The role of Nrg1-ErbB signaling in promoting Schwann cell myelination

By Neeraja Syed

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Dr. Haesun A. Kim

and approved by

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Abstract

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Myelination is important for rapid saltatory conduction of nerve signals along the axons. Under various pathological conditions, myelin is lost and rebuilding myelin is required for functional recovery. In the PNS, myelination is regulated by axonal signals. Therefore, enhancing the promyelinating signals could be used as a therapeutic strategy to improve myelin repair by endogenous or transplanted myelinating glial cells such as Schwann cells. Recent studies identified Nrg1-type III as an important promyelinating signal that regulate Schwann cell myelination. Therefore, we hypothesized that providing an ectopic Nrg1-type III signal in a soluble form would increase the myelinating potential of Schwann cells. Since Nrg1-type III is normally expressed by axons as a membrane bound protein, in Aim 1 we first characterized the signaling function and the therapeutic potential of soluble Nrg1-type III in Schwann cells. Results from this study show that soluble Nrg1-type III elicits a promyelinating effect in-vitro: it increases myelin gene expression and enhances myelination on axons that express low levels of Nrg1-type III. However, when used at high concentrations it has a contrasting effect and inhibits myelination. Remyelination in the adult PNS is often incomplete: the myelin segments are thin and short. To determine the cell type that contributes to the defect, we

characterized the myelination potentials of Schwann cells and neurons isolated from adult PNS in Aim 2. Results show that while adult Schwann cells myelinate normally, the axons provide insufficient promyelinating signals by expressing low levels of Nrg1-type III. In Aim 3, we investigated whether providing ectopic Nrg1-type III improves myelination on adult axons. Surprisingly, neither treatment with soluble nor over expressing membrane bound Nrg1-type III was sufficient to enhance myelination on adult axons. Further analysis revealed that adult axons increases Erk1/2 activity in the associated Schwann cells, an inhibitory signal for myelination. Inhibiting those signals significantly increase myelination on adult axons.

Dedication

*This dissertation is dedicated to my mother **Ramarathinam A.** for her unconditional love and support all my life and to the memory of my father **Alagarswamy P.V.***

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Chapter 1

Introduction

1.1 Myelin disorders in the nervous system

The orchestrated interaction between neurons and glial cells in the nervous system provide the environment for rapid saltatory conduction of nerve signals along the long axons. This interaction involves myelination of axons by the associated glial cells, which is performed by Schwann cells and oligodendrocytes in the peripheral and the central nervous system (PNS and CNS), respectively. In the PNS, neurons regulate survival, proliferation and differentiation of the Schwann cells during development (Jessen and Mirsky, 2005). In adults, axonal signals regulate Schwann cell plasticity that is required for nerve repair under pathologic conditions (Fricker et al., 2011). Reciprocally, Schwann cells regulate the survival of axons during development and regeneration (Edgar and Garbern, 2004). In the CNS, oligodendrocytes and axons also rely on each other for their survival (Barres et al., 1993, Griffiths et al., 1998). Due to the neurotrophic effect provided by the associated glial cells, loss of myelin is detrimental to the neuronal survival, resulting in severe sensory and motor deficits.

In humans, development of demyelinating and dismyelinating neuropathies is associated with hereditary alterations in myelin gene expression, inflammation by infectious agents, autoimmunity, or the exposure to toxic chemicals. Charcot-Marie-Tooth (CMT) disease is a common hereditary myelin neuropathy of the PNS. Various forms of CMT are developed by mutations in different genes and they are classified as CMT1, CMT2, CMT3, CMT4, AR-CMT2, and HSN (Suter and Scherer, 2003). Among them, CMT1 is the most prevalent myelin neuropathy and it is developed by Schwann

cell demyelination. CMT1 is further categorized into different subtypes based on mutations in specific genes such as PMP22 (CMT1A and HNPP), P0 (CMT1B), LITAF (lipopolysaccharide induced tumor-necrosis factor (TNF)-α factor) (CMT1C), and GAP junction protein (CMT1X) (Suter and Scherer, 2003). Autoimmunity in the PNS is associated with development of Guillain-Barre syndrome (GBS) and it is thought to be triggered by acute viral or bacterial infections. The infectious agents are believed to induce the production of antibodies against glycolipids and gangliosides of PNS myelin leading to demyelination. (Kountouras et al., 2008, Pithadia and Kakadia, 2010).

Leukodystrophy is the inherited myelin neuropathy of the CNS and is classified into different types: Pelizaeus-Merzbacher disease (PMD) is a hypo-myelinating leukodystrophy (HLD) caused by mutation in PLP1 gene (Garbern, 2007). Mutation in connexin 47 gene causes HLD 2 (Uhlenberg et al., 2004) while mutations in ABCD 1 gene, a peroxisomal transporter protein, causes X-linked adrenoleukodystrophy (X-ALD)(Baes and Aubourg, 2009). Multiple Sclerosis (MS) is an autoimmune disease in the CNS that results in development of severe and widespread demyelinated lesions in the white matter of the brain and spinal cord. During the early course of the disease, the immune attack on the CNS myelin is transient and the remyelination occurs, however as the disease progresses, demyelination dominates which leads to axonal degeneration and neuronal death resulting in severe neurological deficits (Compston and Coles, 2008).

In both the PNS and the CNS, demyelination of axons is followed by some degree of remyelination by the endogenous glial cells. For example, in the PNS, once the myelin debris is cleared up by macrophages and de-differentiated Schwann cells (Bruck et al., 1996, Bruck, 1997), the glial cells associate and remyelinate the axons at the lesion.

However, the remyelinated segments are often reduced in the myelin thickness and the internodal length (Beuche and Friede, 1985, Bardosi, 1987).

In MS patients, axons at the lesion site are remyelinated by the endogenous oligodendrocyte precursor cells (OPCs) (Gensert and Goldman, 1997), however, chronic demyelination results in poor remyelination characterized by thinner and shorter myelin segments (Ludwin, 1980). Recurrent demyelination results in fewer number of oligodendrocytes (Johnson and Ludwin, 1981) and insufficient remyelination, eventually causing axon degeneration (Dubois-Dalcq et al., 2005). Therefore, identifying a strategy to rebuild myelin in demyelinated lesions is an important therapeutic objective for addressing neurodegenerative diseases.

1.2 A Possible strategy to promote remyelination

The strategy of using transplanted myelin-forming cells to remyelinate demyelinated axons was first conceived when it was reported that after peripheral nerve implantation in the spinal cord, CNS axons entered the nerve implant and became myelinated by the Schwann cells (Cajal RY, 1928). More recently, experimental transplantation using Schwann cells, olfactory ensheathing cells, oligodendrocytes, and stem cells (embryonic and adult) have provided promising effects on re-growing injured axons into their original targets and rebuilding the myelin (Duncan, 2005). Among these cells, Schwann cells have been considered as the most promising candidate for the autologous transplantation therapy for following reasons. First, Schwann cells can spontaneously remyelinate axons at the demyelinated lesions in both the PNS and the CNS (Felts and Smith, 1992). Second, human Schwann cells can be easily purified from

peripheral nerve biopsies of the patients and expanded in culture (Avellana-Adalid et al., 1998). Furthermore, remyelinated segments generated by transplanted Schwann cells at the MS lesions may not be targeted by immune cells, thus resistant to recurrent demyelination. Moreover, transplanted Schwann cells have been shown to improve regeneration and functional recovery of injured axons in various animal models of spinal cord injury (Blakemore, 1977, Duncan et al., 1981, Baron-Van Evercooren et al., 1992).

Despite these major advancements in the development of the transplantation therapy, remyelination by Schwann cells is often incomplete. Studies have shown that transplanted Schwann cells remyelinate less than 50% of the demyelinated lesion and following nerve regeneration after injury. The resulting myelin segments are often short and thin (Kohama et al., 2001, Lankford et al., 2002). Incomplete remyelination is also observed in the PNS (Beuche and Friede, 1985). Therefore finding a strategy to enhance the remyelination capacity of transplanted Schwann cells will greatly improve its repair function in demyelinated lesions.

1.3 Neuregulin and Schwann cell myelination
1.3.1 Neuregulins and erbB receptors:

Neuregulins (Nrg) are a family of growth factors derived from Nrg genes. In mammals, four Nrg genes (1, 2, 3, and 4) have been identified. Among them, Nrg1 has been well characterized and studied. From a single Nrg1 gene at least 35 different Nrg1 isoforms are generated by different promoter usage and alternative splicing. They are classified into type I, II, III, IV, V, and VI based on their unique N-terminal domains (Falls, 2003). The isoforms type I, II, and III have been extensively studied, however,

type IV, V, and VI were only recently identified and their functions remain unknown. Type I, II, and III Nrg1 isoforms possess an epidermal growth factor (EGF) like domain that is necessary and sufficient to activate erbB receptors (Holmes et al., 1992, Lu et al., 1995). However, they differ in their amino terminal sequences. Type I isoforms possess an immunoglobulin like domains and type II isoforms, also known as GGF, possess a kringle domain along with immunoglobulin (Ig)-like domains on their N-terminus. On the other hand, type III isoforms possess a cysteine rich domain (CRD) which serves as a second transmembrane domain (Buonanno and Fischbach, 2001, Falls, 2003). These isoforms are initially made as transmembrane proteins and then they undergo proteolytic processing by metalloproteases such as BACE and TACE (Horiuchi et al., 2005, Hu et al., 2006, Velanac et al., 2012). After proteolytic processing, type I and II isoforms are shed from the cell membrane and function as a paracrine signals. In contrast, due to its CRD domain Nrg1-type III remains tethered to the cell membrane and functions as a juxtacrine signal (Falls, 2003).

In the PNS, Nrg1 are expressed by the neurons and regulate development of the associated Schwann cells. The axonal Nrg1 transduces signals by binding to erbB receptors, a family of single transmembrane receptor tyrosine kinases (RTKs), on the Schwann cell surface. The ErbB family receptors include erbB1, erbB2, erbB3 and erbB4. Among them, Schwann cells express erbB2 and erbB3 (Levi et al., 1995). Nrg1 binds to erbB3 with high affinity and induces conformational changes in the receptor leading to heterodimerization with erbB2. ErbB3 lacks a catalytically active kinase domain, however, heterodimerization activates tyrosine kinase domain on erbB2 which subsequently lead to tyrosine phosphorylation of the cytoplasmic domains of both

receptors (Pinkas-Kramarski et al., 1996). Phosphorylated tyrosine residues then recruit a variety of adaptor proteins and enzymes to activate intracellular downstream signaling pathways.

1.3.2 Neuregulin in Schwann cell myelination:

Schwann cells are derived from multipotent neural crest cells. During PNS development, neural crest cells migrate from the tip of the neural tube folding to different pathways that determine the lineage development of various cell types. The population of neural crest cells that migrate towards a lateral ventral direction give rise to Schwann cell lineage. When migrating neural crest cells associate with developing axons, they develop into Schwann cell precursors (SCPs) which occurs around embryonic days (E) 14-15 in rats (mouse E 12-13). At this stage, SCPs interact with sensory and motor neurons which support the survival and further differentiation of the SCPs into immature Schwann cells (Jessen et al., 1994) which occurs around E 15-17 (mouse E 13-15). Immature Schwann cells at this stage proliferate and begin to sort axons based on the axon diameter. Large diameter axons are sorted individually and form 1:1 association with the immature Schwann cells which later become pro-myelinating Schwann cells. During the early post natal development, promyelinating Schwann cells convert into myelinating Schwann cells and wrap their membranes around axons to form multi layered compact myelin sheath. Multiple small diameter axons are ensheathed by non myelinating Schwann cells and together form Remak bundles (Jessen and Mirsky, 2005).

Among multiple extrinsic factors, Nrg1s have been shown to play a key role in Schwann cell development (Jessen and Mirsky, 2005). In mice lacking all Nrg1 isoforms,

the number of SCPs was severely reduced in the peripheral nerve and died during embryogenesis. A similar phenotype was observed in mice lacking erbB2 or erbB3 receptors in Schwann cells (Riethmacher et al., 1997, Britsch et al., 1998). However, most severe phenotype was observed in erbB3 mutant mice with complete absence of SCPs (Meyer and Birchmeier, 1995, Riethmacher et al., 1997, Britsch et al., 1998). Overall, above evidences suggest that Nrg1-erbB signaling play a key role during development of the Schwann cell lineage.

It has long been known that axonal signals regulate Schwann cell myelination. Recent studies have identified Nrg1-type III as a key promyelinating signal that is required for myelination. In co-culture studies, axons from Nrg1-type III knock out neurons failed to become myelinated while Nrg1-type III heterozygous axons were hypomyelinated. Ectopic expression of the membrane bound rescued the myelination defect in Nrg1-type III knock out neurons. The same study also showed that forced expression of Nrg1-type III into SCG neurons, which are normally ensheathed but non-myelinated, changed their fate to a myelinated phenotype (Taveggia et al., 2005). *In-vivo* studies using transgenic mice showed that over expression of Nrg1-type III increased myelin sheath thickness (Michailov et al., 2004). Altogether, these studies show that Nrg1-type III acts as an instructive signal for Schwann cell myelination and the levels of myelination is graded to the amount of Nrg1-type III expressed on the axons.

1.3.3 Nrg1-erbB downstream signals that regulate myelination

Phosphorylated tyrosine residues on erbB2 and erbB3 receptors recruit p85 subunit of PI3-kinase, Grb2, shc, and PLCγ. Recruitment of p85 subunit activates the

PI3-kinase /Akt pathway whereas Grb2 and shc activate Ras/Raf/Erk1/2 pathway. Activation of PLCγ leads to calcineurin/NFAT signaling (Kao et al., 2009, Fricker and Bennett, 2011). Functional outcomes of these pathways in Schwann cell development have been extensively characterized. In this section, I describe the functions of individual erbB downstream signaling pathways in regulating Schwann cell myelination.

PI3-kinase

Receptor tyrosine kinase bound PI3-kinase catalyzes the enzymatic conversion of PIP2 (phosphatidylinositol (4, 5) bisphosphate) to PIP3 (phosphatidylinositol (3, 4, 5) triphosphate) which in turn activates the downstream kinase Akt. Cytosolic protein PTEN (phosphatase and tensin homolog), a cytosolic phosphatase, reverses this reaction by dephosphorylating PIP3, acting as a major negative regulator of PI3-kinase/Akt signaling pathway.

In Schwann cell monocultures, adeno viral mediated expression of dominant negative PI3-kinase blocks the expression of myelin genes during Schwann cell differentiation (Ogata et al., 2004). In DRG- Schwann cell co-cultures, inhibition of PI3-kinase at the early stages of myelination completely blocked the subsequent Schwann cell myelination, however, inhibition at the later stages had no effect, indicating the requirement of PI3-kinase at the initial stages of myelination (Maurel and Salzer, 2000). Furthermore, over expression of catalytic subunit of PI3-kinase (p110) enhanced Schwann cell myelination in co-cultures, suggesting the role of the PI3-kinase as a positive regulator of myelination (Ogata et al., 2004).

Studies using homozygous and heterozygous Nrg1-type III mutant mice have identified the link between expression levels of the axonal Nrg1-type III and activation levels of PI3-kinase in Schwann cells. When Schwann cells were stimulated with neurite membranes prepared from Nrg1-type III$^{-/-}$, Nrg1-type III$^{+/-}$ and wild type neurons, Akt activation levels were reduced in Schwann cells incubated with Nrg1-type III$^{+/-}$ neurons whereas no Akt activation was observed with Nrg1-type III$^{-/-}$ neurons. These evidences suggest that the level of myelination may be determined by the levels of the Nrg1-type III induced Akt activation (Taveggia et al., 2005).

Evidences from several *in-vivo* studies confirmed the role of PI 3-kinase pathway in Schwann cell myelination. Over production of PIP3 in Schwann cells by PTEN deletion leads to hypermyelination of the PNS axons (Goebbels et al., 2010). shRNA mediated knock down of Akt in sciatic nerve at earlier stages of myelination resulted in shorter and thinner myelin sheaths (Cotter et al., 2010). Interestingly, however, constitutive activation of Akt in Schwann cells was not sufficient to increase Schwann cell myelination (Flores et al., 2008), indicating the involvement of other PIP3-dependent signaling pathways in promoting myelination.

Ras/Raf/Erk1/2

Another major signaling pathway which is activated by Nrg1-erbB signaling is the Ras/Raf/Erk1/2 pathway. *In-vitro,* it has been shown that adeno viral mediated expression of dominant negative Ras promotes Schwann cell differentiation into myelinating phenotype by expressing myelin proteins such as MAG, P0, MBP and pro-myelinating transcription factors such as Oct-6 and Krox-20 (Ogata et al., 2004). A recent

in-vivo study showed that Ras/Raf/Erk1/2 activation is sufficient to induce Schwann cell demyelination and dedifferentiation in the adult PNS. Subsequent reversal of the Ras/Raf/Erk1/2 activation to the basal levels promotes remyelination to resume (Napoli et al., 2012), suggesting that Ras/Raf/Erk1/2 activation negatively regulates Schwann cell differentiation and myelination.

On the contrary to the above findings other studies have illustrated the role of the Ras/Raf/Erk1/2 as a positive regulator of Schwann cell differentiation. It has been shown that Nrg- 1 induced Ras/Raf/ Erk1/2 activation is required to elevate the expression of a pro-myelinating transcription factor Krox-20 (He et al., 2010). In support to this study, two groups have recently reported that deletion of Erk1/2 in SCPs disrupts Schwann cell myelination without affecting immature Schwann cell proliferation (Newbern et al., 2011). Another group observed that Schwann cell specific deletion of non receptor tyrosine phosphatase Shp2, an upstream effector of Erk1/2, blocks Schwann cell differentiation resulting in hypomyelination (Grossmann et al., 2009). Overall, data from all the above studies targeting Ras/Raf/Erk1/2 signaling pathway show the dual function of Erk1/2 in regulating myelination, one that inhibits and the other that promotes the process.

PLC-γ

Nrg-1 induced PLCγ activation has been shown to regulate Schwann cell myelination. Active PLCγ triggers calcium influx into Schwann cells that activates a phosphatase calcineurin. Calcineurin activates the transcription factor NFAT which synergistically acts with Sox 10 to induce the expression of Krox-20 and other myelin

proteins. The importance of the calcineurin-NFAT signaling pathway is confirmed *in-vivo*: Mice lacking the regulatory subunit of calcineurin exhibit hypomyelination in the PNS (Kao et al., 2009), suggesting its role in myelination.

p38 MAPK

We have shown recently that p38 MAPK functions as a negative regulator of Schwann cell differentiation: enforced p38 MAPK activation blocks cAMP-induced expression of Krox 20 and myelin proteins, but induces expression of c-Jun. As expected of its role as a negative signal for myelination, inhibition of p38 MAPK in co-cultures promotes myelin formation by increasing the number as well as the length of individual myelin segments. p38 MAPK concomitantly acts as a signal that induces Schwann cell demyelination and dedifferentiation. Schwann cell demyelination is mediated by p38 MAPK activation in myelinating co-cultures and the inhibition blocks the degenerative effect on the myelin. Furthermore, ectopic activation of p38 MAPK is sufficient to induce myelin breakdown and drives differentiated Schwann cells to acquire a phenotype that resembles immature Schwann cells. Altogether, p38 MAPK is an important regulator of Schwann cell plasticity and differentiation (Yang et al., in press).

1.3.4 Transcription factors that control Schwann cell myelination:

Nrg1 regulates the expression of transcription factors such as Oct-6, Krox-20, NF-κB, Yy1 and NFAT on its own or in coordination with other intracellular signaling pathways. During development of the Schwann cell lineage, Oct-6, a member of the POU domain transcription factor family, appears first in SCPs and its expression level

gradually increases until the first post natal week when myelination is initiated. Then expression gradually decreases afterwards and disappears completely in adults (Blanchard et al., 1996, Zorick et al., 1996). Forced continuous expression of Oct-6 in Schwann cells *in-vivo* has been shown to reduce the expression of myelin proteins P0 and PMP-22 and causes hypomyelination (Ryu et al., 2007), indicating that the temporal expression of Oct-6 is critical for Schwann cells to progress into a myelinating phenotype. Supporting this, Schwann cell specific deletion of Oct-6 resulted in delayed myelination *in-vivo* (Jaegle et al., 1996). Brn-2 is another POU domain transcription factor expressed at the time of Oct-6 during Schwann cell development. Ectopic expression of Brn-2 has been shown to rescue the myelination defect in Oct-6 null mice. In addition, deleting both Oct-6 and Brn-2 results in a severe delay in myelination (Jaegle et al., 2003). These observations indicate that Oct-6 and Brn-2 function during a narrow window of time during Schwann cell development to promote the transition from the pro-myelinating to the myelinating phenotype. Oct-6 expression is tightly under the control of Nrg1-type III present on the axonal membrane. It has been shown *in-vitro* co-cultures that complete absence of axonal Nrg1-type III leads to failure in the expression of Oct-6 in the associated Schwann cells (Taveggia et al., 2005).

Krox-20 is another key transcription factor required for Schwann cell myelination. Krox-20 expression is induced by Oct-6 and Sox-10 (Ghislain and Charnay, 2006, Reiprich et al., 2010). Krox-20 is detected 24-36 hours after the expression of Oct-6 and continues to present throughout life in myelinating Schwann cells (Zorick et al., 1996). Studies with Krox-20 null mice show that in the absence of Krox-20, Schwann cells associated with large caliber axons failed to myelinate and are arrested at a pro-

myelinating stage (Topilko et al., 1994). In adult mice, disrupting Krox-20 expression in Schwann cells leads to demyelination, indicating that Krox-20 function is also critical for maintenance of myelin sheath in adults (Decker et al., 2006). All the above observations show that Oct-6 and Krox-20 are the major regulators of myelination.

Nrg1-type III also activates transcription factor NFAT through calcineurin mediated signaling as mentioned earlier. NFAT is involved in promoting myelination by acting synergistically with Sox-10 to induce the transcription of Krox-20. Deletion of the regulatory subunit of calcineurin in Schwann cells and sensory neurons *in-vivo* resulted in hypomyelination. However, deleting it in sensory neurons alone did not affect myelination, indicating that calcineurin regulates myelination through NFAT in Schwann cell autonomous manner (Kao et al., 2009).

There is also evidence showing that Nrg1-type III induces Krox-20 expression by activating another transcription factor Yy1 through Erk1/2 signaling. Conditional ablation of Yy1 in Schwann cells resulted in hypomyelination. However, over expression of Krox-20 in Yy1 deficient Schwann cells rescued myelin protein expression in Schwann cells indicating that Yy1 is the upstream regulator of Krox-20. It was also shown that the MEK inhibitor U0126 inhibited the Nrg1 mediated Yy1 phosphorylation, which is required to induce Krox-20 expression. Altogether, the above evidences show that Nrg1 mediated Erk1/2 signaling phosphorylates Yy1 and which subsequently regulate the expression of Krox-20 (He et al., 2010).

NFκB is another transcription factor that is required for myelination and its activation is under the control of axonal Nrg1-type III. Using *in-vitro* myelinating co-cultures, it has been shown that inhibition of NFκB by a chemical inhibitor or expression

of the inhibitory subunit resulted in complete block of Schwann cell myelination. In those cultures, induction of Oct-6 was completely inhibited, indicating that NFκB functions upstream of Oct-6. The same group also identified that axonal Nrg1-type III activates NFκB and inhibiting the erbB2 and erbB3 receptor activation in Schwann cells reduced the NFκB activation (Limpert and Carter, 2010).

The transcription factor c-Jun, activated by JNK1/2 signaling pathway, has shown to act as a negative regulator of myelination. c-Jun expression is down regulated at the time of initiation of myelination and forced expression inhibited myelination in *in-vitro*. c-Jun expression is up regulated after nerve injury and c-Jun deficient mice exhibited the delay in Schwann cell dedifferentiation after nerve injury, indicating that c-Jun drives Schwann cell dedifferentiation both *in-vitro* and *in-vivo* (Parkinson et al., 2008)

1.4 Research Aims

In this study, we hypothesized that enhancing a promyelinating signal would promote the myelination potential of adult axons. Focusing on the pro-myelinating function of the Nrg1-type III on Schwann cells, the goals of this study are; 1) to characterize the signaling property of soluble Nrg1-type III and determine its role during Schwann cell myelination 2) to characterize the myelination properties of Schwann cells and axons from adult PNS. To address this, we have following research aims.

Aim1 (Chapter 2): To study the signaling functions of recombinant soluble Nrg1-type III

It has been shown that the axonal Nrg1-type III is a key factor that provides an instructive signal for Schwann cell myelination and the level of myelination is graded to the amount of Nrg1 expressed on the axons(Michailov et al., 2004, Taveggia et al., 2005). Therefore, we hypothesized that enhancing the Nrg1-type III signal in Schwann cell would promote the myelination potential. For a therapeutic purpose, use of a soluble form would provide an advantage over the use of a membrane bound Nrg1-type III. However, the signaling function and the effect of soluble Nrg1-type III on myelination are not known. To address this we used the *in-vitro* myelinating co-culture system to analyze the signaling functions of soluble Nrg1-type III. Data from Aim1 show that soluble Nrg1-type III elicits a promyelinating function in Schwann cell- DRG neuron co-cultures. It promotes myelination on Nrg1-type III+/- neurons but fails to induce myelination on Nrg1-type III -/- neurons. An interesting finding in this study is that GGF, a soluble Nrg1-type II isoform, previously regarded as negative regulator of myelination also

elicits a promyelinating function when used at low concentrations suggesting that the promyelinating effect is not isoform specific. At high concentrations, both type II and type III isoforms inhibit myelination in a manner dependent on Erk1/2 activation.

Aim 2 (Chapter 3): To compare and characterize axons and Schwann cells from adult and developing PNS

Remyelination of adult axons by Schwann cells is often incomplete suggesting that the properties of adult and embryonic axons could be different. It has been shown that levels of Nrg1-type III expression gradually decreases postnatally (Taveggia et al., 2005). Therefore we hypothesized that the amount of Nrg1-type III expressed on adult axons is not sufficient to induce complete myelination. Alternatively, it is also possible that the adult Schwann cells have lost their intrinsic ability to fully myelinate the associated axons. To address this, we first compared the myelination potentials of the Schwann cells prepared from adult and neonatal rats and then we also compared the myelination properties of adult and embryonic neurons. We investigated the Nrg1-type III expression by adult and embryonic neurons. Finally we analyzed the activation levels of erbB2 and Akt in Schwann cells induced by adult and embryonic neurons. Data from this part of the study show that myelination on adult axons is much reduced compared to the embryonic ones while adult Schwann cells retain their myelination potential. Therefore, axons are the source of insufficient myelination. Furthermore, adult neurons express low levels of Nrg1-type III compared to embryonic neurons and adult neurons have a decreased ability to activate erbB2 and Akt in the associated Schwann cells.

Aim 3 (Chapter 4): To determine whether enhancing Nrg1-type III signaling improves myelination on adult axons

In this study, we hypothesized that the myelination of adult axons can be improved by enhancing Nrg1 signaling in the associated Schwann cells. To address this, we tested the effect of soluble Nrg1-type III on myelination using adult DRG-Schwann cell co-culture system. We also ectopically expressed the membrane bound Nrg1-type III in adult axons to determine whether increasing the Nrg1-type III levels would rescue the myelination defect. Data from this part of the study show that neither soluble nor membrane bound Nrg1-type III signal enhance myelination of adult axons. Interestingly, we observe that activation levels of Erk1/2 is increased in adult DRG-Schwann cell co-culture during the early stages of myelination, Furthermore, inhibition of the Erk1/2 activation in adult DRG and Schwann cell co-culture enhances myelination.

Chapter 2 - *To study the signaling functions of recombinant soluble Nrg1-type III*

Introduction

Schwann cell development in the peripheral nervous system (PNS) is dependent on contact mediated signaling between Schwann cells and the associated axons. The Nrg1 and erbB ligand-receptor system lends itself to the task of close contact signaling, as PNS neurons express Nrg1 family ligands and Schwann cells express erbB2 and erbB3, which heterodimerize to form functional Nrg1 receptors (Falls, 2003). Members of Nrg1 family growth factor are initially expressed on the PNS axons as transmembrane proteins, which are then shed from the membrane by proteolytic processing. All isoforms, type I, II and III, contain an epidermal growth factor (EGF)-like signaling domain in the extracellular domain that is necessary and sufficient for activating erbB receptors on the Schwann cell surface (Holmes et al., 1992, Lu et al., 1995) . They differ, however, in the sequences N-terminus to the EGF-domain; the type I and II isoforms contain an Ig-like domain whereas type III isoform contains a cysteine-rich domain, which functions as a second transmembrane domain. As a result, Nrg1 type I and II are released and function as paracrine signals after proteolytic cleavage, whereas Nrg1-type III remains tethered to the axonal membrane and mediates juxtacrine signaling (Falls, 2003).

Recent studies have shown that membrane-bound Nrg1-type III plays an essential role in initiating and regulating myelination in the PNS. Axons that lack Nrg1-type III expression fail to become myelinated (Taveggia et al., 2005). Over expression of Nrg1-type III in neurons results in hypermyelination, indicating that the levels of myelination are determined by the amount of Nrg1 type III expressed on individual axons (Michailov et al., 2004, Taveggia et al., 2005). In contrast, soluble Nrg1-type II has been shown to inhibit Schwann cell myelination and promote de-differentiation (Zanazzi et al., 2001).

Since both Nrg1 isoforms bind and activate erbB receptors, it is unclear how they elicit opposing effects on myelination. It is possible that juxtacrine signaling provided by the membrane-bound Nrg1 is required for myelination whereas paracrine signaling by soluble isoforms prevents the process. Alternatively, the isoform-specific effects may be mediated by differential activation of the ErbB receptor downstream signaling pathways.

To address the issue, we investigated the effect of Nrg1-type III, presented in a paracrine manner, on Schwann cell myelination. We report that soluble Nrg1-type III elicits a pro-myelinating function in Schwann cell-neuron co-cultures. It increases myelination of Nrg1-type III$^{+/-}$ neurons but fails to myelinate Nrg1-type III$^{-/-}$ neurons. Interestingly, low concentrations of Nrg1-type II also promote myelination, suggesting that the effect is not isoform specific. At high concentrations, both Nrg1 inhibit myelination in a manner dependent on Mek/Erk activation. Altogether, our data suggest that the pro-myelinating function of Nrg1 is not limited to the juxtacrine signal and can be provided by a paracrine signal in a concentration-dependent manner. Finally, our studies suggest that there may be two distinct steps of Schwann cell myelination: an initial phase dependent on membrane-bound Nrg1 signaling and a later phase that can be promoted by soluble Nrg1 isoforms.

Materials and Methods

Antibodies

For immunofluorescence analysis, monoclonal antibody (SMI94) to myelin basic protein (MBP) (Covance, Emeryville, CA) was used at 1: 300 dilutions. For Western blot analysis, monoclonal antibody to phospho-Akt (Cell signaling, Danvers, MA) and polyclonal antibody to phospho-Erk1/2 (Promega, Madison, WI) were used at 1:1000 and 1:5000, respectively. Polyclonal antibodies to Akt (Cell Signaling, Danvers, MA) and Erk1/2 (Promega, Madison, WI) were used at 1:1000 and 1:5000, respectively. Polyclonal antibodies to erbB, phosphoerbB2, erbB3 and phospho-erbB3 were used at 1:500 (Santa Cruz, Biotech, Santa Cruz, CA). Polyclonal antibody to Krox 20 (Covance, Emeryville, California) was used at 1:500, polyclonal antibody to MBP (Chemicon, Billerica, MA) was used at 1:3000, polyclonal chicken antibody to P0 (Chemicon, Billerica, MA) was used at 1:1000, polyclonal antibody to c-Jun (Santa Cruz Biotech, Santa Cruz, CA) was used at 1:400 and monoclonal antibody to α-actin (Sigma-Aldrich, St. Louis, MO) was used at 1:5000 dilution. Polyclonal antibody to Nrg1-type III (Santa Cruz Biotech, Santa Cruz, CA) was used at 1:500.

Type-II, Type-III and EGF-domain Neuregulin-1

Recombinant human sensory and motor neurons derived factor (rhSMDF, type III Nrg1) was purchased from R&D systems (Minneapolis, MN). Recombinant human glial growth factor-II (rhGGF-II, type II Nrg1) was obtained from Acorda (Hawthorne, NY) under a material transfer agreement. In this study, rhSMDF and rhGGF-II are referred simply as soluble Nrg1-type III and GGF, respectively. The soluble Nrg1-type III was the

peptide with N-terminus 296 amino acid residues containing both the EGF domain and CRD domain (Ho et al., 1995). The GGF was the peptide with N-terminus 519 amino acid residues containing the EGF like domain and the Ig-like domain (Marchionni et al., 1993). Both Nrg1 peptides lacked the transmembrane and cytoplasmic domains. The recombinant human EGF like domain fragment of Nrg1 (EGF-Nrg1) was obtained from R&D system.

Primary rat Schwann cell culture

Schwann cells were prepared from sciatic nerves of newborn rats (1–2 Days old) as described previously (Brockes et al., 1979). For routine culture, Schwann cells were grown in Dulbecco's modified Eagle's medium (DMEM) (Mediatech, Manassas, VA) with 10% Fetal Bovine Serum (FBS) (Mediatech, Manassas, VA) supplemented with 10 ng/ml of EGF-Nrg1 and 5 μM Forskolin (Sigma, St. Louis, MO). Cells between passages 2 and 4 were used in all experiments described in the text.

In vitro myelination: Dorsal root ganglion (DRG) neuron-Schwann cell co-cultures

Dissociated DRG neurons were prepared from embryonic day (E) 15.5 rat embryos or E13.5 mouse embryos as described previously (Eldridge et al., 1987)and plated onto type-1 rat tail collagen coated 12 mm glass coverslips at a density of 1.25 DRG/coverslip. Five to six hours later, the cultures were flooded with neurobasal medium (Mediatech, Manassas, VA) supplemented with B27 (GIBCO, Carlsbad, CA),

0.08% glucose, NGF (50 ng/ml) and a mixture of 15 μM 5-fluorodeoxyuridine (FdUr)(Sigma-Aldrich, St. Louis, MO) and Uridine (Sigma-Aldrich, St. Louis, MO). Cultures were maintained in the medium for additional 2–3 days in order to remove proliferating non-neuronal cells then switched to fresh medium without FdUr and Uridine mixture and maintained until the DRG axons reached the periphery of the coverslips. After the axonal networks were established, Schwann cells were plated onto the neurons at a density of 150,000 cells/coverslip in Minimal Essential Medium (MEM) (Mediatech, Manassas, VA) supplemented with 10% FBS, 0.08% glucose and 50 ng/ml NGF. Five to seven days later, cultures were switched to myelinating medium: Minimal Essential Medium (MEM) supplemented with 10% FBS, 0.08% glucose, NGF (50 ng/ml) and ascorbic acid (50 μg/ml). Eleven days later, myelination was assessed by immunostaining for MBP. Soluble Nrg1 or U0126 was added at the time of switching to the myelinating medium and replenished every two days.

Schwann cell proliferation in co-cultures

Bromo-deoxy-uridine (BrdU; 10 μM) was added to the co-cultures at the time of the Nrg1 treatment. Sixteen hours later, cultures were fixed in 4% Para-formaldehyde (PFA) for 20 minutes and then washed 3 times in PBS. The PFA fixed cultures were treated with 2N HCl for 15 minutes at 37 °C. Cells were washed 3 times in 0.1M borate buffer (pH8.5) over a 10 minute period and then washed 3 times in PBS in the same manner. Cultures were incubated in blocking solution (5% normal goat serum supplemented with 0.3% Triton X 100) for 60 minutes and incubated with monoclonal BrdU antibody prepared in blocking solution for overnight at 4°C. Cy2 conjugated-goat

anti mouse secondary antibody was added for 1 hour and before mounting, cells were incubated with DAPI for 1 minute to visualize nuclei. Percentage of BrdU incorporated Schwann cells was determined by counting the number of BrdU-positive nuclei among the DAPI-positive nuclei of the axon associated Schwann cells.

In vitro myelination: Superior cervical ganglion (SCG) neuron-Schwann cell co-cultures

Dissociated SCG neurons were prepared from postnatal day 1–2 rats as described previously (Johnson, 2001) and plated onto collagen-coated 12 mm glass coverslips at a density of 0.5 SCG/coverslip. Next day, the cultures were flooded with neurobasal medium supplemented with B27, 0.08% glucose, NGF (50 ng/ml) and a mixture of FdUr and Uridine (10 μM) and maintained in the same medium for additional 2–3 days in order to remove proliferating non-neuronal cells. The cultures were switched back to fresh medium without FdUr and Uridine mixture and maintained until the axons extend out to the periphery of the coverslips. Schwann cells were plated onto the neurons at a density of 150,000 cells/coverslip and maintained in MEM supplemented with 10% FBS, 0.08% glucose and 50 ng/ml NGF until the Schwann cells populate the axons (about 7–10 days). Myelination was initiated by placing the cultures in myelinating medium as described for DRG-Schwann cell co-culture. Five to six weeks later, myelination was assessed by MBP immunostaining. Soluble Nrg1-type III was added at the time of placing the cultures in myelinating medium and replenished every two days.

Immunofluorescence staining for MBP

DRG-Schwann cell or SCG-Schwann cell co-cultures were rinsed in phosphate buffered saline (PBS) and then fixed in 4% paraformaldehyde for 20 minutes. After washing with PBS, samples were permeabilized in ice-cold methanol for 25 minutes and then incubated in blocking solution (5% normal goat-serum + 0.3% Triton X100) for 1 hour at room temperature. This was followed by incubation with primary antibody MBP prepared in blocking solution for overnight. After washing 3 times with PBS, samples were incubated with Alexa 488 conjugated goat anti-mouse secondary antibody (Molecular Probes, Eugene, OR) for 45 minutes. Cell nuclei were stained with DAPI. After mounting, MBP+ myelin segments were examined by epifluorescence on a Nikon E800 microscope.

Assessment of myelination (Myelin Index)

Myelin index was determined as an average-fold increase in the number of myelin segments formed in experimental cultures compared to the control cultures. To account for the variability in myelin formation in individual cultures, we established three or four individual coverslips for each experimental condition, and myelin segments, visualized by MBP immunostaining, were counted on all coverslips. For control cultures, pairwise comparisons were made between all coverslips within the group, yielding 9 or 16 ratios per experiment. Ratios from multiple, independent experiments were then averaged and the value was expressed as the myelin index ± SEM for control. A value close to 1 indicates minimal variation in myelination within the control group. For the myelin index in an experimental group, a similar strategy was used to make pairwise comparisons

between three or four experimental coverslips and the control coverslips within an experiment. The experimental to control ratios from multiple experiments were then averaged, yielding the myelin index ± SEM for each experimental group.

Measurement of myelin segment length

Following immunostaining for MBP, MBP+ myelin segments from 10-20 random fields per coverslip were photographed using a 40X objective lens. The digitized images were analyzed using Metamorph software (MDS Analytical Technology) for measuring lengths of individual myelin segments. A total of 200 to 700 individual segments were analyzed from three separate experiments.

Statistical analysis

Student t-test and One way ANOVA followed by post hoc analysis was performed for all experiments using Sigma Stat 3.5 software.

Western blot analysis

To prepare cell lysates, co-cultures were washed twice in PBS and then lysed in 300 μl of ice-cold lysis buffer (20 mM Tris HCl pH7.4, 1% NP-40, 10% Glycerol, 2.5 mM EGTA, 2.5 mM EDTA, 150 mM NaCl, 20 μM leupeptin, 10 μg/ml aprotinin, 1 mM phenylmethane sulphonyl fluoride (PMSF) and 1 mM sodium orthovanadate). Lysates were cleared by centrifugation for 15 min at 14,000 rpm in the cold and the protein concentration of the supernatants was determined. Twenty μg of the lysates were size-

fractionated on 10% SDS-polyacrylamide gel and transferred onto PVDF membrane. After blocking in 5% milk, the membranes were incubated with appropriate primary antibodies prepared in 5% BSA in TBST (TBS + 0.05% Tween 20). After incubating with HRP-conjugated secondary antibodies, the protein bands were visualized by enhanced chemiluminescence (ECL).

Preparation of adult DRG neuron cultures

The dissociated DRG neurons were prepared from adult rats as described previously with modification (Burkey et al., 2004). Briefly, the spinal column of an adult rat was removed and incisions were made along the ventral and the dorsal surface to expose DRG neurons. The DRG neurons were then removed and collected in a 100-mm Petri dish containing L-15 media (Invitrogen, Carlsbad, CA). After trimming off the attached nerve strings, DRG neurons were dissociated in 0.25% collagenase for 2 hours at 37°C then dissociated mechanically by trituration using a narrow-bore glass Pasteur pipette. The Myelin debris and endogenous Schwann cells were removed by passing the dissociated DRG neurons through two layers of BSA gradient (5% and 10%) twice by centrifugation at 115xg for 4 minutes. The pellet containing DRG neurons were then suspended in Neurobasal medium supplemented with B27, 0.08% glucose and 50 ng/ml of NGF (Harlan Bioproducts, Indianapolis, Indiana) and plated onto Matrigel-coated 12 mm glass coverslips at a density of 2.5 DRG neurons per coverslip. Five to six hours later, the cultures were flooded with Neurobasal medium (Mediatech, Manassas, VA) with supplements as above and 15 µM 5-fluorodeoxyuridine (FdUr) (Sigma-Aldrich, St. Louis, MO) and Uridine (Sigma-Aldrich, St.Louis, MO). Cultures were maintained in the

medium for 6-7 days until the non-neuronal cells were removed and the axons extended out to the periphery of the coverslips.

Preparation of neurite membrane fraction from neuron cultures

Neurite membrane fractions were prepared as described previously (Maurel and Salzer 2000). Briefly, dissociated DRG neurons were prepared as described above in 35mm culture dishes until the axons extend out to the periphery. Neurons were scraped off the plate, homogenized in PBS using a Dounce homogenizer (Wheaton, USA) and centrifuged at 80xg for 20 minutes at 4°C to remove any cell debris and collagen. The supernatant was collected and the membrane fractions were collected by ultracentrifugation at 35,000xg for 1 hour at 4°C. After determining the protein concentrations, an equal amount of adult and embryonic neurite membrane fractions were centrifuged (200 X g, 10 minutes, 4°C) onto serum starved rat primary Schwann cells. After incubating at 37°C for 20 minutes, cell lysates were prepared, size fractionated on SDS-PAGE and then subjected to Western blot analysis.

In vitro myelination: Adult DRG neuron-Schwann cell co-cultures

The dissociated DRG neurons were prepared from adult rats using the modified protocol which was described previously by Burkey et al.,2004. Briefly, the adult rat was sacrificed and the spinal column was removed. Then, the cut through both dorsal and ventral surfaces was made to expose the DRG neurons. The DRG neurons were plucked and the attached nerve strings were trimmed. The isolated DRG neurons were dissociated in 0.25% collagenase for 1 hour at 37°C followed by 30 minutes incubation with 0.25%

trypsin. Then, they were dissociated and passed through the two layer BSA gradient (5% and 10%) twice by centrifugation at 800 rpm for 5 minutes to remove the myelin debris and endogenous Schwann cells. The pellet containing DRG neurons were then suspended in neurobasal medium supplemented with B27, 0.08% glucose, NGF (50 ng/ml) and plated onto matrigel coated 12mm glass coverslips at the density of 2 DRG neurons/coverslip. From this point onwards, the protocol described in the embryonic DRG/SC co-culture system was followed to setup the adult DRG/SC myelinating co-cultures. Eleven days after initiating myelination, the cultures were fixed and immunostained with MBP.

Lentivirus generation and infection

A cDNA encoding HA-tagged Nrgβ1a type III was first subcloned from pcDNA+HA-tagged Nrgβ1a type III (a gift from Talmage lab) into lentiviral vector pLL 3.7-GFP (addgene) in the place of GFP. Lentiviral vector pLL 3.7-GFP was used as a control. The lentiviral vector was transfected into 293FT cells together with a packaging plasmid psPAX2 and an envelop plasmid pMD2.G using calcium phosphate (Invitrogen). 48 hours later, the viral supernatant was harvested and infected into neurons (270μl viral soup + 30 μl NB) for 16-18 hours and then the infected neurons were placed in NB media and maintained in the same media. One week later, three coverslips were used to collect cell lysates to confirm the HA-tagged Nrg1-type III expression and rest of the coverslips were fixed in 4% PFA and immunostained with HA antibody. For the co-cultures one week after infecting the neurons, Schwann cells were plated. Approximately five days later, myelination was initiated by the addition of ascorbic acid and then followed the same protocol described earlier for embryonic DRG /Schwann cell co-culture.

Results

Soluble Nrg1-type III enhances Schwann cell myelination

To assess the role of soluble Nrg1-type III during myelination, we used a well-established *in-vitro* myelinating culture system in which Schwann cells were co-cultured with dorsal root ganglion (DRG) neurons and induced to myelinate by adding ascorbic acid to the culture medium (Eldridge et al., 1987). As a source of soluble Nrg1-type III, we used the extracellular N-terminal 296 amino acid residues containing the EGF domain and the CRD domain (Ho et al., 1995). This recombinant Nrg1 protein has been shown to activate ErbB receptors and the downstream signaling pathways in Schwann cells (Taveggia et al., 2005). Primary rat Schwann cells were seeded onto DRG neurons and allowed to proliferate in association with the axons. At the time of initiating myelination (Day 0), cultures were treated and continuously maintained in the presence of the soluble Nrg1-type III (0.1 or 1 nM). Eleven days later, cultures were fixed and immunostained for myelin basic protein (MBP) to visualize myelin segments. Representative images of the cultures are shown in Figure 1A. Cultures treated with 1 nM of soluble Nrg1-type III appeared to have a substantial increase in the number of myelin segments compared to non-treated (NT) control cultures. Quantification of the result is shown in Figure 1B: cultures treated with 1 nM soluble Nrg1-type III had a 2.9-fold increase in the number of myelin segments over control. In contrast, soluble Nrg1-type II (GGF) (Marchionni et al., 1993) inhibited myelination on DRG neurons in a dose-dependent manner (Figure 1A and 1C) as shown previously (Zanazzi et al., 2001). The recombinant EGF like domain of Nrg1 (EGF-Nrg1) had a similar effect as GGF resulting in a 2.3 fold decrease and almost a complete inhibition in myelination when used at 0.3 nM and 0.6 nM respectively (Myelin index for NT, 1.28 ± SE 0.31; 0.3 nM EGF-domain, 0.56 ± SE 0.09; 0.6 nM

EGF-domain, 0.005 ± SE 0.003). None of the Nrg1 had a significant effect on Schwann cell proliferation in co-cultures (Figure 1D).

To further analyze the myelination-promoting effect of soluble Nrg1-type III, we compared the numbers and lengths of myelin segments formed over time during the course of myelination in control and Nrg1 treated cultures. In control cultures, the number of myelin segments increased steadily following the addition of ascorbic acid (Day 0) as expected (Figure 1E). In cultures treated with soluble Nrg1-type III, the number increased more rapidly resulting in a 2.1-, 1.8- and 2.3-fold increase over the control on day 7, 11 and 15, respectively. There was also a significant increase in the myelin segment length: 86 μm, 107 μm and 112 μm in control cultures versus 116 μm, 142 μm and 141 μm in soluble Nrg1-type III treated cultures on the corresponding days (Figure 1F). Together, these results suggest that soluble Nrg1-type III elicits a pro-myelinating function in Schwann cells.

Soluble Nrg1-type III rescues the myelination defect on Nrg1 type III-deficient neurons

Mice deficient in Nrg1-type III expression exhibit defects in Schwann cell myelination. In heterozygous mutant mice, neurons are thinly myelinated and Schwann cells express reduced levels of myelin proteins and pro-myelinating transcription factors (Michailov et al., 2004, Taveggia et al., 2005). When DRG neurons from wild type, Nrg1-type III$^{+/-}$ and Nrg1-type III$^{-/-}$ neurons were co-cultured with Schwann cells under myelinating conditions, there was a marked decrease in the level of myelination in Nrg1-type III$^{+/-}$ compared to wild type cultures (Figure 2A), confirming the previous *in-vivo*

findings (Michailov et al., 2004, Taveggia et al., 2005). Neurons from Nrg1-type III$^{-/-}$ mice failed to myelinate as reported previously (Taveggia et al., 2005).To determine whether soluble Nrg1-type III could rescue the myelination defects in these neurons, wild type or mutant co-cultures were treated with increasing concentrations of soluble Nrg1-type III (0.1, 0.3 and 1 nM) and myelination was assessed as above. We found that soluble Nrg1-type III at 0.3 and 1 nM restored myelination in Nrg1-type III$^{+/-}$ cultures to wild type levels (Figure 2B). Representative images are shown in Figure 2C. A similar result was obtained when the number of myelin segments was normalized against the number of Schwann cells, indicating that the improved myelination was not due to an increase in Schwann cell number following soluble Nrg1-type III treatment (Figure 2D). In addition, there was no obvious cell death in both control and Nrg1 treated cultures (data not shown). Soluble Nrg1-type III also increased the myelin segment length in Nrg1 type-III$^{+/-}$ cultures to the wild type level (Figure 2E). Soluble Nrg1-type III, however, failed to induce myelination on Nrg1-type III$^{-/-}$ neurons at all concentrations tested (data not shown), in agreement with an earlier report (Taveggia et al., 2005). Therefore, ectopic stimulation with soluble Nrg1-type III rescues myelination defects on Nrg1-type III$^{+/-}$, but not Nrg1-type III$^{-/-}$ neurons.

The pro-myelinating function of soluble Nrg1 type III is accompanied by an increase in Krox-20 and PI3-kinase activation

Schwann cells in Nrg1-type III$^{+/-}$ mice express low levels of pro-myelinating transcription factors Krox 20 and Oct 6 (Taveggia et al., 2005).To determine whether the myelination-rescue by soluble Nrg1-type III was accompanied by an increase in the transcription factor expression, we assessed the levels of Krox 20 as well P0, a myelin-

related protein, in co-cultures treated with the soluble Nrg1-type III. Wild type co-cultures, under non-myelinating (- ascorbic acid) and myelinating conditions (+ ascorbic acid), served respectively as negative and positive controls. Under myelinating condition, P0 and Krox20 expression levels increased in wild type cultures, whereas the levels remained low in Nrg1-type III$^{+/-}$ cultures. In the presence of soluble Nrg1-type III, the expression levels increased in Nrg1 type III$^{+/-}$ cultures (Figure 2F). Membrane-bound Nrg1-type III on the axonal surface is the key Nrg1 isoform that activates the PI3-kinase pathway in associated Schwann cells (Taveggia et al., 2005). Since activation of PI3-kinase and the effector Akt is crucial for initiating myelination (Maurel and Salzer, 2000, Ogata et al., 2004), we asked whether Akt activation is decreased in Nrg1 type III$^{+/-}$ co-cultures and subsequently whether soluble Nrg1-type III could increase the activation level. As shown in Figure 2G, under myelinating condition, the level of active Akt (p-Akt) was substantially decreased in Nrg1-type III$^{+/-}$ co-cultures compared to the wild type. Treatment with soluble Nrg1-type III subsequently increased the p-Akt level. These results indicate that the pro-myelinating function of soluble Nrg1-type III is associated with an increase in Krox-20 and activation of the key signaling pathway required for myelination.

Soluble Nrg1-type III is sufficient to induce myelination on normally non-myelinated sympathetic neurons

Small diameter axons, such as those of sympathetic neurons, do not become myelinated but ensheathed due to the low levels of Nrg1-type III expression; forced expression of the type III isoform into these neurons converts them into myelinated ones,

highlighting the instructive role of the axonal Nrg1 in determining the myelination state of the PNS neurons (Taveggia et al., 2005). Therefore, we asked whether soluble Nrg1-type III was sufficient to induce myelination on normally non-myelinated axons. Sympathetic neurons of the SCG were prepared from postnatal day 2 rat pubs and co-cultured with Schwann cells. These neurons expressed lower levels of Nrg1-type III compared to the neurons of DRG (Figure 3A) as shown previously (Taveggia et al., 2005). Once the Schwann cells expanded along the axons, the cultures were maintained under myelinating condition in the continuous presence of soluble Nrg1-type III (0.1 or 1 nM) for 6 weeks, then fixed and immunostained for MBP. Images and quantification of the cultures are shown in Figure 3B. In control cultures, only a handful of myelin segments were formed despite the lengthy myelination period, which is expected of the low-myelination property of Schwann cells associated with sympathetic neurons. On the contrary, a significant number of myelin segments were formed in cultures treated with the soluble Nrg1-type III. This result indicates that the pro-myelinating function of soluble Nrg1-type III is sufficient to induce myelination on normally non-myelinated small diameter axons.

Inhibition of Mek1/Erk activation blocks the inhibitory function of GGF on myelination

The myelin-promoting effect of soluble Nrg1-type III contrasts with the inhibitory effect of GGF and the EGF-Nrg1 on myelination (Figure 1). Since we did not observe any noticeable differences in either erbB2 or erbB3 activation kinetics in Schwann cells treated with the neuregulins (Figure 4A), we hypothesized that the isoform specific

effects on myelination might be due to differential activation of the receptor downstream signaling pathways. Previous studies have implicated the Ras/Raf/Erk and PI3 kinase pathways, respectively, as negative and positive regulators of myelination and suggested that the balance between the two may determine the myelination state of the Schwann cells (Maurel and Salzer, 2000, Harrisingh et al., 2004, Ogata et al., 2004). Therefore, we compared the activation states of the two pathways in co-cultures treated with increasing concentrations of EGF-Nrg1, GGF or soluble Nrg1-type III. Cell lysates were prepared 45 minutes following the Nrg1 treatment under myelinating condition and the presence of the phosphorylated proteins was determined by Western blot analysis (Figure 4B). Activation of erbB2 and Akt appeared at concentrations as low as 0.1 nM with all three Nrg1 and increased thereafter in a dose-dependent manner. While 0.3 and 0.6 nM of EGF-Nrg1 and GGF, respectively, were sufficient to activate Erk1/2, a much higher dose (10 nM) was needed for soluble Nrg1-type III to induce Erk1/2 activation. At 1 nM (boxed lanes), one of the concentrations tested for myelination in Figure 1, the inhibitory Nrg1s (GGF and EGFNrg1) activated both Akt and Erk1/2 whereas Nrg1-type III predominantly activated Akt. To determine whether the Erk activation was associated with the inhibitory effect of GGF, co-cultures were treated with GGF along with increasing concentrations of U0126, a specific inhibitor of Erk1/2 activator Mek1/2, and maintained under myelinating condition. Western blot analysis presented in Figure 5A shows that U0126 successfully inhibited GGF-induced Erk1/2 activation in a dosage-dependent manner while it had no effect on Akt activation (+GGF lanes). Next, we assessed the effect of Mek1/2 inhibition on myelination (Figure 5B and C). Addition of U0126 to co-cultures reversed the inhibitory effect of GGF, indicated by the dose-

dependent increase in myelination. This result suggests that Mek1/2 and possibly the downstream effector Erk1/2 is likely to mediate the inhibitory effect of GGF on myelination. This result is in agreement with the previous report that growth factor-induced Mek1/2 activity inhibits myelin gene expression in Schwann cells (Ogata et al., 2004). It is also interesting to note that a continuous treatment with U0126 alone, in the absence of GGF, was sufficient to increase myelination (Figure 5D). This result suggests that the endogenous Mek/Erk activity in co-cultures is likely to work in parallel with GGF to provide additional negative signal for myelination. To determine a possible mechanism by which GGF blocks myelination, we investigated the expression levels of c-Jun, a transcription factor recently described as an inhibitor of Schwann cell differentiation and myelination (Parkinson et al., 2008). Western blot analysis on lysates prepared from co-cultures revealed that GGF treatment increased expression of c-Jun protein (Figure 5E). Subsequent treatment with U0126 decreased the c-Jun level in a dose-dependent manner, indicating that the GGF-induced c-Jun expression was dependent on Mek/Erk activity. The decrease in c-Jun was also accompanied by an increase in myelin basic protein expression. These results suggest that the inhibitory effect of GGF on myelination is associated with its induction of c-Jun through the activation of the Mek/Erk pathway.

Soluble Nrg1-type III inhibits myelination at high concentrations in a Mek/Erk-dependent manner

We observed that soluble Nrg1-type III began to activate Erk in the co-cultures when used at high concentrations (10nM) (Figure 4B), suggesting a possibility that it

might elicit a negative effect on myelination when the concentration was increased. A series of myelination assays showed that myelination was indeed inhibited in cultures treated with a high-concentration soluble Nrg1-type III (Figure 6A and B), which was in contrast to the pro-myelinating function at lower doses demonstrated in Figures 1 through 3. As with GGF, the inhibitory effect was dependent on Mek/Erk activation as co-treatment with U0126 blocked the inhibitory effect and increased myelination. These results suggest that the pro-myelinating function of soluble Nrg1-type III might not be isoform specific, but rather concentration-dependent.

GGF promotes Schwann cell myelination at low concentrations

Next, we investigated whether GGF, previously regarded as the Nrg1 isoform that inhibits myelination, would elicit a similar concentration-dependent dual effects as seen with soluble Nrg1-type III. To address this, we first assessed the concentration-dependent Akt and Erk activation kinetics in co-cultures treated with low concentrations of GGF, ranging from 0.0005 nM and to 1 nM. As shown in Figure 7A, Akt activation increased at doses as low as 0.0005 nM, whereas, a noticeable increase in Erk activity did not appear until 0.3 nM of GGF was used. Consequently, the differential activation of Akt and Erk at the low concentrations generated a narrow range of doses, i.e. between 0.0005 and 0.01 nM, in which Akt was the predominant pathway activated in response to GGF (boxed in the quantification blots in Figure 7A). Myelination assay using co-cultures showed that within the low concentration range, GGF elicited a pro-myelinating effect in a dose-dependent manner: 1.5-, 2.3-, 2.2- and 2.8-fold increase in myelination at 0.0005, 0.001, 0.003 and 0.01 nM of GGF, respectively, compared to the control (Figure 7B). At

higher concentrations, GGF inhibited myelination, coinciding with the appearance of Erk activation as shown in Figure 1. Low doses of GGF were also able to increase myelination on Nrg1-type III$^{+/-}$ neurons (Figure 7C), indicating a direct pro-myelinating effect similar to that of soluble Nrg1-type III. Altogether, these results show that ectopic stimulation with soluble Nrg1 can both promote and inhibit myelination and the isoform-independent binary choice is determined by a dose-dependent differential activation of the downstream signaling pathways in Schwann cells.

Discussion

Ectopic stimulation with soluble Nrg1-type III promotes myelination

Myelination is a complex cellular process that proceeds in several distinct stages. Prior to initiating myelination, Schwann cells align themselves along the axons and begin the process of radial sorting adopting a 1:1 relationship with the axons to be myelinated. Concomitantly, Schwann cells become polarized by positioning themselves between the axon and the basal laminae establishing two distinct membrane domains. This is followed by continuous extension and migration of the inner Schwann cell membrane around the circumference of the axon and lastly, compaction of the membrane by exclusion of the cytoplasm to the cell periphery. The membrane-bound Nrg1-type III is a key axonal protein required for myelination in the PNS, however the molecular mechanisms by which it regulates and promotes myelination remain unclear. A recent study has suggested two distinct roles of the axonal Nrg1 during myelination (Taveggia et al., 2005). In neurons lacking Nrg1-type III expression (Nrg1-type III$^{-/-}$), Schwann cells do not ensheath or segregate the axons properly and, as a result, fail to myelinate, indicating that the Nrg1 function is essential during the pre-myelination process. The study has also shown that expression of pro-myelinating transcription factors in Schwann cells is sensitive to the amount of Nrg1-type III expressed on the associated axons, thus suggesting a direct role of Nrg1 in myelination. By demonstrating that the myelination defect on Nrg1-type III$^{-/-}$ neurons could not be rescued by ectopic stimulation with soluble Nrg1-type III, the study further emphasized the importance of the "axonal" Nrg1 in initiating myelination. Therefore, an intriguing finding of the present study is the rescue of the myelination defect in Nrg1-type III$^{+/-}$ cultures by soluble Nrg1-type III

(Figures 1 and 2). Unlike Nrg1-type III$^{-/-}$ neurons, large axons of Nrg1-type III$^{+/-}$ neurons are ensheathed and sorted normally by the Schwann cells but poorly myelinated (Michailov et al., 2004, Taveggia et al., 2005). Addition of soluble Nrg1-type III, as shown in this study, then increases expression levels of Krox20 and myelin proteins leading to enhanced myelination. These data suggest that soluble Nrg1-type III elicits a pro-myelinating function, independent of the previous sorting process that is likely to require membrane-bound axonal Nrg1-type III. This notion is further supported by the finding that soluble Nrg1-type III also promotes myelination on axons of normally non-myelinated SCG neurons (Figure 3) that express low levels of Nrg1 type (Taveggia et al., 2005) but are properly ensheathed and segregated by the associated Schwann cells.

Axonal Nrg1-type III is required for the early events of myelination

The rescue of myelination by soluble Nrg1-type III of Nrg1-type III$^{+/-}$ but not of Nrg1-type III$^{-/-}$ neurons implies the exclusive requirement of the juxtacrine Nrg1 signaling during early events of axonal segregation and ensheathment. The mechanism by which membrane-bound Nrg1 regulates early axon-Schwann cell interaction remains unknown. It is possible that activation of Nrg1-ErbB receptor signaling at the Schwann cell-axon interface is important for establishing the initial polarity prior to myelination. In polarized epithelial cells, domain specific activation of ErbB2 regulates differentiation phenotype (Ramsauer et al., 2006). It is also possible that Nrg1-ErbB cooperates with other cell surface receptors or adhesion molecules that function at the axon-glial interface. Neurotrophin receptor p75 is required for myelination and localized to the adaxonal domain through its interaction with polarity protein Par-3 (Chan et al., 2006).

Several cell adhesion molecules including cadherins and Nectin-like proteins have also been shown to mediate axon-Schwann cell interaction at this junction and are required for myelination (Wanner et al., 2006, Maurel et al., 2007, Spiegel et al., 2007). Whether the ErbB receptor complex interacts with these proteins at the junction or whether the interaction has a functional significance during early events of myelination remains unknown. It is also possible that the spatial opposition between the Nrg1-ErbB at the adaxonal and the basal lamina-integrin signal at the abaxonal junction is required for the pre-myelination process (Benninger et al., 2007, Nodari et al., 2007).

Soluble Nrg1 can both promote and inhibit myelination: a binary choice determined by the concentration

In the PNS, GGF has been regarded as the Nrg1 isoform associated with the Schwann cell injury response that inhibits myelination (Huijbregts et al., 2003). Thus, an unexpected result of this study was the myelination-promoting effect of GGF when used at low concentrations. Similar observation was made with soluble Nrg1-type III, which, at high concentrations inhibited myelination while at low doses, promoted the process. This is an intriguing finding as it demonstrates that independent of the isoforms, soluble Nrg1 can elicit two contrasting biological functions under the same cellular context solely based on the amount presented to the cell. This can be explained by a concentration-dependent differential activation of the receptor downstream signaling effectors in Schwann cells shown by all three Nrg1 isoforms: our data show that the pro-myelinating function of Nrg1 is observed at concentrations that preferentially activate Akt, while the transition into the inhibitory role at higher concentrations coincides with the appearance

of Erk1/2 activation. Consequently, inhibition of the Nrg1-induded Erk1/2 activity reverses the inhibitory effect on myelination. These results support the previous notion that the overall balance between PI3-kinase and Ras/Raf/Erk activation determines the myelination state of Schwann cells (Ogata et al., 2004). Alternatively, it is also possible that Nrg1 might have an indirect effect on the signaling events that occurs in neuron, through the associated Schwann cells, thus regulating myelination (Esper and Loeb, 2004). In the CNS, while Nrg1-type III function is not required for initiating myelination, its ectopic expression in CNS neurons nevertheless enhances myelination (Brinkmann et al., 2008). Soluble Nrg1 also promotes myelination in oligodendrocyte progenitor (OPC)-DRG co-cultures; however it does not exhibit an inhibitory effect on oligodendrocyte myelination (Wang et al., 2007). This suggests a difference in the mechanisms by which myelination is regulated in oligodendrocytes and Schwann cells.

The inhibitory function of Nrg1 on myelination is likely to be mediated through activation of the Ras/Raf/Erk pathway

The Ras/Raf/Erk pathway plays an inhibitory role during Schwann cell differentiation by blocking myelin gene expression (Harrisingh et al., 2004, Ogata et al., 2004). However, its direct role during myelination has not been elucidated. Here we show that treatment with Mek1/2 inhibitor blocks the inhibitory role of ectopic soluble Nrg1 and restores myelination, suggesting that the inhibitory role of Nrg1 is likely to be mediated through its activation of the Mek/Erk pathway. The mechanism by which Mek/Erk activation inhibits myelination is unclear. A possible mechanism includes suppression of myelin gene expression as described above. Supporting this, our data

show that Mek1/2 inhibition increase MBP expression in GGF treated cultures (Figure 4).

It is also possible that the Mek/Erk pathway regulates the expression of transcription

factors involved in Schwann cell differentiation and myelination. Recently it has been

shown that c-Jun functions as a negative regulator of the myelination program in the

PNS (Parkinson et al., 2008). Accordingly, we find that the inhibitory effect of GGF is

accompanied by an increase in c-Jun expression, which is suppressed when Mek is

inhibited. Therefore, suggesting that the Mek/Erk function on myelination is in part

mediated through induction of c-Jun. Another interesting finding of the present study is

the presence of an intrinsic Mek/Erk dependent signal that serves as a negative regulator

of myelination. This was shown in experiments in which treatment of normal myelinating

co-cultures with the Mek inhibitor promoted myelination. The nature of the signal that

contributes to the Mek/Erk activity during myelination is presently unknown, although it

is likely to be axonal in origin, independent of the axonal Nrg1-type III (Taveggia et al.,

2005). Several Mek/Erk activators, such as FGF-2 and PDGF, are expressed on PNS

neurons and their receptors are found on Schwann cells (Hardy et al., 1992, Eccleston et

al., 1993, Oellig et al., 1995, Grothe and Wewetzer, 1996) Treatment with FGF-2 down-

regulates myelin gene expression and inhibits myelination *in-vitro* ((Zanazzi et al., 2001).

It will be of great interest to assess the regulatory roles of these growth factors during

myelination in the PNS.

Therapeutic potential of soluble Nrg1

The rebuilding of myelin in demyelinated lesions in the nervous system by

transplanting exogenous myelin-forming glial cells is a concept that has been explored

and tested for many years. Schwann cells offer the possibility of autologous transplantation as they are easily obtained and expanded in culture, and myelinate when transplanted in demyelinated lesions (Duncan et al., 1981, Baron-Van Evercooren et al., 1992, Morrissey et al., 1995). However Schwann cell remyelination of adult axons is often incomplete, resulting in the formation of thinner myelin sheathes and shorter internodes compared to normal nerves (Beuche and Friede, 1985, Kohama et al., 2001, Lankford et al., 2002). The pro-myelinating effect of soluble Nrg1-type III presented in this study is significant as it provides a potential therapeutic strategy for improving myelination by Schwann cells. However, it should be cautioned that concentrations above the threshold level could have a devastating consequence on the pathologic condition. Further understanding of the inhibitory role of Ras/Raf/Erk pathway on myelination might provide insights into developing a combined strategy for improving myelination.

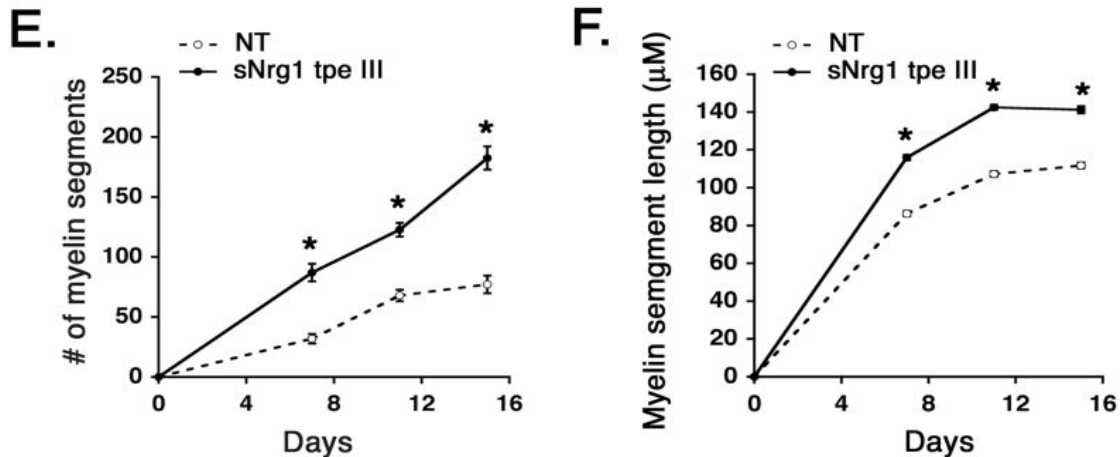# Figure 1: Soluble Nrg1 type III promotes SC myelination
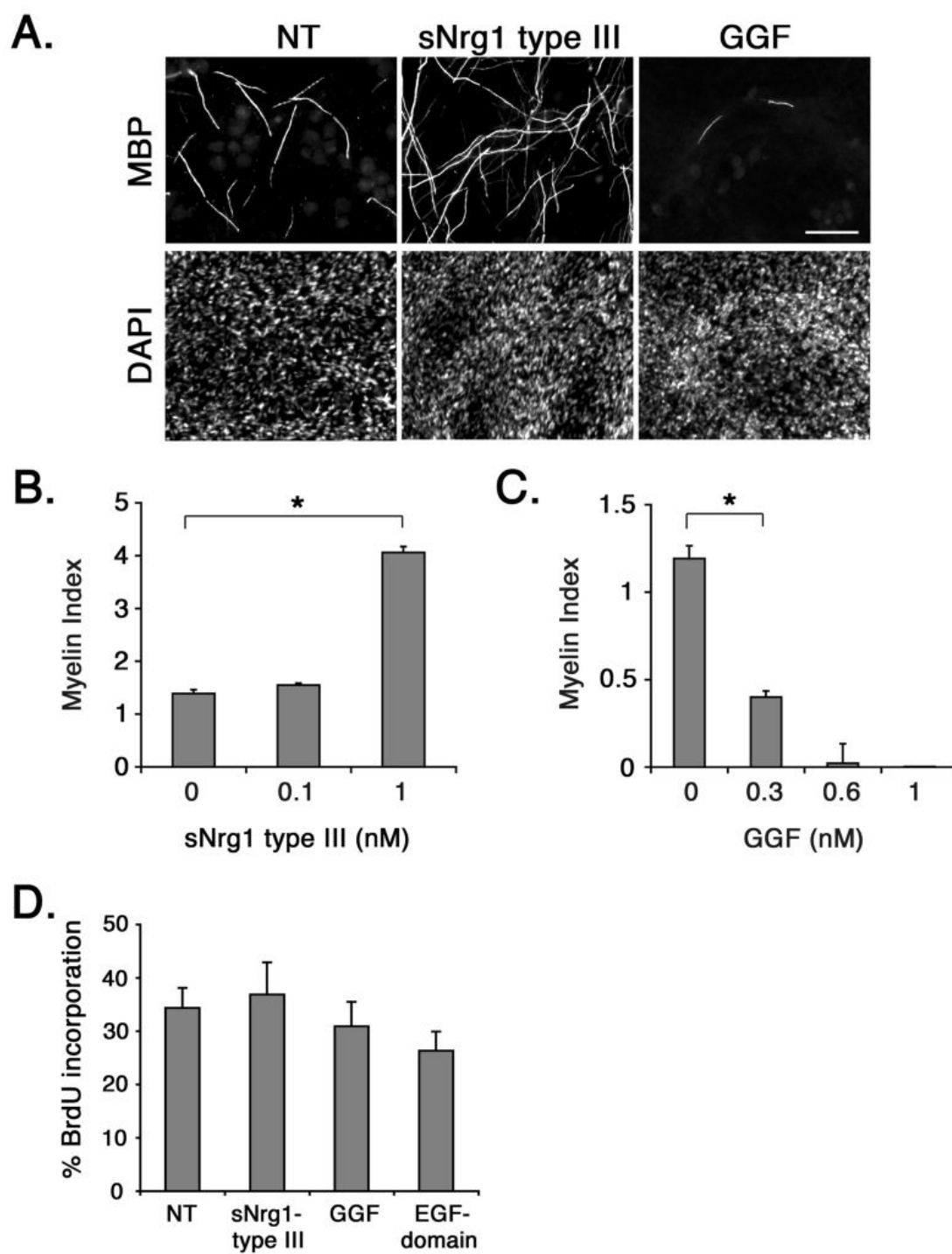
Figure 1.
Soluble Nrg1-type III promotes Schwann cell myelination. (A) Images of myelin segments formed in cocultures treated with soluble Nrg1-type III or GGF at 1 nM. Nrg1 was added at the time of initiating myelination and remained for 11 days, at which time the cultures were fixed and immunostained for MBP and DAPI. Scale bar: 100 μm. (B) Quantification of myelination in cultures treated with soluble Nrg1-type III at 0.1 and 1 nM. (C) Myelination in the presence of GGF (0.3, 0.6 and 1 nM). For (B) and (C), the data were collected from 10–12 coverslips/condition, from three separate experiments. (D) Schwann cell proliferation in co-cultures. Two hundred thousand Schwann cells were seeded onto dissociated DRG neurons. Three days later, the co-cultures were treated with Nrg1 as indicated. Sixteen to eighteen hours later, BrdU was added and 24 hours later the cultures were processed for BrdU immunostaining. There was no significant change in Schwann cell proliferation in cultures treated with soluble Nrg1-type III, GGF or EGF-Nrg1 (p = 0.427). The means ± SEM were determined from five coverslips/condition from two separate experiments. (E) Upon initiating myelination (Day 0), co-cultures were treated with or without soluble Nrg1-type III (1 nM) and the number of total myelin segments was counted on Day 7, 11 and 15. There was a significant increase (*: p<0.001) in the number of myelin segments in soluble Nrg1-type III treated cultures compared to the non-treated (NT) control. (E) The lengths of individual myelin segments were measured from myelinating co-cultures, maintained with or without (NT) soluble Nrg1-type III (1 nM) for 11 days. There was also a significant increase (* p<0.001) in the myelin segment length (mean ± SEM, n = 200) in cultures treated with soluble Nrg1-type III at all time points.

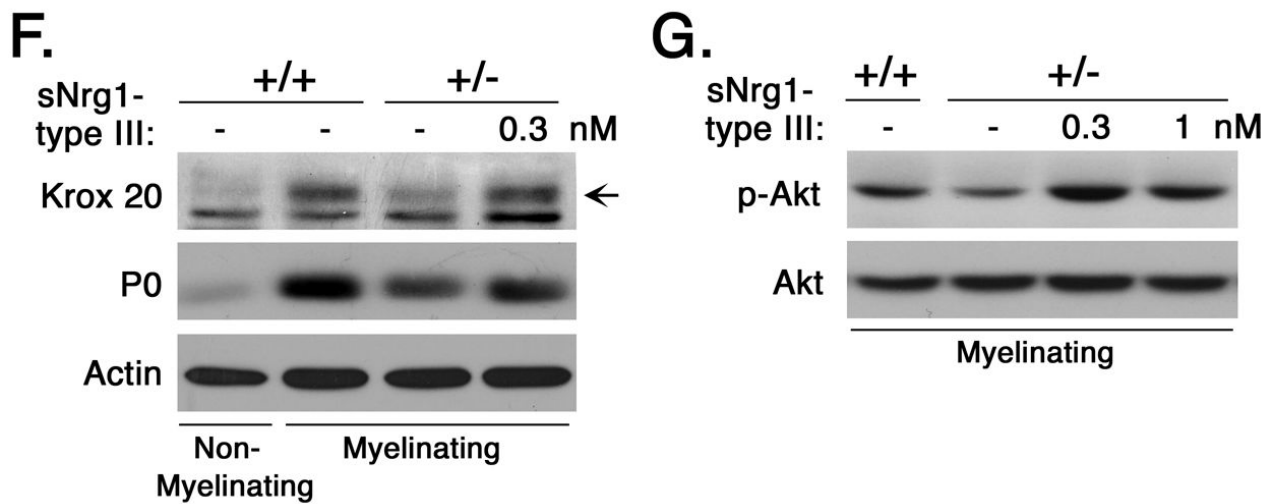**Figure 2: Soluble Nrg1-type III rescues the myelination defect on Nrg1 type III+/−neurons**
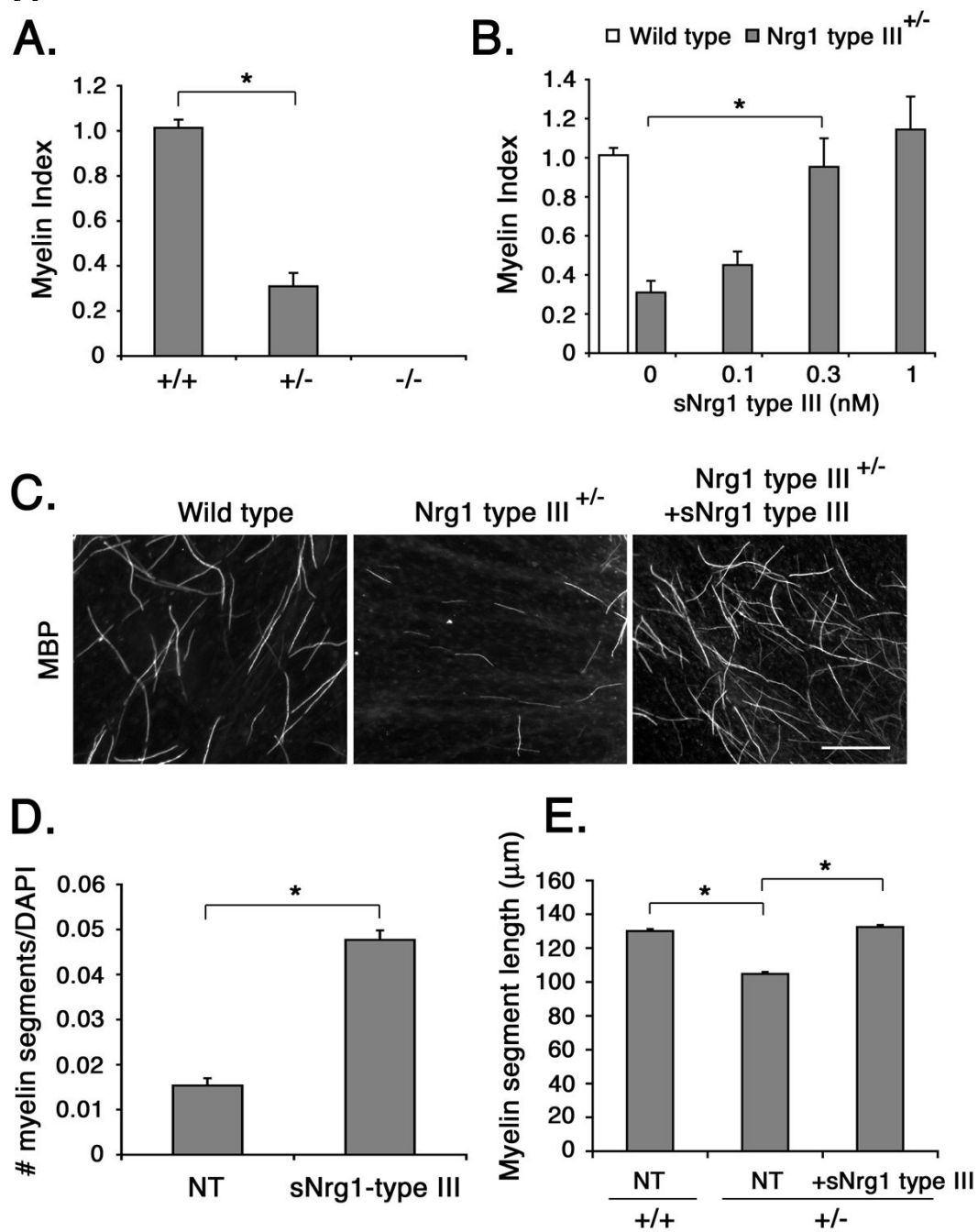
Figure 2.
Soluble Nrg1-type III rescues the myelination defect on Nrg1-type III$^{+/-}$ neurons. (A) DRG neurons from wild type, Nrg1 type III$^{+/-}$ and Nrg1 type III$^{-/-}$ mouse embryos were co-cultured with rat Schwann cells and maintained in myelinating media for 11 days. Quantification of the number of myelin segments showed a significant decrease (*: p<0.001) in myelination in Nrg1-type III$^{+/-}$ cultures whereas no myelination was observed on Nrg1-type III$^{-/-}$ neurons. (B) Myelination in Nrg1-type III$^{+/-}$ co-cultures (grey bars) treated with soluble Nrg1-type III. Wild type cultures were used as control (white bar). In (A) and (B), data shows the means ± SEM for each condition from three independent experiments. (C) Images of myelin segments formed in wild type and Nrg1-type III+/− cultures treated with soluble Nrg1 type III (1 nM). Scale bar: 100 μm. (D) The number of myelin segments per DAPI-positive Schwann cell nucleus in 11-day myelinating Nrg1-type III$^{+/-}$ co-cultures. A significant increase (*: p<0.001) in myelination is seen in cultures treated with soluble Nrg1-type III. The means (± SEM) from three independent experiments are shown. (E) The mean (± SEM) individual myelin segment lengths in wild type (+/+) and Nrg1-type III heterozygous (+/−) co-cultures on Day 11 after initiating myelination. Treatment with soluble Nrg1-type III increased the myelin segment length in Nrg1-type III$^{+/-}$ cultures to the wild type level (n=700, *: p < 0.001). (F) Soluble Nrg1-type III increases Krox 20 expression in Nrg1 type III+/− co-cultures. Lysates were prepared from Nrg1-type III$^{+/-}$ co-cultures maintained in the presence of soluble Nrg1 type III (0.3 nM) for 11 days under myelinating condition and Krox 20 and P0 expression was determined by immunoblotting. Wild type cultures under non-myelinating and myelinating condition served as negative and positive controls. (G) Soluble Nrg1-type III increases Akt activation in Nrg1-type III$^{+/-}$ co-cultures. Wild type ($^{+/+}$) or Nrg1-type III$^{+/-}$ co-cultures under myelinating condition were treated with soluble Nrg1-type III as indicated. Forty-five minutes later, cell lysates were prepared and p-Akt levels were detected by immunoblotting.

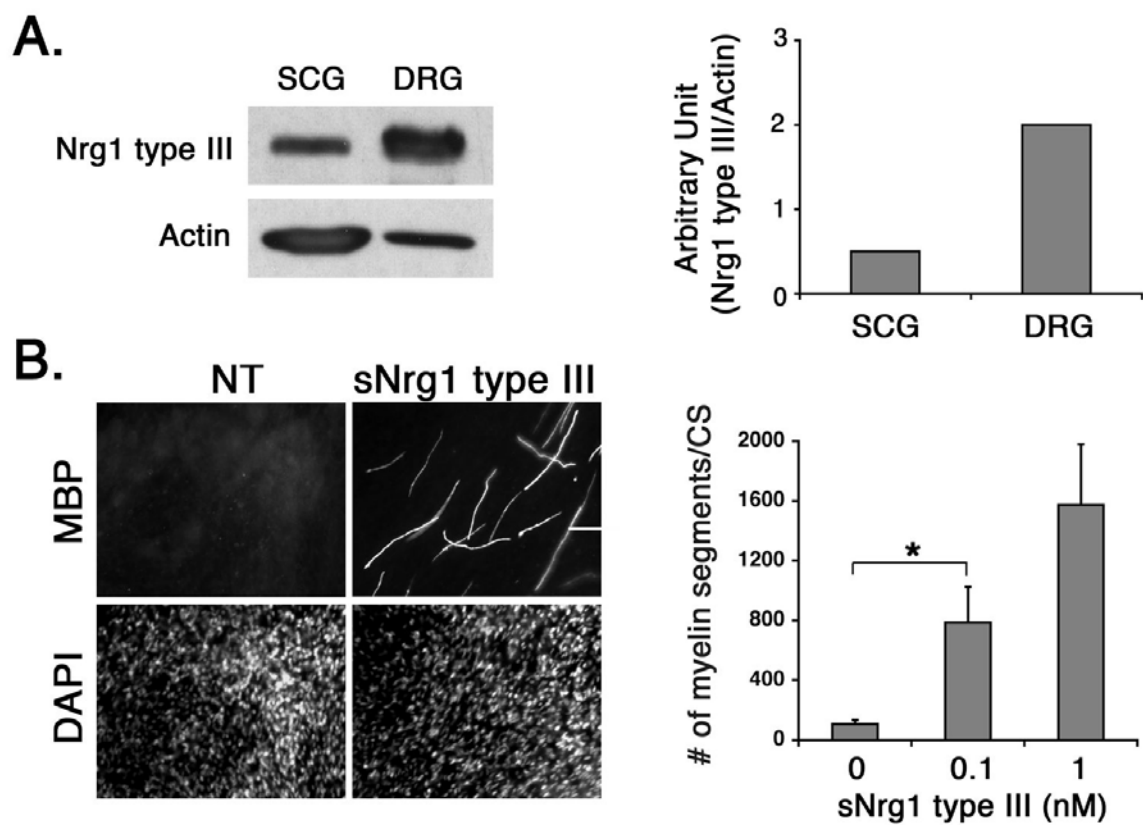**Figure 3: Soluble Nrg1-type III induces myelination on normally non-myelinated SCG neurons**

Figure 3.
Soluble Nrg1-type III induces myelination on normally non-myelinated SCG neurons.
(A) Expression levels of Nrg1-type III in SCG and DRG neurons. Cell lysates were
prepared from purified SCG and DRG neurons grown on coverslips and the Nrg1-type III
levels were determined by immunoblotting. Quantification is shown on the right, in
which the levels of Nrg1-type III are normalized against the levels of actin. (B) Schwann
cell-SCG co-cultures were maintained in myelinating media in the absence (NT) or
presence of soluble Nrg1-type III (1 nM) for 6 weeks. Cultures were fixed and
immunostained for MBP and DAPI. Scale bar: 100 μm. Quantification of the result is
shown on the right. Treatment with soluble Nrg1-type III (1nM) induced myelination on
SCG neurons, in a dose-dependent manner (*: $p < 0.002$). The means ± SEM from 12
coverslips/condition, from four independent experiments are shown.

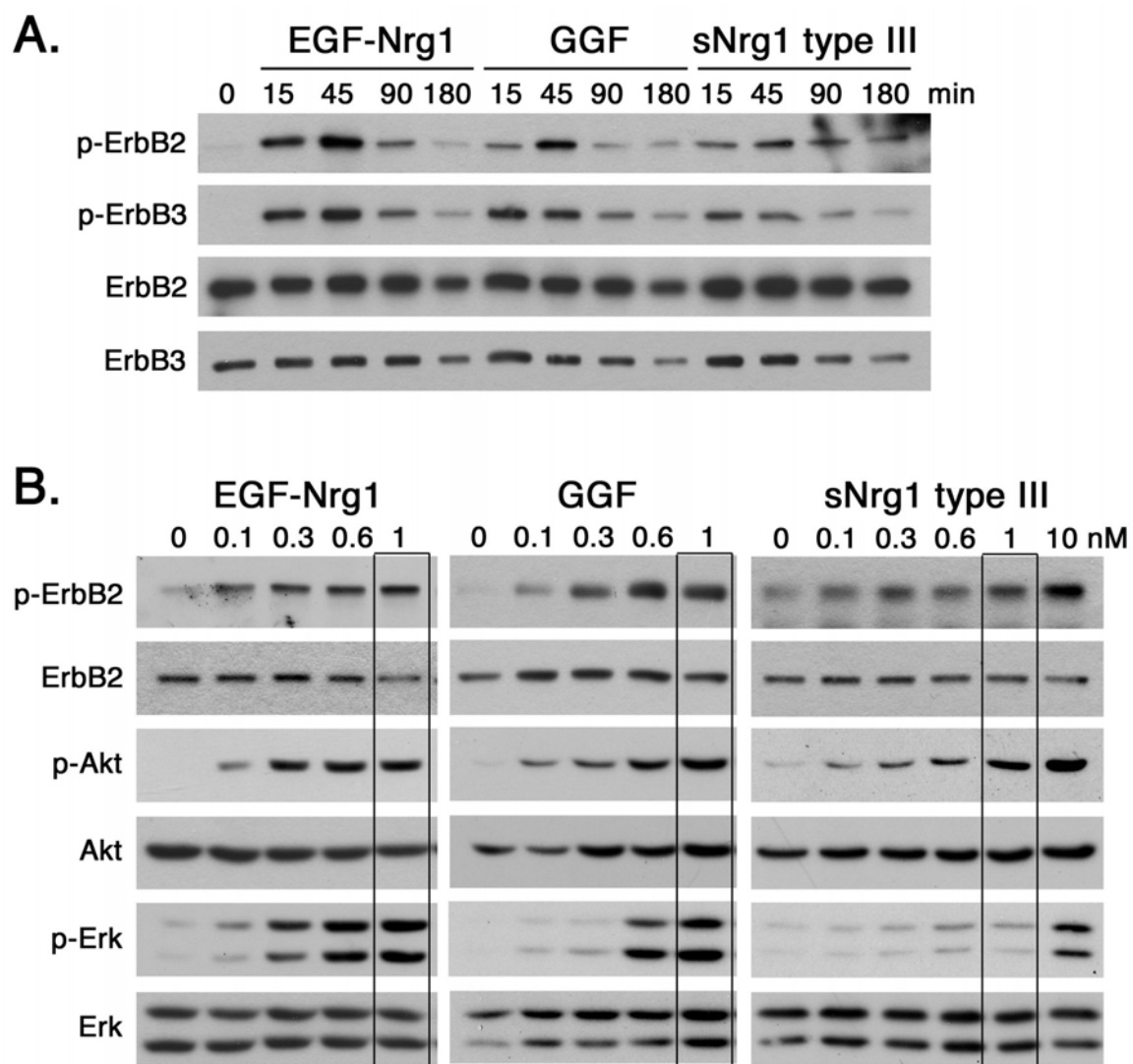**Figure 4: Differential activation of Akt and Erk in Schwann cell-DRG co-cultures**

Figure 4.
Differential activation of Akt and Erk in Schwann cell-DRG co-cultures (A) Schwann cells were treated with EGF-Nrg1, GGF or soluble Nrg1-type III at 1 nM and cell lysates were prepared at indicated times. Activation states of erbB2 and erbB3 were determined by immunoblotting using activation state specific antibodies (p-ErbB2, p-ErbB3). All three Nrg1 isoforms show similar receptor activation kinetics for both receptors. (B) Activation states of erbB2, Akt and Erk in Schwann cell-DRG co-cultures under myelinating condition. Co-cultures were treated with EGF-Nrg1, GGF or sNrg1 type III at indicated doses and forty-five minutes later, cell lysates were collected for immunoblotting. Boxed lanes show the states of erbB2, Akt and Erk activation at 1 nM, a concentration at which EGF-Nrg1 and GGF inhibited myelination while soluble Nrg1-type III promoted myelination, as shown in Figure 1.

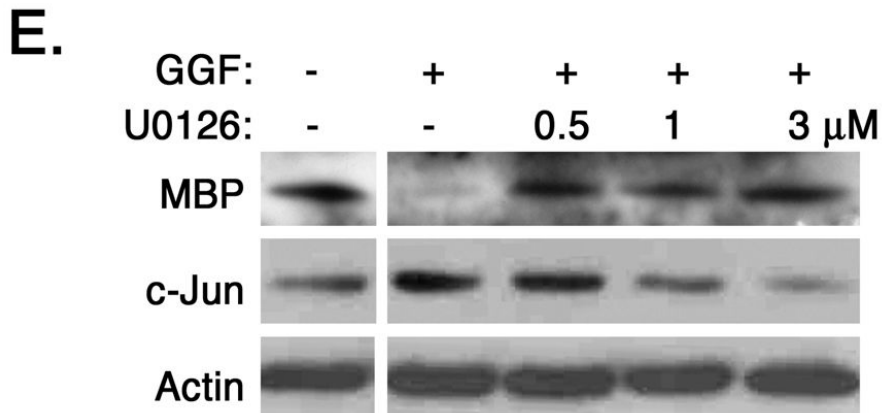**Figure 5: Inhibitory effect of GGF on myelination is blocked by inhibition of the Mek/Erk activation**
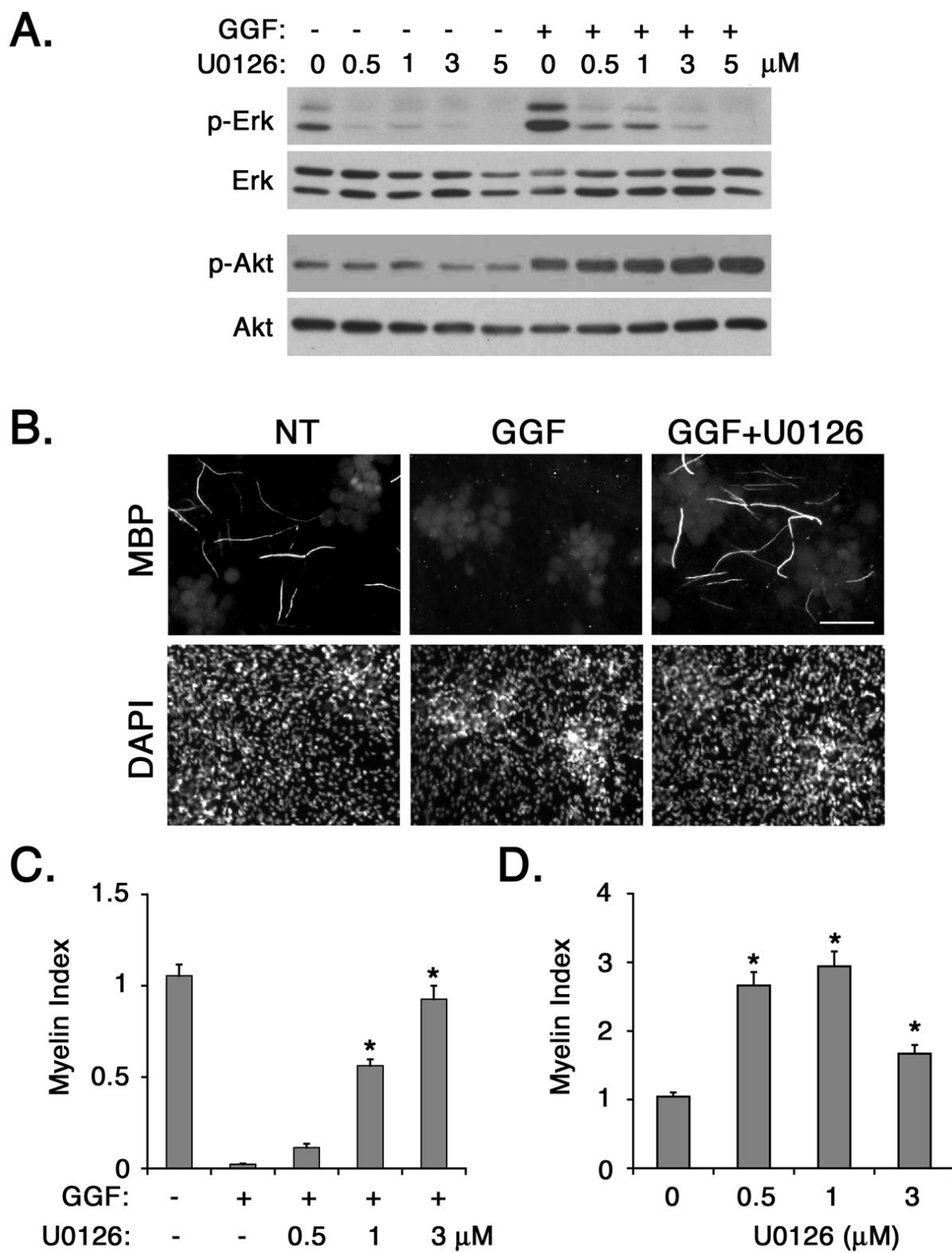
Figure 5.
Inhibitory effect of GGF on myelination is blocked by inhibition of the Mek/Erk activation. (A) Inhibition of GGF-induced Erk activation in co-cultures. Schwann cell-DRG co-cultures were pre-treated with U0126 for 30 minutes at indicated doses followed by GGF treatment (0.6 nM). Forty-five minutes later, cell lysates were prepared and the levels of p-Erk and p-Akt were determined. Treatment with U0126 inhibited both endogenous and GGF-induced Erk activation without affecting Akt activation. (B) Images of MBP+ myelin segments in 11-day myelinating co-cultures maintained with GGF alone or in combination with U0126 (1 nM). NT: no-treatment. (C) Quantification of myelination in cultures treated with GGF and increasing doses of U0126 (0.5, 1 and 3 μM). Treatment with U0126 abolished the inhibitory effect of GGF and increased myelination (*: p< 0.001). The means (± SEM) were determined from 10 coverslips per condition from two independent experiments. (D) In the absence of GGF, co-cultures were treated with increasing concentration of U0126 under myelinating condition and myelination was analyzed as above. A significant increase in myelination was observed in cultures treated with U0126 (*: p< 0.001). (E) Inhibition of GGF-induced Mek/Erk activation is accompanied by a decrease in c-Jun and an increase in MBP expression. Co-cultures were maintained under myelinating condition in the presence of GGF alone or in combination with U0126 (0.5, 1 and 3 nM) for 11 days and the cell lysates were analyzed for MBP and c-Jun expression. Levels of actin are shown as loading control.

Figure 6: Soluble Nrg1-type III inhibits myelination at high concentrations in a manner that is dependent on Mek/Erk activation
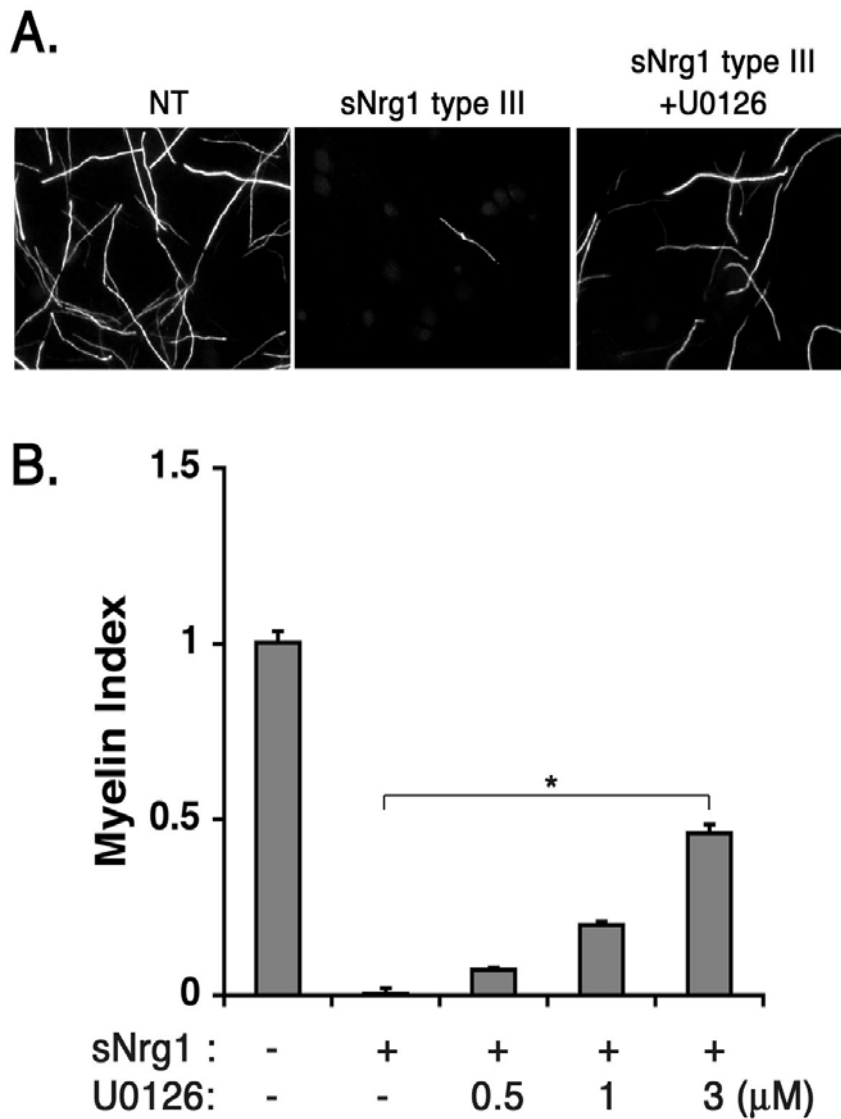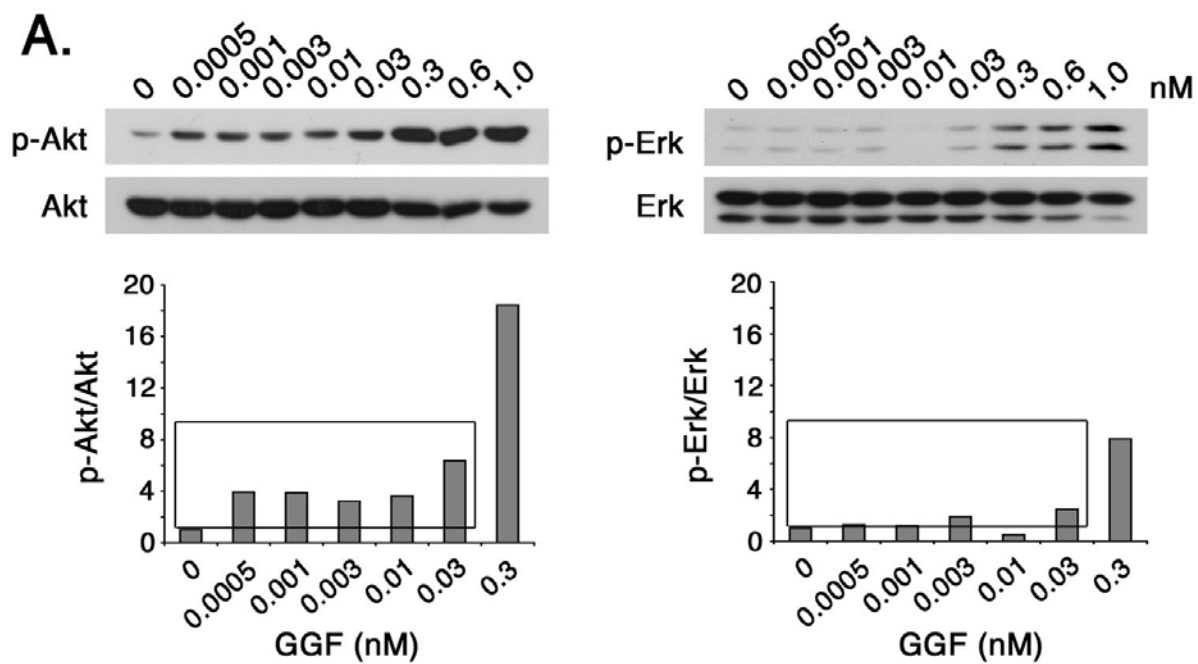

Figure 6.

Soluble Nrg1 type III inhibits myelination at high concentrations in a manner that is dependent on Mek/Erk activation. Co-cultures were maintained in myelinating media in the presence of 10 nM soluble Nrg1 type III in the absence or presence of U0126 (0.5, 1 and 3 nM). Eleven days later, myelination was evaluated. Images of the cultures are shown in (A) and quantification of the result is shown in (B). Soluble Nrg1 type III almost completely inhibited myelination. Co-treatment with U0126 reversed the inhibitory effect in a dose-dependent manner (*: p < 0.001)

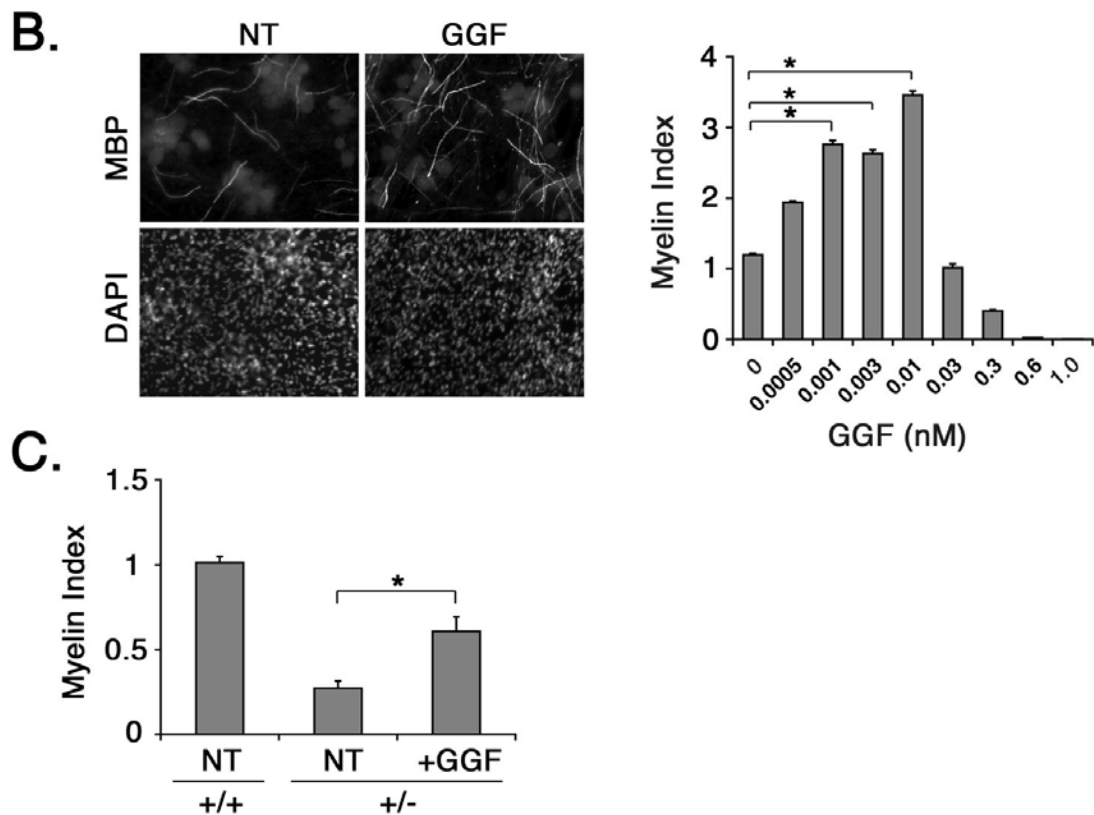**Figure 7: GGF promotes myelination at low concentrations**

Figure 7.

GGF promotes myelination at low concentrations. (A) Schwann cell-DRG co-cultures were treated with increasing concentrations of GGF ranging from 0.0005 to 1 nM. Forty-five minutes later the cell lysates were prepared and the levels of Erk and Akt activation were determined by immunoblotting. Quantifications of the blots are shown at the bottom. A window of concentrations that shows predominant Akt activation is shown (boxed). (B) Co-cultures were treated with different concentrations of GGF (0.0005, 0.001, 0.003, 0.01, 0.03, 0.3, 0.6, and 1nM) for 11 days under myelinating condition then fixed and immunostained for MBP and DAPI. Images of the control and cultures treated with 0.01nM of GGF are shown along with the quantification of the result on the right. A significant increase in myelination was seen in cultures treated with GGF at concentrations between 0.0005 and 0.01 nM compared to the control (*: $p < 0.001$). At 0.3 nM and higher, GGF inhibited myelination. The means from 12 coverslips/condition, from four separate experiments are shown. (C) Low concentration of GGF (0.01 nM) significantly increased myelination on Nrg1 type III+/− neurons (*: $p < 0.001$).

Error bars show ± SEM.

Chapter 3 - *To compare and characterize axons and Schwann cells from adult and developing PNS*

Introduction

Transplantation of myelinating glial cells, which have a potential to provide a favorable environment to regenerate and remyelinate injured axons, is one of the promising approaches for successful nerve repair after nerve injury. Glial cells such as olfactory ensheathing cells, oligodendrocytes, and Schwann cells from adult and embryos have been tested to determine their abilities to promote remyelination in adult axons (Nogradi, 2000) (Woodhoo et al., 2007). Among them, transplanted Schwann cells induce regeneration of the injured axon and promote remyelination efficiently (Li et al., 2007). Since then, Schwann cells have been considered a good candidate for efficient rebuilding of myelin at the demyelinated lesions. Schwann cells, that are isolated from the peripheral nerves of the patient and grafted at the lesion site also promoted remyelination in adult axons, suggesting a possibility for autologous transplantation.

Although exogenously transplanted Schwann cells remyelinate the CNS axons, the process is never complete. Intermittent demyelinated areas at the remyelinated region along with short and thin myelin segments have been observed (Dubois-Dalcq et al., 2005). Similar incomplete remyelination has also been observed on regenerated adult PNS axons after nerve injury (Friede and Samorajski, 1967). These observations suggest that the intrinsic properties of adult axons may be different form the developing nerve. It is possible that adult axons may not provide sufficient pro-myelinating signals to associated Schwann cells resulting in incomplete remyelination. Alternatively, it is possible that Schwann cells prepared from mature nerves may have lost their ability to remyelinate efficiently.

To address this, we first compared the myelination potentials Schwann cells prepared from adult and neonatal nerves. We also compared the myelination properties of adult and embryonic neurons. Since the axonal Nrg1-type III is the major regulator of Schwann cell myelination, we tested the possibility that adult axon may not express sufficient amount of Nrg1-type III required for myelination. Finally, we analyzed whether there are differences in the activation levels of erbB2 and Akt in Schwann cells associated with adult and embryonic axons.

Data from this part of the study show that adult Schwann cells myelinate normally, however the level of myelination on adults is reduced, suggesting that adult axons are the source of insufficient myelination. Furthermore, adult neurons express low levels of Nrg1-type III compared to embryonic neurons. Finally, low levels of Nrg1-type III on adult axons have a decreased ability to activate erbB2 and Akt.

Results

Adult Schwann cells myelinate normally in-vitro

To determine the myelination property of adult Schwann cells, Schwann cells were isolated from the sciatic nerves of adult and new born rats (1-2 days old). The adult and neonatal Schwann cells were seeded onto embryonic DRG neurons and allowed to proliferate in association with axons. Ascorbic acid was added to the co-culture to initiate myelination and eleven days later, the co-cultures were fixed and immunostained for MBP expression. The neonatal Schwann cells co-cultured with embryonic DRG neurons were myelinated robustly as expected. Adult Schwann cells co-cultured with embryonic DRG neurons also formed myelin at a comparable level as neonatal Schwann cells. Representative images are shown in Figure 8A. Quantification of the cultures showed that there was no significant difference between adult and neonatal Schwann cells in myelinating embryonic DRG neurons (Figure 8B). Taken together these data indicate that adult Schwann cells do not show any defect in myelinating the associated axons.

Myelination on adult axons is reduced compared to embryonic axons

Next I asked whether adult axons provide sufficient instructive signals to the associated Schwann cell for myelination. To address this, DRG neuron cultures were prepared from adult rats and embryos. To ensure that equal numbers of adult and embryonic neurons were plated, the plating densities of the DRG neurons were normalized to the number of neurons within each DRG. For example, 0.8 embryonic DRG and 2 adult DRG neurons, when dissociated, yielded a similar number of neurons in culture. Once established, adult and embryonic neurons were seeded with neonatal

Schwann cells and myelination was assessed as above. In control cultures with embryonic neurons, the axons were myelinated normally, as expected. In adult neuron cultures, myelination was much reduced compared to the control. The representative images are shown in Figure 9A. Quantification of results showed that there was approximately 5 fold decrease in myelination of adult axons (Figure 9B; p=>0.001).

To further confirm the defect in myelination, the number of myelin segments formed in co-culture was normalized against the number of Schwann cells. This is to rule out the possibility that difference in myelination might have been due to the difference in the Schwann cell number (see also the section below). There were an average of 0.039 and 0.0067 myelin segment per Schwann cells (DAPI) in control and adult DRG co-cultures, respectively, indicating a decrease in myelination on adult axons (Figure 9C, p=>0.001). Altogether, these data suggest that adult axons, not the adult Schwann cells, contribute to the decrease in myelination.

Schwann cells align and proliferate normally on adult axons

To further characterize the intrinsic property of the adult axons, we investigated whether the axons were capable of supporting alignment and proliferation of the associated Schwann cells prior to the onset of myelination. For this, the neonatal Schwann cells were first infected with adeno-viruses expressing GFP. Forty eight hours later, GFP expressing Schwann cells were plated onto adult and embryonic DRG neurons. Two days later, the co-cultures were fixed and immunostained for GFP (to visualize Schwann cells) and Tuj1 (to visualize axons). On the axons of embryonic DRG, Schwann cells aligned along the axis of the Tuj1 positive axons, acquiring a bipolar

morphology (Figure 10A, arrows). Similar morphology was observed in Schwann cells co-cultured with adult DRG neurons. These results demonstrate that adult DRG neurons do not affect the association of Schwann cells with their axons.

To determine whether adult axons support Schwann cell proliferation, neonatal Schwann cells were seeded onto adult and embryonic DRG neuron cultures. Two days later BrdU was added and ten hours later the cultures were fixed and immunostained for BrdU to identify the proliferating Schwann cells. The result showed that there was no significant difference in the percentage of BrdU incorporated Schwann cell associated with adult or embryonic axons (Figure 10B). This data shows that Schwann cells associated with adult axons proliferate normally. Taken together, data from the above experiments suggest that Schwann cells align and proliferate normally on adult axons.

Nrg1-type III expression is reduced in adult neurons

The onset of myelination is regulated by the presence of Nrg1-type III on the axons. Since adult axons could support the previous two events (alignment and proliferation) required for myelination, we investigated whether adult axons are defective in providing the promyelinating signal to the Schwann cells. In this study, we compared the expression levels of Nrg1-type III in adult and embryonic axons by western blot analysis. As shown in Figure 11, adult DRG neurons appeared to express lower levels of Nrg1-type III compared to the embryonic DRG neurons. Quantitative Western blot analysis showed that the amount of Nrg1-type III expressed in adult neurons was approximately three-fold lower than that of embryonic neurons. This data shows that

adult axons express low levels of Nrg1-type III, an axonal signal which is required for Schwann cell myelination.

Axon-induced Nrg-ErbB signaling is reduced by adult neurons

Axonal Nrg1-type III is the key activator of PI3-kinase in Schwann cells that is crucial for myelination. To determine whether the low levels of Nrg1-type III expressed on the surface of adult axons affected erbB2 and PI 3-kinase activation, Schwann cells, were stimulated with neurite membrane fractions prepared from adult and embryonic DRG neurons and the activation levels of ErbB2 and Akt were determined. Soluble Nrg1, a known activator of erbB receptors and Akt in Schwann cells was used as a positive control. Membrane fractions from embryonic DRG neurons stimulated erbB2 and Akt as expected. On the contrary, adult membrane fractions did not appear to increase the activation levels above the basal level (Figure 12). Therefore, adult axons which express low levels of Nrg1-type III also fail to increase the erbB-Akt activation, an important promyelinating signal necessary to induce myelination.

Discussion

Axons, not the Schwann cells, are the source of insufficient myelination in the adult PNS

Studies with autologously transplanted Schwann cells provided a positive prospect to efficient nerve repair and restoration of nerve conduction after nerve injury. The Schwann cells can be easily isolated from the peripheral nerves of the patient, expanded *in-vitro* and grafted at the lesion site (Morrissey et al., 1995), ruling out the possibility of graft rejection. Despite all these benefits, remyelination of both PNS and CNS axons by transplanted Schwann cells is not complete (Friede and Samorajski, 1967, Kohama et al., 2001, Lankford et al., 2002). The reason is unknown, although there are some possibilities that may be considered. Adult axons might not provide sufficient pro-myelinating signals to associated Schwann cells to attain optimum myelination. Another possibility is that adult Schwann cells might not be as efficient in myelinating axons as their counterparts of developing nerve. An interesting finding from this current study was that adult Schwann cells myelinated embryonic DRG axons as efficiently as neonatal Schwann cells whereas myelination of adult DRG axons by Schwann cells was reduced compared to the embryonic DRG axons (Figures 8 and 9), implying that axons contributed to the insufficient myelination in adults.

Reduced Nrg1-type III levels lead to decreased Nrg1-ErbB signaling in adults

Since Nrg1-type III is the key factor that grades Schwann cell myelination (Taveggia et al., 2005), one can speculate that alterations in Nrg1-type III expression in adult axons may be the source for this myelination defect. Our data also demonstrate that adult neurons in culture express reduced amount of Nrg1-type III compared to embryonic neurons. This is in agreement with the previous study which showed that expression levels of Nrg1-type III is reduced postnatally in developing sciatic nerve (Taveggia et al., 2005) . Axonal Nrg1-type III is the key activator of PI 3-kinase/Akt, a positive regulator of myelination (Taveggia et al., 2005, Goebbels et al., 2010). Our data show that reduced axonal Nrg1-type III present on adult axons resulted in reduced activation of Akt in Schwann cells (Figure 12), suggesting that this could be the possible reason for impaired myelination (Syed and Kim, 2010).

Reduced Nrg1 on adult axons does not impair Schwann cell proliferation and alignment

Nrg1-erbB signaling is also important for Schwann cell association and proliferation of Schwann cells along the axons. Therefore, it is possible that low levels of Nrg1-type III on adult axons could affect these early events that precede myelination. Our data show that the amount of Nrg1-type III expressed on the surface of the adult axons is sufficient to support Schwann cell proliferation and proper association along the axons

(figure 10). Therefore, the incomplete myelination observed in adults is not due to the secondary effect of defect in association and proliferation of Schwann cells.

Altogether, results from this part of the study suggest that the reduced Nrg1-type III on adult axons is the limiting factor for myelination. Therefore a therapeutic strategy to increase the Nrg1-erbB signaling in transplanted Schwann cell may improve remyelination and restore nerve conduction.

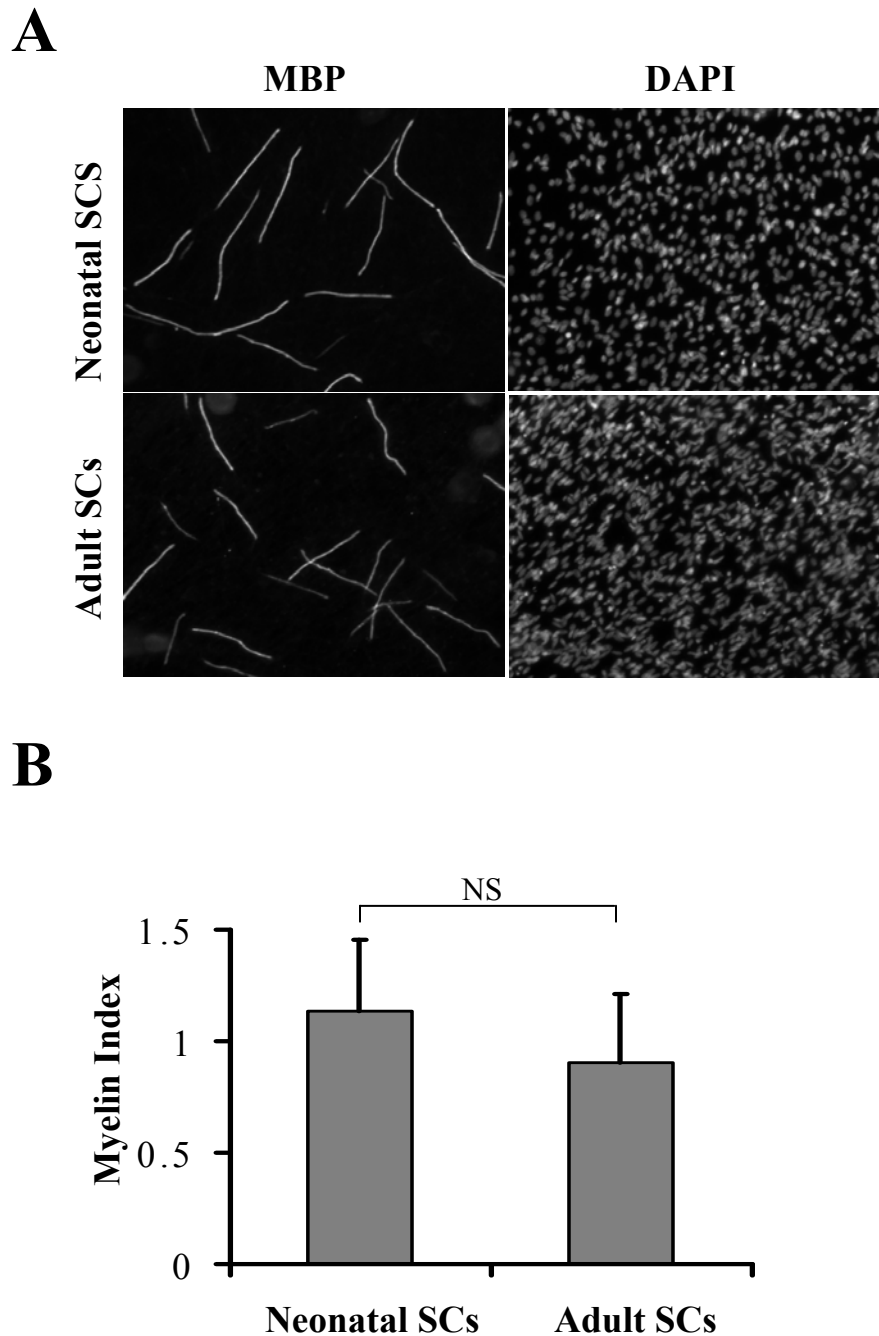**Figure 8: Adult Schwann cells myelinate normally *in-vitro***

A

B

Figure 8:
 Adult Schwann cells myelinate normally *in-vitro*
(A) Images of myelin segments formed in embryonic DRG neurons co-cultured with neo-natal or adult Schwann cells. (B) Quantification of myelin segments formed in the co-cultures shows that there is no significant difference (p=0.684) between neo-natal and adult Schwann cells in myelinating embryonic DRG neurons. The means ± SEM from 3 coverslips/condition from two independent experiments are shown.

Figure 9: Reduced myelination on adult axons compared to embryonic axons
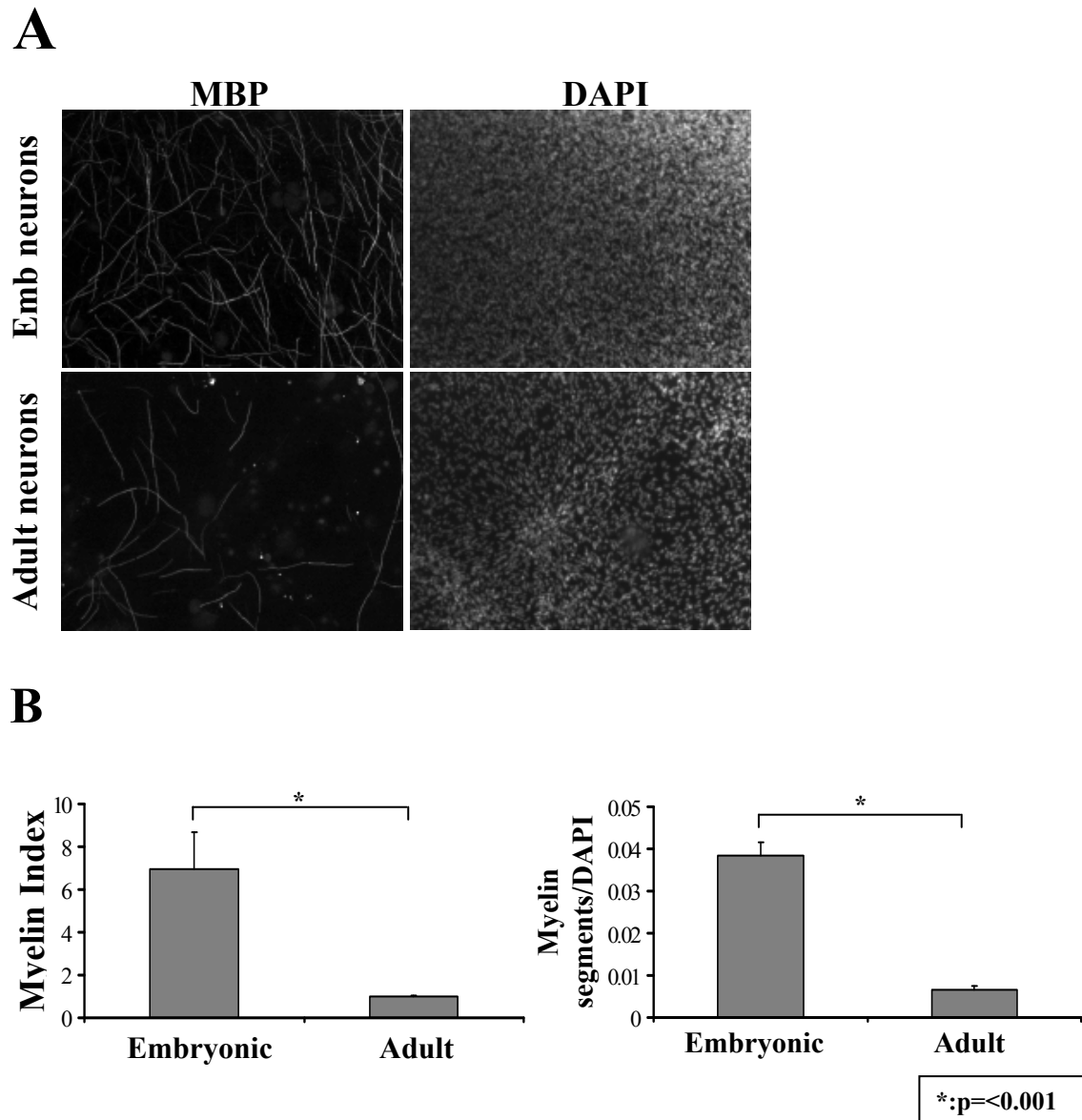
A

B

*:p=<0.001

Figure 9:
Reduced myelination on adult axons compared to embryonic axons
(A) Images of myelin segments formed in embryonic or adult DRG neurons co-cultured with neo-natal Schwann cells. (B) Quantification of the number of myelin segments formed (Myelin index) and quantification of the number of myelin segments per DAPI-positive Schwann cell nuclei (Myelin segments / DAPI) showed that there was a significant reduction in myelination of adult DRG axons by associated neo-natal Schwann cells (*p=<0.001). The means ± SEM from two independent experiments (3-4 coverslips/condition) are shown.

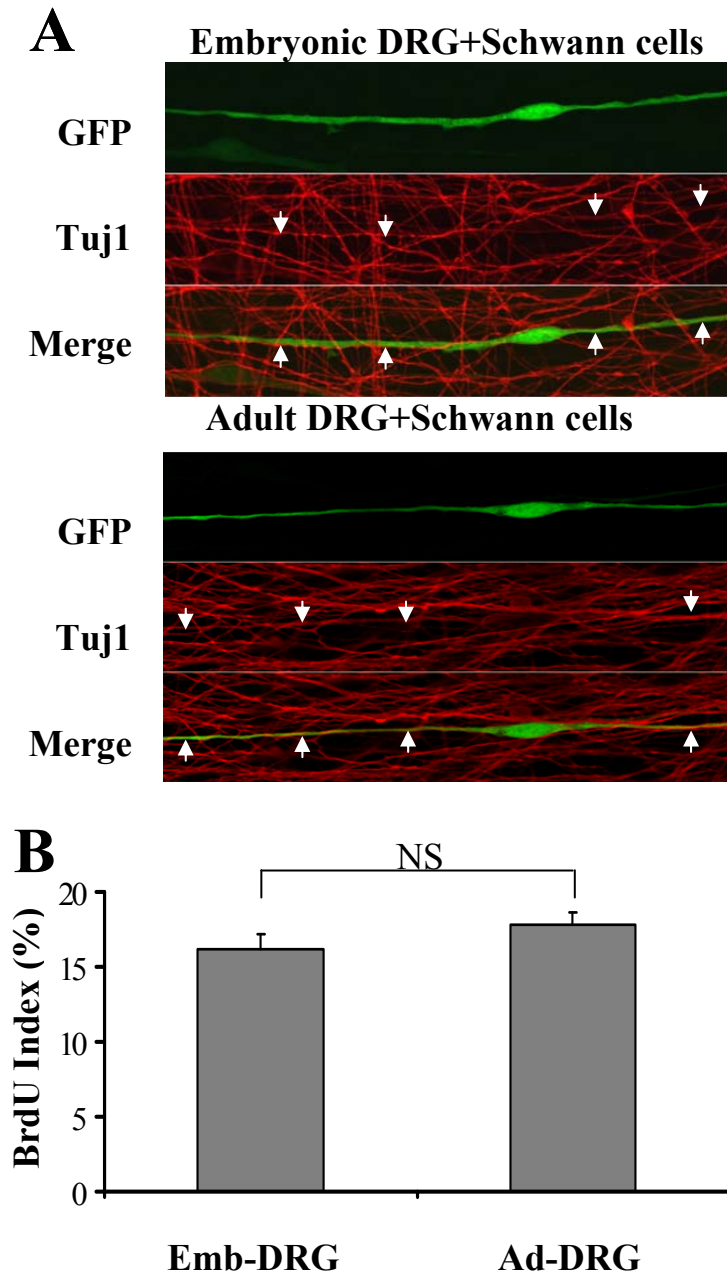# Figure 10: Schwann cells align and proliferate normally on adult axons

Figure 10:
Schwann cells align and proliferate normally on adult axons

(A) GFP-labelled neonatal Schwann cells were plated onto adult or embryonic DRG neurons and three days later cultures were fixed and immunostained with GFP (to label Schwann cells) and Tuj1 (to label neurons). Images showed that the Schwann cells aligned normally on adult DRG axons. (B) No significant difference in proliferation between Schwann cells associated with adult or embryonic DRG neurons (*p=0.201). The means ± SEM from two independent experiments (3 coverslips/condition) are shown.

Figure 11: Nrg1-type III expression is reduced in adult neurons

Figure 11:
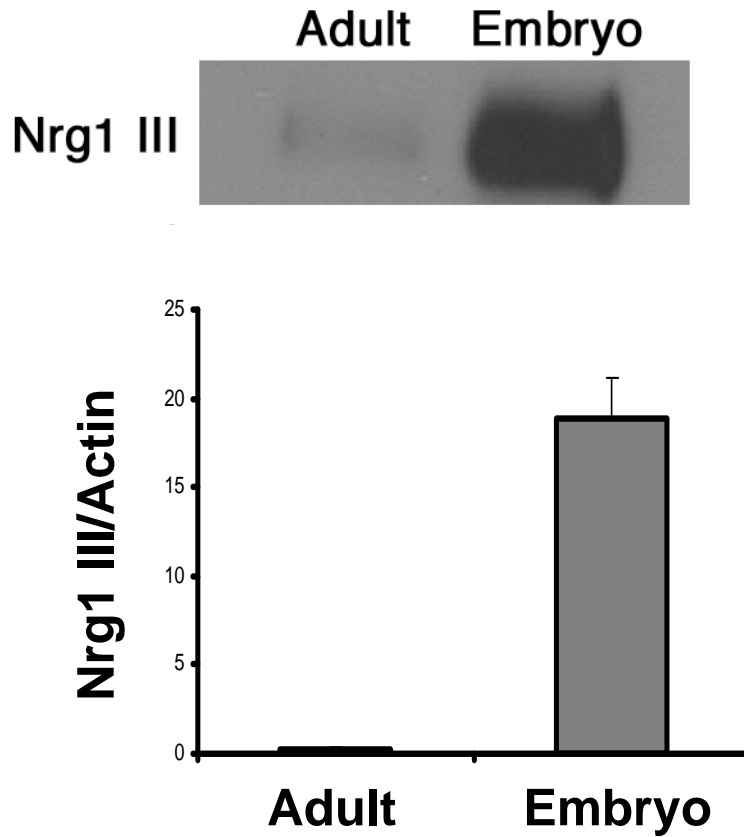Expression of Nrg1 type III in adult and embryonic neurons. Cell lysates were prepared from purified adult and embryonic neuron only cultures and subjected to Western blotting analysis. Quantification of the result normalized by the amount of actin showed that Nrg1-type III expression was reduced in adult neurons compared to embryonic neurons (Quantification was done by combining three separate experiments).

Figure 12: Axon induced Nrg1-ErbB signaling is reduced by adult neurons

A
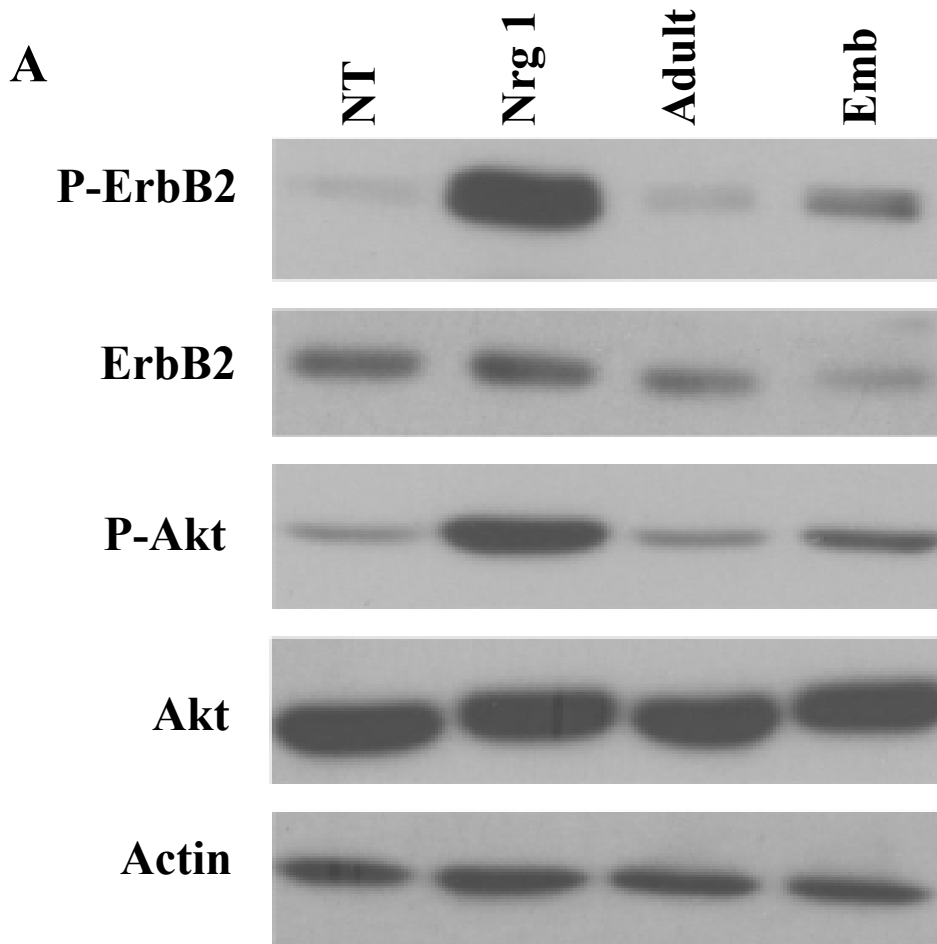
Figure 12:

Axon induced Nrg1-erbB signaling is reduced by adult neurons

A) Neurite membrane fractions from adult and embryonic neurons were prepared and plated onto Schwann cell mono-culture. Twenty minutes later, cell lysates were collected and subjected to Western blot analysis. The levels of ErbB2 and Akt activation are lower in Schwann cells treated with adult neurite membrane fractions. Actin was used as a load control.

Chapter 4 - *To determine whether enhancing Nrg1-type III signaling improves myelination of adult axons*

Introduction

Demyelinating neuropathies result in chronic demyelination and disturbances in the propagation of electrical nerve signals along the axons. Functional recovery can only be achieved by promoting efficient remyelination. Experimental transplantation of Schwann cells has shown a promising effect on re-growing injured axons into their original targets and rebuilding the myelin (Blakemore, 1977, Duncan et al., 1981, Baron-Van Evercooren et al., 1992). However, both in the CNS and the PNS, the remyelination by transplanted Schwann cells is incomplete (Beuche and Friede, 1985, Kohama et al., 2001, Lankford et al., 2002) as described earlier. Data from Chapter 3 showed that adult axons express low levels of Nrg1-type III and this could be a possible reason for insufficient Schwann cell remyelination in adults. Previously, we have shown that soluble Nrg1-type III promotes myelination of axons with low levels of axonal Nrg1-type III (Syed et al., 2010). Ectopic expression of the membrane bound Nrg1-type III also enhance myelination both *in-vivo* and *in-vitro* (Michailov et al., 2004, Taveggia et al., 2005). Therefore, we investigated whether enhancing Nrg1-type III signaling would improve myelination in adults.

For this study, we used two experimental strategies: 1) To increase Nrg1 signaling in adult DRG co-culture by treatment with soluble Nrg1-type III, 2) To increase the axonal Nrg1-type III expression in adult neurons by lenti viral mediated transduction. Surprisingly, data from this part of the study show that neither soluble nor membrane bound Nrg1-type III was sufficient to enhance myelination of adult axons. Further analysis revealed that presence of an inhibitory signal in addition to the low Nrg1-type III expression contributes to the impaired myelination of adult axons.

Results

Soluble Nrg1-type III does not enhance myelination on adult axons

We have shown previously that soluble Nrg1-type III elicits a promyelinating effect in a dosage dependent manner (Chapter 2). Soluble Nrg1-type III can also rescue the myelination defect on axons with low levels of the axonal membrane bound Nrg1-type III. To determine whether soluble Nrg1-type III enhances myelination on adult axons myelinating co-culture were established using DRG neurons from adult rats. Control cultures were prepared using embryonic DRG neurons. Both cultures were seeded with neonatal Schwann cells. At the time of initiating myelination, the cultures were treated with 0.03, 0.06, and 0.6 nM of soluble Nrg1-type III, concentrations that were shown to enhance myelination on embryonic DRG cultures. Cultures without Nrg1-type III treatment served as controls. Eleven days later, cultures were fixed and immunostained for MBP. In embryonic DRG cultures, soluble Nrg1-type III enhanced myelination (Figure 13A) as we have shown previously (Chapter 2). In adult DRG co-cultures, however, the soluble Nrg1-type III did not have any significant effect on myelination (Figure 13 B). These observations show that soluble Nrg1-type III is not sufficient to enhance myelination on adult axons.

Expression of membrane bound Nrg1-type III does not enhance myelination on adult axons

Ectopic expression of the membrane bound Nrg1-type III increases myelination both *in-vivo* and *in-vitro* (Michailov et al., 2004, Taveggia et al., 2005). Since the soluble

Nrg1-type III was not able to promote myelination on adult axons, we investigated whether ectopically expressed membrane bound Nrg1-type III could enhance myelination on the adult axons. To address this, cDNA encoding a full length Nrg1-type III (HA tagged) was cloned into a lentiviral vector. Lentiviral vector harboring GFP without the insert was used as a control. The viral vectors were transfected into 293 FT cells to generate lentiviruses. To confirm lentiviral mediated expression of the Nrg1-type III, we first infected Hela cells with the lentiviruses and determined the transgene expression by Western blot analysis. Using an antibody against the HA-tag, a ~135 kDa band, the expected size of Nrg1-type III, was detected in cells infected with Nrg1-type III virus (Figure 14A). This data shows the successful lentiviral driven expression of exogenous HA-tagged Nrg1-type III in Hela cells.

Next, embryonic and adult DRG neurons were infected with lentiviruses expressing HA-tagged Nrg1-type III. One week later, the cell lysates were prepared and subjected to Western blot analysis. In order to distinguish the endogenous Nrg1-type III from the transgenic protein, the lysates were probed with an antibody to the c-terminus of Nrg1 and HA. In control cultures, Nrg1-type III expression was low in adult neurons compared to the embryonic DRG neurons as shown previously (Chapter 3). Nrg1-type III was expressed more robustly in the Nrg1-type III infected adult and embryonic neurons compared to the controls (Figure 14 B). These observations show that the lentiviral mediated infection drive the expression of exogenous membrane bound Nrg1-type III in adult and embryonic neurons. To further confirm the expression of exogenously expressed Nrg1-type III in neurons, the embryonic DRG neurons were infected with lentiviruses carrying HA-tagged Nrg1-type III as described above. Ten days later, the

cultures were fixed and immunostained for HA and neurofilament (NF). The images in figure 14 C showed that the exogenously expressed HA-tagged Nrg1-type III was expressed in infected neurons.

Ectopically expressed Nrg1-type III on the axonal membrane is expected to increase the erbB-PI 3-kinase signaling in the associated Schwann cells. To investigate this, adult DRG neurons expressing the Nrg1-type III transgene were seeded with Schwann cells and nine days after initiating myelination, the activation levels of erbB2 and Akt were determined. Western blot analysis showed that there was a noticeable increase in the activation levels of ErbB2 and Akt in co-cultures, expressing Nrg1-type III, compared to the control cultures in which neurons were infected with lentiviruses expressing GFP alone (Figure 15). This result demonstrates that exogenously expressed Nrg1-type III is functional and activates the erbB-PI3-kinase in the associated Schwann cells.

To determine whether the expression of full length Nrg1-type III could enhance myelination on adult axons, control and Nrg1-type III infected neurons were co-cultured with Schwann cells and myelination was assessed by MBP immunostaining. Quantification of myelin segments showed (Figure 16B) that there was no significant difference between control (pLL-GFP) and the Nrg1-type III expressing neurons (pLL-Nrg1-type III) in promoting Schwann cell myelination. The representative images are shown in Figure 16A. Therefore, increasing the amount of membrane bound Nrg1-type III on the axonal membrane is not sufficient to enhance myelination on adult axons.

Increased activation of negative signals in adult DRG neuron-Schwann cell co-culture

Our data so far show that increasing the promyelinating Nrg1 signal is not sufficient to enhance myelination on adult axons. Since Schwann cell myelination is achieved by the balance between positive and negative regulators (Jessen and Mirsky, 2008), it is possible that myelination on adult axons is impaired by an axonal signal that activate negative regulators of myelination in the associated Schwann cells. Supporting this hypothesis, we observed that there was an elevated level of Erk1/2 activation in adult DRG neuron co-cultures, especially during the early stage of myelination (Figure 17). Previous studies including our own showed that increased Erk1.2 activation inhibited myelination (Harrisingh et al., 2004, Ogata et al., 2004, Syed et al., 2010). Therefore, increased Erk1/2 activation could be the possible negative signal that prevents the enhancement of myelination on adult axons.

To elucidate the role of active Erk1/2 in adults, we used U0126 (1 nM), an inhibitor of the upstream Mek1/2. Adult DRG co-cultures were treated with U0126 at the time of initiating myelination and maintained in the presence of the inhibitor. Eleven days later, the co-cultures were fixed and immunostained for MBP. There was a significant increase in myelination of adult axons in the presence of U0126; approximately two-fold increase in myelination was observed when Erk1/2 activation was inhibited in co-cultures (Figure 18). This result shows that increase in the activation of Erk1/2, a negative signal for myelination, contributes to the low myelination property of the adult axons. Our data also shows that inhibition of the negative signal rather than improving the promyelinating signal may provide efficient signaling for improving myelination in adults.

Discussion

Enhancing Nrg1-type III signaling on adult axons alone is not enough to promote myelination

The objective of this study was to find a way to enhance myelination of adult axons by Schwann cells in-vitro. Since adult neurons have limited potential to induce axonal Nrg1-erbB signaling (Chapter 3), a major signaling pathway that is required for myelination, we hypothesized that treatment with soluble Nrg1-type III could enhance myelination in adult DRG neuron co-culture. Despite the pro-myelinating effect on embryonic neurons soluble Nrg1-type III did not recapitulate on adult axons (Figure 13). This result shows that the signaling mechanism of myelination during development and in adult may be different.

Previous study has shown that axonal Nrg1-type III plays an important role during remyelination in adult (Jessen and Mirsky, 2005, Fricker et al., 2011). Therefore, we speculated that ectopic expression of Nrg1-type III on the surface of adult axon. Interestingly, ectopic Nrg1-type III also failed to promote myelination on adult axons (figure 16). This observation raised questions regarding the inability of Nrg1-type III to promote myelination in adults: 1) Nrg1 signaling alone may not be sufficient to induce remyelination in adults or; 2) other growth factors may regulate remyelination in adults. For example, IGF signaling activates PI3-kinase/Akt and promotes the expression of myelin genes *in-vitro* (Ogata et al., 2004). IGF mRNA expression is up regulated in the peripheral nerve after sciatic nerve injury (Pu et al., 1995) ; 3) adult axons provide negative signals to Schwann cells, thus suppresses the positive pro-myelinating signals.

Considering the importance of axonal Nrg1 during regeneration and remyelination (Fricker et al., 2011), the third possibility would be a considerable interest to analyze further.

Myelination is regulated by negative signals

Balance between positive and negative signals regulate myelination during development (Jessen and Mirsky, 2008). However, the axonal signals that activate the negative signals in Schwann cells are not clearly understood. Based on their function during Schwann cell demyelination and dedifferentiation, Erk1/2, JNK, and p38 MAPK have been considered as the negative regulators of myelination. In our study, we detected elevated levels of active Erk1/2 at the time of initiation of myelination (Figure 17), possibly blocking the promyelinating signals in adults.

Activated Erk1/2 is a negative signal that inhibits myelination in adult DRG co-culture

The elevated Erk1/2 activity during the early stages of myelination in adult DRG co-cultures raises the possibility that inhibiting the Erk1/2 activation might promote myelination. When we blocked Mek1/2 in co-cultures using a pharamacological inhibitor U0126 we were able to increase myelination on adult axons (Figure 18). During regeneration and remyelination in adult PNS after injury, Erk1/2 activity is increased (Yamazaki et al., 2009). Our data suggest that the high levels of activated Erk1/2 may contribute to the incomplete remyelination after injury. *In-vivo* inhibition of active Erk1/2 and determining its effect on remyelination is of a great interest for the future study.

The signal that activate Erk1/2 in Schwann cells is unknown . Several Mek/Erk activators, such as Ig-Nrg1, FGF-2 and PDGF, are expressed on PNS neurons and their receptors are found on Schwann cells (Hardy et al., 1992; Eccleston et al., 1993; Oellig et al., 1995; Grothe and Wewetzer, 1996)(Carroll et al., 1997). *In-vitro* experiments showed that treatment with FGF-2 down-regulates myelin gene expression and inhibits myelination. Ig-Nrg1 also inhibits myelination and induces demyelination (Zanazzi et al., 2001). PDGF treatment has been shown to suppress the expression of myelin associated proteins (Ogata et al., 2004). All these observations suggest that axonally expressed growth factors could provide negative signals to Schwann cells during myelination.

The other negative regulators such as p38 MAPK and JNK are also activated in Schwann cells following peripheral nerve after injury. Prior to remyelination, for example, the activity of p38 MAPK is sustained up to 28 days after injury well into the remyelination phase (Yamazaki et al., 2009). Therefore it is possible that p38 MAPK may also serve as a negative regulator that inhibits Schwann cell myelination. Supporting this we have shown recently that inhibition of p38 MAPK during the course of myelination promotes myelination *in-vitro* (Yang et al., in press). It would be interesting to understand the effect of p38 MAPK inhibition on myelination of adult axons.

Altogether, our data suggest that enhancing Nrg1-type III signaling in combination with inhibiting Erk1/2 activation might promote optimum remyelination and functional recovery in adult.

Figure 13: Soluble Nrg1-type III does not enhance myelination on adult axons
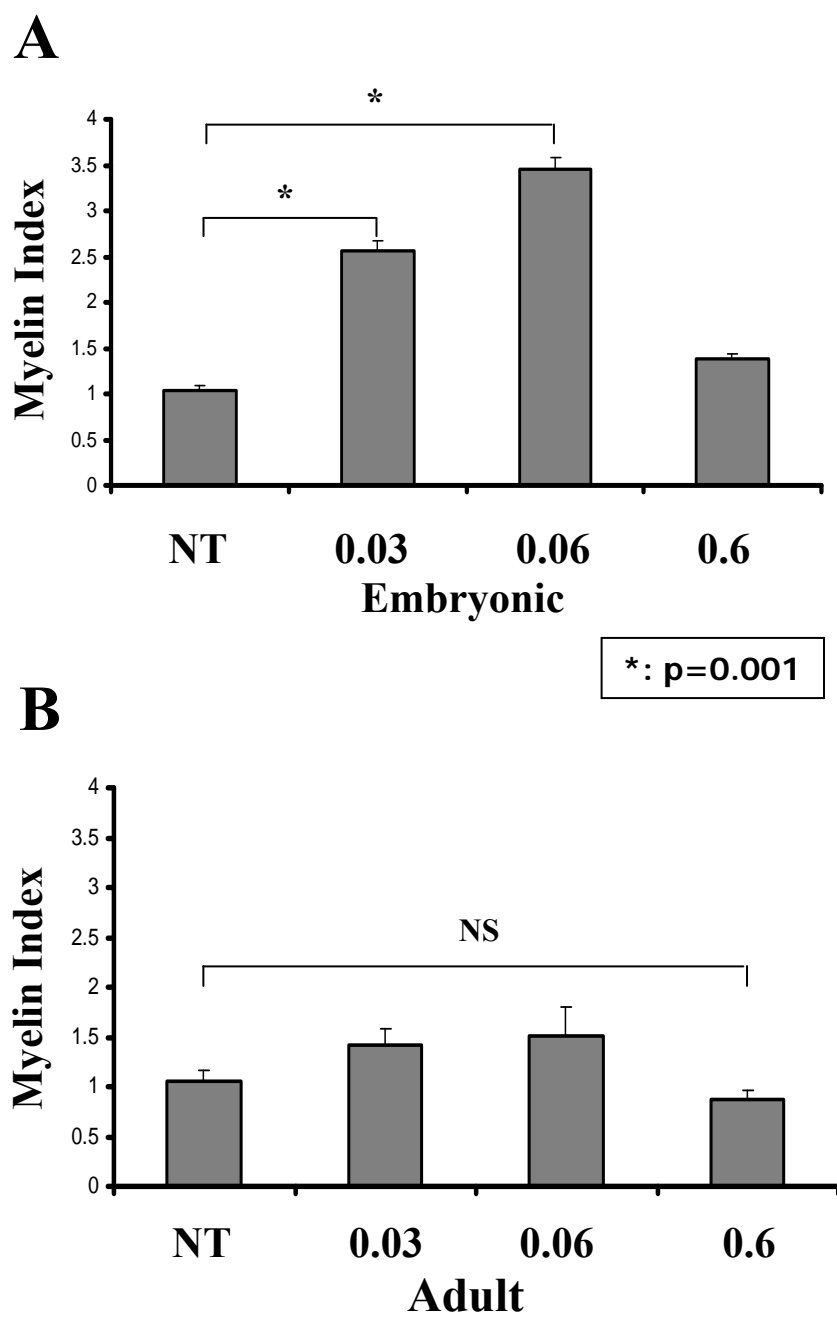

A

*: p=0.001

Figure 13:

 Soluble Nrg1-type III does not enhance myelination on adult axons

(A). Quantification of embryonic NRG/ Schwann cell co-cultures treated with 0.03, 0.06, and 0.6nM of soluble Nrg1-type III. Statistically significant difference was observed between the control and the treatments with soluble Nrg1-type III in promoting myelination (p=0.001). The means ± SEM from two independent experiments (3-4 coverslips/condition) are shown. (B). Quantification of adult DRG / Schwann cell co-cultures treated with indicated concentrations of soluble Nrg1-type III. No significant difference was observed between control and soluble Nrg1-type III treatments (p=0.064). The means ± SEM from three independent experiments (3-4 coverslips/condition) are shown.

Figure 14: Nrg1-type III expression in Hela cells and DRG neurons
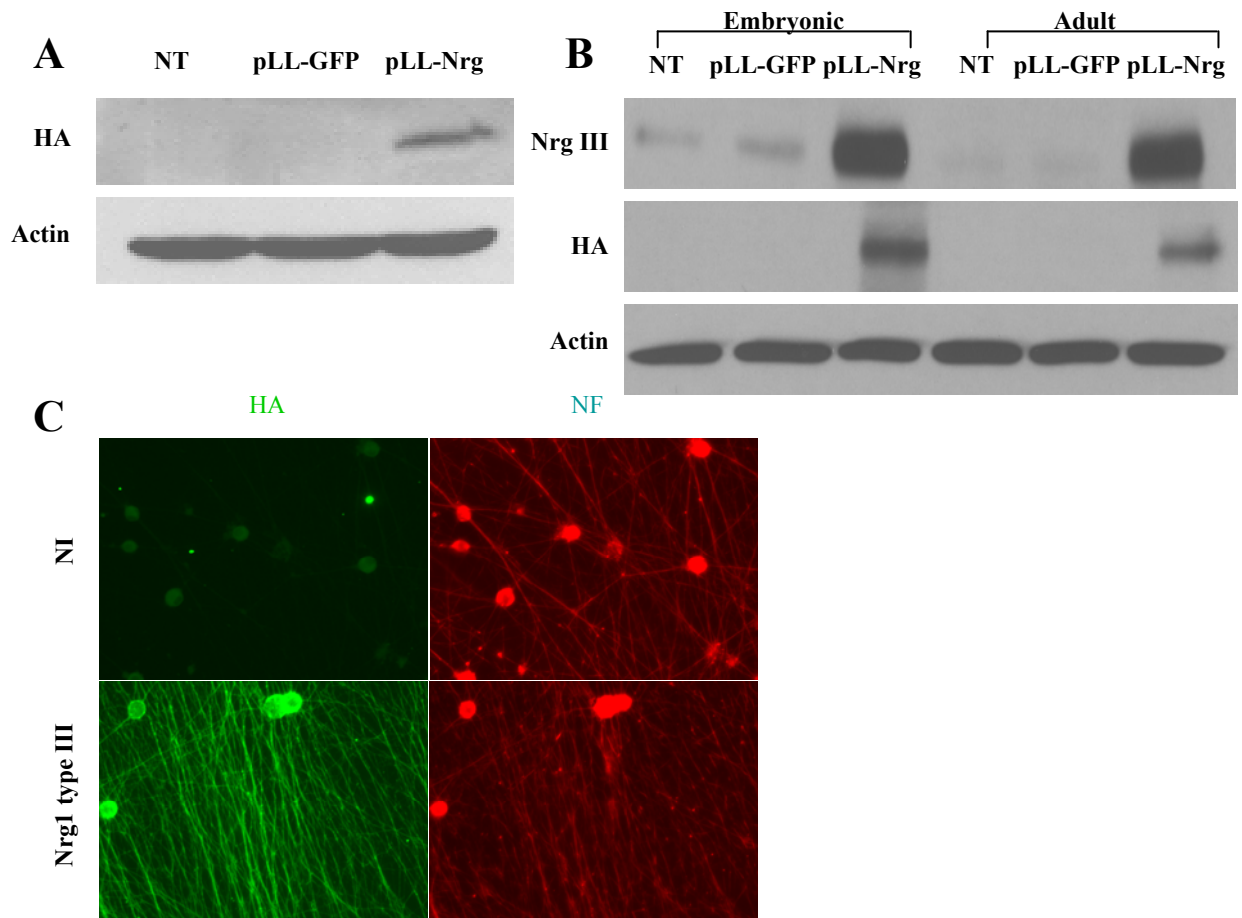
Figure 14: Nrg1-type III expression in Hela cells and DRG neurons
(A) cDNA encoding a full length Nrg1-type III (HA tagged) was cloned into a lentiviral vector pLL 3.7 (plentilox 3.7) (pLL-Nrg) and lentiviruses were generated. pLL 3.7 harboring GFP without the insert was used as a control (pLL-GFP). Hela cells were infected with lenitviruses expressing HA-tagged Nrg1-type III. Three days later, lysates were prepared from infected Hela cells and subjected to Western blot analysis. HA expression was observed only in the cultures infected with lentiviruses carrying Nrg1-type III (B). Adult and embryonic DRG neurons were infected with lentiviruses expressing HA-tagged Nrg1-type III. One week later, cell lysates were made and subjected to Western blot analysis. Similar HA expression was observed. To distinguish the endogenous Nrg1-type III expression from transgenic protein, lysates were probed with the antibody c-terminus to Nrg1. As expected, adult neurons expressed low levels of Nrg1-type III compared to embryonic neurons in control cultures. Lentiviral infection appeared to increase the Nrg1-type III expression both in adult and embryonic DRG neurons. Actin served as a load control. (C) Embryonic DRG neurons were infected with

lenitviruses expressing HA-tagged Nrg1-type III. Ten days later, the cultures were subjected to immunostaining. HA expression was observed only in the cultures infected with lentiviruses carrying Nrg1-type III. NF (neurofilament) to label neurons.

Figure 15: Ectopic Nrg1-type III expression in neurons

pLL-GFP pLL-NRG
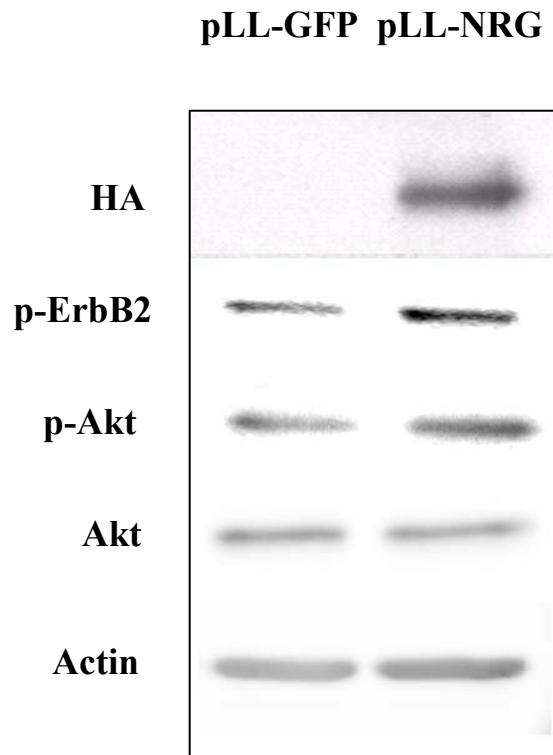
Figure 15:
Ectopic Nrg1-type III expression in neurons
Adult DRG neurons were infected with lentiviruses expressing HA-tagged Nrg1-type III, one week later Schwann cells were plated and myelination was initiated. Nine days later, the lysates were made from the co-cultures and probed with p-ErbB2, ErbB2, p-Akt, Akt, and actin. There was an increase in the activation levels of ErbB2 and Akt in the co-cultures, where neurons were infected with HA-tagged Nrg1-type III, showing that ectopically expressed Nrg1-type III increased the activation of Nrg1-ErbB signaling. Actin served as a load control. (Data from one experiment is shown)

Figure 16: Membrane bound Nrg1-type III does not enhance myelination on adult axons
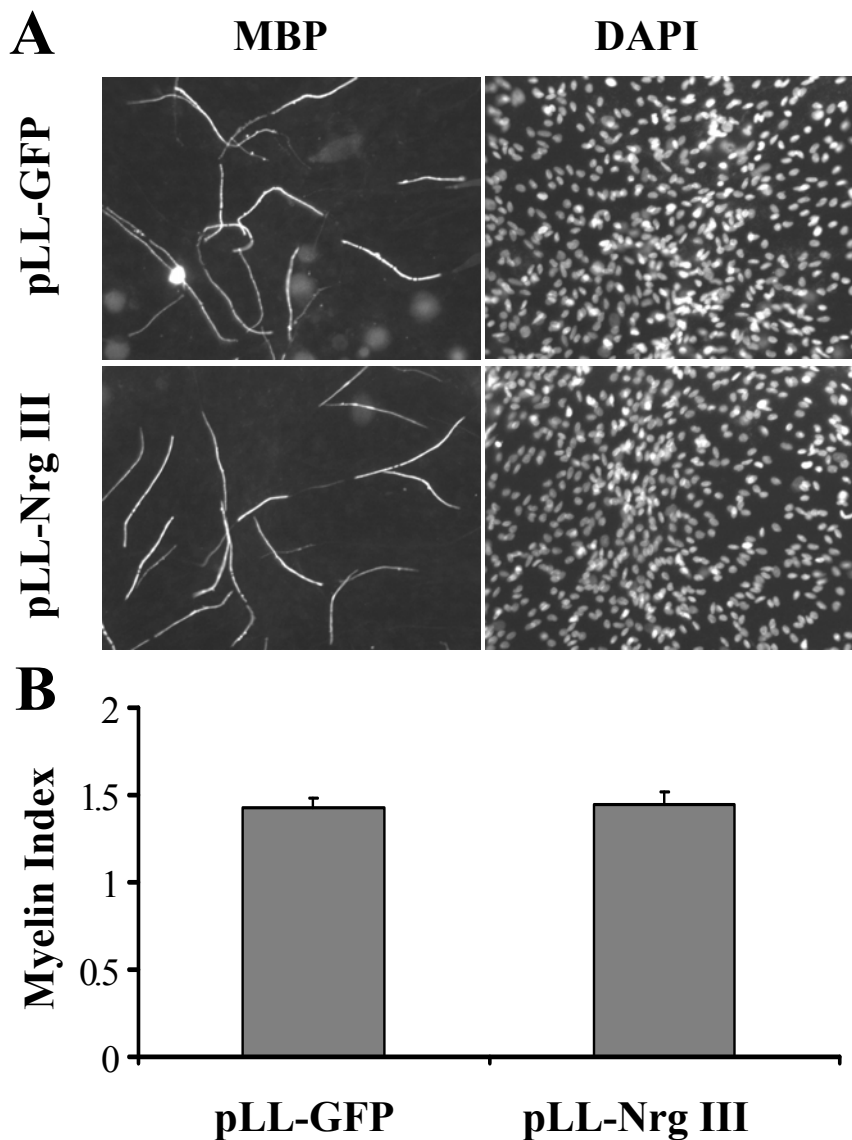
Figure 16:

Membrane bound Nrg1-type III does not enhance myelination on adult axons
(A) Images of myelin segments formed in adult DRG neuron/Schwann cell co-cultures infected with lentiviruses carrying pLL-GFP (control) and pLL-Nrg1-type III. (B). Quantification showed that there was no significant difference between control (pLL-GFP) and pLL-Nrg1-type III infected cultures (p=0.847). The means ± SEM from two independent experiments (3 coverslips/condition) are shown.

Figure 17: Increased Erk1/2 activation in Schwann cells associated with adult axons
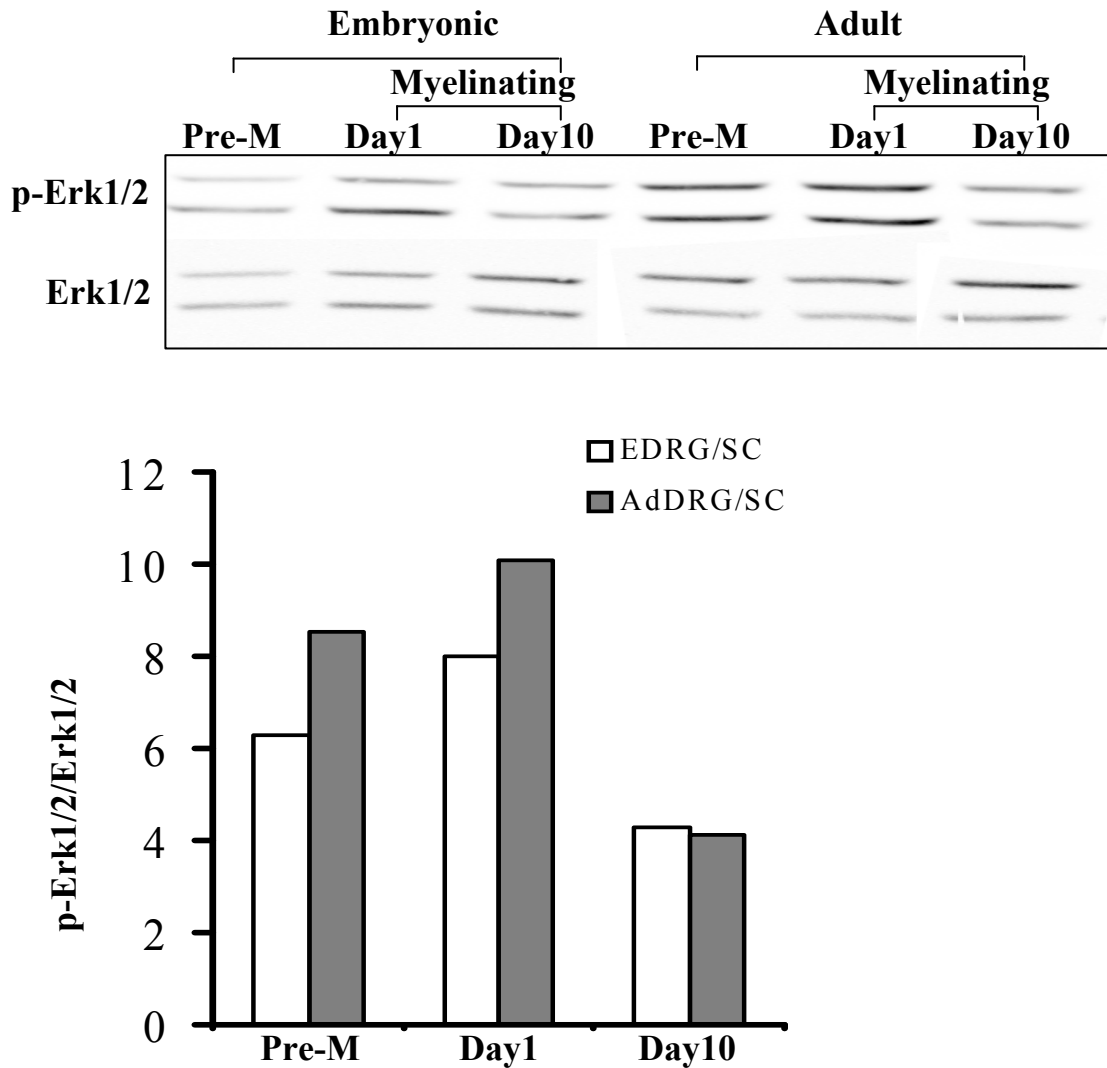
Figure 17:
Increased Erk1/2 activation in Schwann cells associated with adult axons.
Cell lysates were collected from embryonic and adult DRG neuron / Schwann cell co-cultures at pro-myelinating stage, one day after initiating myelination (Day 1), and ten days after initiating myelination (Day 10). Western blot analysis showed that there was elevated Erk1/2 activation during the time of initiation of myelination in adult DRG neuron and Schwann cell co-culture. Quantification of results from two experiments is shown at the bottom.

Figure 18: Inhibiting Erk1/2 activation enhances myelination on adult axons

Figure 18:
Inhibiting Erk1/2 activation enhances myelination on adult axons
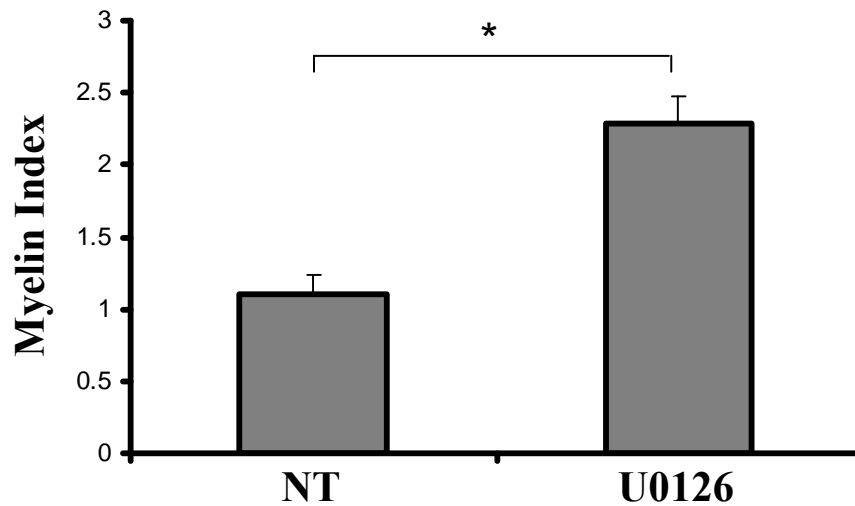Quantification of number of myelin segments formed in adult DRG neuron / Schwann cell co-culture showed that there was a significant increase in the number of myelin segments formed in the cultures treated with 1nM of U0126 compared to the control (*:p<0.001). The means ± SEM from two independent experiments (3coverslips/condition) are shown.

Chapter 5

Conclusion and Future Directions

Schwann cell development and myelination depend on contact-mediated signaling between Schwann cells and the associated PNS axons. Unlike oligodendrocytes in the CNS, myelinating Schwann cells in the PNS dedifferentiate into an immature Schwann cell-like phenotype after nerve injury and provide a permissive environment for the injured axons to regenerate and reinnervate their target. When these Schwann cells contact regenerating axons, they redifferentiate and remyelinate and this eventually leads to successful functional recovery. Apart from the remyelination in the PNS axons, spontaneous Schwann cell remyelination of CNS axons has also been reported (Felts and Smith, 1992). This observation has raised the possibility that Schwann cells could be an alternative source to repair CNS tracts. However, the remyelination by Schwann cells in adults is incomplete both in the PNS and the CNS.

The objective of this study was to understand whether enhancing Nrg1-ErbB signaling could improve remyelination in adults. Previous studies have shown that soluble Nrg1 such as GGF inhibits Schwann cell myelination (Zanazzi et al., 2001) whereas the membrane bound Nrg1-type III promotes myelination (Michailov et al., 2004, Taveggia et al., 2005). This has indicated that the pro-myelinating function of Nrg1-type III is mediated by juxtacrine signals activated at the Schwann cell – axon interface. In Chapter 2, we have investigated the role of soluble Nrg1-type III as a paracrine signal in promoting Schwann cell myelination. The data showed that soluble Nrg1-type III promoted myelination by increasing both the number of myelin segments formed and the internodal lengths of individual myelin segments, indicating that the

soluble Nrg1-type III not only improves myelin initiation but also promotes myelin maturation. This is an important finding since it suggests a therapeutic potential of soluble Nrg1-type III in promoting remyelination in adults, which often results in formation of immature myelin segments. Another intriguing finding was the concentration dependent biphasic effect of soluble Nrg1 on Schwann cell myelination independent of the Nrg1 isoforms. The data we obtained showed that soluble Nrg1-type III promoted myelination at low concentrations and inhibited myelination when the dosage was increased (Figure 1 and 6). A similar dose-dependent biphasic effect was observed with GGF, previously regarded as a negative regulator of Schwann cell myelination (Figure 7). Biochemical analysis revealed that the inhibitory effect of soluble Nrg1 observed at high concentrations coincided with the appearance of active Erk1/2 (Figure 4). On the other hand, activation of PI 3-kinase pathway was induced early at low concentrations and persisted throughout. This indicates a dose-dependent differential activation of Nrg1 signaling in Schwann cells. When the cultures were treated with Mek/Erk inhibitor, the inhibitory effect of Nrg1 was reversed, confirming that the Nrg1 function was mediated through the activation of the Ras/Raf/Erk pathway. Soluble Nrg1-type III also enhanced myelination on axons of DRG neurons from Nrg1-type III$^{+/-}$ mice that are thinly myelinated (Figure 2). Furthermore, treatment with soluble Nrg1-type III was sufficient to induce myelination of normally unmyelinated axons of the SCG neurons. Overall, the data from chapter 2 suggest that the promyelinating function of Nrg1 is not limited to the juxtacrine signal and can be provided by a paracrine signal in a concentration-dependent manner.

Following injury to the PNS, remyelination of regenerating axons is crucial for achieving functional recovery of the injured axons. However, as mentioned earlier, in adult animals Schwann cell remyelination is often incomplete. This could be due to the differences in the intrinsic properties of adult and embryonic cells of the PNS that adult Schwann cells and/or axons have reduced ability to myelinate or become myelinated, respectively. In chapter 3, we compared the myelination properties of embryonic and adult Schwann cells and neurons in the co-culture system. When Schwann cells isolated from neonatal or adult rats were co-cultured with embryonic DRG neurons, there was no significant difference in myelination (Figure 8). However, when the myelination properties of axons from embryonic or adult DRG neurons were compared, adult axons exhibited significantly reduced ability to become myelinated (Figure 9). Biochemical analysis of adult and embryonic neurons revealed that the expression level of Nrg1-type III, axonal protein required for myelination, was significantly reduced in adult neurons (Figure 10). Reduced Nrg1-type III expression in adult neurons did not affect the Schwann cell alignment and proliferation along the axons indicating that the defect is localized at the stage of myelination (Figure 11). Reduced Nrg1-type III expression in adults correlated with reduced activation of ErbB2 and Akt in the associated Schwann cells (Figure 12). Overall, adult neurons express low levels of Nrg1-type III and provide insufficient promyelinating signals to the Schwann cells and this might be a possible reason for defective myelination on adult axons.

In Chapter 4, we analyzed the possible strategies to enhance myelination on adult axons. Unlike the promyelinating effect on embryonic neurons, soluble Nrg1-type III did not promote myelination in adult DRG neuron co-culture. Similar results were obtained

when membrane bound Nrg1-type III was ectopically expressed in neurons to enhance myelination. As mentioned earlier, Schwann cell myelination is regulated by the balance between positive and negative regulators. Biochemical analysis showed that at the time of initiating myelination, the levels of active Erk1/2 were high in adult DRG neuron co-culture compared to the embryonic DRG neuron co-culture. Furthermore, inhibiting Erk1/2 activation by Mek1/2 inhibitor was sufficient to enhance myelination in adults.

Molecular mechanisms of soluble and membrane bound Nrg1-type III in Schwann cell myelination

During PNS development, immature Schwann cells interact with axons. This interaction activates multiple signaling pathways in Schwann cells which lead to Schwann cell proliferation and sorting of axons based on their diameter. These two events are the initial events for Schwann cell myelination to proceed further. Among the Nrg1 isoforms expressed in the PNS, Nrg1-type III has been reported as a key signal that mediates the initial events of myelination. For example, deletion of soluble Nrg1 isoforms type I and type II does not affect myelination *in-vivo* (Meyer et al., 1997) whereas in the absence of Nrg1-type III Schwann cells do not proliferate or sort the axons and as a result they fail to myelinate the axons (Taveggia et al., 2005).

In our study, we analyzed the myelination promoting effects of Nrg1-type III provided in a soluble form and found that while it enhances myelin formation on both normal and Nrg1-type III$^{+/-}$ neurons, it fails to induce myelination on Nrg1-type III$^{-/-}$ neurons. (Syed et al., 2010). On the other hand, ectopic expression of membrane bound Nrg1-type III in Nrg1-type III$^{-/-}$ cocultures rescues the myelination defect indicating the

exclusive requirement of the juxtacrine membrane bound Nrg1-type III signaling for initiating the early events of myelination (Taveggia et al., 2005, Syed et al., 2010). The molecular mechanism that selectively requires membrane bound Nrg1-type III during the early events of myelination remains unclear. It is possible that the Nrg1-type III-erbB signal at the adaxonal junction collaborates with other co-receptors or growth factors to promote the early events of myelination. Supporting this, various cell surface and intracellular proteins at the axon-Schwann cell junction have been reported to be involved in the initiation of myelination. Chan et al., (2006) have shown that asymmetric distribution of cell polarity protein Par-3 at the axon-Schwann cell junction recruits p75 receptor to the area, which in turn binds to BDNF expressed by neurons to induce myelin proteins. Short hairpin RNA (shRNA) mediated disruption of Par-3 expression in Schwann cells disrupts the recruitment of p75 and the subsequent myelination process (Chan et al., 2006). Cell adhesion molecules like Nectin like (Necl) proteins are distributed along the internode of the axon-Schwann cell junction. It has been reported that heterophilic interaction between Necl-1 expressed on axons and Necl-4 expressed on Schwann cells regulates Schwann cell myelination. Knockdown of Necl-4 in Schwann cells by shRNA inhibits myelination in co-cultures. This demonstrates the function of Necl-4 in initiation of Schwann cell myelination (Maurel et al., 2007, Spiegel et al., 2007). However, whether membrane bound Nrg1-type III signaling interacts with Par-3, Necl-4, or other proteins at the axon-Schwann cell junction remains to be investigated. It is also interesting to note that while deletion of Par-3 or Necl-4 in Schwann cells block myelination they do not affect Schwann cell proliferation and sorting, the earlier events that are affected in the absence of Nrg1-type III.

In the absence of Nrg1-type III, Schwann cells fail to properly associate with axons. Therefore, the juxtacrine Nrg1-type III signal is likely to play a role during events prior to myelination. We have observed that addition of soluble Nrg1-type III is not sufficient to reestablish axon-Schwann cell association in Nrg1-type III deficient co-culture, indicating the unique role of the membrane bound Nrg1-type III. Our data show that once the Schwann cells associate with axons and myelination is initiated by the membrane bound Nrg1-type III, addition of soluble Nrg1 promotes the subsequent events, thus enhancing myelin formation: soluble Nrg1 expression increases myelin protein expression and lengthens the internodal length. Results from our study distinguish the role of juxtacrine and paracrine signals during myelination. While membrane bound Nrg1-type III is a key signal which is required at each stage of Schwann cell development like proliferation, sorting, and association of Schwann cells with axons, Nrg1-type III in its soluble form elicits a promyelinating effect which is dependent on the presence of the membrane bound Nrg1-type III.

Differential activation of Erk1/2 with high and low doses of soluble Nrg1

An interesting finding from this study is that soluble Nrg1, depending solely on the availability of the amount of ligand, elicits two contrasting cellular functions through the same receptor complex independent of the isoforms. Based on the data obtained from this study we are proposing a model that at low concentrations, soluble Nrg1 preferentially activates Akt and promotes myelination, while at high concentrations, it activates both Erk1/2 and Akt pathways and inhibits myelination. This phenomenon of

exerting different phenotypic responses by the same ligand is found in other growth factors. For example, PDGF, at lower concentrations supports cell migration whereas at higher concentrations, it switches from supporting cell migration to cell proliferation (De Donatis et al., 2008).

The mechanism by which soluble Nrg1 differentially activates Erk1/2 pathway at different concentrations is not known. It is possible that in response to low and high concentrations of soluble Nrg1, erbB receptors are phosphorylated at different tyrosine residues, leading to recruitment of distinct adaptor proteins and activation of different downstream signaling pathways. This can be tested by treating the Schwann cells with low and high concentrations of soluble Nrg1 and comparing the differential receptor phosphorylation by phosphopeptide mapping (Sweeney et al., 2000).

Alternatively, it is possible that different doses of the ligand differentially modulate the receptor endocytic pathway thus eliciting distinct physiological responses. It has been shown that PDGF at low concentrations induces clathrin-mediated endocytosis of the PDGF receptor whereas at high concentrations the internalization shifts toward a clathrin-independent/caveloar pathway (De Donatis et al., 2008). EGF receptor also follows different endocytic pathways depending on the concentrations of EGF. High concentrations of EGF preferentially induce clathrin-independent/caveloar mediated endocytosis whereas low concentrations induce clathrin mediated endocytosis (Sigismund et al., 2005). Therefore, it is possible that erbB receptor may also follow different endocytic pathways in response to low and high doses of ligand and differentially activate Erk1/2 pathway.

It is also possible that different doses of soluble Nrg1-type III modulate the kinetics of Erk1/2activation, In PC12 cells, it has been shown that NGF (Nerve growth factor) at low and high concentrations induces transient and sustained Erk1/2 activation respectively. Transient activation involves SOS dependent Ras activation, followed by c-Raf and MEK activation, which leads to phosphorylation of Erk1/2. Phosphorylated Erk1/2, as a negative feedback mechanism, phosphorylates SOS leading to the dissociation of SOS from the adaptor protein Grb2 and subsequently decreases the Ras activation. During sustained Erk1/2 activation, NGF mediated TrkA phosphorylation activates Rap1, followed by B-Raf and MEK activation, leading to sustained Erk1/2 activation. In these experiments, low and high concentrations of NGF determine the transient and sustained activation of Erk1/2 (Sasagawa et al., 2005). In cancer cells, it has been reported that a stimuli that activates Erk1/2 also activates MKP-1 (Mitogen activated protein kinase phosphatase – 1), which dephosphorylates Erk1/2 and terminates its activation. However, at high concentrations of stimuli, the activated Erk1/2 can trigger MKP-1 degradation via the ubiquitin-proteasome pathway, thereby attaining sustained Erk1/2 activation (Lin et al., 2003). Therefore, in Schwann cells, it is possible that soluble Nrg1 at high concentrations activates the Rap1/B-raf pathway, which leads to increased Erk1/2 phosphorylation; or activated Erk1/2 degrades the phosphatase that is involved in dephosphorylating Erk1/2 and accomplishes increased Erk1/2 activation. In our studies, the level of Erk1/2 activation was determined at only one time point (45 minutes after the Nrg1 treatment), thus the transient Erk1/2 activation might have been missed. Detailed time course experiments would determine the kinetics of Erk1/2 activation by varying doses of soluble Nrg1-type III.

Transcriptional regulation of Schwann cell myelination by soluble Nrg1

At the transcriptional level, Schwann cell myelination is regulated by the balance between positive (Oct-6, Krox-20, Sox-10, and NFκB) and negative regulators (C-Jun, Notch, Sox-2, Pax-3, and Id2) (Jessen and Mirsky, 2008). Expression of transcription factors is regulated by cell surface receptors that activate cytoplasmic down stream signaling pathways. In our study, we found that low concentrations of soluble Nrg1-type III induce the expression of promyelinating transcription factor Krox-20 whereas at high concentrations it induces the expression of c-Jun, a negative regulator of Schwann cell myelination (Syed et al., 2010). The transcriptional regulatory mechanism by which soluble Nrg1 regulates Krox-20 or c-Jun expression is not yet known. It is possible that low doses of soluble Nrg1-type III induce the expression of Oct-6, which then acts synergistically with Sox-10 to induce the expression of Krox-20. Studies with Nrg1-type III knock out mice have shown that Oct-6 expression is tightly regulated under the control of Nrg1-type III present on the axonal membrane. (Taveggia et al., 2005). It has also been shown that Oct-6 in combination with Sox-10 binds to the MSE (Myelinating Schwann cell Element) element of the Krox-20 gene and activate the expression of Krox-20 protein, which eventually induces the expression of myelin proteins (Ghislain and Charnay, 2006).

In our study, we found that the inhibitory effect of soluble Nrg1 on myelination is associated with c-Jun expression. Recently, Napoli et al., have reported that Erk1/2 activation induced the expression of c-Jun *in-vivo* during Schwann cell dedifferentiation (Napoli et al., 2012). The molecular mechanism by which c-Jun negatively regulates myelination is not clear. It is possible that in the cocultures treated with soluble Nrg1,

increased c-Jun expression suppresses the transcription of Krox-20 gene. Recently, it has been reported that Schwann cell specific forced activation of c-Jun in DRG neuron cocultures suppresses the expression of Krox-20 and as a result, it inhibits Schwann cell myelination. From this study, it was concluded that effective initiation of myelination depends on the suppression of c-Jun by Krox-20 (Parkinson et al., 2004) and forced expression of c-Jun inhibits the myelination process. In the same study, they also showed that c-Jun regulates Sox-2 levels (Parkinson et al., 2008). Enforced Sox-2 expression has been reported to inhibit Krox-20 mediated myelin gene expression and myelination in cocultures (Le et al., 2005). Therefore, the inhibitory effect of soluble Nrg1 on myelination might be associated with its induction of c-Jun through the activation of the Erk1/2 pathway, which in turn activates the expression of Sox-2 and suppresses the expression of Krox-20. Other transcription factors that negatively regulate myelination such as Pax-3, Notch, Id2 and Id4 are all repressed during the myelination process. Whether c-Jun regulates the expression of the above mentioned transcription factors is not known. Determining the expression profiles of these transcription factors at different doses of soluble Nrg1-type III may provide insights into understanding the possible mechanism by which soluble Nrg1 negatively regulates myelination.

Effect of combined treatment of enhancing Nrg1 signaling and inhibiting Erk1/2 activation on myelination of adult axons

We show that inhibition of negative signal such as Erk1/2 enhanced myelination in adult DRG neuron co-culture. However, the combined effect of enhancing positive signal and inhibiting negative signal on Schwann cell myelination still remains to be

addressed. This can be tested by adding soluble Nrg1-type III and the Mek1/2 inhibitor during the course of myelination to determine the levels of myelination as compared to the effect of Nrg1 or Mek1/2 inhibitor treatment alone. Alternatively, Nrg1 signaling can also be enhanced by lentiviral mediated expression of the membrane bound Nrg1-type III in adult DRG neurons. The Mek1/2 inhibitor will be added during the course of myelination and the effect on Schwann cell myelination will be analyzed.

The source of Erk1/2 activity that inhibits myelination is unknown since both Schwann cells and neurons were exposed to Mek1/2 inhibitor in co-cultures. This can be tested by conditional deletion of Erk1/2 in neurons or Schwann cells and determining the effect on myelination. Although the possibility that neuronal Erk1/2 inhibits myelination by the associated Schwann cells exists, it is more likely that the Erk1/2 in Schwann cells plays a more significant role in eliciting the inhibitory effect, since Schwann cell specific activation of Erk1/2 alone was sufficient to induce Schwann cell demyelination and dedifferentiation (Napoli et al., 2012).

Effect of other negative signals in myelination of adult axons

Although our study focused mainly on Erk1/2, other negative signals of Schwann cell myelination should also be taken into consideration while considering a therapeutic strategy to improve remyelination. For example, our recent study shows that p38 MAPK acts as a negative regulator of Schwann cell myelination (Yang et al., in press). Activation of JNK in Schwann cells has also been shown to block myelination (Parkinson et al., 2008). It will be of interest to determine whether these signals are up regulated in the adult PNS and to investigate the effect of combined inhibition on Schwann cell

myelination. It is also possible that cross talk between the signaling pathways negatively regulates myelination. For example, in primary human fibroblasts, it has been shown that activation of Erk1/2 leads to activation of p38-MAPK (Wang et al., 2002).

Therapeutic potential of Mek1/2 inhibitor in promoting remyelination in adults

In recurrent Guillain-Barre syndrome (GBS) patients, the demyelinated areas often get remyelinated by the endogenous Schwann cells. However, the remyelinated segments are immature as seen during remyelination of regenerating axons following physical injury to the PNS (Pithadia and Kakadia, 2010). Similar immature myelin segments were observed in a conditional mouse model of Charcot-Marie-Tooth disease type-1A (Perea et al., 2001). Interestingly, elevated levels of activated Erk1/2 have been observed in Schwann cells in both inherited and infectious disease models of PNS neuropathy (Tapinos et al., 2006, Fischer et al., 2008). Since our data show that inhibition of Erk1/2 enhances myelination in adults, it is possible that this strategy may improve remyelination and would provide a new therapeutic opportunity to treat demyelinating neuropathies of the PNS.

It is possible that Erk1/2 activity is important during developmental myelination but not during myelin repair in adults. Caution should be taken since recent studies have shown that apart from its role as a negative regulator, Erk1/2 also acts as a positive regulator of Schwann cell myelination during development (Newbern et al., 2011, Napoli et al., 2012). This study raises question of developmental stage specific role of Erk1/2 in Schwann cells. This can be tested by conditionally knocking out Erk1/2 in adults and

comparing the remyelination potential of Schwann cells. It is also possible that threshold levels of active Erk1/2 may determine Schwann cell myelination. Since our data shows that elevated Erk1/2 activation at the early stage of myelination in adult DRG co-cultures compared to the embryonic DRG co-cultures, it is possible that adult axons intrinsically developed the potential to activate high levels of Erk1/2 in associated Schwann cells. If this is the case, Schwann cell specific ectopic activation of Erk1/2 in-vivo is expected to block developmental myelination in the PNS.

Potential axonal signal that activates Erk1/2 in adults

Our study shows that adult neurons suppress myelination by Schwann cells in a manner that is dependent on Erk1/2 activation. The axonal signal that activates Erk1/2 in the associated Schwann cells is not known. Previous studies have reported that Nrg1-type II, FGF-2, and PDGF are expressed on the PNS neurons (Hardy et al., 1992, Eccleston et al., 1993, Oellig et al., 1995, Grothe and Wewetzer, 1996) and their expression is increased in the PNS after nerve injury. The expression levels of receptors for Nrg1, FGF-2, and PDGF have also been shown to increase in distal Schwann cells that are undergoing dedifferentiation (Oya et al., 2002, Jungnickel et al., 2006). Since these growth factors are capable of activating Erk1/2 and other signaling pathways that are known to inhibit or promote myelination in Schwann cells it has been speculated that they may function as negative axonal signal for Schwann cell myelination. It is unknown whether neurons in the adult PNS express high levels of these growth factors or the receptors. To address this, the expression pattern of Nrg1-type II, FGF-2, and PDGF in

embryonic vs. adult neurons has to be determined. This will provide insights into understanding the potential axonal signal that activates Erk1/2 in adults.

Proposed model to improve remyelination in adults

Based on the data obtained from this thesis work, I propose a model in Figure 19 to improve remyelination in adults. Results from our Mek1/2 inhibitor study demonstrate that inhibition of Erk1/2 activation alone is sufficient to enhance myelination on adult axons. Nonetheless, combined treatment of Nrg1-type III (to activate PI3-kinase/Akt signaling) and Mek1/2 inhibitor (to inhibit Erk1/2 activation) might promote remyelination in adults. Finally, performing this study *in-vivo* might provide insights into developing a combined strategy for improving remyelination in adults.

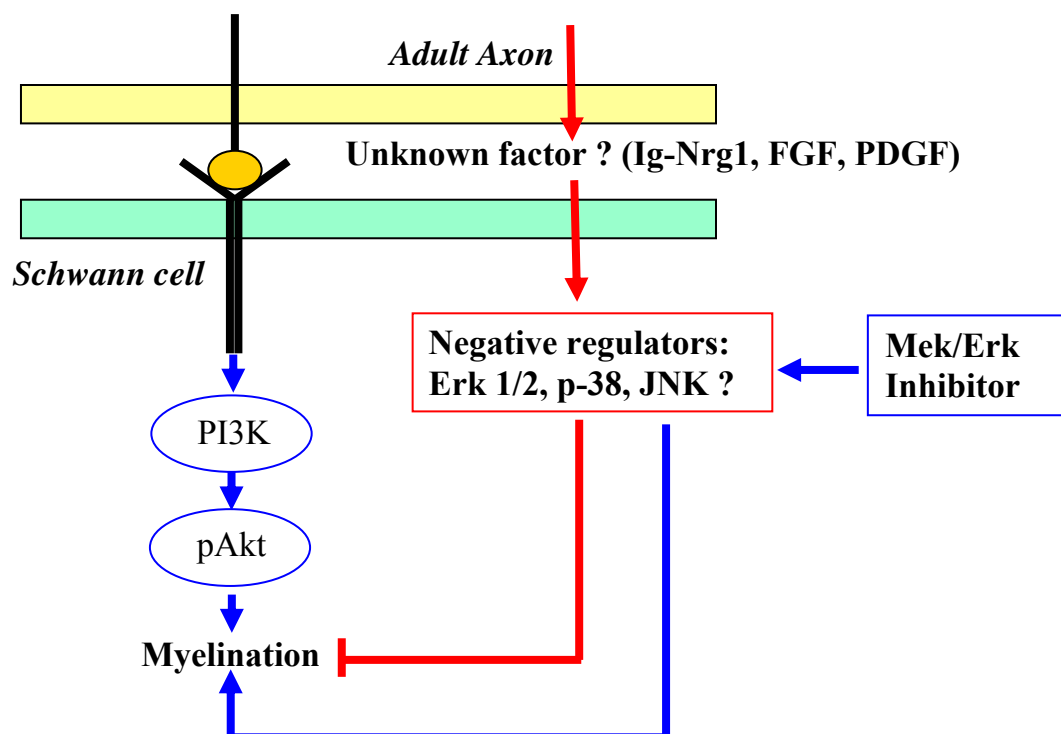# Figure 19: Proposed model to improve remyelination in adults

Figure 19: Proposed model to improve remyelination in adults. Our data suggest that apart from the increase in positive signal (Akt) induced by Nrg1-type III, an inhibitory signal (active Erk1/2) from adult axons inhibits the myelination process. Inhibition of active Erk1/2 enhances myelination on adult axons.

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Curriculum vitae

GENERAL INFORMATION

Name:	Neeraja Syed
Affiliation:	Department of Biological Sciences
	Rutgers, the State University of New Jersey
Address:	195 University Ave
	Boyden Hall, Room 206
	Newark, NJ 07102
Phone:	(609) 284 2659
E-mail:	neerajas@pegasus.rutgers.edu

EDUCATION:

2004-2012	Ph.D., Rutgers, the State University of New Jersey
	Newark, New Jersey (Cell Biology)
1997-2000	M.B.A. Bharadhidasan University, India
1995-1996	Dip. In Biotech. Madurai Kamaraj Univesity, India
1991-1994	B.Sc. Madurai Kamaraj Univesity, India

GRANTS

New Jersey commission on Spinal Cord Research-Individual Research Grant
Period: December 15, 2009 – December 30, 2011

RESEARCH EXPERIENCE

2004-present	Rutgers, the State University of New Jersey.
	Graduate student (Advisor: Dr. Haesun Kim)
	The role of Nrg1-ErbB signaling in promoting Schwann cell myelination.
1996-2003	SPIC Science Foundation, India
	Research Assistant
	Isolation, Characterization and cloning of α-amylase, protease and lipase.

TEACHING

2004-2009	Teaching assistant for the following courses:
	General Biology, Foundation of Biology, Mammalian Physiology, Microbiology and Genetics.
	Rutgers, the State University of New Jersey.

SKILLS

Experimental techniques:

PCR, RT PCR, cloning, southern and northern hybridization, western blot, and immunostaining. Generation, handling and use of recombinant viruses (retrovirus, adenovirus, and lentivirus) under BL2 safety level for *in vitro* experiments. Extensive experience in establishing following myelinating co-cultures:
1. Embryonic DRG neurons with neonatal Schwann cells or adult Schwann cells
2. Adult DRG neurons with neonatal Schwann cells or adult Schwann cells
3. Sympathetic cervical Ganglion neurons with neonatal Schwann cells.

TRAINING

The Spinal cord injury research methods training at W. Keck center for Neuro Science, Newbrunswick, NJ - August 2011.

PUBLICATIONS

1. **Syed N**, Reddy K, Yang DP, Taveggia C, Salzer JL, Maurel P, and Kim HA. Soluble neuregulin-1 has bifunctional, concentration-dependent effects on Schwann cell myelination. *Journal of Neuroscience*, 2010. 30(17): p. 6122-6131.

2. **Syed N** and Kim HA. Soluble neuregulin and Schwann cell myelination: A therapeutic potential for improving remyelination on adult axons Molecular and Cellular Pharmacology, 2010. 2(4):p. 161-167.

3. Yang DP, Kim J, **Syed N**, Tung YJ Bhaskaran A, Mindos T, Mirsky R, Jessen KR, Maurel P, Parkinson DB and Kim HA. p 38 MAPK activation triggers demyelination and functions as a negative regulator of Schwann cell differentiation and myelination. *Journal of Neuroscience*, 2012. 32(21): p. 7158-68.

Poster presentation

1. **Syed N**, Reddy K, Yang DP, and Kim HA. Double-edged sword: concentration-dependent effects of neuregulin-1 on schwann cell myelination. 2008 Society for Neuroscience Abst.
2. **Syed N** and Kim HA. The role of Nrg1-ErbB signaling in myelination of adult axons. 2011 Society for Neuroscience Abst.