ErbB2/ErbB3 through an interaction, direct or indirect, that involves the extracellular domain of Necl-1 and not the Fc portion that is fused to it in the Necl-1-Fc construct.

PTPN13 is a phosphatase that has been shown to down-regulate ErbB3 phosphorylation [181]. We detected trace amounts of PTPN13 in all ErbB3 IP lanes, which did not appear to be dependent on the treatment of Schwann cells with Necl-1-Fc and stimulation by rhNrg1-EGFD.

Cell adhesion molecules Necl-2 and Necl-4 (Fig. 13; solid triangle) have previously been reported to form a complex with the ErbB3 receptor [161, 162]. We did not, in our experimental setup, co-IPed Necl2 and Necl-4 with ErbB3, in all experimental conditions. The band detected in the ErbB3 IP lanes for Necl-4 (open triangle) corresponds to the heavy chain of the mouse anti-ErbB3 antibody that was used for the co-immunoprecipitation.

DISCUSSION

In this chapter, we investigated the role of cell adhesion molecule Necl-1 during Schwann cell myelination. Necl-1 is expressed at the surface of the axons of DRG neurons [112, 145] while its heterophilic ligands Necl-2 and Necl-4 [145, 165] are expressed by Schwann cells and are localized to the adaxonal membrane that is in contact with the axon [112, 145]. Therefore, it is considered that the Nectin-like proteins promote axo-glial interactions through Necl-1/Necl-2 and Necl-1/Necl-4 associations. In support of this interpretation is the phenotype that is observed in the Necl-4 knockout mouse [24]: the adaxonal membrane of the myelinating Schwann cells is no longer in close apposition with the underlying axon.
Moreover, the delayed onset of myelination observed in this knockout mouse [24] is also in agreement with the in vitro data, which showed that Schwann cells whose Necl-4 function has been abolished or perturbed fail to progress to the pro-myelinating stage [145] and are stopped at the one-full-turn association with an axon, failing to spiral wrap further to form compact myelin sheathes [112].

Considering the strong heterophilic interaction between Necl-1 and Necl-4 [165], it is interesting that the analysis of two independent Necl-1 knockout mice [24, 167] did not reveal any phenotypes affecting PNS myelination. These results may suggest at least four different possibilities: 1) Necl-1 is dispensable for PNS myelination, 2) compensatory mechanisms are involved, 3) Necl-1 does not have the same pro-myelinating function as Necl-4 does, or 4) Necl-1 provides opposite regulatory mechanisms, pro-myelinating or inhibitory, depending on its binding partners on the Schwann cells. It is also possible that the time frame of the in vivo analyses [7 days [167] to 60 days [24, 167]] does not correspond with the time frame of Necl-1 function or observable phenotype. A case in point is the E-cadherin knockout mouse, which has a delayed onset of myelination observable at postnatal day p2 that is recovered from by post-natal day p14 [182].

We therefore used an in vitro approach to determine a possible role of Necl-1 that was not detected by the in vivo approach. We used the long-established Schwann cell / DRG myelinating co-culture system [183] that has been central to the identification of key molecules and processes governing axo-glial interactions, domain formation and PNS myelination [101, 184] [31, 145, 185]. We show strong evidence that Necl-1 may provide an inhibitory regulatory mechanism to Schwann
cell myelination. Removing Necl-1 expression from DRG neurons results in a striking increase in the number of myelinated segments (Fig. 6). Conversely, over-expressing Necl-1 on the axonal surface of DRG neurons results in an almost complete inability for Schwann cell to form myelin segments (Fig. 7).

An increase in Edu incorporation, indicative of an increase in Schwann cell proliferation, was also observed concomitant with an increase in myelination (Fig. 9). To be able to differentiate, cells need to exit the cell cycle. Therefore, it is unlikely that the effect of axonal Necl-1 on Schwann cell myelination can be attributed to a regulation of the cell cycle. The effect of axonal Necl-1 could then be a simple consequence of an increase in Schwann cell numbers resulting in an increase in the number of myelin segments. However the result obtained with SCG neurons whose axons do not support myelination, both in vivo and in vitro [178, 186, 187] precludes this possibility. We found that NGF-dependent SCG neurons express much higher levels of Necl-1 compared to NGF-dependent DRG neurons (Fig. 8). Most strikingly, decreasing the expression of Necl-1 in these SCG neurons induced the formation of a significant numbers of myelin segments (Fig. 8). NGF-dependent SCG and DRG neurons in purified cultures, i.e. in the absence of Schwann cells, have neurites of similar diameter [188, 189]. However, to account for a possible difference in surface-to-volume ratio between both types of neuron in the co-culture system that could be due to axon diameter differences, the expression levels of Necl-1 were normalized to actin (axonal cytoplasm) or TrkA (axonal plasma membrane). Necl-1 levels were still higher in SCG lysates, regardless of the mode of normalization: 1.7 versus 1.8, actin versus TrkA,
respectively (Fig. 10). This suggests that the difference in expression of Necl-1 between SCG and DRG neurons is not dependent on differences in surface-to-volume ratio, and may represent differences in transcriptional, post-transcriptional, and/or translational controls between myelinated and non-myelinated fibers. These results therefore suggest that the amount of Necl-1 expressed at the surface of axons is sufficient to alter the fate of the Schwann cells in contact with these axons. In particular, it suggests that, at least in vitro, high levels of axonal Necl-1 are inhibitory to Schwann cell myelin formation whereas low levels would be permissive.

In that respect it is interesting to note the significant decrease in Necl-1 expression in the 4.1B knockout mouse, which exhibits an about 3-fold increase in the number of small caliber axons (1 µm) being myelinated in the sciatic nerve [111], as well as a slight, but significant, hyper-myelination across all axon diameters [111, 190]. This is of course an indirect correlation, and it is quite possible that the phenotype observed in the 4.1B knockout mice is not related to the decrease in axon-derived Necl-1. However, along with the in vitro data, it does shed lights on the apparent lack of phenotype in the PNS of the Necl-1 knockout mouse [24, 167]. Indeed one would predict a hyper-myelinating phenotype in the Necl-1\(^{-/-}\) mouse. A major difference between the 4.1B\(^{-/-}\) and the Necl-1\(^{-/-}\) mice is the time frame at which EM analyses were done: 4 to 13 months (4.1B\(^{+/+}\)) and 7 days to 2 months (Necl-1\(^{+/+}\)). While the difference in g-ratio, in 4.1B\(^{-/-}\) mice, was small at 4 and 6 months, it was reported to increase in mice growing older (13 months, [190]). It is therefore possible that an overall difference in g-ratio may not have
been detectable in 2-month old Necl-1 knockout mice, but could be in older mice. In addition a detailed analysis of the number of myelinated axons, binned per axon diameter, may reveal an increase in the number of small caliber axons being myelinated, as was shown in the 4.1B\textsuperscript{−/−} mice [111]. Of course one cannot preclude a possible role for Necl-1 also at the onset of myelination that, when knocked-out, may produce a transient phenotype no longer observable at p7 (earliest time point of the Necl-1\textsuperscript{−/−} analysis; [24, 167]). Observation at time points earlier than post-natal p7 may reveal phenotypes such as a higher number of axons sorted out in a 1:1 relationship or a decreased g-ratio in the Necl-1\textsuperscript{−/−} mice. Since the Necl-1\textsuperscript{−/−} mice generated by Park et al. (2008) and Golan et al. (2013) are pan knockouts, an analysis of the SCG sympathetic track to detect myelinated fibers would also be informative.

To assess whether the \textit{in vitro} inhibitory effect of Necl-1 may be relevant \textit{in vivo}, we attempted to evaluate if Necl-1 expression by purified DRG neurons \textit{in vitro} was within physiological levels. The quantitative analysis shown in Fig. 11 indicates that while Necl-1 levels in DRG are higher than that of p0 sciatic nerve extracts, they are 50% lower than that of p30 nerve extracts. Because of the presence of Schwann cells and fibroblasts in the sciatic nerve, as well as the large increase in myelin proteins levels during the myelination process \textit{in vivo}, this comparison is therefore quite relative. It is also not possible to determine cell type contribution. However, the results suggest that Necl-1 \textit{in vitro} levels are likely to be within the range of \textit{in vivo} levels and that the \textit{in vitro} results on myelination may therefore be physiologically relevant. An \textit{in vivo} determination of the relative
amount of Necl-1 on axons of similar calibers, myelinated versus non-myelinated, as determined by electron microscopy, would be informative. Conversely, it would be similarly informative to determine whether small caliber axons, which are either ensheathed or poorly myelinated in the sciatic nerve, express higher levels of Necl-1 compared to the well-myelinated large caliber axons. The current Necl-1 antibodies however are not suitable for EM studies.

The lack of phenotype in the Necl-1−/− mice, as well as the increase in myelination in the culture system after ablating Necl-1 expression, could also result from the expression of another cell adhesion molecule compensating for the loss of Necl-1. A possible candidate is Necl-3, which, as far as we know, is the only other known ligand for Necl-4 [145, 165]. Our results however show that Necl-3 expression by DRG neurons, at least in vitro, is not affected by the absence or over-expression of Necl-1. In addition, we have previously shown that the extracellular domain of Necl-4 fails to bind to Necl-1 deficient DRG neurons. This further underscores the lack of significant amount and up-regulation of Necl-3 or other potential compensating ligands for Necl-4, in Necl-1 deficient DRG neurons [145].

Given that axonal Necl-1 has a negative impact on both Schwann cell proliferation and myelination, the data suggest that Necl-1 may interfere with a pathway that is common to both biological outcomes. On the axon, type III Nrg1 is the key molecule regulating Schwann cell proliferation and myelination [31, 93], through the activation of the PI3 kinase/Akt signaling cascade [32, 139, 140]. Therefore two possibilities come to mind. i) Necl-1 affects type III Nrg1 expression
and/or localization to the axonal plasma membrane, which however does not appear to be the case since type III Nrg1 level are not significantly affected in neurite preparations from Necl-1 knockdown and over-expressing DRG neurons (Fig. 10). Or ii) Necl-1 affects activation of the ErbB receptors. Consistent with this latter possibility is our finding that the extracellular domain of Necl-1 significantly inhibits the rhNrg1-EGFD–mediated activation of ErbB3 on Tyr\textsuperscript{1289}, a docking site for the p85 subunit of PI3 kinase [191], and that of downstream PI3 kinase effector Akt (Figure 12A-B). Interestingly, this inhibitory effect is specific to the PI3 kinase / Akt pathway insofar that the activation of the Mek/Erk1/2 pathway by Nrg1 is not particularly affected by Necl-1 (Figure 12C). This is corroborated by the lack of effect on ErbB2 phosphorylation on Tyr\textsuperscript{1248} (Figure 12D), which couples ErbB2 to the Ras/Raf/Erk signaling cascade [192-194].

These results suggest that the extracellular domain of Necl-1 decoupled the Akt and Erk intracellular pathways that were activated by rhNrg1-EGFD and the ErbB2/ErbB3 receptors. A similar finding has been described with Herstatin, which uncouples the Akt and Erk pathways that are activated by EGF and the EGF receptor (EGFR, [195]). The specific mechanism(s) that impart this apparent specificity to Necl-1 on Nrg1-activated pathways is (are) unclear. Although it does not appear to be the mode of action for Herstatin [195] one possible mechanism is that the extracellular domain of Necl-1 affects the apparent concentration of, or alters the binding affinity of the EGF domain of Nrg1 to ErbB3. An alternative is that, as with Herstatin [195], Necl-1 prevents heterodimerization of ErbB2 and ErbB3 upon ligand binding to ErbB3. Our coIP experiments however, done in
conditions of maximum activation of the Akt and Erk pathways (10 nM of rhNrg1-EGFD; see Figure 12), suggest that the extracellular domain of Necl-1 does not impair ErbB2/ErbB3 interaction upon rhNrg1-EGFD binding, since no noticeable differences in the coIP of ErbB2 with ErbB3 were observed between Necl-1-Fc and hu-IgG conditions (Figure 13). This finding also suggests that the extracellular domain of Necl-1 does not affect the binding of rhNrg1-EGFD to ErbB3 since the stabilization of the interaction between ErbB2 and ErbB3 results from a change in conformation that is mediated upon ligand binding to ErbB3 [131]. These data are in agreement with that of Kawano et al. (2009) whose results also suggest that the interaction of Necl-2 with ErbB3 does not prevent ligand binding to ErbB3 and recruitment of ErbB2. Even in the event of a disruption of ErbB2/ErbB3 dimerization, it would be difficult to draw a definitive conclusion. Indeed, while Herstatin has been shown to prevent dimerization of the EGF receptor, along with a decrease in overall EGFR phosphorylation [131], only the Akt pathway was affected, without any effect on the Erk pathway. This result strongly suggests that Herstatin binding to the EGFR probably affected the EGFR pattern of phosphorylation, leaving the docking site for Grb2 (involved in Erk pathway activation, [196]) unaltered. Interestingly it is what we observed with Necl-1 (Figure 12), a specific decrease in phosphorylation of Tyr^{1289} on ErbB3 (docking site for the p85 subunit of PI3 kinase; [191]), while the specific site for activation of the Erk pathway, Tyr^{1248} on ErbB2, was unaffected. A similar differential phosphorylation resulting in differential signaling has been described for ErbB4, albeit induced by different Nrg1 ligands [197]. One mechanism that would alter ErbB
phosphorylation profile and impact phospho-tyrosine docking site usage is the recruitment to ErbB complexes of specific regulatory components. For example, phosphatases such as Ptpruz and PTPN13 have been shown to repress the phosphorylation of ErbB2 [163], ErbB3 [161, 162] and ErbB4 [198]. Interestingly, PTPN13 was recently shown to interact with Necl-2 and Necl-4 [161, 162], ligands for axonal Necl-1 that are both present on Schwann cells [112, 145]. Necl-2 and Necl-4 were also shown to interact with ErbB3 through their extracellular domains. This interaction does not prevent ErbB2 recruitment upon ligand binding to ErbB3 [161]. We did detect PTPN13 in our ErbB3 coIP experiments (Figure 13), and this pull-down was not affected by the presence of Necl-1-Fc. As mentioned earlier, ErbB2 was also coIPed, confirming that PTPN13 is present in ErbB2/ErbB3 complexes. As anticipated we also coIPed Necl-1-Fc. Surprisingly however, neither Necl-2 nor Necl-4 were detected, which raises the question on how both Necl-1 and PTPN13 are associated with ErbB2/ErbB3 in Schwann cells. Since Necl-2 and Necl-4 were not detected in the control IP (non-specific human IgG in place of Necl-1-Fc; Figure 13), it is unlikely that Necl-1-Fc displaced their interaction with ErbB3. Either both Necl-2 and Necl-4 do not interact significantly with ErbB receptors in an endogenous setting (compared to ectopic overexpression for the reported interactions), or interactions were disrupted during the coIP process. While that latter possibility cannot be ruled out, it is unlikely since the antibody used for the coIP recognizes an intracellular epitope on ErbB3, and ErbB2 and Necl-1-Fc coIP (extracellular interactions) were not prevented. PTPN13 contains five PDZ domains, and it being coIPed in absence of Necl-2 and Necl-4,
suggests that additional components may be involved.

To summarize, our \textit{in vitro} studies strongly support a function for Necl-1 in PNS myelination. While the molecular regulatory mechanisms are not clearly understood, the extracellular domain of Necl-1 has the potential to interfere with the activation of the ErbB3/PI3K/Akt signaling cascade, which is the pro-myelinating pathway in the PNS. Interestingly the Erk1/2 pathway, which has inhibitory properties, was unaffected. The selective inhibitory effect of Necl-1 may represent a regulatory mechanism that help Schwann cells integrate the neuregulin signal to switch between different biological outcomes such as proliferation, survival, ensheathment and myelination. There remains to determine the relevance of these finding to \textit{in vivo} myelination, since analyses of Necl-1\textsuperscript{-/-} mice have not detected any phenotypes. The lack of phenotype is not however contradictory in itself. Since Necl-1 is dramatically up-regulated at post-natal day 30, when the process of active myelination is over, Necl-1 may not provide an inhibitory mechanism at the onset of myelination, but later on during myelin maintenance. An analysis of the Necl-1 knockout mice at older age, as was done on the 4.1B\textsuperscript{-/-} mice, may be informative.
**Figure 1**

**Organization of myelinated axons CNS vs PNS.** One Schwann cell forms only one myelin segment around one axon, whereas oligodendrocytes form multiple segments (up to about 40) around the same and multiple axons. In the PNS, myelin segments are also surrounded by a basal lamina that is not present around the myelin segments made by oligodendrocytes in the CNS. Finally the axonal plasma membrane at the nodes of Ranvier is not fully “naked” and in direct contact with the extracellular medium. In the PNS, the axonal plasma membrane is covered by microvilli that are protruding from the edge of the outer layer of the myelin sheath. However in the CNS, oligodendrocytes do not extend microvilli and the node is surrounded by an extension from specialized astrocytes called perinodal astrocytes. (© Hyosung Kim, 2017)
Steps of Schwann cell differentiation. Myelinating and non-myelinating Schwann cells in the PNS are derived from the neural crest cell. Prior to the association period (usually before E18 in rodent), Schwann cell undergoes migration stages (before E16.5 in rodent) moving from DRG to peripheral site of body. Myelinating Schwann cell starts associating with a large caliber axon by segregating it from axon bundle through a process called “axonal radial sorting”. Non-myelinating Schwann cell segregates small caliber axons from an axon bundle by multiple association, storing it into Remak bundle. (© Patrice Maurel, 2017)
**Organization of myelinating Schwann cells.** A. The spiral-wrapped plasma membrane compacts to form the compact myelin sheath. This represents about 99% of the zone of contact between the glial cell and the myelinated axon. The inner (adaxonal) and outer (abaxonal) layers are, however, not compacted. B. The inner and outer membranes of the internode express different sets of molecules that allow the myelinating and myelinated Schwann cells to interact with the axon and the extracellular matrix. This localized repartition of various molecular components across the radius of the myelinating Schwann cell/axon unit is referred to as radial polarity.
**Longitudinal structural compartmentalization of axon by myelination.** Molecules of the node include Na\(^+\) channel, NrCAM, Neurofascin-186, Ankyrin G, βIV-spectrin. Axonal membrane in the paranode contains Contactin and Caspr, which bind to Neurofascin-155 on the paranodal side of myelin. Juxtaparanode can only be distinguished by distribution of multiprotein complex containing TAG-1, Caspr2, PDZ, 4.1B, Kv1.1, and Kv1.2. Necl-4 along the internode is expressed by Schwann cell and forms a complex with Necl-1 on axonal membrane. (© Hyosung Kim, 2017)
Type III Nrg1 is a membrane bound isoform of Nrg1. Type III Nrg1, which is a key growth factor on the axons, regulates Schwann cell myelination. Type III Nrg1 is one of the splicing variant of Nrg1 family. Because of cysteine-rich domain (CRD), type III Nrg1 remains tethered on the membrane after metalloproteinase cleavage, therefore it is a juxtacrine signal. The signal from Type III Nrg1 on axons will be picked up by ErbB3 receptor on Schwann cells, which in turn initiates the activation of the PI3K/Akt pathway.
Axonal Necl-1 is not necessary for the myelination of DRG axons by Schwann cells in an *in vitro* coculture system. DRG neurons were infected with lentivirus encoding either a shRNA to a non-specific sequence (shLuc), or shRNAs (shNecl1 #1 and #2) specific to Necl-1. **A**: Constructs expressed GFP, whose detection by immunostaining in the neurite network demonstrates efficient transduction. Controls (Ctrl) are noninfected cultures. All cultures were coimmunostained for neurofilament (red). Scale bar = 50 µm. **B**: Western blot analysis of DRG neuron cultures demonstrating the effective knockdown of Necl-1 expression (shNecl-1 #1 and #2) in DRG neurons. Noninfected DRG neurons (ctrl) and DRG neurons infected with the shLuc lentivirus serve as controls. **C**: Representative images of myelinating cocultures established with Schwann cells added onto control DRG neurons (Ctrl, shLuc) or onto DRG neurons devoid of Necl-1. (shNecl-1 #1). Myelin segments were detected by immunostaining for MBP (red); Schwann cell nuclei were labeled with DAPI (blue). **D**: Quantitation of the effect of Necl-1 knockdown (shNecl-1 #1, #2) in DRG neurons on Schwann cell myelination. Controls are noninfected (Ctrl) and shLuc-infected.
DRG neurons. Mean ± SEM from $n = 4$ (Ctrl, shLuc, shNecl-1 #1) and $n = 3$ (shNecl-1 #2) independent experiments. One-way ANOVA ($P = 0.0018$) followed by Bonferroni post-hoc analysis (n.s. = not significant; ** $P < 0.01$).
**Figure 7**

Increased expression of axonal Necl-1 impairs myelination by Schwann cells in a myelinating co-culture system. **A:** DRG neurons were infected with a lentivirus driving the expression of Necl-1 (HA-Necl-1) at the surface of axons, as shown by live immunodetection of the HA tag (red). **B:** Representative images of myelinating cocultures established with Schwann cells added to control DRG neurons (Ctrl = non-infected; pLenti = infected with a construct expressing GFP), or to neurons over-expressing Necl-1 (HA-Necl-1). Myelin segments were immunostained for MBP (red); Schwann cell nuclei were labeled with DAPI (blue). Scale bar = 50 µm. **C:** Quantitation of the effect of Necl-1 over-expression in DRG neurons on Schwann cell myelination. Controls are noninfected (Ctrl) and pLenti-infected DRG neurons. Mean ± SEM from n = 4 independent experiments. One-way ANOVA (P = 0.0036) followed by Bonferroni post-hoc analysis (n.s. = not significant; *** P < 0.001).
**Figure 8**

**SCG neurons are myelinated in the absence of Necl-1.**  
A: Lysates of NGF-dependent DRG, and NGF-dependent SCG neurons (three independent lysates, respectively) were fractionated by Western blotting and quantitatively analyzed.  
B: While both types of neurons express Necl-1, the levels of expression are about 2-fold higher in SCG neurons. Student’s t-test (** $P = 0.0019$; *** $P = 0.0004$).  
C: Schwann cells form myelin segments (MBP immunostained red) when associated with SCG neurons that were first infected with shRNA lentiviruses against Necl-1 (shNecl-1 #1). Controls were noninfected SCG neurons (Ctrl) and SCG neurons infected with the non-specific shLuc construct.  
D: Quantitation of the number of myelinating segments. Mean ± SEM from $n = 3$ independent experiments. One-way ANOVA ($P = 0.0008$ followed by Bonferroni post-hoc analysis (n.s. = not significant; ** $P < 0.01$).
Knockdown of Necl-1 expression in DRG neurons promotes axon-mediated Schwann cell proliferation. **A**: Schwann cells were seeded onto control DRG neurons (Ctrl, shLuc) or DRG neurons knocked-down for Necl-1 expression (shNecl-1 #1). After 24 h, the cocultures were treated with Edu and Schwann cell proliferation was assessed by immunostaining for incorporated Edu (green). **B**: Quantitation of Edu incorporation as a % of Edu-positive nuclei among DAPI-stained nuclei. Values represent the mean ± SEM from n = 3 independent experiments. One-way ANOVA (P = 0.0316) followed by Bonferroni post-hoc analysis (n.s. = not significant; *, P < 0.05).
Necl-1 levels do not affect expression of Type III Nrg1 and Necl-3 on neuritis of DRG neurons.

Neurite membranes, prepared from control DRG neurons (pLL, pLenti) or DRG neurons knockdown for (shNecl-1) or over-expressing (HA-Necl-1) Necl-1, were analyzed by quantitative Western blotting for the expression of type III Nrg1 and Necl-3, a ligand for Schwann cell Necl-4. pLL is the vector control for the shRNA data, whereas pLenti is the vector control for the over-expression data. Data was normalized using TrkA as a neurite membrane marker. No significant changes were observed. Values represent the mean ± SEM from n = 3 independent experiments. Student’s t-test (n.s. = not significant).
Comparative assessment of Necl-1 expression in vitro and in vivo. Lysates of Schwann cells (Sc), DRG neurons (DRG), post-natal day 0 (p0, birth) and day 30 (p30) sciatic nerves were qualitatively (panel A) and quantitatively (panel C) analyzed for the expression of Necl-1. Two bands were detected at 43kDa (lower band indicated by open triangle) and 47 kDa. Neurofilament (NFM) and GFAP were used as DRG neuron and Schwann cell specific markers, respectively. PCR analysis (panel B), done on DRG and Schwann cell cDNA, detected a specific 118bp product for the canonical (Can), 43kDa Necl-1, in DRG neurons only. No band (129bp) for a computationally predicted 47 kDa splice variant (X1) was detected in Schwann cells. HPRT1 is used as control. The 43 kDa was used for the quantitation shown in panel C, normalized to NFM. Values represent the mean ± SEM from n = 3 independent experiments.
Necl-1 inhibits Nrg1-mediated activation of ErbB3 and Akt in Schwann cells. Schwann cells were first maintained in defined media for 24hrs and then incubated with Necl1-Fc at various concentrations (0 nM = solid line; 10 nM = dash line; 20 nM = dotted line) for 30 min., before stimulation with rhNrg1-EGFD (Nrg1; 0, 2.5, and 10 nM). Phosphorylated and total levels of Akt, ErbB3, Erk, and ErbB2 (blots A-D, respectively) were analyzed by quantitative Western blotting. Mean ± SEM from n = 4 (ErbB2, ErbB3) and n = 7 (Akt, Erk1/2) independent experiments. In all Necl-1-Fc conditions, rhNrg1-EGFD stimulated the activation of ErbB2, ErbB3, Akt, and Erk1/2 (Two-way ANOVA, \( P < 0.0001 \)). However, increasing concentrations of Necl1-Fc do significantly reduce by about 50% the effect of rhNrg1-EGFD stimulation (Two-way ANOVA, \( P < 0.0001 \)). Necl-1-Fc has no inhibitory effect on the activation of ErbB2 (Two-way ANOVA, \( P = 0.1012 \)) and Erk1/2 (Two-way ANOVA, \( P = 0.8292 \))
**ErbB3 and ErbB2 coimmunoprecipitates with Necl-1 –Fc.** Schwann cells were first maintained in defined media for 24 h and then incubated with Necl-1-Fc (20 nM) for 30 min, before stimulation with rhNrg1-EGFD (Nrg1; 10 nM). Human IgG (hu-IgG) is used as a control for the specificity of the Necl1-Fc interaction. ErbB3 immunoprecipitated complexes were then analyzed for the presence of ErbB2, Necl-1-Fc, Necl-2, Necl-4, and PTPN13. For each condition, three lanes were run: “in” represents the input lanes (2% of material use for IP), “Δ” represents the depleted material after IP (2% of material used for IP, after the IP), and “IP” represents the immunoprecipitated lanes. The solid triangle indicates Necl-4, whereas the open triangle corresponds to the heavy chain of the mouse anti-ErbB3 antibody used for the coimmunoprecipitation.
CHAPTER IV

The Schwann cell-specific form of Necl-2 is a negative regulator of myelination in vitro

INTRODUCTION

Necl-2 is highly expressed in epithelial tissue [151, 199] and is not detectable in non-epithelial cells such as fibroblasts and L cells. Its expression is however not strictly limited to epithelial cells, as Necl-2 is expressed by non-epithelial cells such as glial cells and neurons. These are however cells whose precursors were, during their development, migratory cells before their final differentiation.

The loss of Necl-2 expression has been observed in many cancers derived from epithelial [200], neuronal [201] and glial cells [202]. The loss of Necl-2 has also been associated with autism spectrum disorder [203]. Several studies have shown that Necl-2 promote cell-cell adhesion through heterophilic and homophilic interactions. The current consensus is that Necl-2 functions as a tumor suppressor through the regulation of cell-cell adhesion.

Increased proliferation has also been observed in cancer cells affected by a lower level or absence of Necl-2 expression. Other studies have shown that high level of Necl-2 reduces keratinocyte proliferation and motility [204]. In that respect it is interesting to note the domain organization of Necl-2: i) the extracellular domain with three Ig loops to promote cell adhesion; ii) a FERM-binding domain to recruit scaffolding members of the 4.1 family; iii) a PDZ-binding domain to recruit multiple-PDZ adaptor proteins. These characteristics make Necl-2 well-suited to
assemble and stabilize multi-protein complexes, signaling complexes in particular.

Several lines of evidence do suggest that Necl-2 may be involved in the regulation of ErbB2/ErbB3 signal transduction. A Necl-2/ErbB3 cis-interaction has been shown in cells that ectopically overexpress Necl-2. Necl-2 recruits PTPN13 through its PDZ binding domain, a tyrosine phosphatase that can de-phosphorylate ErbB3, and thereby abolish Akt, as well as Rac1 activity [161].

In addition to potentially interact with tyrosine kinase receptors of the ErbB family, Necl-2 can also form a complex in cis with integrin α6β4 [205]. The interaction appears to regulate the assembly/disassembly of integrin α6β4 from hemidesmosomes [205].

While these two types of interactions may provide clues to the molecular mechanisms by which Necl-2 functions as a tumor suppressor, they are of particular relevance to PNS myelination investigations. Indeed Schwann cells express endogenously both ErbB3 (as well as ErbB2) and Necl-2. The activation of ErbB2/ErbB3, and downstream PI3K/Akt signaling cascade by axonal type III Nrg1 is the key event that determine Schwann cell myelinating phenotype [31-33]. α6β4 integrin is not expressed by non-myelinating Schwann cells. Its expression appears at the onset of myelin segment formation [206], and α6β4 localizes in the outer plasma membrane of the Schwann cell [206]. β4 integrin deficiency leads to abnormal myelin folding in old mice [92], suggesting a function for α6β4 in myelin maintenance.

I therefore undertook to examine the role of Schwann cell Necl-2 in myelination, using the myelinating co-culture system. Based on the current
literature on Necl-2 interactions, one could forecast that Necl-2 may be inhibitory to Schwann cell myelination. In this chapter, I provide evidence that, in vitro, Necl-2 indeed negatively regulates the activation of the pro-myelinating signal Akt by axonal contact, but not by soluble Nrg-1 treatment. This inhibitory effect of Necl-2 on Akt correlates with an observed inhibitory effect of Necl-2 on Schwann cell myelination.

RESULTS

The Schwann cell-specific form of Necl-2 is dramatically down regulated as myelination progresses in the PNS.

Both Schwann cells and DRG neurons were previously shown to express Necl-2. While the protein backbone of Necl-2 is detected by Western blot as a band with an apparent MW of \( \approx 42 \) kDa, I found that the N-glycosylated form of Necl-2 runs at different apparent MW between Schwann cells and DRG neurons. While the Schwann cell form is detected as band of \( \approx 90 \) kDa, the DRG neuron form is detected at \( \approx 110 \) kDa and 75 kDa (Fig. 14A).

I then performed an analysis of the expression pattern of Necl-2 in the developing sciatic nerve, from post-natal day 0 (p0) to 30 (p30), at which time PNS myelination is considered complete (Fig. 14B). Myelination was detected by the increased expression of myelin protein zero (Mpz), a cell adhesion molecule that is required for the formation of compact myelin sheathes. Interestingly the level of expression of the Necl-2 band at 90 kDa (Schwann cell form), while high at the onset of myelination (p0 to p4), dramatically decreased as myelination progressed (p7 to p30). The level of expression of the Necl-2 band at 75 kDa (DRG neuron
form) did not change overtime. Both Necl-1 and Necl-4 are present from the onset of myelination (p0) onward, and slightly increase overtime as myelination progresses.

While purely correlative, the pattern of expression of Necl-1, Necl-2 and Necl-4 is potentially in line and not contradictory with the hypothesis of two interacting combinations (Necl-1/Necl-2 and Necl1/Necl-4) that would have opposing effects on PNS myelination.

The Schwann cell-specific form of Necl-2 is a negative regulator of Schwann cell myelination in vitro

To further characterize the function of Necl-2 expressed by Schwann cells, I used both loss and gain of function approaches. The loss of function strategy used the lentiviral-mediated delivery of shRNAs specific to Necl-2 (shNecl-2) into Schwann cells. Panel A in Figure 15 demonstrates the efficient abolition of Necl-2 expression in infected Schwann cells, whereas the control lentivirus that expresses the non-specific shRNA against luciferase (shLuc) has no effect on Necl-2 expression. This result underscores the specificity of the shRNA against Necl-2. Schwann cells deficient in Necl-2 expression when then added to DRG neuron cultures to test their ability to form myelin segments. Non-infected and shLuc-infected Schwann cells myelinated equally well (Fig. 15C). Interestingly, co-cultures that were setup with Schwann cells lacking Necl-2 exhibited a 2-fold increase in the number of myelin segments that were formed (Fig. 15B).

This result is in agreement with our hypothesis of a negative role for Schwann cell-specific Necl-2 in PNS myelination.
To corroborate these results, the gain of function strategy used the lentiviral-mediated delivery into Schwann cells of a cDNA encoding the full-length Necl-2 tagged with the flag epitope (Flag-Necl-2). The pLL3.7 vector encoding for the GFP (pLL-GFP) was used as a control for the potential effects of lentiviral transduction and expression of an exogenous protein into Schwann cells. Western blot analyses showed the resulting increase in the levels of expression of Necl-2 in Schwann cells harboring the Flag-Necl-2 constructs (Fig. 16A), whereas expression of the pLL-GFP construct does not affect Necl-2 basal levels of expression (Fig. 16A). Live immunofluorescence detection of the Flag tag also demonstrated the proper targeting of Flag-Necl-2 at the plasma membrane (Fig. 16B). Both non-infected (Ctrl) and pLL-GFP expressing Schwann cells formed numerous and comparable myelin segments when co-cultured with DRG neurons in myelinating conditions (Fig. 16C-D). However, myelinating co-cultures established with Schwann cells expressing higher levels of Necl-2 (Flag-Necl-2) showed a marked 50% decrease in the number of compact myelin sheathed upon immunostaining for MBP.

As with the loss of function approach, these results are also in agreement with the potential negative role of Schwann cell-specific Necl-2 in PNS myelination.

The Schwann cell-specific form of Necl-2 does not affect axon-mediated Schwann cell proliferation

To determine whether Necl-2 that is expressed by Schwann cells is involved in regulating axon-mediated proliferation, we cultured control Schwann cells (non-infected and pLL-GFP), Necl-2-deficient Schwann cells (shNecl-2) and Necl-2-over-expressing Schwann cells (Flag-Necl-2) onto the neurite network of DRG
neuron cultures. Proliferation was assessed by the nuclear incorporation of Edu.

As shown in Fig. 17, the ablation (panel A) or over-expression (panel B) of Necl-2 in Schwann cells did not result in any statistical difference in the nuclear incorporation of Edu by Schwann cells, which was estimated at 38% on average across all experimental conditions.

The Schwann cell-specific form of Necl-2 negatively regulates the activation of Akt in the context of axon-glia interaction

The activation of the PI3 kinase/Akt signaling cascade by axon-bound type III Nrg1 is necessary to Schwann cell myelination [32, 139, 164]. Another major pathway activated by Nrg1, with important implication to PNS myelination, is the Mek/Erk signaling cascade [32, 140, 141, 144, 164].

To determine whether the expression or not of Necl-2 by Schwann cells could have an impact on the activation of these signaling pathways by axonal contact, Necl-2-deficient or Necl-2-over-expressing Schwann cells were seeded onto the neurite network of DRG neurons. Controls were Schwann cells transduced with the shLuc or pLL-GFP constructs, respectively. Quantitative Western blotting was used to assess for the phosphorylation status of ErbB2, ErbB3, Akt, Erk and PDK1. In order to perform statistical analyses across multiple replicate experiments, the data was normalized as follow: i) normalization of the phosphorylated band to the total band, for any given protein analyzed and ii) then normalization of every single experimental time points to its control counterpart (for example, the experimental data at time point 24 hrs is normalized to the control data at time point 24 hrs, and experimental data at time point 48 hrs is normalized
to control at time point 48 hrs). Finally, signaling pathway activity was considered in two contexts: i) non-myelinating conditions: co-cultures were incubated for 24 and 48 hours after Schwann cells seeding (Figs. 18-19) and ii) myelinating conditions: myelination was initiated by the addition of ascorbic acid 3 days after Schwann cells seeding (day 0) and maintained for 5 days (Figs. 20-21).

In the absence of expression of Necl-2 by Schwann cells, the levels of phosphorylation of Akt at both the $T^{308}$ and $S^{473}$ residues, in the non-myelinated co-cultures, are significantly higher than those detected in non-myelinated co-cultures setup with Schwann cells over-expressing Necl-2 (Fig. 18A-B). Phosphorylation of Akt on $T^{308}$ is mediated by PDK1 [133]. Concomitant with the increase in Akt phosphorylation on $T^{308}$, we also noticed an increase in the phosphorylated status of PDK1 on residue $S^{241}$ (Fig. 18 A-B).

Surprisingly, we did not detect any changes in the phosphorylation status of the ErbB2 (Tyr$^{1248}$) and ErbB3 (Tyr$^{1289}$) tyrosine kinases that are upstream of Akt activation (Fig. 19 A-B). Similarly, we also did not detect any significant modification sin the levels of phosphorylation of Erk1/2 (Thr$^{202}$/Tyr$^{204}$) (Fig. 19 A-B).

Similarly in myelinating conditions (Figs. 20-21), Akt at $T^{308}$ and $S^{473}$, and PDK1 at $S^{241}$ are highly phosphorylated in co-cultures setup with Necl-2-deficient Schwann cells (Fig. 20A-B). The phosphorylation status of ErbB2 (Tyr$^{1248}$), ErbB3 (Tyr$^{1289}$) and Erk1/2 (Thr$^{202}$/Tyr$^{204}$) were not significantly affected in co-cultures setup with Necl-2-over-expressing Schwann cells (Fig. 21 A-B).

The Schwann cell-specific form of Necl-2 does not affect the activation of Akt
by soluble forms of neuregulin

To determine whether the absence or expression of Necl-2 in Schwann cells could have an impact on the activation of these signaling pathways by soluble forms of Nrg-1, Schwann cells (Necl-2-deficient, Necl-2-over-expressing, shLuc and pLL-GFP controls) were stimulated for 10 minutes with the EGF domain of the recombinant human Nrg1 (rhNrg1-EGFD), at 10 nM. As mentioned earlier, the EGF-like domain of neuregulin is necessary and sufficient to activate the ErbB receptors and downstream signaling cascades [117] with kinetics that, within the time frame used in our experiments (10 minutes of treatment), are similar to that of full-length neuregulins [125, 180]. Quantitative Western blotting was used to determine the phosphorylated levels of Akt (S473), Erk1/2 (Thr202/Tyr204), ErbB3 (Tyr1289) and ErbB2 (Tyr1248).

In all experimental conditions ErbB2, ErbB3, Akt and Erk1/2 were strongly activated by rhNrg1-EGFD (Fig. 22-23; for all conditions, compare time points 15 to 60 minutes with the time point 0 (not stimulated)). However, for all molecular components analyzed, the extent of phosphorylation was not significantly changed between Necl-2-deficient (red line) and Necl-2-over-expressing (green line) Schwann cells (Fig. 22-23).

Necl-2 does not co-immunoprecipitates with the ErbB2 and ErbB3 tyrosine kinase receptors.

PTPN13 is a phosphatase that has been shown to down-regulate ErbB3 phosphorylation [181]. Interestingly, in the context of ectopic expression in HEK293 cells and in the A549 cancer cell line [161], PTPN13 has been shown to interact
with the PDZ-binding domain of Necl-2. Within that context, the extracellular domain of ErbB3 has also been shown to interact with the extracellular domain of Necl-2 [161]. Since Necl-2, ErbB2 and ErbB3 are naturally expressed in Schwann cells, we tested i) the possibility for a Necl-2 complex with the ErbB receptors in Schwann cells and ii) the possibility that the formation of such a complex could be neuregulin-dependent (Fig. 24). Schwann cells, treated or not with rhNrg1-EGFD (-Nrg1, + Nrg1) were lysed and immunoprecipitated for Necl-2 (Fig. 24A). As the Necl-2 antibody was generated in chicken, an immunoprecipitation with a non-specific IgY antibody was also performed to test for not specific pull-downs. While Necl-2 was specifically IPed by the chicken anti-Necl-2 antibody, only trace amounts of ErbB2 could be detected, and no ErbB3 was co-IPed (Fig. 24A). Treating Schwann cells with rhNrg1-EGFD had no effects (Fig. 24A). We corroborated further these results by performing reciprocate co-IPs with ErbB2 and ErbB3 (Fig. 24B and 24C, respectively). ErbB2 and ErbB3 were strongly immunoprecipitated by their respective antibodies. As expected, we noticed an increase in ErbB3 (ErbB2 IP) or ErbB2 (ErbB3 IP) co-IP upon neuregulin treatment. However in both cases Necl-2 was not co-immunoprecipitated.

Altogether these results strongly suggests that Necl-2, ErbB2 and ErbB3, PTPN13 (Fig. 25) at endogenous levels in Schwann cells, do not participate in a common complex, either through direct or indirect interactions.

The levels of expression of Necl-2 affect Schwann cell morphology

While preparing Schwann cells over-expressing or lacking Necl-2, changes in morphology were observed. To better determine the nature of these
morphological changes, Schwann cells (shLuc and pLL-GFP controls, Necl-2 deficient, Necl-2 over-expressing) were seeded at low density (10,000 cells per cm²) onto a laminin-1 substrate, to avoid changes due to cell-cell contact. Phalloidin staining of the actin stress fibers was used to visualize changes in Schwann cells morphologies (Fig. 26-27) 20 hrs after plating. Two striking changes were observed. First, at least 80% of the Schwann cells deficient in Necl-2 presented a dramatic decrease in the intensity of the phalloidin staining (Fig. 26A,B), suggestive to a substantial decrease in the formation of stress fibers in these cells. Second, the number of lamellipodia was 2.5-fold higher in Schwann cells expressing higher levels of Necl-2 compared to the pLL-GFP control Schwann cells (Fig. 27C,D).

**Down-regulation of Necl-2 increase the Rac1 activity by Schwann cells**

Given that the level of Necl-2 in Schwann cells has such strong impact on morphological changes, it is most likely that small GTPase activity will be regulated by the level of Necl-2. Small GTPase activity assay will be my focus to address the molecular mechanism behind these morphological changes. These preliminary findings provide another potential mechanisms for Necl-2 to regulate Schwann cell myelination. They strongly suggest that Necl-2 may regulate the dynamics of the actin cytoskeleton during myelination. Considering the impact on stress fibers and lamellipodia, we are currently assessing whether Necl-2 regulate the activity of RhoA and Rac1. Ongoing studies are also assessing how Necl-2 may affect Schwann cell migration, axonal sorting and wrapping. Our study shown that, Necl-2 deficient Schwann cells grown on laminin substrates for three hours, then
perform Glisa assay specific for Rac1. We found that it’s about 30% increase of Rac1 in Necl-2 deficient Schwann cells grown on laminin substrate (Fig. 28).

**DISCUSSION**

Necl-2 is expressed at the surface of Schwann cells and localizes to the membrane that is in contact with the axon, under the compact myelin sheath. It is not present at the nodes of Ranvier and paranodal domains [112, 145]. Necl-2 has two ligands, itself and Necl-1, both of which are expressed on the axon [112, 145]. Necl-2 can therefore mediate two types of axo-glial interactions, homophilic and heterophilic, which may elicits different biological responses by both the Schwann cells and the DRG neurons.

In this chapter, I investigated the role of cell adhesion molecule Necl-2 expressed by Schwann cells during myelination in an *in vitro* culture system. Ablation of Necl-2 expression from Schwann cells results in a striking increase in the number of myelinated segments (Fig. 15). Conversely, the over-expression of Necl-2 on the Schwann cell surface results in a substantial inability by Schwann cells to form myelin segments (Fig. 16). Edu incorporation was not affected (Fig. 17), which suggests that Necl-2 in Schwann cells is not involved in regulating the exit from the cell cycle, a pre-requisite to cell differentiation. In the absence of an effect on Schwann cell proliferation, it is unlikely that the effect of Schwann cell Necl-2 on myelination could reflect a simple consequence of an increase in Schwann cell numbers resulting in an increase in myelin segments, and *vice-versa*. These results therefore suggest that the amount of Necl-2 at the surface of the Schwann cell may be sufficient to alter the fate of the Schwann cells when in
contact with axons. In particular it suggests that, at least in vitro, high levels of Schwann cell Necl-2 are inhibitory to Schwann cell myelin formation whereas low levels would be permissive.

In that respect it is interesting to note the changes in the levels of expression of Necl-2 in the sciatic nerve (PNS) during the first 30 days of postnatal development (Fig. 14). Necl-2 is strongly expressed during the first 4 to 7 days after birth, and then the levels quickly decline to be “almost” undetectable by postnatal day p30. “Almost”, as Necl-2 is undetectable due to a choice of exposure that would not overly saturate the blot in the lanes for early time point samples. Necl-2 is still present at p30 and in adulthood as was shown by Maurel et al (2007) and Spiegel et al (2007). The Western blot however does reflect to the dramatic changes in the representation of Necl-2 in the sciatic nerve as it develops. It is interesting to note that during that time window of development, p0 to about p10, Schwann cells are present at different stages of development. Immature Schwann cells are still largely present, performing late radial sorting [80], and Schwann cells that have sorted out axons to a 1:1 association only start to form compact myelin sheathes (p1 to p30). This compaction requires the Schwann cells to increase the production of myelin proteins involved in compaction such as Mpz, MBP and PMP22. The temporal correlation of the dramatic increase in the expression of such markers (see Mpz in Fig. 14) with the dramatic decrease in Necl-2 expression is therefore potentially in line with, and not contradictory with the hypothesis that Necl-2 may be inhibitory to Schwann cell myelination, as the in vitro findings suggest.
PNS myelination is regulated by axon-derived and basal lamina-derived signals. Most important is the axon-bound type III Nrg1 signals, which has been shown to be the on/off switch controlling whether a Schwann cell forms myelin or becomes an ensheathing Schwann cell [31, 93]. The type III Nrg1 signal is transduced by receptor tyrosine kinases ErbB2 and ErbB3, activating three downstream effector pathways; i) the PI3K/Akt pathway, the MAPK pathway, and the PLC-γ pathway. While all three have been implicated in PNS myelination [94, 128, 207], the activation of the PI3K pathway is pivotal to Schwann cell development. It regulates Schwann cell proliferation, survival, and most importantly the onset of myelination [31-33, 140]. Most recently, the in vivo manipulation of Akt activity strongly demonstrated that Akt regulates axon wrapping and myelin sheath thickness in the PNS [139]. Interestingly I found that the levels of phosphorylation of Akt on both T^{308} and S^{473} residues are strongly dependent, in co-cultures non-myelinating and myelinating conditions, on the levels of expression of Necl-2 by Schwann cells. High levels of Necl-2 inhibited Akt activation, while low levels increased Akt activation (Fig. 18, 20). These data are in line with the recent finding that Necl-2, at least in the context of in vitro ectopic expression, may interact with, and de-phosphorylate ErbB3 by recruiting tyrosine phosphatase PTPN13 to the complex. This interaction results in a decrease in Akt, as well as Rac1 activation [161]. Surprisingly however there were no significant changes in ErbB2 and ErbB3 phosphorylation status (Fig. 19, 21). We also could not detect, by co-immunoprecipitation (coIP) experiments, the presence of Necl-2 in ErbB2/B3 complexes, and vice versa (Fig. 24), although we could co-IP ErbB2
with ErbB3 upon Nrg1 stimulation, suggesting that our IP conditions were probably adequate. Yet Necl2 levels similarly regulated PDK1, which phosphorylates Akt on T\textsuperscript{308}. As both Akt and PDK1 are recruited to the plasma membrane by PI\textsubscript{(3,4,5)}P3 interacting with their respective pleckstin homology (PH) domain, it suggests that PI\textsubscript{(3,4,5)}P3 production may have been affected. It will be interesting to perform a quantitative analysis on the levels of PI\textsubscript{(3,4,5)}P3 in Necl-2-deficient Schwann cells. As this data is derived from co-cultures, one of course cannot preclude the possibility of an effect on Akt activity that is present in the DRG neurons.

Another interesting finding was that that effect of Necl-2 on the activation of Akt and PDK1 was context-dependent. While Akt and PDK1 activation was perturbed in the context of axo-glial interactions, it was not in the context of purified Schwann cells stimulated by soluble neuregulin-1 (Fig. 22). While this may support the possibility that what was observed in the co-culture settings was from within the DRG neurons, one must remember the findings obtained by adding the extracellular domain of Necl1 to the Schwann cells: a concentration-dependent decrease in Akt activation by soluble neuregulin-1 as the amount of Necl-1 was increased. Necl-1 is expressed by the axon and is likely to mediate some of the axo-glial interactions through a Necl-1/Necl-2 heterophilic interaction \[112, 145, 165\]. Therefore the difference between co-culture and purified Schwann cells experimental setups may reflect the effect of Necl-1 on Schwann cells.

Rac1 activity has been shown to control the formation of lateral lamellipodia in Schwann cells. This process appears to be largely dependent on signaling
through the basal lamina and β1 integrin-based receptors on the Schwann cells [72, 208]. Rac1 is important for the proper regulation of the morphogenetic movements associated with radial sorting [208]. Rac1 is also regulated by ErbB receptors, through the interaction with the guanidino exchange factor PI(3,4,5)P3-dependent Rac exchanger 1 protein, or P-Rex1 [209] and other members of the Dbl family of guanine-nucleotide exchange factors [210]. Interestingly, research has been shown that Akt can directly activate Rac1, independently of the PI(3,4,5)P3-dependent effect of PI3K on GEFs of Rac1, suggesting a possible direct connection between Akt, Rac1 and myelin wrapping[139]. Interestingly, I found that Rac1 is also decreased in Necl-2–deficient Schwann cells, along with a decrease in Akt activity (Fig. 28) It is therefore possible that Necl-2 may regulate some aspects of Schwann cell morphogenetic movements, and the dramatic changes observed in Schwann cell morphology when over-expressing or when deficient in Necl-2 expression (Fig. 26, 27) do support that hypothesis. Also in line with this hypothesis is that Necl2 is highly expressed from p0 to about p7, a time of active radial sorting, i.e. a time of active Schwann cell remodeling that is, in part, dependent on Rac1 appropriate regulation [208]. In culture, Schwann cells expressing high levels of Necl-2 form numerous lamellipodia, instead of the usual one or two that are present on Schwann cells with endogenous levels of Necl-2 (Fig. 27). This may indeed reflect a possibility for the high levels of Necl-2 in the early postnatal sciatic nerves to regulate radial sorting by regulating the formation of the lamellipodia needed to separate axons into smaller bundles until a 1:1 association is established. Interestingly, Schwann cells expressing low or no Necl-
2 have a completely different morphology, and are particularly affected in their ability to maintain the network of stress fibers (Fig. 26). While the process of spiral wrapping is not well understood at the molecular level, it was recently shown that maintaining a labile acting cytoskeleton was important for the formation of the myelin sheath [211]. The dramatic decreased in Necl-2 as the formation of compact myelin increases, and therefore as more and more Schwann cells undergo spiral wrapping, may therefore potentially suggest a function of Necl-2 is that process.

To summarize, my in vitro studies of Necl-2 strongly support a function for Necl-2 expressed by Schwann cells in PNS myelination. The molecular mechanisms are yet not clearly understood. However, Necl-2 has the potential to interfere with the activation of the PI3K/Akt signaling cascade, which is the pro-myelinating pathway in the PNS. Necl-2 expressed by Schwann cells can also alter the morphology of Schwann cells in vitro, in particular the formation of lateral lamellipodia and the stability of the actin cytoskeleton. The correlation between the time-course of expression of Necl-2 in the developing sciatic nerve, along with the effects of Necl-2 on Schwann cells morphology in vitro, strongly support a function of Necl-2 in regulating radial sorting and spiral wrapping.
**Necl-2 may interact with Necl-1 to regulate myelination in the earlier stage of developing rat sciatic nerves.**

**A.** Necl-2 has at least two isoforms at the PNS system. **B.** Before birth, Schwann cells actively sort out axons and continue this process up to P5, to establish a 1:1 relationship prior to formation of the compact myelin sheath, as shown by the expression of the myelin marker myelin protein zero (MPZ). While Necl-2 is expressed at high levels at P0, note the dramatic down-regulation right after P4. The expression of Necl-2 is down-regulated when myelin gene expression is increasing, which may support the idea of Necl-2 as a negative regulator of myelination. Necl-4 and Necl-1 are upregulated with myelination.
Schwann cell specific Necl-2 is not necessary for Schwann cell myelination in vitro.

A. Western blot analysis demonstrates efficient knock-down of Necl-2 expression in Schwann cells infected with virus encoding shRNA against Necl-2 (shNecl-2). B. Representative images of myelinating co-cultures in which Schwann cells were non-infected (Ctrl), infected with a virus encoding shRNA against luciferase sequence (shLuc) and with shNecl-2. C. Quantification of the effect of Necl-2 knockdown in Schwann cells on myelination. Mean ± SEM from n = 4 (Ctrl, shLuc, shNecl-2) independent experiments. One-way ANOVA (P < 0.05) followed by Bonferroni post-hoc analysis (n.s. = not significant).
Increased expression of Schwann cell specific Necl-2 impairs Schwann cell myelination \textit{in vitro}. \textbf{A-B}. Western blot analysis and Immunostaining demonstrate efficient overexpression of Necl-2 in Schwann cells infected with virus encoding full-length Necl-2 tagged with Flag (oxNecl-2). \textbf{D}. Representative images of myelinating co-cultures in which Schwann cells were non-infected (Ctrl), infected with a virus encoding the vector and with oxNecl-2. \textbf{C}. Quantification of the effect of Necl-2 overexpression in Schwann cells on myelination. Mean ± SEM from \(n = 3\) (Ctrl, vector, oxNecl-2) independent experiments. One-way ANOVA \((P < 0.05)\) followed by Bonferroni \textit{post-hoc} analysis (n.s. = not significant).
Necl-2 regulates myelin formation independently of axon-mediated Schwann cell proliferation. A. Knockdown or B. overexpressed Necl-2 Schwann cells were seeded onto control DRG neurons. After 24 h, the cocultures were treated with Edu and Schwann cell proliferation was assessed by immunostaining for incorporated Edu (red). This assay suggests that removal or overexpression of Necl-2 in Schwann cells does not affect axon-contact-mediated proliferation of these Schwann cell proliferation. Quantitation of Edu incorporation as a % of Edu-positive nuclei among DAPI-stained nuclei. Values represent the mean ± SEM from (N = 4, knockdown; N = 3, overexpression) independent experiments. One-way ANOVA followed by Bonferroni post-hoc analysis. There is no significance of these comparison.
Necl-2 in Schwann cells negatively regulates the activation of Akt and PDK1 in the Schwann cell - DRG neuron co-culture system. A-B. Schwann cells (controls, knockdown or over-expressing Necl-2), were seeded onto axons of purified DRG neuron, and signaling pathway activation was assessed 24 and 48 hours later. C-E. After normalizing to its vector control, removing Necl-2 from Schwann cells results in substantial increase in both Akt T308 (p= 0.0311), S473 (0.0218) and PDK1 S241 (p=0.0021) compared to overexpressing Necl-2 in Schwann cells. Mean ± SEM from n = 4 (Knockdown) and n = 3 (Overexpression) independent experiments.
Necl-2 in Schwann cells does not regulate the activation of ErbB3, ErbB2, and Erk1/2 in the Schwann cell - DRG neuron co-culture system. A-B. Schwann cells (controls, knockdown or over-expressing Necl-2), were seeded onto axons of purified DRG neuron, and signaling pathway activation was assessed 24 and 48 hours later. C-E. After normalizing to its vector control, removing Necl-2 from Schwann cells does not regulate both ErbB3, ErbB2 and Erk1/2 compared to overexpressing Necl-2 in Schwann cells. Mean ± SEM from n = 4 (Knockdown) and n = 3 (Overexpression) independent experiments.
Necl-2 in Schwann cells negatively regulates Akt and PDK1 in the early stage of myelination in vitro. **A-B.** Schwann cells (controls, knockdown or over-expressing Necl-2), were seeded onto axons of purified DRG neuron, and signaling pathway activation was assessed day 0 and day 5 after vitamin C treatment. **C-E.** After normalizing to its vector control, removing Necl-2 from Schwann cells results in substantial increase in Akt T308 (p=0.0189), S473 (p=0.0006) and PDK1 S241 (p=0.0003) compared to overexpressing Necl-2 in Schwann cells. Mean ± SEM from n = 4 (Knockdown) and n = 3 (Overexpression) independent experiments.
Necl-2 in Schwann cells does not regulates ErbB3, ErbB2 and Erk1/2 in the early stage of myelination in vitro. A-B. Schwann cells (controls, knockdown or over-expressing Necl-2), were seeded onto axons of purified DRG neuron, and signaling pathway activation was assessed day 0 and day 5 after vitamine C treatment. C-E. After normalizing to its vector control, removing Necl-2 from Schwann does not regulate both ErbB3, ErbB2 and Erk1/2 compared to overexpressing Necl-2 in Schwann cells. Mean ± SEM from n = 4 (Knockdown) and n = 3 (Overexpression) independent experiments.
Necl-2 in Schwann cells does not regulate the activation of Akt mediated by soluble Nrg1 in vitro. A, C. Schwann cells (controls, knockdown or over-expressing Necl-2), were stimulated with Nrg1, and signaling pathway activation was assessed at time 0, 15 mins, 60 mins and 180 mins after Nrg1 stimulation. B, D. After normalizing to its vector control, removing Necl-2 from Schwann cells (red line) results in no significant difference in the activation of Akt T308, and S473 compared to overexpressing Necl-2 in Schwann cells (green line). Mean ± SEM from n = 3 (Knockdown) and n = 3 (Overexpression) independent experiments.
Necl-2 in Schwann cells does not regulate the activation of ErbB3, ErbB2, and Erk1/2 mediated by soluble Nrg1 in vitro.  

A-B. Schwann cells (controls, knockdown or over-expressing Necl-2), were stimulated with Nrg1, and signaling pathway activation was assessed at time 0, 15 mins, 60 mins and 180 mins after Nrg1 stimulation.  

C-E. After normalizing to its vector control, removing Necl-2 from Schwann cells (red line) results in no significant difference in the activation of ErbB3, ErbB2, and Erk1/2 compared to overexpressing Necl-2 in Schwann cells (green line).  

Mean ± SEM from n = 3 (Knockdown) and n = 3 (Overexpression) independent experiments.
Necl-2 in Schwann cells does not form a complex with ErbB3 and ErbB2. A. Schwann cells, treated or not with rhNrg1-EGFD (-Nrg1, + Nrg1) were lysed and immunoprecipitated for Necl-2. As the Necl-2 antibody was generated in chicken, an immunoprecipitation with a non-specific IgY antibody was also performed to test for not specific pull-downs. While Necl-2 was specifically IPed by the chicken anti-Necl-2 antibody, only trace amounts of ErbB2 could be detected, and no ErbB3 was co-IPed. Treating Schwann cells with rhNrg1-EGFD had no effects. B. We corroborated further these results by performing reciprocate co-IPs with ErbB2 and ErbB3. ErbB2 and ErbB3 were strongly immunoprecipitated by their respective antibodies. As expected, we noticed an increase in ErbB3 (ErbB2 IP) or ErbB2 (ErbB3 IP) co-IP upon neuregulin treatment. However in both cases Necl-2 was not co-immunoprecipitated. 2% = input lane; Δ = depleted input after coIP.
Necl-2 in Schwann cells does not form a complex with PTPN13. A. Schwann cells, treated or not with rhNrg1-EGFD (-Nrg1, +Nrg1) were lysed and immunoprecipitated for Necl-2. As the Necl-2 antibody was generated in chicken, an immunoprecipitation with a non-specific IgY antibody was also performed to test for not specific pull-downs. While Necl-2 was specifically IPed by the chicken anti-Necl-2 antibody, only trace amounts of PTPN13 could be detected, and even can be detected in IgY control IP. Treating Schwann cells with rhNrg1-EGFD had no effects. B. We corroborated further these results by performing reciprocate co-IPs with PTPN13. PTPN13 were strongly immunoprecipitated by their respective antibodies and yet Necl-2 was not co-immunoprecipitated. 2% = input lane; Δ = depleted input after coIP.
Representative images of control Schwann cell (shLuc) or Schwann cells knockdowned for Necl-2 (shNecl-2), cultured on a laminin-1 substrate. Schwann cells (shLuc and Necl-2 deficient) were seeded at low density (10,000 cells per cm²) onto a laminin-1 substrate, to avoid changes due to cell-cell contact. Phalloidin staining of the actin stress fibers was used to visualize changes in Schwann cells morphologies 20 hrs after plating. Quantification of the numbers of Schwann cells that at least 80% of the Schwann cells deficient in Necl-2 presented a dramatic decrease in the intensity of the phalloidin staining (N = 3), suggestive to a substantial decrease in the formation of stress fibers in these cells.
Representative images of control Schwann cells (vector; expresses GFP) or Schwann cells overexpressing Necl2 (oxNecl-2), cultured on a laminin-1 substrate. Schwann cells (pLL-GFP controls and Necl-2 over-expressing) were seeded at low density (10,000 cells per cm²) onto a laminin-1 substrate, to avoid changes due to cell-cell contact. Phalloidin staining of the actin stress fibers was used to visualize changes in Schwann cells morphologies 20 hrs after plating (N = 3). Quantification of the numbers of Schwann cells that the number of lamellipodia was 2.5-fold higher in Schwann cells expressing higher levels of Necl-2 compared to the pLL-GFP control Schwann cells.
Down-regulation of Necl-2 increase the Rac1 activity by Schwann cells cultured on a laminin-1 substrate. Schwann cells (vector and Necl-2 deficient) were seeded at low density (150,000 cells per 3.8 cm²) onto a laminin-1 substrate, to avoid changes due to cell-cell contact. After 3 hours seeding, when Schwann cells attach well to the laminin-1 substrate. GLISA assay was performed to detect the activation of Rac1 and RhoA by Schwann cells cultured on a laminin-1 substrate for 3 hrs. Normalization was done with the activity of total Rac1 and RhoA of Necl-2 deficient cells over vector control cells. The activity of Rac1 is about 30 % increase of Rac1 in Necl-2 deficient Schwann cells grown on laminin substrate (N = 3), and yet there is no significant difference of the activity of RhoA.
CHAPTER V

Conclusion and Future Directions

The Nectin-like cell adhesion molecules have been shown to be expressed along the internode of the myelin sheathes in the PNS [112, 145]. Their particular enrichment at the zone of contact between the plasma membrane of the myelinating Schwann cell and the plasma membrane of the axon, make then suitable candidate to mediate axo-glial interactions. Certainly the expression of Necl-4 on the Schwann cells, and its heterophilic binding partner Necl-1 on the axon does support that view. In vitro [112, 145] and in vivo [24] studies demonstrating the pro-myelinating function of Schwann cell Necl-4 further strengthen this hypothesis.

The expression of Necl-2 by Schwann cells and by DRG neurons however render the studies on Necls function in PNS myelination rather complex. Indeed, Necl-2 is also a ligand for Necl1, and can also interact homophilically. There are therefore potentially at least 3 forms of axo-glial interactions mediated by the Necls along the internode: Necl-1/Necl-4, Necl-1/Necl-2 and Necl-2/Necl-2 [145].

The sometime confounding effects of multiple hetero- and hemophilic interactions that are supported by cell adhesion molecules, and which may be providing compensatory mechanisms have hampered many in vivo studies of cell adhesion molecules functions. The phenotypes from knockout model animals are often subtle, transient, or even absent, even though in vitro studies strongly support a role for these cell adhesion molecules [24, 102, 167, 182].

I therefore resorted to the well-established in vitro model of PNS myelination,
which has been instrumental over the past 35 years in all major discoveries in PNS myelination.

The results obtained in the study of axonal Necl-1 were surprising and unexpected. Indeed as the ligand for Schwann cell Necl-4, and as Necl-4 been shown, *in vitro* but *in vivo* as well, to be needed for proper Schwann cell myelination, the expected results were to found that axonal Necl-1 was pro-myelinating as well. The experiments however have strongly demonstrated the opposite, that Necl1 is inhibitory to Schwann cell myelination, at least *in vitro*.

This finding can be reconciled if one considers that Necl-1 has another ligand on Schwann cells, Necl-2, and that the interaction Necl-1/Necl-2 has been shown to be weaker than the Necl-1/Necl-4 interaction [145, 165]. One could hypothesize that as the levels of Necl-1 are low on the axon, the stronger Necl-1/Necl-4 interaction is favored, leading to myelination. When the levels of Necl-1 are increased, the Necl-1/Necl-2 interaction can happen, and be favored if the Necl-1/Necl-4 become saturated. (It is important to note that while absolute changes in each Necl have been observed in the developing sciatic nerves, the relative amount of each Necl in relation to the others is not known to date). This Necl-1/Necl-2 would be hypothesized to have a negative impact on Schwann cell myelination. A consequence of this hypothesis is that Schwann cell Necl-2 must be inhibitory to Schwann cell myelination. That is what I have demonstrated in Chapter IV, and is further supported by the findings that Necl-2 negatively regulates the activation of the pro-myelinating PI3K/Akt pathway.

This work must however be further extended as some questions remain.
i) For example, in the co-culture setup, is it the PI3K/Akt pathway within the Schwann cells or within the DRG neurons that is affected. It is important, as the activation of this pathway by soluble Nrg1 in isolated Schwann cells is not affected.

ii) As I showed that the extracellular domain of Necl-1 down regulates the activation of the PI3K/Akt pathway, as well as the activation of ErbB3, one can ask whether the apparent discrepancy between the co-culture and the isolated Schwann cells data reflects the presence and function of axonal Necl-1 in the co-culture setup. Additional experiments, where Necl-2-deficient Schwann cells are co-cultured with Necl1-over-expressing DRG neurons, or vice versa Necl-2-over-expressing Schwann cells are co-cultured with Necl-1-deficient DRG neurons might be informative.

Ultimately, what is (are) the function(s) of the Necls in the developing sciatic nerves, and how can they affect the process of myelination? Indeed myelination in vitro provides a more-or-less black and white answer, determined by counting the number of myelinated segments, that while strongly demonstrating that these molecules are involved, the exact step (radial sorting, wrapping etc.) that is regulated is not necessarily clear.

Type III Nrg1, signaling through the ErbB2 and ErbB3 receptors, is the on/off switch to myelination in the PNS [31, 93]. The levels of type III Nrg1, and therefore the levels of PI3K/Akt activation in the Schwann cells dictate whether a Schwann cells become ensheathing, or become a myelinating Schwann cell. Furthermore, the levels of type III Nrg1 also determine the myelin thickness once myelination starts. However myelination in rodents does not start until birth, and becomes
prominent by postnatal day p4/p7, when Necl-2 decreases. What is (are) the mechanism(s) that prevent the Schwann cells to form compact myelin before birth? Could the high level of expression of Necl-2 from p0 to abut p7 maintain the Schwann cells in an immature phenotype, conducive to the extensive radial sorting that is still ongoing? Would it be accomplished by preventing Nec-1 to interact with Necl-4 (whose expression actually increase with myelination; Fig. 14)?

Another interesting question to address is whether Necl-2 may be involved directly in regulating radial sorting and spiral wrapping. This possibility is raised by the fact that high levels of Necl-2 promote the formation of lamellipodia. It has been suggested that these lamellipodia, under the regulation of Rac1 activity, are necessary to the radial sorting process [208]. Necl-2 expression is high in the sciatic nerve during the time frame of active radial sorting (Fig. 14), and low at the time of spiral wrapping. Regarding spiral wrapping, it was recently shown that the actin cytoskeleton must be in constant flux [211]. The finding that Schwann cells deficient in Necl-2 have a strongly decrease levels of stress fibers is therefore intriguing, and warrants further investigation. While this last experiment was done on a laminin substrate, one should consider that the Schwann cell is normally integrating signals form the basal lamina and the axon. What would be the effect of “sandwiching Necl-2-deficient, or Necl-2 over-expressing Schwann cells between lamin-1 and Necl-1?"

The in vitro studies of Necl-1, Necl-2 and Necl-4 strongly support a function of these cell adhesion molecules in regulating events of PNS myelination. Manipulating their levels of expression is highlighting some of the molecular
mechanisms that may be affected and involved. Interestingly, these molecular mechanisms have already been shown to be important players to different aspects of myelination. This is only the beginning, and I strongly believe that further studies will elucidate how the Nectin-like cell adhesion molecules regulate Schwann cell myelination in the peripheral nervous system.


35. Greenfield, S., et al., Protein composition of myelin of the peripheral
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