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Axogial communication mediated by soluble neuregulin-1 and bdnf

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AXOGIAL COMMUNICATION MEDIATED BY SOLUBLE NEUREGULIN-1 AND BDNF

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DEDICATION

This work is dedicated to my family: my wife Rong Sun, parents and lovely daughter SuSu, for their support, understanding and unconditional love. I could not achieve my dream without them.

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PREFACE

Precisely orchestrated communication between axons and glial cells is critical for normal development of both central and peripheral nervous system. Part of this communication comes from a family of alternatively spliced, neuron-derived growth and differentiation factors produced by the neuregulin-1 (*NRG1*) gene. NRG1 has been shown to have multiple important functions on the maintenance and development of both neurons and glia. NRG1 isoforms are produced as transmembrane precursors (proNRG1) that are subsequently proteolytically cleaved to both soluble and membrane-bound proteins. All isoforms have an EGF-like domain that is essential for erbB receptor activation. Majority of soluble forms have a unique N-terminal, positively charged heaprin-binding domain (HBD) that targets the protein to the cell surfaces rich in developmentally expressed heparan sulfate proteoglycans (HSPGs). This dissertation will focus on the developmentally specific roles of soluble NRG1 in regulating early Schwann cell development, and how the interplay between soluble NRG1 and neurotrophins such as brain-derived neurotrophic factor (BDNF) can mediate this function *in vivo*.

In Chapter I, the introduction will be given about some necessary background of NRG1, neurotrophic factors and Schwann cell development as well as the diversity of HSPGs, followed by the clarification of the rational of this project. In Chapter II, evidence will be presented suggesting that the potential usage of NRG1's heparin-binding domain can increase the fusion protein's targeting specificity through the interaction with specific

heparan sulfate in the extracellular matrix, and thus allow us to generate a novel potent NRG1 antagonist that could effectively compete with endogenous NRG1 on an even footing to block its activity. This new regent would not only enable more effective studies of NRG1's functions in both development and diseases, but also set an example for the future development of biopharmaceuticals with enhanced tissue-targeting specificity and minimal toxicity. In chapter III, evidence from the studies with this new regent and genetic modulations of soluble NRG1 signaling will reveal the precise developmentally specific *in vivo* functions of soluble NRG1 on Schwann cell precursors' survival, proliferation and their differentiation into immature Schwann cells. Further *in vivo* evidence presented in this chapter shows that reciprocal signaling between axon-derived soluble NRG1 and Schwann cell-secreted BDNF at the axoglial interface is critical for the early Schwann cell development. Finally, a detailed model about how this positive feedback loop through soluble NRG1 and BDNF regulate the development of Schwann cell precursors during axon-glial communication, will be discussed in chapter IV, which also will tie the body of work together by describing the significance and implications of the novel NRG1 antagonist and soluble NRG1-mediated axoglial signaling for both normal development and a variety of neurological disorders including demyelinating diseases and peripheral neuropathy.

Understanding of *in vivo* functions of soluble NRG1 on Schwann cell development as well as the molecular and cellular mechanisms, by which it is mediated by neurotrophic factors, is of great interest due to the critical roles NRG1 plays during normal development and its potentials for the therapeutics of human diseases.

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CHAPTER I

INTRODUCTION

In the nervous system, the function of a neuron is to communicate with other cells through its functionally distinct domains including cell body, the axon and the dendrites. This cell communication requires recruiting other supporting cells called glia, through interacting with them. The glial cells which closely follows the outgrowth of axons, support neuron maintenance and later may insulate axons by myelination process for optimal and rapid signal transduction in both peripheral and central nervous system. On the other hand, neurons can regulate glia behavior such as survival, proliferation and differentiation. In the peripheral nervous system (PNS), Schwann cells can form either myelination or just ensheathment around axons in respond to axon-derived instruction. Furthermore, sensory neurons in Dorsal Root Ganglia (DRG) which contact with Schwann cells in peripheral nervous system may also enter the central nervous system (CNS) to communicate with another type of glial cells, oligodendrocytes, in which both cross-talks at axoglial interface are very important for the normal functions of the nervous systems. So far, a lot of research evidence has shown that, at molecular level, the communication between neurons and glial cells is mediated by membrane bound and/or soluble factors which may be temporally and spatially expressed both by the neuron and the glial cells. This axon-glial communication is critical for growth and differentiation of both cell types and it would eventually influence the whole developmental process of both peripheral and central nervous system. The disruption of this communication has

been strongly implicated in many neurodegenerative and demyelinating diseases (Esper et al., 2006).

Neuregulin-1 (NRG1) isoforms are growth and differentiation factors derived from neurons, and their functions in nervous system development as well as neurological diseases have been studied extensively. However, the mechanisms by which the functions of NRG1 on glial cell development are regulated, has not been fully understood. Previous *in vitro* studies from our lab have shown that Schwann cell or target derived neurotrophic factors can promote the localized release of soluble NRG1 from neurons or nerve endings respectively through PKC pathway (Loeb et al., 2002; Esper and Loeb, 2004,2009). So in this thesis work, I hypothesize that the reciprocal signaling between axon-released soluble NRG1 and Schwann cell-derived neurotrophic factors could mediate functional axon-glial communication *in vivo*, and the locally released soluble NRG1 as a part of this reciprocal regulatory loop, may play a critical role in regulating Schwann cell development during early embryonic development.

Molecular structure of neuregulin-1

The neuregulin-1 (NRG1) is a large family of growth and differentiation factors, with a wide range of important functions during the development of heart and nervous system as well as in pathogenesis of human diseases ranging from breast cancer to schizophrenia (Lupu et al., 1996; Buonanno and Fischbach, 2001; Falls, 2003; Esper et al., 2006; Mei and Xiong, 2008). The *NRG1* gene encodes at least 31 isoforms through a combination of different promoter usage and post-transcriptional alternative splicing (Steinthorsdottir et al., 2004; Mei and Xiong, 2008). The original nomenclature of NRG1

isoforms is based on their biological activities when they are first discovered, such as ARIA (Acetylcholine Receptor inducing Activity), GGF (Glial Growth Factor), SMDF (Sensory and Motor neuron Derived Factor). Currently, alternatively splicing isoforms of NRG1 proteins are subdivided into six types (I-VI) according to their distinct structure and function of N-terminal region (Mei and Xiong, 2008), while they all have a highly conserved and tightly folded epidermal growth factor-(EGF) like domain, which is alone necessary and sufficient for the binding and activation of erbB receptors (Buonanno and Fischbach, 2001; Falls, 2003). NRG1 isoforms are first expressed as trans-membrane precursors (proNRG) that then undergo proteolytic cleavage to produce either released soluble (type I-II, IV-VI) forms or membrane-bound (type III) form (Fig.1). The cleavage is catalyzed by transmembrane proteases including tumor necrosis factor-α converting enzyme (TACE/ADAM17), β-site of amyloid precursor protein cleaving enzyme (BACE) and meltrin beta (ADAM19) (Horiuchi et al., 2005; Willem et al., 2006; Mei and Xiong, 2008). As a result, NRG1 type III still remains at the membrane after proteolytic cleavage because of the existence of cysteine-rich domain (CRD) that works as a second trans-membrane domain, while other soluble NRG1 isoforms can be shed from cell membrane to become soluble proteins and function through paracrine signaling during the cell-cell communication. These isoforms have significant difference in both processing and spatial or temporal expression pattern that are precisely regulated by axoglial communication and neuronal activity, so they could take over different *in vivo* functions during embryonic development (Meyer et al., 1997; Eilam et al., 1998; Esper et al., 2006).

NRG1 isoforms mediate their effects by binding and activating the erbB family of

receptor tyrosine kinases, specifically erbB2, erbB3, and erbB4 (Buonanno and Fischbach, 2001; Citri et al., 2003). NRG1 can bind to erbB3 or erbB4, followed by the formation of homo- or hetero-dimeric receptors such as erbB2-erbB3, erbB2-erbB4, erbB3-erbB4, erbB4-erbB4, leading to the receptor tyrosine trans-phosphorylation and activation of downstream signaling including mitogen-activated protein kinase (MAPK) and phosphatidylinositol 3-kinase (PI3K) pathways (Yarden and Sliwkowski, 2001) that could be involved in many processes during neural development and pathogenesis of some cancers (Fig.2). The erbB receptors are expressed in a variety of tissues and cell types, including neurons, nerve innervation targets and glial cells during the development of nervous system. In Schwann cells, erbB4 is minimally expressed and therefore erbB2-erbB3 heterodimer is most popular receptor activation form to mediate the functions of NRG1 on Schwann cell development (Birchmeier and Nave, 2008).

The common feature shared by most secreted soluble NRG1, except type VI, is to have an extracellular spatially-separated heparin-binding domain (HBD) that is also called the Immunoglobulin (Ig)-like domain (Fig.1). NRG1's HBD is unique from other HBDs because of its disulfide-linked C2 immunoglobulin domain structure that allows it to be capable of maintaining the structural specificity for high affinity heparin binding (Loeb, 2003). This C2 loop consists of a long stretch of alternating positively-charged amino acids and is separated from the EGF-like domain by a glycosylated spacer. So the net result is that a highly positively charged heparin binding domain can specifically target released NRG1 protein to extracellular matrix rich in negatively charged heparin-sulfate proteoglycans (HSPGs), which could lead to the precise tissue-specific localization for multiple functions through the erbB receptor activation during the

development of nervous system (Loeb et al., 1999; Li and Loeb, 2001; Pankonin et al., 2005) (Fig.2).

Interaction of soluble NRG1 and heparan-sulfate proteoglycan

Heparan-sulfate (HS) is one of major glycosaminoglycan (GAG) side chains that attach to proteoglycan (PG) core proteins. HSPGs have been suggested to be one of the most information-condensed biological molecules in nature and are found ubiquitously and abundantly in the extracellular matrix on every cell surface of most tissues (Turnbull et al., 2001) (Fig.2). The diversity of HSPGs results from both the existence of a large number of core proteins that serve to distribute them to different regions of the extracellular space, and the generation of the enormous variety of negatively charged, sulfated HS structures that could occur during HS biosynthesis in the endoplasmic reticulum and the Golgi apparatus as well as the modification of sulfation pattern by specific extracellular sulfatases (Dhoot et al., 2001; Esko and Selleck, 2002). In addition to the immense diversity of their molecular structure, biologically-driven expression and degradation of HSPGs in a cell/tissue-type-specific manner is precisely regulated by distinct physiological or pathological signals both temporally and spatially, leading to the activation of restricted signaling pathways that is consistent and reproducible at the right time and place (Couchman, 2010).

The highly specific, but low affinity interactions between heparin-binding domain of proteins and HSPGs in the extracellular matrix has served to concentrate the ligands or viruses near their high affinity cell-surface receptor for sustaining the signaling, as well as the protection from proteolysis (Sadir et al., 2004). For example, fibroblast growth

factor (FGF), one of the most extensively studied heparin-binding growth factor, binds to specific heparan sulfates not only for selective tissue targeting, but also to enhance its signaling through membrane receptors (Friedl et al., 2001; Mohammadi et al., 2005). Similarly, adeno-associated virus type 2 (AAV-2) is also using specific cell-surface HSPGs for cell recognition and can infect human dendritic cells and activate T cells through heparin-binding (Manno et al., 2006; Vandenberghe et al., 2006). Therefore, HSPGs are natural and highly specific “targets” for a wide variety of heparin-binding proteins, growth factors and viruses that have evolved to fully exploit the properties of HSPGs for specific tissue/cell targeting and exerting their functions on the regulation of cell behaviors and biological processes.

Our laboratory have shown that the interaction of NRG1’s HBD with HSPGs can facilitate the localization of NRG1, resulting in the targeted protein deposition and potentiation of NRG1 signaling in specific regions of the developing peripheral and central nervous system (Meier et al., 1998; Loeb et al., 1999). The accumulation of NRG1 at HSPG-rich synaptic basal lamina of neuromuscular junction provides a sustained source of ligands for erbB receptor activation that is important for the induction of postsynaptic acetylcholine receptor gene expression in muscle during chick embryonic development (Li and Loeb, 2001).

In addition, previous studies have demonstrated that different sulfate groups that are localized at different portion of glycosaminoglycan in HSPGs through the expression of specific synthetic enzymes, have distinct ability in binding to soluble NRG1, demonstrating that the differential presentation of HSPG sulfation pattern in the different tissues or cell types, may contribute to the regulation of soluble NRG1 localization and

functions (Nogami et al., 2004; Pankonin et al., 2005). Both the completely desulfated heparin and the De-*N*-sulfated sugars were unable to bind NRG1, while removing the 2-O- and 6-O- sulfate groups reduced NRG1-heparing binding at a lesser extent, suggesting a critical importance of specific sulfation pattern in HSPG for the accumulation of NRG1 on the cell surface. Taken together, all these evidence demonstrate that NRG1 has a unique, structurally distinct, and highly cell/tissue-specific heparin-binding domain that is critical for the targeted distribution of the protein and the initiation of optimal signal transduction.

Because of NRG1's wide range of functions during embryonic development and the strong mitogenic effect in cancers, blocking soluble NRG1 signaling has become an attractive target for both developmental studies and therapeutics (Mendelsohn and Baselga, 2000; Li et al., 2004a; Esper et al., 2006; Montero et al., 2008). Currently, although some soluble NRG1 antagonists could work in cell culture at the high concentration, they can not block the signaling effectively *in vivo*, because they are soluble and not able to concentrate on the same cell surface as endogenous soluble NRG1 does through specific HSPGs binding, which provides a rational to develop a novel effective antagonist with better targeting specificity to block soluble NRG1 signaling. Therefore, in this thesis work, by taking advantage of the naturally specific interaction between NRG1's HBD and HSPGs, I have worked with other colleagues in the lab to generate and characterize a novel fusion protein called HBD-S-H4 that fuses the human NRG1's heparin-binding domain to the soluble ectodomain of human erbB4 (HER4/H4) receptor, and it has the enhanced targeting specificity and can be used as a dominant negative NRG1 antagonist both *in vitro* and *in vivo*, which will be described in

the Chapter II.

Neuregulin-1 and Neurotrophic factors in the peripheral nerve development

NRG1 isoforms are highly expressed by different types of neurons and have multiple important functions in the development and maintenance of the peripheral nervous system including Schwann cells, synapses at neuromuscular junctions (NMJs) and somatosensory system (Fischbach and Rosen, 1997; Falls, 2003; Birchmeier and Nave, 2008; Mei and Xiong, 2008). Transmembrane NRG1 precursor (proNRG1) is transported down axons from neuron cell body and then concentrated in the synaptic basal lamina of NMJs, where soluble heparin-binding forms of NRG1 have been released from motor nerve endings after proteolytic cleavage and activated erbB receptors on the postsynaptic muscle membrane of neuromuscular synapses, resulting in up-regulation of the expression and insertion of muscle acetylcholine receptors (AChRs) required for the proper synaptic transmission (Buonanno and Fischbach, 2001; Li and Loeb, 2001; Li et al., 2004b). Other studies have also indirectly implicated the important roles of soluble NRG1 in the early development of the sympathetic nervous system and the induction of muscle spindle differentiation *in vivo* (Britsch et al., 1998; Hippenmeyer et al., 2002).

In the developing peripheral nerves, neuronal axons, once they first emerge from the spinal cord into the periphery, are always surrounded and supported by Schwann cells that are major glial cells in peripheral nervous system and responsible for the myelination process around axons to facilitate the rapid electrical signal transduction from the cell body to the axon terminal (Jessen and Mirsky, 2005). Schwann cell lineage is originated from neural crest stem cells that first show up at the dorsal part of neural tube and soon

migrate along either ventral or lateral direction. Cells towards ventral side of the neural tube will become Schwann cell precursors (SCPs) that could be differentiated into immature Schwann cells and eventually develop into mature Schwann cells (myelinating or non-myelinating cells).

At the axoglial interface, neuron derived NRG1 plays a critical role in regulating Schwann cell survival, proliferation and differentiation as well as migration at multiple developmental stages of Schwann cell lineage (Dong et al., 1995; Ciutat et al., 1996; Grinspan et al., 1996; Mahanthappa et al., 1996; Meintanis et al., 2001; Winseck et al., 2002; Jessen and Mirsky, 2005; Lyons et al., 2005; Birchmeier and Nave, 2008; Yamauchi et al., 2008). Recently, lots of evidence has shown that membrane-bound type III NRG1 is critical for the Schwann cell myelination (Michailov et al., 2004; Taveggia et al., 2005; Nave and Salzer, 2006). The protein level of this NRG1 isoform determines the differentiation or ensheathment fate of immature Schwann cells and regulates the thickness of myelin sheath around axons through activating specific signal pathways such as phosphatidylinositol-3-kinase (PI-3K) pathway (Maurel and Salzer, 2000; Chen et al., 2003; Ogata et al., 2004; Chen et al., 2006). At early stages of Schwann cell development, NRG1 signaling is required for the migration of neural crest stem cell past the DRG location to the ventral side of neural tube so that Schwann cell precursors could be generated (Dong et al., 1995; Garratt et al., 2000; Wolpowitz et al., 2000). Exogenous soluble NRG1 can rescue Schwann cells from both normal-occurring and nerve injury-induced apoptosis *in vivo* (Kopp et al., 1997; Winseck et al., 2002; Winseck and Oppenheim, 2006). Moreover, Knocking out all NRG1 isoforms or their erbB2/3 receptors leads to a almost complete loss of neural crest cell-derived Schwann cell

precursors as well as the deficiency of sympathetic gangliogenesis (Meyer and Birchmeier, 1995; Meyer et al., 1997; Riethmacher et al., 1997; Morris et al., 1999; Woldeyesus et al., 1999). In contrast, mice with specific disruption of the type III NRG1 isoform show that a reduced number of SCPs are still present in ventral nerve area and would be differentiated into Schwann cells along growing axons at the later stage (Wolpowitz et al., 2000), suggesting the important *in vivo* functions of soluble NRG1 on regulating Schwann cell precursors' survival and differentiation. Taken together, NRG1 provides a critical axonally-derived signaling for promoting Schwann cell development and regulating myelination process. However, so far, the precise, developmentally specific roles of endogenous soluble NRG1 in regulating Schwann cell development and myelination are still not clear.

The neurotrophic factors are a family of survival and differentiation factors that are produced in a variety of tissues, most notably in glia and neuronal targets such as skin and muscle (Diamond et al., 1992; Copray and Brouwer, 1994; Jessen and Mirsky, 1999; Garratt et al., 2000). The first identified neurotrophic factor Nerve Growth Factor (NGF) followed by its other family members Brain-Derived Neurotrophic Factor (BDNF), Neurotrophin-3 (NT-3), and Neurotrophin-4 (NT-4). Other target-derived neurotrophic factors include glial-cell derived neurotrophic factor (GDNF), hepatocyte growth factor (HGF) and ciliary neurotrophic factor (CNTF) etc. Each of them has been shown to promote the survival and differentiation of a variety of neuron types both *in vitro* and *in vivo* through the activation of receptor tyrosine kinase including Trk family receptors (Lewin and Barde, 1996; Gilbert, 2000). NGF signals are mediated by TrkA receptor, BDNF and NT-4/5 are the ligands for TrkB, and TrkC is the main receptor for NT-3. Upon

binding their specific ligands, the Trk receptors homo-dimerize and auto-phosphorylate each other to transduce an intracellular signal. There has been a great deal of attention to the roles of target-derived neurotrophic factors in supporting survival of neurons that send their axons into the periphery (Snider, 1994; Snider and Wright, 1996). Meanwhile, Schwann cells are another major source of a variety of neurotrophic factors including BDNF, NT-3, GDNF, CNTF, PDGF, and IGF-2 (Jessen and Mirsky, 1999; Garratt et al., 2000; Mirsky et al., 2002), and the roles of Schwann cell-derived neurotrophic factors in the regulation of developing peripheral nerves are still poorly understood. Actually, Knockout critical components of NRG1-erbB signaling in mice shows the loss of Schwann cells followed by the dramatic death of the sensory and motor neurons that they support (Meyer and Birchmeier, 1995) (Riethmacher et al., 1997; Morris et al., 1999; Woldeyesus et al., 1999; Wolpowitz et al., 2000), suggesting that the survival of developing neurons depends on not only target-derived neurotrophic factors, but also trophic supports from Schwann cells which are associated with peripheral axons along the course to their targets. Because neuronal survival is mediated in part by Schwann cell secreted neurotrophic factors, it seems reasonable to postulate a bidirectional signaling network between NRG1 and neurotrophic factors at the axon-Schwann cell interface.

Now it is becoming clear that the functions of neurotrophic factors go well beyond neuronal survival. In DRG sensory neuron cultures, NGF, BDNF and NT-3 have direct effects on Schwann cell myelination through TrkA, p75NTR and TrkC receptor respectively (Chan et al., 2001; Cosgaya et al., 2002; Chan et al., 2004; Ng et al., 2007). The axotomy of the peripheral nerve produces an impressive up-regulation of BDNF,

NT-4 and NGF suggesting an important function for these neurotrophic factors in nerve regeneration as well as in development (Funakoshi et al., 1993). In addition, the injection of GDNF into rats leads to the proliferation and myelination of non-myelinating Schwann cells around some of the very small caliber C-fiber axons (Hoke et al., 2003). Similarly, we have demonstrated that in the neuromuscular synapses, neurotrophic factors produced by postsynaptic muscles promote activity-dependent soluble NRG1 release from presynaptic nerve terminal and the addition of both BDNF and GDNF is able to rescue NRG1 expression after the blockade of synaptic activity, suggesting a feedback loop between presynaptic NRG1 release and postsynaptic expression of neurotrophic factors (Loeb et al., 2002). Above all, we have extended the study of bidirectional communication between neurotrophic factors and NRG1 signaling to axon-glial interface, and found that Schwann cell derived neurotrophic factors, including BDNF and GDNF, promote the rapid local release of soluble NRG1 from axons, through protein kinase C-δ (PKC-δ) activation, in both motor and sensory neuron cultures (Esper and Loeb, 2004,2009). So likely, the study of the interplay between neuron-derived soluble NRG1 and Schwann cell-secreted neurotrophic factors will help to better understand the axon-glial communication in both development and diseases. In this thesis work, I will specifically focus on the *in vivo* roles of reciprocal signaling between soluble NRG1 and BDNF in regulating Schwann cell early development including their survival, differentiation and proliferation at axoglial interface.

CHAPTER II

DEVELOPMENT OF A NOVEL ANTAGONIST FOR SOLUBLE NEUREGULIN-1

SUMMARY

A major goal of this project is to develop a potent antagonist of soluble NRG1 signaling for its functional studies *in vivo,* and challenge the limitation of selectively targeting drugs to diseased tissues in the current biopharmaceutical development that has been solved by growth factors and viruses through targeting tissue-specific cell-surface heparan-sulfates. Neuregulin-1 (NRG1), as a growth factor important in both nervous system development and cancer, has a unique heparin-binding domain (HBD) that targets to cell surfaces expressing its erbB2/3/4 receptors. We have harnessed this natural targeting ability of NRG1 by fusing NRG1's HBD to soluble ectodomain of human erbB4 (HER4/H4). This fusion protein retains high-affinity heparin binding to heparin and to cells that express heparan sulfates resulting in a more potent NRG antagonist. *In vivo*, it is targeted to peripheral nerve segments where it significantly blocks the activity of NRG1 as a Schwann cell survival factor. This novel antagonist not only provide a new way to block NRG1 signaling and allows us to directly study its function *in vivo*, but also demonstrates the utility of NRG's heparin-binding domain in biopharmaceutical targeting with enhanced specificity.

METHODS

Construction of fusion proteins

All fusion proteins were derived entirely from human sequences. The extracellular domain of HER4 receptor (H4) corresponds to 99-2042bp of the human HER4 NM_005235 mRNA. The sequence from 99-173bp encodes a 25 amino acid signal sequence that was incorporated onto the N-terminus of all constructs for secretion and protein expression. H4 was amplified by PCR and then inserted into pMH vector (Boehringer Mannheim) between KpnI and EcoRI to generate the H4-HA construct. The HBD (532-849bp) and HBD-S (532-1023bp) domains of NRG β1 form (NM_013964) were amplified from the plasmid HARIA $PATH_2$ (gift of Dr. Tejvir Khurana, University of Pennsylvania). Either HBD-S or the HBD domain alone was inserted into pMH-H4-HA in the KpnI site to generate HBD-S-H4-HA and HBD-H4-HA, respectively. H4-HBD-HA was subcloned by inserting the HBD domain into pMH-H4-HA between the EcoRI and BamHI sites. For the HBS-S-H4 and H4 fusion proteins, the HA-tag in both constructs was replaced by a His-tag using PCR and they were subcloned into pMH between NdeI and BamHI site.

Expression and purification of fusion proteins

HEK293 cells were transfected with the four recombinant constructs using lipofectamine 2000 according to the manufacturer (Invitrogen). The G418 geneticin (Invitrogen) was then added at a concentration of 400μg/ml to the HEK293 cells to select the positive transfected cells. Stable cell lines were selected following three weeks of

G418 selection. Stably transfected HEK293 cells were diluted and plated in 96 well plates to yield single positive clones. The single clones that expressed the highest level of each fusion proteins (confirmed by western blot using HA antibody) were then maintained in culture media with 200μg/ml geneticin. To express and purify His-tagged HBS-S-H4 and H4 proteins, HEK293 freestyle suspension cell line was transfected with both constructs by 293fectin reagent (Invitrogen) following manufacturer's instruction. Media from 7-8 day old transfected cell culture was then collected by centrifugation and fusion proteins were purified using a nickle column (Qiagen) for His-tagged proteins, followed by a heparin-Sepharose column (Sigma) for HBD-S-H4 or a second nickle column for H4. Purity was assessed by silver stained gels and protein quantification was determined using the Bradford Assay (Pierce).

Silver staining

Purity of recombinant proteins was assessed by resolving 250ng of HBD-S-H4 and H4 fusion proteins on a 7.5% reducing SDS-polyacrylamide gel followed by silver staining using the SilverSNAP Stain Kit (Pierce) following manufacturer's instruction.

L6 assay

Fusion proteins were pre-mixed with 75pM NRG at room temperature for 30 minutes and then applied to L6 myotubes in 48-well plates for 45 minutes at 37°C. In some cases, HBD-S-H4 or H4 was added to L6 myotubes first, incubated for 45 minutes, then washed with medium 3 times, and challenged with 75pM NRG for 45 minutes. The erbB receptor phosphorylation (p185) assay was performed as described previously (Esper and Loeb,

2004) using the phosphotyrosine antibody (pY, 4G10; Upstate Biotechnology). The blot was stripped and reprobed with erbB2 and erbB3 (Neomarkers) antibodies for the quantitation of total erbB proteins present in the lysate. Band intensity was quantified with Metamorph Image analysis software (Universal Imaging) (Li et al., 2004a). Recombinant NRG protein, corresponding to amino acids 14-276, was purchased from R&D Systems.

CHO cell binding assay and immunofluorescent staining

HBD-S-H4 and H4 were conjugated with biotin by a biotin protein labeling kit (Solulink) following the manufacturer's instructions. $1x10^4$ wild-type CHO or CHO-pgsD677 cells that lack heparan sulfate (ATTC#: CRL-2244) were plated in each well of 96-well plate or chamber-slide and cultured for 3 days. Some cells were treated with 2 units/ml heparinase (Sigma) or media for 3 hours and then incubated with 50nM biotin-conjugated HBD-S-H4 or H4 at room temperature for 2 hours. Cells were washed with PBS for three times. For the cell binding assay, strepavidin-horseradish peroxidase (HRP) was added to live cells in 96 -well plate and incubated for 30 minutes. Signals were measured by addition of chemiluminescence reagents (Perkin-elmer) and using a microplate luminometer. For staining, cells were fixed in 4% paraformaldehyde for 30 minutes and strepavidin-HRP and tyramide-alexa fluor 647 (Invitrogen) were used to visualize the signal.

Heparin binding assay

Optimem I (Invitrogen) was incubated with transfected HEK293 cell lines for 2 days

before the conditioned media containing each of four recombinant HA-tagged constructs were collected and passed through a heparin-Sepharose column (Sigma). The flow-through was collected and the column was washed with PBS. The binding proteins were then eluted with increasing concentrations of NaCl (0.25, 0.3, 0.4, 0.5, 0.6 and 1M). The flow-through and elution fractions were analyzed by western blotting with an anti-HA antibody (Covance) to determine the extent of heparin binding for each protein.

A heparin-binding plate (BD bioscience) was coated with 150µl/well of 100µg/ml heparin (Sigma) overnight at room temperature (Mahoney et al., 2004). Wells were blocked with 0.2% gelatin/PBS for 1 hour at 37°C. Biotin-conjugated HBD-S-H4, H4 or IgG was added to each well at the concentration from 0 to 150nM and incubated for 2 hours at 37°C. Wells were then washed three times with PBS and incubated with avidin-HRP (Sigma) for 30 minutes. Binding was detected by adding substrate p-nitrophenyl phosphate (pNPP) and the absorbance was measured at 405nm. Dissociation constant (Kd) of the saturation curve was calculated by a online Kd calculator:

www.invitrogen.com/site/us/en/home/support/research-tools/kd-calculator.html

Chicken eggs and in ovo treatment

Fertilized chicken eggs were obtained from Michigan State University Poultry Farms (East Lansing, MI) and incubated at 37°C in a Kuhl rocking humidified incubator (Flemington, NJ) at 50% humidity. Chicken embryo experiments were performed with approval of Institutional Animal Care and Use Committee at Wayne State University. Either HBD-S-H4 or H4 proteins were dissolved in sterile saline containing 0.2% BSA to

prepare 600nM concentration in total volume of 200μl solution, and applied daily to embryos at E5 and E6 through a small hole on choroallantoic membrane (Winseck et al., 2002). Treatment with the identical volume of saline without recombinant proteins was used as a control. The eggs were sealed after each treatment with clear packing tape and embryos were sacrificed at E7 for immunostaining as described below.

Immunostaining

Chicken embryos were fixed with 4% paraformaldehyde overnight. After washing briefly with PBS, embryos were placed in 30% sucrose and cut transversely at 12μm on a cryostat. Immunofluorescence was performed as described previously (Ma et al., 2009). Sections were incubated with antibodies to chicken NRG 183N (Loeb et al., 1999)(1:100) or human NRG AD03 (Pankonin et al., 2009) (1:300, Assay Designs) and Schwann cells 1E8 (1:10, Developmental Studies Hybridoma Bank) or 6xHis-tag (1:100, Abcam) in blocking solution (10% normal goat serum, 0.2% TritonX-100 in PBS) for overnight at 37°C, followed by the incubation with goat anti-mouse or anti-rabbit alexa-fluor 546 (1:250, Invitrogen). For AD03 or His-tag immunostaining, biotin conjugated goat anti-rabbit (1:500, Perkin-elmer) or HRP conjugated goat anti-mouse (1:100, Invitrogen) was used as secondary antibody and signal was detected using a tyramide signal amplification kit (Invitrogen) following the manufacturer's instructions. Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assays were performed with in situ cell death kit (Roche) after the incubation of secondary antibodies for AD03 and 1E8 double-labeling. Some sections were treated with 1M NaCl/PBS at 37°C for 90 minutes as described previously to disrupt ionic interactions before the

immunostaining procedure.

Quantitative analysis

Digital images were obtained with a Nikon Eclipse 600 epifluorescent microscope with a Princeton Instruments Micromax 5 MHz cooled CCD camera. Metamorph Image analysis software (Universal Imaging) was used to quantify the percentage of apoptotic Schwann cells in the area of ventral nerve root at the lumbar level of spinal cord. Regions of interests were first defined using a Schwann cell marker staining for a non-biased selection of ventral nerve regions. These nerve segments were further divided in half at the midpoint between the spinal cord and the union of the motor and sensory axons. The total number of Schwann cells in each nerve segment was quantified by counting the number of full nuclei in each of these regions of interest using the nuclear dye DAPI. This was achieved by dividing total pixel area of signal thresholded for the nuclear signal by the average pixel area for an individual Schwann cell nucleus. Manual counts were used to validate this method. The number of TUNEL positive Schwann cell nuclei was then counted in both proximal and distal nerve areas. 12-20 sections for each animal, and at least 5 animals for each condition were quantified. Statistical significance was defined as $p < 0.05$ using a two-tailed student's *t* test.

RESULTS

Optimal fusion of neuregulin's heparin-binding domain (HBD) to the human erbB4 receptor (HER4/H4)

As the first step, fusion proteins were generated (performed by my colleague, Qunfang Li & Mark Pankonin) to determine the optimal arrangement of the HBD to retain high affinity heparin-binding (Fig. 3*A*). The HBD was inserted either C-terminal (H4-HBD) or N-terminal (HBD-H4 and HBD-S-H4) to H4. The HBD-S-H4 contains the natural-occurring glycosylated spacer domain (S) from NRG placed between the HBD and H4 domain (Fischbach and Rosen, 1997). Heparin-sepharose chromatography was used to compare the heparin binding ability of each relative to the H4 protein alone by determining the salt concentration required to elute it from the column. While the HBD-S-H4 and H4-HBD proteins bind the heparin column, the H4 and HBD-H4 constructs did not (Fig. 3*A*). Interestingly, the construct with the spacer, that most closely resembles native NRG (HBD-S-H4), had the highest heparin binding affinity requiring 1M NaCl for elution. This may be due to the physical separation of the HBD from H4 that could either allow an optimal protein conformation necessary for heparin binding or prevent steric inhibition between the two domains. The ability of each construct to block NRG-induced receptor activation was then determined based on its ability to block NRG induced erbB receptor tyrosine phosphorylation (p185) in rat L6 cells (Fig. 3*B*). Consistent with the heparin binding results, antagonist potency paralleled their heparin-binding activities. While H4 and HBD-H4 had little effect on reducing receptor phosphorylation, HBD-S-H4 and H4-HBD significantly inhibited erbB receptor activation.

The quantification of phosphorylated erbB protein normalized to total erbB2 protein, revealed that HBD-S-H4, is the most potent antagonist to block NRG1-induced erbB activation with the reduction of the p185 signal by approximately 80%. These pre-screening results demonstrate that the isolated HBD retains high-affinity heparin binding activity, even when fused to another protein, resulting in a NRG antagonist with significantly improved blocking potency.

HBD-S-H4 is targeted to cell surface heparan sulfates.

Based on the above pre-screening results, my colleague, Haiqian An, has generated highly purified HBD-S-H4 and native H4 His-tagged fusion proteins in HEK293 suspension cells (Fig. 4*A*), and then, in this part of my thesis work, I continure to characterize biochemical properties of the fusion proteins as well as their cell/tissue binding ability and *in vivo* blocking activity.

In order to demonstrate the enhanced blocking activity of HBD-S-H4 is due to its ability to concentrate on cell surfaces, I pre-treated L6 cells with either HBD-S-H4 or H4 and then vigorously washed to remove any antagonist not bound to the cell surface. They were then challenged with soluble NRG as shown in Fig. 4B. Cells pre-treated with HBD-S-H4 were resistant to NRG activation, whereas no effect was observed with H4 treatment.

To further confirm that the accumulation of HBD-S-H4 protein on cell surface is mediated by the interaction of HBD and HSPGs, CHO cells that do and do not express heparan sulfates on their surfaces, are used to compare the binding of biotinylated HBD-S-H4 onto wild-type CHO cells without and with heparinase treatment and to

pgsd677 mutant CHO cells that lack the ability to synthesize heparan sulfate (Lidholt et al., 1992). While the HBD-S-H4 construct adhered to the wild type CHO cells, the H4 construct without heparin-binding domain did not. Similarly, the absence of heparan sulfate in mutant cells or heparinase treatment of wild type cells significantly decreased HBD-S-H4 binding with minimal fluorescence intensity (Fig. 5*A*). Similarly, chemiluminescence assay after adding the fusion proteins to these cells shows that HBD-S-H4 has significantly enhanced binding ability to wild type CHO cells, compared to either the treatment on mutant CHO cells or the H4 treatment (Fig. 5*B*). Finally, a binding assay was used to determine the affinity of HBD-S-H4 to heparin. While both H4 and IgG had minimal binding, HBD-S-H4, bound to heparin-coated plates saturating at 60nM and a calculated dissociation constant (Kd) of 14.7nM (Fig. 5*C*). All these results demonstrate that the increased potency of HBD-S-H4's cell-surface binding is in part due to its ability to specifically interact with heparan sulfate and concentrate on cell surfaces where it exhibits sustained activity.

In vivo targeting to peripheral nerve segments induces Schwann cell apoptosis

Within the developing spinal cord, NRG1 isoforms are highly expressed in motor and sensory neurons and have critical functions in peripheral nerve development, including Schwann cell proliferation, survival and myelination (Jessen and Mirsky, 2005; Nave and Salzer, 2006; Birchmeier and Nave, 2008). In order to determine if HBD-S-H4 targets the same regions of the developing nervous system as endogenous NRG1, we compared the tissue distribution of exogenously added HBD-S-H4 in chicken embryos to the endogenous expression pattern of chick NRG1 during embryonic development (Fig. 6, 7).

HBD-S-H4 was localized on tissue sections of these embryos using an antibody (AD03) that only recognizes the human extracellular domain of NRG (huNRG) and does not cross-react with endogenous chicken NRG (chNRG) (Pankonin et al., 2009). Within the spinal cord, HBD-S-H4 became concentrated in the same region of the spinal cord and peripheral nerve as endogenous chNRG. The fusion protein also accumulated outside the nervous system in regions that do not normally express NRG1. This distribution outside the nervous system is likely due to its adherence to developmentally-expressed HSPGs (Fig. 6). In peripheral nerve area, no huNRG immunoreactivity was present in the saline or H4 treated animals, while double-labeling the ventral nerve root shows that the same regions of peripheral nerve where chNRG was concentrated, bound high levels of HBD-S-H4 (Fig. 7). Slight differences in patterns may reflect mild protein sequence differences between the human and chicken HBD. High salt treatment, which interrupts NRG1-HSPG ionic interactions in the extracellular matrix, removed the HBD-S-H4 immunoreactivity suggesting the interaction of the fusion protein's HBD with HSPGs is important for the HBD-S-H4's accumulation on the specific cell surface *in vivo* (Fig. 7). Consistently, immunostaining with a His-tag antibody shows that only HBD-S-H4, but not H4 became concentrated in this nerve area, although both fusion proteins have His-tag on their C-terminus (Fig. 8). All these results demonstrate that systemically administered HBD-S-H4 is targeted to and concentrated on the same regions of the developing nervous system as endogenous NRG1 through its heparin-binding domain, thereby making it a useful tool to study the roles of matrix-bound NRG1 in peripheral nerve development.

Previous studies have shown that NRG1 signaling is critical for Schwann cell survival

during early embryonic development (Winseck et al., 2002; Jessen and Mirsky, 2005). Given its ability to concentrate in these developing nerves, the *in vivo* efficacy of HBD-S-H4 was determined by counting apoptotic Schwann cell number in the ventral nerve root. HBD-S-H4 treatment significantly increased the number of apoptotic Schwann cells, while H4 had no effect (Fig. 9*A, B*). Analysis of distal nerve regions with maximal HBD-S-H4 staining shows maximal Schwann cell apoptosis, corresponding to the regions rich in endogenous chNRG1. In comparison, much less dying Schwann cell number was found in the proximal nerve regions that also shows low level of both HBD-S-H4 and chNRG1 accumulation. Taken together, these findings demonstrate that HBD specifically targets HBD-S-H4 to the same nerve segments as endogenous chick NRG1 where it effectively antagonizes NRG's actions as a Schwann cell survival factor *in vivo*.

DISCUSSION

In this chapter, I characterized the heparin-binding ability and blocking activity of HBD-S-H4, a novel potent NRG1 antagonist, providing both *in vitro* and *in vivo* evidence that NRG1's HBD can retain heparin-binding specificity when fused to other polypeptides and demonstrating the efficacy of a novel and highly effective way, not only to block soluble NRG1 signaling, but also to enhance the protein drug's tissue/cell-targeting specificity. The fusion protein that we generated and purified, not only targets the same regions of the developing nervous system where endogenous NRG1 binds, but also effectively blocks NRG1 activity leading to excessive Schwann cell apoptosis. This result affirms NRG1's *in vivo* role as a Schwann cell survival factor and provides a new reagent to examine the roles of released forms of NRG1 in both development and diseases.. Although HBD-S-H4 was designed to block the activity of soluble NRG1 by taking advantage of its natural targeting system, we can not exclude the possibility that it also could block erbB2/3 activation induced by membrane-bound, CRD form of NRG1 when the fusion protein gets into the extracellular space of Schwann cells that closely contact with axons at the axoglial interface after systematically treatment. So, the precise, developmentally specific *in vivo* functions of soluble NRG1 will be further studied and discussed in the following Chapter III, by using the combination of this novel antagonist and other genetic tools.

Previously, similar efforts have been made to generate fusion proteins with heparin-binding domains. Fusion of the heparin-binding domain of vascular endothelial growth factor A (VEGF-A) and heparin-binding epidermal growth factor (HB-EGF) to

VEGF-E and insulin-like growth factor-1 (IGF-1), respectively, can maintain their biological activities with less unwanted side effects by increasing the binding specificity on the cell surfaces (Heil et al., 2003; Tokunou et al., 2008). However, these HBDs are relatively simple with short stretch of positive-charged amino acids in the domain, and thus containing not much information about binding specificity. In comparison, NRG1's HBD has a unique disulfide-linked C2 immunoglobulin loop capable of maintaining a structure necessary for tissue/cell type specific heparin binding with high affinity leading to precise protein tissue distribution during both development and diseases (Loeb, 2003). This key feature of the domain has set NRG1 apart from other growth factors and viral heparin-binding proteins in the ability of heparin-binding.

Interestingly, in the fusion protein, a glycosylated spacer peptide that also exists in natural soluble NRG1 structure appears to be required to keep the HBD away from the HER4 domain and maintain maximal biological activity of both domains. Fusion of NRG1's HBD to a soluble HER4 receptor not only converts it into a heparin-binding protein, but also dramatically potentiates its ability to block soluble NRG1 signaling through the binding of specific HSPGs in the extracellular matrix and the concentration on cell surface. This concentration not only provides sustained antagonist activity, but also targets the antagonist to the same tissues where the agonist is expressed, thus placing it on a more equal footing. Recently, in collaboration with others, we have successfully shown that the intrathecal injection of HBD-S-H4 is able to block NRG1 signaling and reduce microglial cell proliferation and chemotaxis that directly contribute to the development of neuropathic pain after sciatic nerve injury, and thus lead to the improvement of pain-related behaviors including mechanical pain hypersensitivity and

cold allodynia in the rat model (Calvo et al., 2010; Calvo et al., 2011). Therefore, this novel entirely “humanized” fusion protein is a powerful targeted antagonist that could be a useful research tool to study NRG1’s multiple *in vivo* critical functions during development, as well as be used in disease states where NRG1 signaling is deleterious (Ma et al., 2009).

CHAPTER III

RECIPROCAL AXON-GLIAL SIGNALING THROUGH SOLUBLE NEUREGULIN-1 AND BDNF REGULATES EARLY SCHWANN CELL DEVELOPMENT

SUMMARY

During peripheral nervous system development, successful communication between axons and Schwann cells is required for proper function of both myelinated and non-myelinated nerve fibers. Alternatively-spliced proteins belonging to the neuregulin1 (*NRG1*) gene family of growth and differentiation factors are essential for Schwann cell survival and peripheral nerve development. While recent studies have strongly implicated membrane-bound NRG1 forms (type III) in the myelination at late stages, little is known about the role of soluble, heparin-binding forms of NRG1 in regulating early Schwann cell development *in vivo*. These forms are rapidly released from axons *in vitro* by Schwann cell-secreted neurotrophic factors, and, unlike membrane-bound forms, have a unique ability to diffuse and adhere to heparan sulfate-rich cell surfaces. Here, we show that axon-derived soluble NRG1 translocates from axonal to Schwann cell surfaces in the embryonic chick between days 5-7, corresponding to the critical period of Schwann cell precursor's survival and differentiation. Down-regulating endogenous type I/II NRG1 signaling either with a targeted NRG1 antagonist or by HBD-specific shRNA, blocks their differentiation from precursors into immature Schwann cells and increases programmed cell death, while up-regulating NRG1 rescues Schwann cells. Exogenous BDNF also promotes Schwann cell survival through promoting the local release of axonal NRG1. Consistently, increased Schwann cell death occurs both in trkB knock-out

mice and after knocking-down axonal trkB in chick embryos, which can then be rescued with soluble NRG1. These findings suggest a localized, axoglial feedback loop through soluble NRG1 and BDNF critical for early Schwann cell survival and differentiation.

METHODS

Chick eggs and in ovo treatment

Fertilized chicken eggs were obtained from Michigan State University Poultry Farms (East Lansing, MI) and incubated in a Kuhl rocking incubator (Flemington, NJ) at 50% humidity. Daily treatments of recombinant human NRG1-β1 extracellular domain (aa 2-246, #377-HB, R&D systems), recombinant human BDNF (aa 129-247, #248-BD, R&D systems) or the recombinant NRG1 antagonist (HBD-S-H4) on chick embryos were performed as described previously (Loeb et al., 2002; Winseck et al., 2002; Ma et al., 2009). In brief, 5μg NRG1, 1μg BDNF, 10 or 20μg HBD-S-H4 were each prepared in saline containing 0.2% BSA, and added onto the chorioallantoic membrane through a small hole in the air sac without damaging underlying blood vessels for two consecutive days. Staging of chick embryos was determined according to Hamburger-Hamilton (HH) stage series (Hamburger and Hamilton, 1951): E4 (stage 24); E5 (stage 26-27); E6 (stage 28-29); E7 (stage 30-31).

TrkB Mice

TrkB-heterozygous mice were generously provided by M. Barbacid (Klein et al., 1993). Homozygous timed-pregnant mutant embryos of either sex were obtained by heterozygous mating and harvested at E12.5 or E13.5 (the day when the vaginal plug was observed, is designated as embryonic day 0). 4-5 separate litters were collected at each stage. Mouse embryo experiments were performed with approval of Institutional Animal Care and Use Committee at Wayne State University.

Chick in ovo electroporation and shRNA knockdown

Type I proNRGβ1a cDNA with a myc-tag at the C-terminus was subcloned into the pMES vector downstream from the chick β-actin promoter with IRES-EGFP (Krull, 2004). shRNA for the heparin-binding domain of chick NRG1 and chick trkB were designed and cloned into the pSilencer 1.0-U6 expression vector (Ambion) according to manufacturer's instructions. Three shRNAs against different regions of each cDNA were tested and the shRNA with the best efficacy *in vitro* was selected for *in ovo* electroporation. The target sequence selected for chick heparin-binding domain (HBD) of soluble NRG1 was AAGCTAGTGCTAAGGTGTGAA, and for chick trkB was AAGGAGCTATATTGAATGAGT. The pCAX vector expressing EGFP was used for co-electroporation with other plasmids to visualize electroporated cells (George et al., 2007). The final concentration of each plasmid was 3μg/μl. The plasmid DNA was electroporated unilaterally into the ventral part of the neural tube at the lumbar level at E2.5 (HH stage 15-16) as previously described (Eberhart et al., 2002). Electrodes were placed ventral-dorsal across the neural tube and pulsed for five times at 35V for 50ms with a square-wave pulse generator (Intracept TSS10, Intracel Ltd.). Embryos were collected from E5 to E7, and only those with strong GFP expression were processed for further analysis.

Immunostaining, TUNEL and BrdU

Chicken and mouse embryos were fixed with fresh 4% paraformaldehyde overnight. Embryos were then equilibrated in 30% sucrose after rinsing quickly with PBS and

mounted in OCT (Tissue-Tek). Frozen sections were cut transversely at 14μm and placed on Superfrost slides (Fisher). Immunofluorescence was performed at the lumbar level as described previously (Loeb et al., 1999; Ma et al., 2009). Sections were incubated with antibodies at the following dilutions: chicken soluble NRG1 ectodomain (183N, rabbit polyclonal, 1:200) (Loeb et al., 1999); P0 (1E8,1:5), AP2α (3B5, 1:10), BrdU (G3G4, 1:100), and neurofilament (3A10,1:10) (Developmental Studies Hybridoma Bank, University of Iowa); GFP (ab6662 1:100) and p75 (ab70481, 1:100) (Abcam); myc-tag (2272, 1:100 Cell Signaling); trkB (sc-12, 1:200, Santa Cruz Biotechnology). Sections were incubated with antibodies in blocking solution (10% normal goat serum, 0.2% TritonX-100 in PBS) overnight at 4 °C, followed by incubation with the corresponding goat anti-mouse or anti-rabbit IgG alexa-fluor antibodies (1:250, Invitrogen) for visualization. O4 (MAB345, 1:100, Millipore) and the trkB antibody were prepared in a blocking solution containing 5% fetal bovine serum in PBS and after overnight incubation, goat anti-mouse IgM secondary antibody was used for O4 detection. For BrdU staining, chick embryos were given 10μg BrdU through the air sac for 3 hours before harvest. Sections were pretreated with 2N HCl to denature DNA for the exposure of BrdU antigen, followed by incubation with blocking solution for 1 hour. Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assays were performed with the in situ cell death-TMR kit (Roche) following the manufacturer's instructions (Ma et al., 2009). Some sections were first treated with 1M NaCl/PBS at 37 °C for 90 minutes as described previously to disrupt ionic interactions between NRG1 and HSPGs (Loeb et al., 1999).

shRNA testing

10^5 COS7 cells were seeded in each well of 8-well chamber slides (BD Bioscience) and co-transfected with type I proNRG1 and EGFP cloned in the pTriex vector (Novagen) together with a given shRNA in pSilencer vector using Lipofectamine 2000 (Life Technologies). The next day, cells were fixed in 4% paraformaldehyde for 30 min and stained for NRG1 (sc-348, 1:100, Santa Cruz Biotechnology) as described above. Nuclei were counter-stained using 4', 6-diamidino-2-phenylindole (DAPI).

Immunoblotting and Quantitative real-time PCR

Spinal cords were harvested from BDNF/saline treated chick embryos or electroporated embryos at E5.5 (HH stage 27), and then processed for protein or RNA extraction. A 5-6 somite-long segment at the lumbar level with high levels of GFP expression was isolated from each embryo as described previously (Liu, 2006) and total protein was extracted separately from the electroporated and control sides, using RIPA lysis and extraction buffer containing 25 mM Tris pH 7.6, 150 mM NaCl, 1% NP40, 1% sodium deoxycholate, 0.1% SDS, and protease inhibitors (Thermo Scientific). Protein samples from 3 animals were used for immunoblotting using antibodies at the following dilutions: NRG1 (sc-348, 1:500, Santa Cruz Biotechnology), β-Actin (A5441, 1:1,000, Sigma), Neurofilament (AB1987, 1:2,000, Millipore), and GFP (ab6556, 1:2,500, Abcam). SuperSignal West Pico Chemiluminescent Substrate (Thermo Scientific) was used for signal detection and each blot was stripped and reprobed with different antibodies. Cos7 cells after transfection were harvested by passive lysis buffer (Promega) and sc-348 antibody was used to detect the expression of exogenous NRG1 (this will detect all

forms of NRG1 with a cytoplasmic tail). Quantification of band intensity was performed using Metamorph image analysis software (Universal Imaging) as described previously (Li and Loeb, 2001; Esper and Loeb, 2004).

For quantitative RT-PCR, total RNA from chick spinal cords after *in ovo* treatment or electroporation, was collected using the RNeasy kit (Qiagen). SuperScript™ First-Strand Synthesis System for RT-PCR (Invitrogen) was used for reverse transcription. Chick HBD-NRG1 transcripts (type I and II) were detected by using the following oligonucleotides directed at the HBD: forward 5'-GACGGACGTCAACAGCAGTTAC; reverse 5'-CAACCTCTTGGTTTTTCATTTCCT; and taqman probe 6FAM-ACACAGTGCCTCCC. For detecting CRD-NRG1 (type III) transcripts, the primers were forward: 5'-ACGGCATCTCAGGCACAAG, reverse: 5'-AAGTGGAAAGTTTTGGAGCAGTTT, and taqman probe: 6FAM-AACAGAAACCAATCTC (ABI). Chicken glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Gg03346990_g1, ABI) was used for normalization. Quantitative PCR data were collected from 5 biological replicates and $\Delta\Delta$Ct was used for calculations.

Imaging and Quantitative analysis

Digital fluorescent images were obtained with a Nikon Eclipse 600 epifluorescence microscope. Confocal stacks of images were acquired from 14μm thick sections with a z-step of 1.05μm using a D-Eclipse C1 confocal system (Nikon), and all representative images are shown as single confocal planes for more precise determination of co-localization. At least 5 sections for each animal (total 5 animals) were used for each

embryonic stage. Metamorph image analysis software (Molecular Devices) was used to quantify co-localization. Quantification of TUNEL positive Schwann cell nuclei was performed as described previously (Ma et al., 2009). 12-20 sections (around 1500 Schwann cell nuclei) at the lumbar level were used to analyze each condition in each animal. shRNA knockdown of trkB was quantified by measuring the intensity of trkB staining divided by the total cell number in the ventral horn that were counted by DAPI staining. At this stage of development the ventral horn consists almost entirely of motor neurons. Statistical significance was defined as $p < 0.05$ using either a one-way ANOVA or a two-tailed student's *t*-test. All data are presented as the mean ± SEM.

RESULTS

Soluble, matrix-bound NRG1 transitions from axons to Schwann cells

Once released from neurons, soluble forms of NRG1 adhere to heparan sulfate rich surfaces through the developmental expression of HSPGs (Loeb et al., 1999). To examine the spatial distribution of soluble NRG1 during early chick axon-glial interactions in the ventral nerve root, we performed confocal microscopy using specific antibodies against the extracellular domain of soluble type I/II NRG1 together with either Schwann cell (P0 protein) (Bhattacharyya et al., 1991) or axonal (neurofilament) markers from E5 to E7 (Fig.10). While at E5, soluble NRG1 is concentrated along motor axons and is not associated with Schwann cells, between E6 to E7, NRG1 becomes progressively localized to Schwann cells (Fig.10*A, B*). Quantitative analysis shows that co-localization of NRG1 immunoreactivity on Schwann cells significantly increases from 25% to 75% between E5 and E7 (Fig.10*C*), with a corresponding decrease in NRG1 axonal localization (Fig.10*D*). In order to show that the NRG1 immunoreactivity seen is indeed due to soluble forms of NRG1 bound to the ECM, we used high salt treatment to disrupt NRG1-HSPGs ionic interactions (Fig.11) (Loeb et al., 1999). This treatment significantly reduced soluble NRG1 immunoreactivity suggesting the protein is bound to developmentally-expressed heparan sulfates along both axons and Schwann cells.

Soluble NRG1 regulates Schwann cell survival in vivo

During early peripheral nerve development, an excess number of Schwann cells are born than are ultimately needed, and their survival has been shown to depend on

axon-derived NRG1 signaling both *in vitro* and *in vivo* during normal development and after axon denervation (Dong et al., 1995; Wolpowitz et al., 2000; Winseck et al., 2002). To investigate further soluble NRG1's functions on Schwann cell survival during this transition of NRG1's localization from axonal to Schwann cell surfaces, we used the novel NRG1 antagonist (HBD-S-H4) that has described in the Chapter II of this dissertation. It can specifically targets the heparan sulfate-rich surfaces that NRG1 binds, by fusing NRG1's heparin-binding domain to a soluble 'decoy' erbB4 receptor with high affinity for NRG1's EGF-like domain (Ma et al., 2009). Treatment with this antagonist results in a dose-dependent increase in Schwann cell death at E7 along both motor and sensory axons (Fig. 12*A, B*). Consistent with previous studies (Winseck et al., 2002), exogenous soluble NRG1 significantly rescues normal-occurring Schwann cell death at both E5 and E6 (Fig.12*C*).

In order to be certain that these survival effects are due to endogenous type I/II NRG1 isoforms, we used chick *in ovo* electroporation to modulate the expression of soluble isoforms unilaterally in the motor axons at lumbar level spinal cord, which has been approved to be feasible approach by comparing GFP expression pattern at the electroporated side to the contralateral side (Fig.13*A, B*). Down-regulation of only soluble NRG1 isoforms in motor neurons using an heparin-binding domain (HBD)-specific shRNA, effectively reduces NRG1 expression at protein level by over 95% *in vitro* (Fig.14*A, B*). Knocking down endogenous soluble NRG1 significantly increased Schwann cell death at E7 (Fig.14*C*). In contrast, over-expression of full-length type I NRG1 with a C-terminal myc-tag in motor neurons rescued Schwann cells from apoptosis at E6, but not E5 (Fig.15*B*). Over-expression of NRG1 was confirmed by

myc-tag staining at the electroporated side (Fig. 15*A*). These results demonstrate that axon-derived, soluble NRG1 isoforms mediate Schwann cell survival *in vivo* in a stage-dependent manner that parallels the expression of developmentally timed NRG1 deposition on Schwann cells.

Soluble NRG1 promotes differentiation of Schwann cell precursors to immature Schwann cells

Schwann cell precursors (SCPs) differentiate into immature Schwann cells that elongate along axons and can then further differentiate into myelinating or non-myelinating Schwann cells, depending on instructions provided by the axon (Taveggia et al., 2005). While type III NRG1 forms contribute to Schwann cell development *in vivo* (Wolpowitz et al., 2000), they are not essential for Schwann cell survival and early differentiation, suggesting a important role for other types of NRG1. Previous *in vitro* studies have shown that SCP survival is more critically dependent on soluble NRG1 signaling than are immature Schwann cells (Jessen and Mirsky, 2005). Given that peak Schwann cell death occurs at E5-E6 in the chick (Ciutat et al., 1996), it seems likely that the *in vivo* survival effects we and others have observed with NRG1 are on SCPs rather than on immature Schwann cells. To confirm this, we measured the transition of SCPs to immature Schwann cells from E4 to E7. While AP2α, a transcription factor marker for SCPs (Jessen and Mirsky, 2005), was down-regulated in motor axon Schwann cells between E4 to E7, it remained highly expressed in the DRG Schwann cells that mature more slowly (Fig.16). S100β is often used as a marker of immature Schwann cells in other species, however, in the chick embryo, it is not expressed until

E13 when the myelination process is initiated (Bhattacharyya et al., 1992). We therefore used an antibody against the lipid antigen O4 as a marker for immature Schwann cell differentiation (Dong et al., 1999). Fig.16 shows that O4 expression turns on rather abruptly in Schwann cells of motor axons at E7, suggesting the normal transition of SCPs to Schwann cells occurs between E6 and E7. When we blocked NRG1 activity using the targeted NRG1 antagonist between E5 and E6, O4 expression at E7 was prevented in both motor and sensory axons, while AP2α expression was not affected (Fig.17). These findings suggest that soluble NRG1 signaling is not only critical for SCP survival, but also for their differentiation into immature Schwann cells.

Soluble NRG1 was formerly called glial growth factor because of its strong mitogenic effect on Schwann cells *in vitro* (Dong et al., 1995; Morrissey et al., 1995). However, its effects on proliferation *in vivo* are less clear with some data suggesting anti-proliferative effects that are stage dependent (Winseck et al., 2002). We therefore next measured Schwann cell proliferation in the presence and absence of the NRG1 antagonist using BrdU labeling. The density of BrdU positive Schwann cells along motor axons in E7 animals treated with the antagonist from E5-E6 in fact is slightly increased, suggesting that NRG1 has a much stronger differentiation rather than proliferation effect on SCPs *in vivo* (Fig.18). This small increase in proliferation rate could be simply due to the presence of more SCPs, even in the presence of increased apoptosis. Thus when NRG1-induced differentiation is disrupted, proliferation is higher from these more mitogenically active SCPs.

Axonal trkB signaling regulates Schwann cell survival through localized soluble NRG1

release

The results above suggest that the number of Schwann cells that survive and differentiate is directly regulated by the amount of soluble NRG1 released during this critical period of development, producing a precise matching of Schwann cells needed for each axonal segment. One way for this matching process to occur is through Schwann cell-derived factors that regulate NRG1 release from axons. We have previously shown that Schwann cell-derived neurotrophic factors, including BDNF and GDNF, promote the rapid release of soluble NRG1 from both motor and sensory axons (Esper and Loeb, 2004). To explore the possibility that BDNF/trkB signaling indirectly modulates Schwann cell survival *in vivo* by promoting the release of soluble NRG1 from axons, we treated embryos with exogenous BDNF. As shown in Fig. 20*A*, this treatment significantly promoted SCP survival at E5. To determine whether this BDNF effect was on axons versus Schwann cells, we determined the location of trkB and p75 receptors (low affinity receptor) in the developing nerve by confocal microscopy (Figs. 19*A, B*). We found that BDNF the receptors trkB and p75 are localized specifically on axons, not Schwann cells. When we down-regulated soluble NRG1 (type I/II) by electroporation, BDNF no longer demonstrated any survival effects (Fig.20*B*), suggesting that BDNF regulates Schwann cell survival indirectly through promoting the axonal release of soluble NRG1.

To investigate further as to whether endogenous trkB signaling is required, we examined the effect of disrupting axonal trkB signaling on Schwann cell survival both by shRNA *in ovo* electroporation in the chick as well in *trkB* knockout mice. In the electroporation experiments, the opposite side of the spinal cord and GFP

electroporation alone were used as controls (Fig. 21*A*). The shRNA against chick trkB produced a significant reduction of trkB immunoreactivity in GFP-positive cells in the ventral horn (Fig.21*A, B*) and was associated with an increase in Schwann cell death at E7. This effect could be rescued with exogenous soluble NRG1 (Fig.21*C*), suggesting that endogenous axonal trkB signaling supports Schwann cell survival through axon-derived soluble NRG1.

Since shRNA knockdowns are never 100% complete, we also examined the effect of the complete absence of trkB at E13.5 in trkB knock-out mice. Knockout mice show a significant increase Schwann cell apoptosis at lumbar level motor axons compared to wild-type littermates (Fig.22*A, B*). E13.5 in the mouse corresponds to the same stage in the chick where the SCP-immature Schwann cell transition occurs (E13-E15) (Jessen and Mirsky, 2005). Prior to this transition, at E12.5, we found no significant difference in Schwann cell death (data not shown), suggesting that Schwann cell survival during this important transition period is selectively regulated by trkB signaling. Taken together, these results support the presence of a positive feedback loop mediated by local signaling of Schwann cell-derived BDNF on axonal trkB that promotes NRG1 release from axons that, in turn leads to the survival of properly positioned Schwann cells.

Modulation of BDNF signaling has no effect on NRG1 expression in motor neurons

The above results suggest that BDNF-trkB signaling regulates Schwann cell survival by promoting the localized release of soluble NRG1 forms, but cannot rule out additional effects of neurotrophic factor signaling on NRG1 synthesis (Loeb and Fischbach, 1997). To determine whether BDNF-trkB signaling *in vivo* also directly affects NRG1 expression

in motor neurons, we first compared NRG1 expression at mRNA level for both HBD (type I/II) and CRD (type III) forms, after either BDNF treatment or electroporation with shRNA-trkB (Fig.23*A*, 24*A*). Transcriptions of both NRG1 isoforms were not significantly affected by the modulation of BDNF-trkB signaling. Similarly, neither BDNF treatment nor trkB knockdown significantly changed the expression of total NRG1 protein in the spinal cord (Fig.23*B, C*; 24*B, C*), suggesting that BDNF-trkB signaling is working locally at the axoglial interface to modulate the release of NRG1 *in vivo*.

DISCUSSION

Although there are extensive *in vitro* and *in vivo* studies that implicate both NRG1 and neurotrophic factor signaling in Schwann cell development and myelination (Chan et al., 2001; Cosgaya et al., 2002; Nave and Salzer, 2006; Ng et al., 2007; Birchmeier and Nave, 2008), in this chapter, I provided the first direct *in vivo* evidence, showing that axon-derived soluble NRG1 is critical for the Schwann cell precursors' survival and their differentiation into immature Schwann cells. Moreover, it has been linked with neurotrophic factor signaling to build a developmental, stage-dependent model of positive feedback loop through soluble NRG1 and BDNF during axon-glial communication that is involved in the regulation of early Schwann cell development (Fig.25).

The *NRG1* gene is perhaps one of the largest and most complex growth factor signaling genes by virtue of the multiplicity of its alternatively spliced form (Falls, 2003). A given neuron can express both membrane-bound (type III) as well as secreted soluble forms (types I/II). Previous studies have shown that soluble heparin-binding forms of NRG1 are highly expressed by both motor neurons in the ventral horn of the spinal cord and sensory neurons in dorsal root ganglia during early embryonic development (Meyer et al., 1997). Our domain-specific antibodies further revealed that neuron-derived proNRG1 can transport along nerves and be presented on the axon membrane, while released soluble NRG1 is accumulated at specific sites through the interaction with HSPGs in the developing nervous system (Loeb et al., 1999). Here, with the antibody that specifically targets soluble NRG1's extracellular domains, we have shown that at

specific developmental stage (after E5), soluble NRG1 translocates and concentrates on the Schwann cell surface by binding to HSPGs in extracellular matrix. A similar role for soluble NRG1 has been proposed for neuromuscular junction development, where presynaptically released soluble NRG1 can promote postsynaptic AChR expression (Sandrock et al., 1997). Once synapses have matured and passed the competitive survival stage of synapse elimination, NRG1 becomes highly concentrated in the synaptic basal lamina through the concentration of agrin and other HSPGs (Li and Loeb, 2001; Loeb et al., 2002; Li et al., 2004b). This process also appears to be controlled by a feedback loop between presynaptic NRG1 and postsynaptic neurotrophic factors. Furthermore, it is also interesting that after sticking to Schwann cells, soluble NRG1 immuno-reactivity is gradually reduced from E6 to E7 in the ventral nerve area, followed by the further decrease to the background level after E9 (Loeb et al., 1999), which could be due to the endocytosis of ligand-receptor complex after NRG1-induced erbB activation and the following degradation of NRG1 in lysosome (Waterman et al., 1998; Carpenter, 2000; Citri et al., 2003).

The current data demonstrates the timing for the normal transition of soluble NRG1 localization at axoglial interface, however, it is still not clear that how efficiently NRG1 precursors will be cleaved *in vivo* once they are presented on the axon membrane during normal development. It could be very quick process, because high salt treatment has reduced the soluble NRG1 immuno-reactivity in ventral nerve area as early as E4 (Loeb et al., 1999), suggesting that the significant portion of soluble NRG1 has been released and associate with extracellular matrix at the early developmental stages. Actually, after proteolytic cleavage, soluble NRG1 could remain localized to the releasing sites by

binding to HSPGs expressed along developing nerves and has been found to be coincident accumulation with HSPGs at neuromuscular synapses (Loeb et al., 1999). So it is possible that the structural change of Schwann cell matrix that becomes more adherent to soluble NRG1 at the later stages may be involved in the NRG1 translocation process. It has been supported partially by double-labeling of agrin, a core protein of HSPG, with soluble NRG1 showing that agrin was highly expressed and co-localized with NRG1 on Schwann cells only after E6, implicating that developmental expressed specific HSPGs in cell matrix may be important to attract and concentrate released NRG1 on the Schwann cell surface for sustaining its signaling during development, just as what we have previously found *in vitro* (Li and Loeb, 2001; Pankonin et al., 2005).

In the developing peripheral nerves, soluble forms of NRG1 are initially associated with axons. Schwann cell precursors migrate along axons intimately and undergo intensive proliferation, producing much more cells than ultimately needed (Winseck et al., 2002; Winseck and Oppenheim, 2006). So their survival is highly dependent on axon-derived trophic factors including NRG1 (Jessen and Mirsky, 2005). Only those cell that are close enough to axons can receive sufficient soluble NRG1 and survive to become immature Schwann cells. Otherwise, they would be eliminated by programmed cell death (PCD). Soluble NRG1 treatment successfully rescued normal-occurring Schwann cell death at both E5 and E6 in this study. However, overexpression of soluble NRG1 precursors on axons could promote SCP survival at E6, but not E5. Together with the developmental deposition of soluble NRG1 at axoglial interface, it suggests that the releasing event is critical for the soluble NRG1's functions on Schwann cell development, and also demonstrate that a potential stage-dependent mechanism by which

axon-derived NRG1 precursors could be efficiently proteolytic cleaved from axon membrane, may only be available after E5. Consistently, knocking-down of endogenous soluble NRG1 signaling with either specific shRNA or NRG1 targeted antagonist, could significantly increase SCP death. Since membrane-bound NRG1 are also expressed in motor neurons and shown to regulate SCP survival *in vivo* (Wolpowitz et al., 2000), SCPs undergoing apoptosis induced by insufficient soluble NRG1 signaling in this study probably are ones not associated with axons. Without the chance to get trophic factors by directly contacting with axons, these cells only can depend on axon-derived soluble NRG1 for survival in a paracrine manner that actually contribute to the rescue of at least 50% of those cells in our data.

Part of the complexity in understanding the functions of various alternatively spliced forms of NRG1 during development comes from having many diverse roles at many different stages. The chick system has a unique advantage that enables the modulation of specific forms of NRG1 in specific regions at specific stages of development. Knockout studies in mice, while sometimes more difficult to interpret, have also lead to significant insights. Knocking out all NRG1 isoforms and their receptors leads to a dramatic loss of neural crest cell-derived Schwann cell precursors (Meyer and Birchmeier, 1995; Meyer et al., 1997; Riethmacher et al., 1997; Morris et al., 1999; Woldeyesus et al., 1999). In contrast, mice specifically deficient in the type III NRG1 isoform show a milder phenotype with the presence of a reduced number of SCPs that still differentiate to Schwann cells and line up along growing axons (Wolpowitz et al., 2000), which supports our observations here that soluble NRG1 isoforms are required for SCP survival.

Our findings in this chapter also suggest that soluble NRG1 regulates both the survival and the differentiation of SCPs. Although it has been well-defined that the transition stage of SCPs to Schwann cells in mice is between E13 and E15 (Jessen and Mirsky, 2005), the timing has not been established in chick embryos. Here we have found that the normal differentiation of SCP to immature Schwann cells happened between E6 and E7 during chick embryonic development, which is paralleled with the timing of soluble NRG1's concentration on Schwann cells. Perhaps one of the most dramatic findings of our targeted NRG1 antagonist was the complete inhibition of O4 expression as a marker of immature Schwann cell differentiation. This effect on differentiation is consistent with previous *in vitro* experiments showing NRG1 signaling accelerates the SCP-Schwann cell transition (Brennan et al., 2000). The developmental down-regulation of AP2α, a marker of SCPs, was not affected by the NRG1 antagonist, suggesting that SCP differentiation to immature Schwann cells is regulated by multiple steps and that NRG1 does not appear to be required for its down-regulation. Given that membrane-bound, CRD form of NRG1 is also essential for SCP development, the differentiation of SCPs that directly contact with axons is probably regulated by both soluble NRG1 and CRD-NRG1 that functions in a juxtacrine manner.

Given the known mitogentic effects of NRG1 on cultured Schwann cells (Dong et al., 1995), one surprising result from the analysis of Schwann cell proliferation measured by BrdU labeling, shows that during the transition of SCPs to immature Schwann cells, NRG1 is anti-proliferative. Treatment with the NRG1 antagonist in fact produced a slight increase, rather than a decrease in Schwann cell proliferation in the developing motor axons. Actually, normal proliferation rate of Schwann cells decreases from SCP to

Schwann cell stage (Winseck et al., 2002), and myelinating Schwann cells' mitogenic activity increases when they are de-differentiated to immature stage after nerve injury or degeneration (Fawcett and Keynes, 1990). The increased proliferation may be attributable to an indirect effect of the strong anti-differentiation effect of the antagonist leading to higher numbers of proliferating SCPs, rather than to the direct regulation of Schwann cell proliferation. Meanwhile, the cancer literature similarly shows both proliferation and differentiation effects of NRG1 on breast epithelial cells that vary as a function of their level of malignant transformation. For example, as breast epithelial cells become more malignant, NRG1 signaling changes from an anti-proliferative to a proliferative effect (Li et al., 2004a).

In this study, we have shown that knocking-down of soluble NRG1 signaling totally blocks the survival effect of BDNF ectopic expression, and soluble NRG1 is able to rescue the Schwann cell death induced by the down-regulation of endogenous axonal trkB signaling. It strongly suggests that endogenous BDNF signaling through axonal trkB receptors regulates SCP survival and differentiation indirectly through modulating the amount of soluble NRG1 released at the axon-glial interface. This finding is consistent with our previous *in vitro* observations showing that BDNF and other neurotrophic factors rapidly promote soluble NRG1 release from axons in neuron culture through protein kinase C-delta (PKC-δ) induced phosphorylation on proNRG1's cytoplasmic tail (Esper and Loeb, 2004,2009). This reciprocal regulatory pathway is stage-dependent and occurs after the period of maximal naturally-occurring SCP death at E5. A key advantage of this localized communication system is that it does not require communication back to the motor neuron cell body to optimize axoglial interactions. Consistently, we found that

the modulation of BDNF-trkB signaling did not significantly change NRG1 mRNA or protein expression in the spinal cord, and may just lead to the improvement of efficiency in NRG1's proteolytic cleavage as what we have found previously. SCPs that survive from programmed cell death, differentiate into immature Schwann cells and receive sustained NRG1 signaling through the deposition of developmentally regulated HSPGs on their cell surface (Fig.25). At later developmental stages, those axons that produce sufficient levels of the type III, membrane-bound CRD-NRG1 are then utilized required to promote axonal myelination (Taveggia et al., 2005).

CHAPTER IV

CONCLUSIONS AND SIGNIFICANCE

The body of work in this dissertation has attempted to develop an understanding of the critical role of reciprocal signaling through soluble neuregulin-1 (NRG1) and brain-derived neurotrophic factors (BDNF) in the regulation of early Schwann cell development *in vivo*. In addition, attempts were also made to develop a novel potent NRG1 antagonist with enhanced targeting specificity through the interaction between heparin-binding domain (HBD) and specific heparan-sulfate proteoglycans (HSPGs) in the extracellular matrix, so that the precise functional roles of soluble NRG1 could be elucidated in both development and diseases.

HSPGs give tissue surfaces a unique molecular signature due to their tremendous diversity, yet specificity in both core protein and glycosaminoglycan (GAG) structures including sulfation patterns (Turnbull et al., 2001; Esko and Selleck, 2002; Kramer and Yost, 2003; Pankonin et al., 2005). This diversity is regulated by highly specific enzymes that regulate their synthesis and modifications through post-translational processing, which results in a spatially- and temporally-regulated means for a given cell surface region to become receptive to specific growth and differentiation factors as well as viruses that have evolved to use of this natural delivery system with the emergence of a number of structurally–distinct heparin-binding domains. The ‘barcode’ provided by HSPGs in the extracellular matrix is often used as low-affinity targeting system that often requires a second, more highly specific receptor-ligand interaction to exert its biological

effects. In the Chaper II, we have taken advantage of growth factor's heparin-binding motifs that are widely used in nature as a source of tissue-specific targeting, to develop a novel NRG1 antagonist by fusing its HBD to soluble HER4 receptor. NRG1's HBD is unique in that it is an easily detachable, stand-alone C2 immunoglobulin domain that can retain its targeting specificity both *in vitro* and *in vivo* when fused to another polypeptide. We found that one of fusion protein constructs originally generated by my colleagues, HBD-S-H4, could work as potential NRG1 signaling blocker. In this thesis work, both *in vitro* and *in vivo* evidence about the characterization of this fusion protein's heparin-binding ability and NRG1 blocking activity, are provided to demonstrate that this completely humanized fusion protein has enhanced tissue-targeting specificity through the concentration on cell surfaces in the same tissue regions as endogenous soluble NRG1, and could be promising as a tool for NRG1's functional studies in development and an anti-NRG1 biopharmaceutical in diseases ranging from chronic pain to cancer (Ma et al., 2009; Calvo et al., 2010; Calvo et al., 2011).

Since this new regent is derived entirely from natural human polypeptide sequences, to test the potential therapeutic effect, we have started to take look at its *in vitro* efficacy of blocking NRG1-induced HER2 receptor activation in some types of human breast cancer cells that are highly proliferative in response to NRG1 (Krane and Leder, 1996; Lupu et al., 1996; Tsai et al., 2003). Previously, we have found that as some human breast epithelial cells become more malignant, they develop autocrine NRG signaling that promotes proliferation (Li et al., 2004a; Yao et al., 2004). The treatment of HBD-S-H4 in MCF10CA1 breast cancer cells effectively blocked both autocrine and paracrine NRG1-induced receptor phosphorylation and thus significantly reduced their

proliferation rate, suggesting that this fusion protein could be useful therapeutically in breast and other cancer cells that proliferate in response to autocrine and paracrine NRG1 signaling (Ma et al., 2009). Actually, a number of efforts have been made to block NRG1-erbB signaling, including the FDA approved humanized monoclonal antibody drug against erbB2 receptor called Trastuzumab (Carter et al., 1992). It have become a clinically effective adjuvant therapy for human breast cancers (Slamon et al., 2001; Vogel et al., 2002), but has to be used at extremely high concentrations that have been linked to cardiac side effects and only works effectively on a subgroup of breast cancer patients that overexpress HER2 receptor (Krauss et al., 2000; Untch et al., 2004). In our experiments, the inhibition level of breast cancer cell growth achieved by HBD-S-H4 treatment was comparable to this currently approved therapeutic antibody (Ma et al., 2009). Further *in vivo* efficacy studies will be needed to determine the therapeutic potential of our novel NRG1 antagonist in human breast cancers.

One of the most important obstacles in the development of biological therapeutics is getting a therapeutic agent to diseased tissues without causing undue toxicity in normal tissues. Recent clinical trial with promising neurotrophic factors, BDNF and GDNF, failed in part because of poor targeting (Thoenen and Sendtner, 2002). Potential protein drugs that delivered to all cells are not helpful, even potentially dangerous for the treatment of human diseases. Here, we have tried to develop a better tissue-specific targeting system for biopharmaceutical development to enhance tissue-specific delivery and produce sustained efficacy by using NRG1's heparin-binding domain. The natural ability of NRG's HBD to concentrate on cell surfaces in a tissue-specific manner may help overcome some of these obstacles for biopharmaceuticals that might otherwise fail due to poor

efficacy and excessive toxicity. GAG diverse structures of HSPGs in the extracellular matrix maintain tissue-specific heterogeneity through highly regulated enzymes that modify the sugar and sulfation patterns needed for the selective targeting of a number of growth factors and viruses, which is critical for the normal development and could be affected by human diseases (Fuster and Esko, 2005; Bishop et al., 2007; Gorsi and Stringer, 2007). Therefore, not only could NRG1's native HBD be a useful targeting vector for tissues that express and/or bind NRG1, but also the subtle modifications in this HBD sequence and other potential HBD sequences could be sufficient to change the tissue-specific targeting specificity in many diseases including neurodegenerative diseases and cancers. So the usage of NRG1's HBD as a novel strategy for selective tissue-targeting with minimal systematic side effects at the same time, could serve a good example on how it is possible to improve therapeutics by targeting biopharmaceuticals to where they are needed with minimal toxicity to other tissues and thus resolves one of the most important obstacles in the development of biological therapeutics.

In chapter III, we provided *in vivo* evidence from the novel NRG1 antagonist treatment and further genetic and biochemical experiments in both chick and mouse embryos, showing that Schwann cell-derived BDNF through axonal trkB receptors stage-dependently induce the axonal release of soluble NRG1 to the developing Schwann cells, and the signaling is stabilized and sustained through the accumulation on the cell surface at the specific developmental stages, which is critical to regulate the survival of Schwann cell precursors as well as their differentiation into immature Schwann cells. So here we have linked together with the functions of neuro-trophic

factors like BDNF and glial-trophic factors like NRG1 on the regulation of Schwann cell development, providing the first *in vivo* evidence for a positive feedback loop between axonal NRG1 and Schwann cell-derived neurotrophins that may not only be important for the axoglial communication, but also be helpful in understanding nervous system diseases that involve the axoglial interface. Figure 25 builds a developmental, stage-dependent model of Schwann cell development that incorporates our findings about soluble NRG1 together with other known roles of membrane-bound NRG1 in regulating the development process of Schwann cell lineage.

To make this model of axoglial signaling complete, some key issues remain to be tested. First, we do not know if any other neurotrophic factors could be also involved in this model and stimulate NRG1 axonal release at the specific developmentally critical period for Schwann cell development. It is necessary to check the potential involvement of GDNF in this stage-dependent axon-glial communication to regulate early Schwann cell development, because it is one of potent Schwann cell-derived neurotrophic factors that can effectively promote soluble NRG1 release form axons in both motor and sensory neuron cultures (Esper and Loeb, 2004). Actually, at the later stage, GDNF can be secreted by immature Schwann cells and support their own survival through autocrine signaling (Jessen and Mirsky, 2005). Second, so far, little is known about the exact mechanisms by which NRG1 precursors can be efficiently cleaved from axon membrane in response to axonal trk receptor activation induced by Schwann cell-derived neurotrophic factors. Although our previous *in vitro* study have shown that the activation of PKC-δ signaling pathway after neurotrophic factor treatment promote NRG1 precursor release and is responsible for the phosphorylation of proNRG1 cytoplasmic tail, it is still

not clear if this phosphorylation event is necessary for the protein conformation change and thus facilitate its cleavage by some protease in the extracellular matrix. It could be elucidated by the further identification of specific phosphorylated residues on proNRG1 cytoplasmic tail combined with three-dimentional structures revealed by x-ray crystallography, and the establishment of the differential expression or activation pattern of PKC-δ at axoglial interface during normal development as well as after the modulation of neurotrophic factor signaling.

Since NRG1 has wide range of functions in both neural development and diseases (Mei and Xiong, 2008), our findings would have implications for better understanding of the mechanisms during axoglial communication that may lead to the development of potential treatments for neurological diseases including demyelinating disease. While in some disease situations it may be advantageous to increase NRG1 signaling, in others, NRG1 signaling may be detrimental and needs to be blocked, which has been achieved by our novel potent targeted NRG1 antagonist. Previous studies have shown that type III NRG1 remains membrane-bound, even after proteolytic cleavage from its precursor (Wang et al., 2000), which make it ideally positioned to determine the ensheathment fate of axons and regulate myelin sheath thickness in peripheral nerves (Michailov et al., 2004; Taveggia et al., 2005). Interestingly, while high concentrations of soluble NRG1 inhibit Schwann cell myelination, low concentrations that switch from Erk to phosphatidylinositol 3-kinase (PI3K) - Akt activation actually promote myelination (Zanazzi et al., 2001; Syed et al., 2010). It seems that the balance between two signaling pathways for myelination is controlled by one molecule. Specific heparan-sulfates in the extracellular matrix may be involved in this process by concentrating small amount of

soluble NRG1 to sustain the signaling and activate the same signaling pathway as membrane-bound NRG1 during the initiation of myelination process. It is really interesting to check the *in vivo* roles of soluble NRG1 and the reciprocal signaling through neurotrophic factors in regulating Schwann cell maturation and myelination at the later developmental stages. So, given the the amount of NRG1 and the intensity of NRG1 signaling are critical for the Schwann cell development, the modulation effect of neurotrophic factor signaling on soluble NRG1 concentration at axoglial interface may have potential for the future therapeutics of demyelinating diseases.

Many evidence have suggested that both NRG1 and neurotrophic factors signaling are important for the peripheral nerve repair (Esper et al., 2006). After axon damage, Schwann cells undergo multiple changes at both molecular and cellular level, and secrete specific neurotrophic factors that could promote axon regrowth (Carroll et al., 1997). Administration of exogenous neurotrophic factors has been used as a potential therapeutic strategy to treat chronic peripheral neuropathies in which the natural repair process can not be achieved (McMahon and Priestley, 1995). Alternatively, manipulation of NRG1 signaling can stimulate nerve regeneration by promoting Schwann cell proliferation and protecting nerves against toxin-induced nerupathy (Oka et al., 2000; ter Laak et al., 2000). Furthermore, it has recently been suggested that once the peripheral nerve forms, NRG1 is dispensable under normal conditions, but critical for nerve repair after injury (Fricker et al., 2011). So the fully understanding of normal axoglial interaction through axon-derived NRG1 and Schwann cell-secreted neurotrophic factors, especially *in vivo* condition, may be important to develop more effective treatment for peripheral neuropathy induced by injury or diseases.

Taken together, our data in this work demonstrated that during the critical period for Schwann cell development, the reciprocal signaling through axonal NRG1 and Schwann cell-derived neurotrophins such as BDNF, is critical for the regulation of Schwann cell precursors' survival and differentiation. Importantly, it suggests that factors that modify the localization and concentration of NRG1 can dramatically alter the biological functions of the ligand. Control of NRG1 signaling through both alternative splicing of membrane-bound and secreted forms, the expression patterns of adherent HSPGs, and local gradients of neurotrophic factors from surrounding cells can thus fine tune NRG1 signaling to achieve its many goals in both development and diseases. A clear understanding of these variables and NRG1 functions during normal development of nervous systems could have important therapeutic implications in a variety of human diseases such as peripheral neuropathy and demyelinating disorders (Loeb, 2007). Therefore, the work presented in this dissertation significantly expands our knowledge and understanding of bidirectional axon-glial communication *in vivo* and may have potential impact on the development of therapeutic strategies for neurological disorders that involve axoglial interface.

APPENDIX A

FIGURES

Figure 1. Molecular structures of NRG1 isoforms

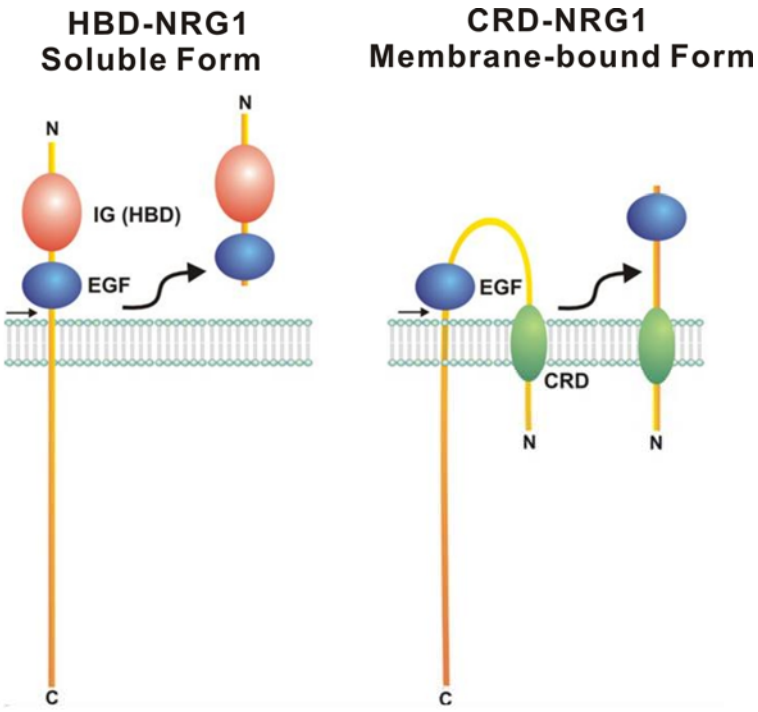


Figure 2. Soluble NRG1 signaling activates erbB receptors through the interaction between HBD and HSPGs

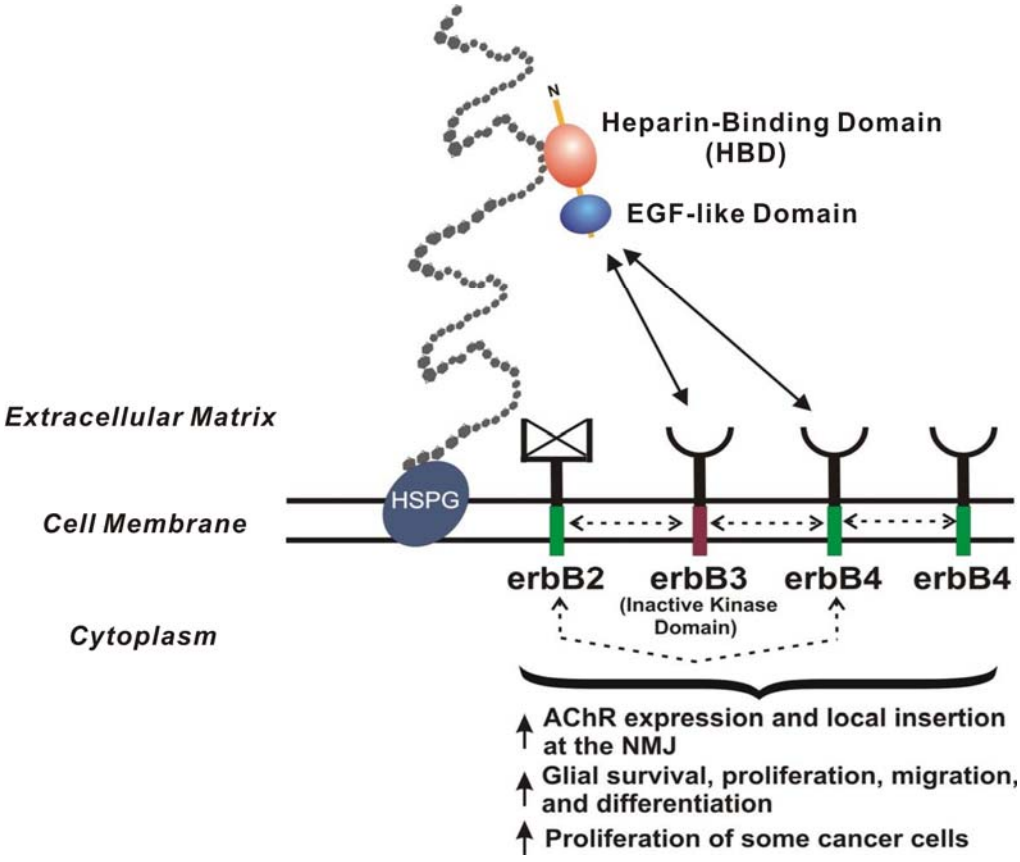


Figure 3. The HBD of NRG1 retains heparin-binding ability and potentiates an HER4 antagonist.

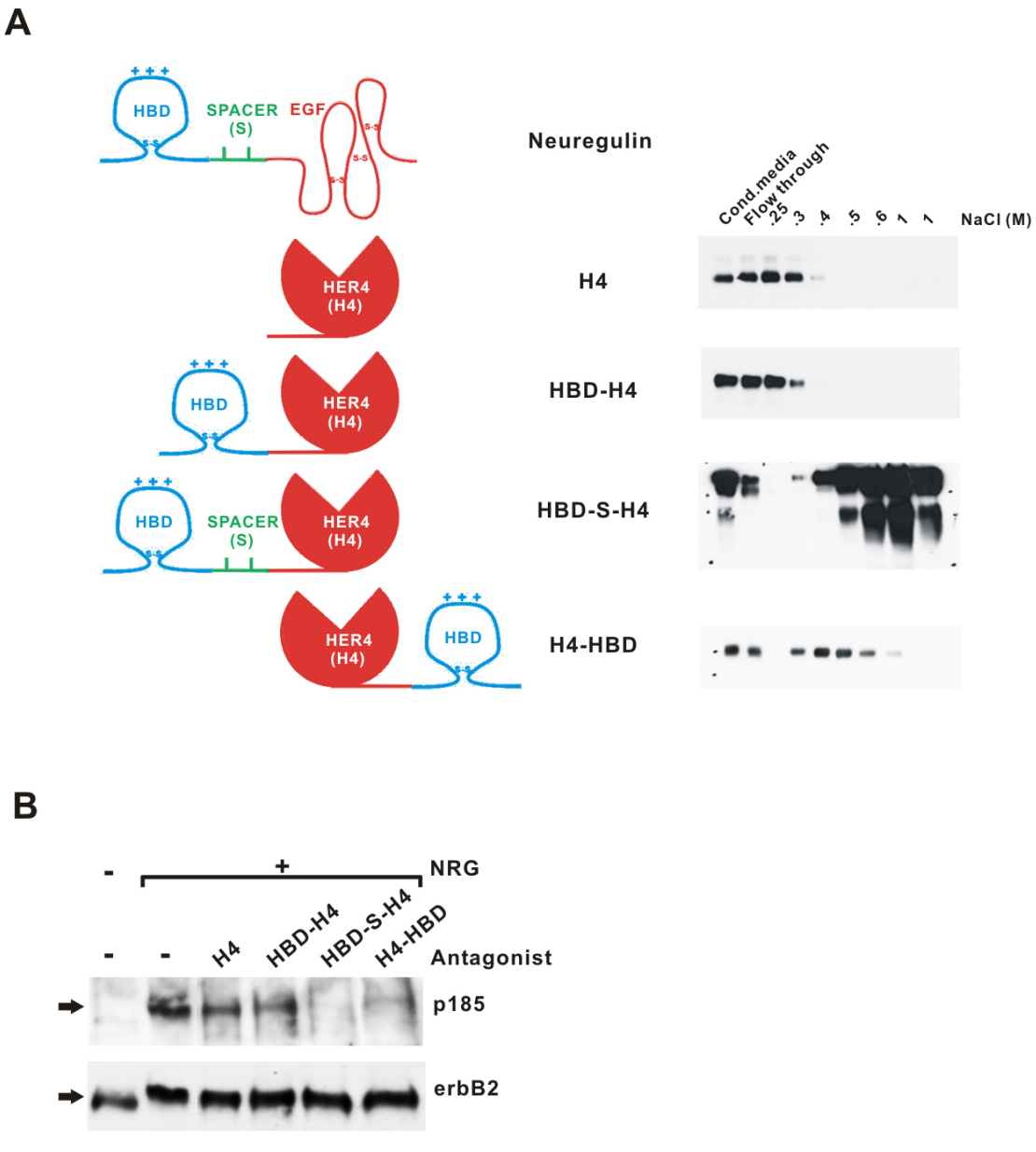


Figure 4. Purified HBD-S-H4 blocks NRG1 induced erbB phosphorylation by concentrating on cell surface.

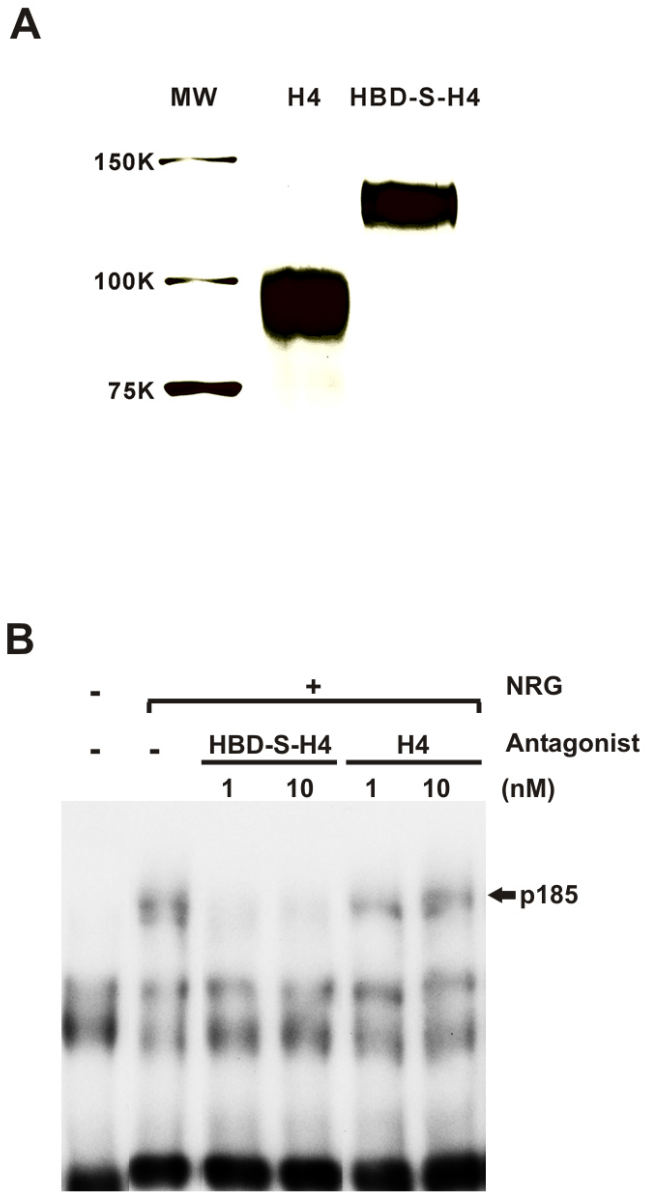


Figure 5. NRG1's HBD targets HBD-S-H4 to cell surfaces through heparan sulfate interactions.

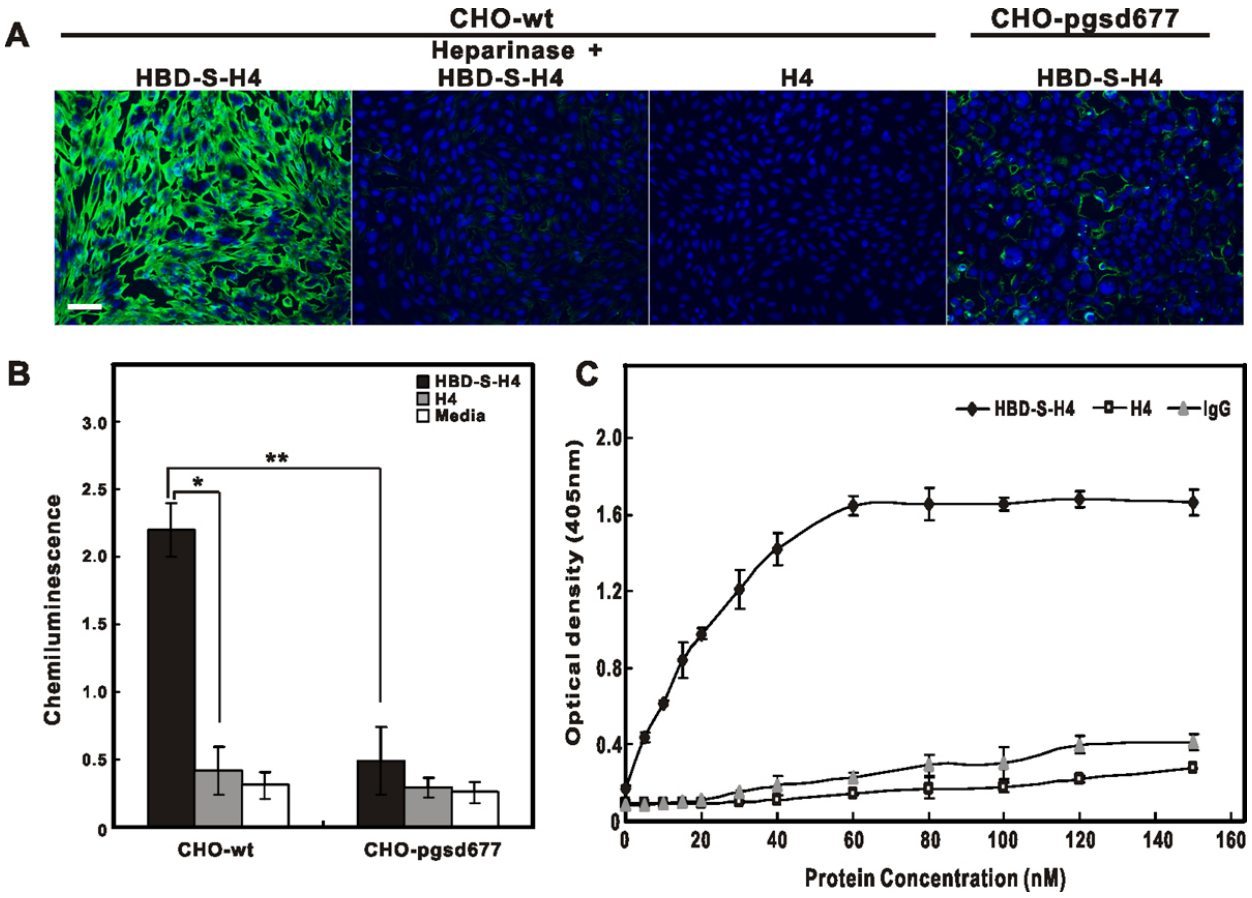


Figure 6. HBD-S-H4 targets to the same regions of the developing spinal cord where NRG1 accumulates

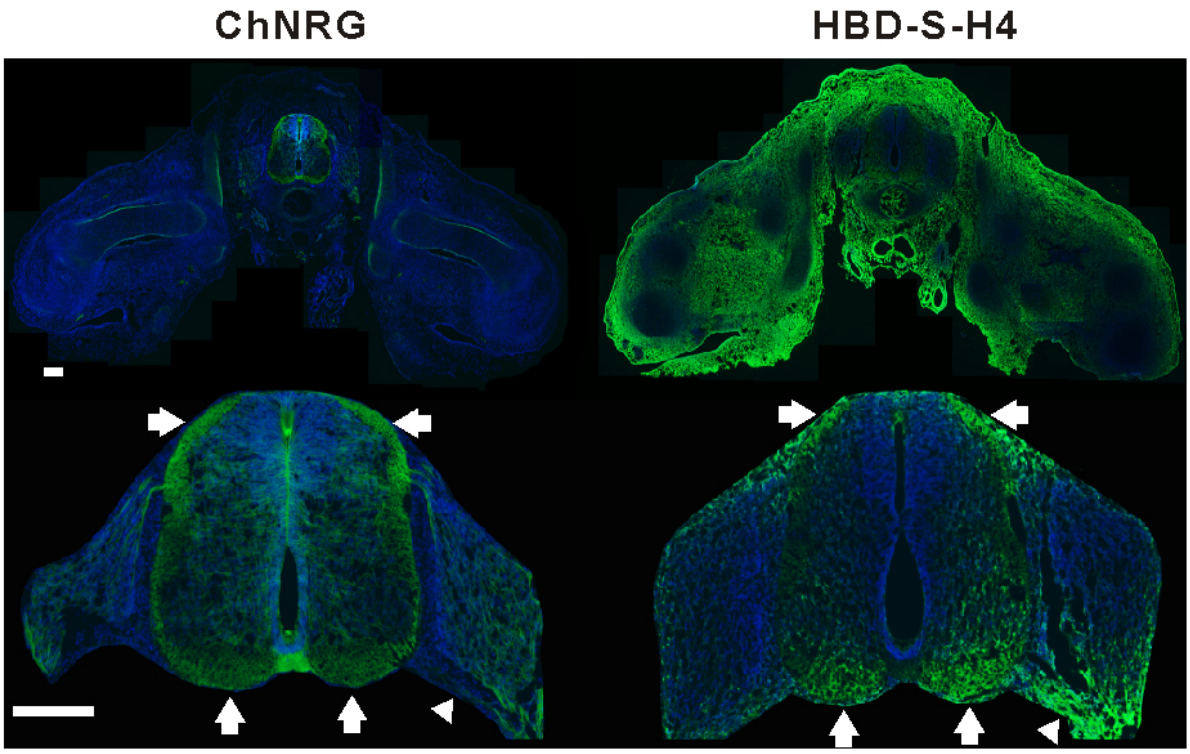


Figure 7. HBD-S-H4 is concentrated in the ventral nerve area through the interaction with HSPGs

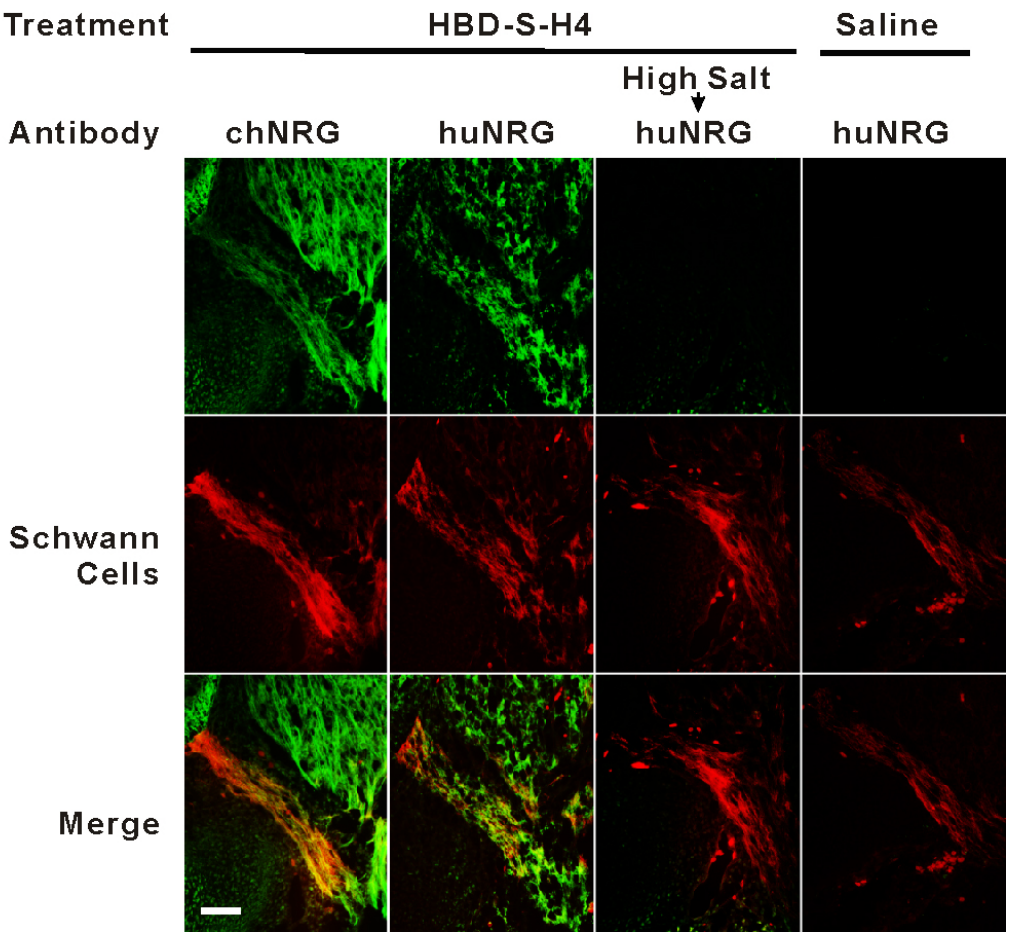


Figure 8. Immunostaining with an antibody against His-tag present in both HBD-S-H4 and H4 constructs.

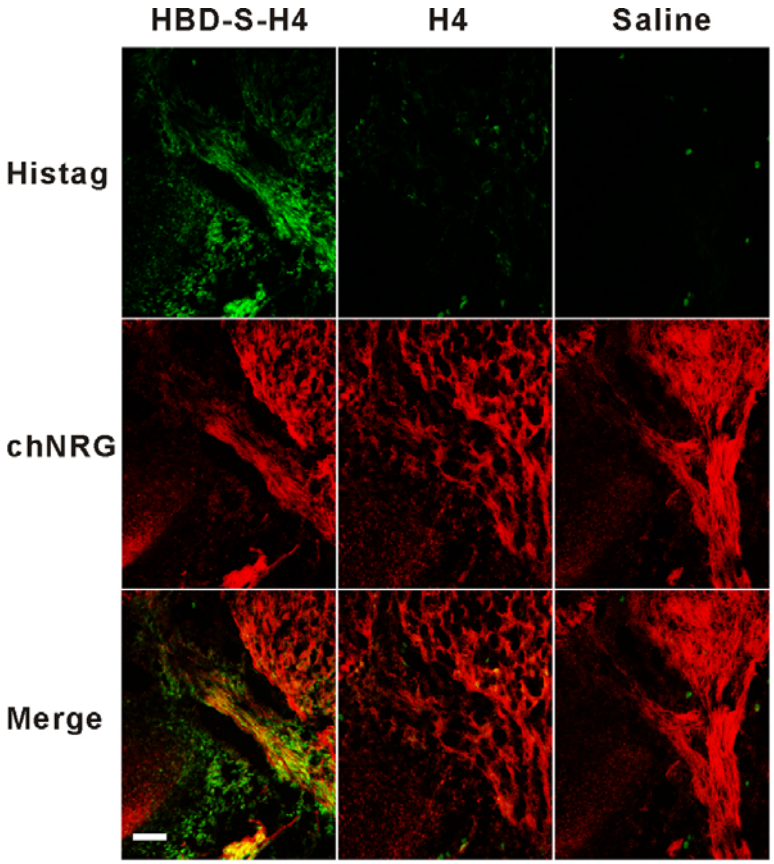


Figure 9. HBD-S-H4 induces Schwann cell apoptosis in the ventral nerve root.

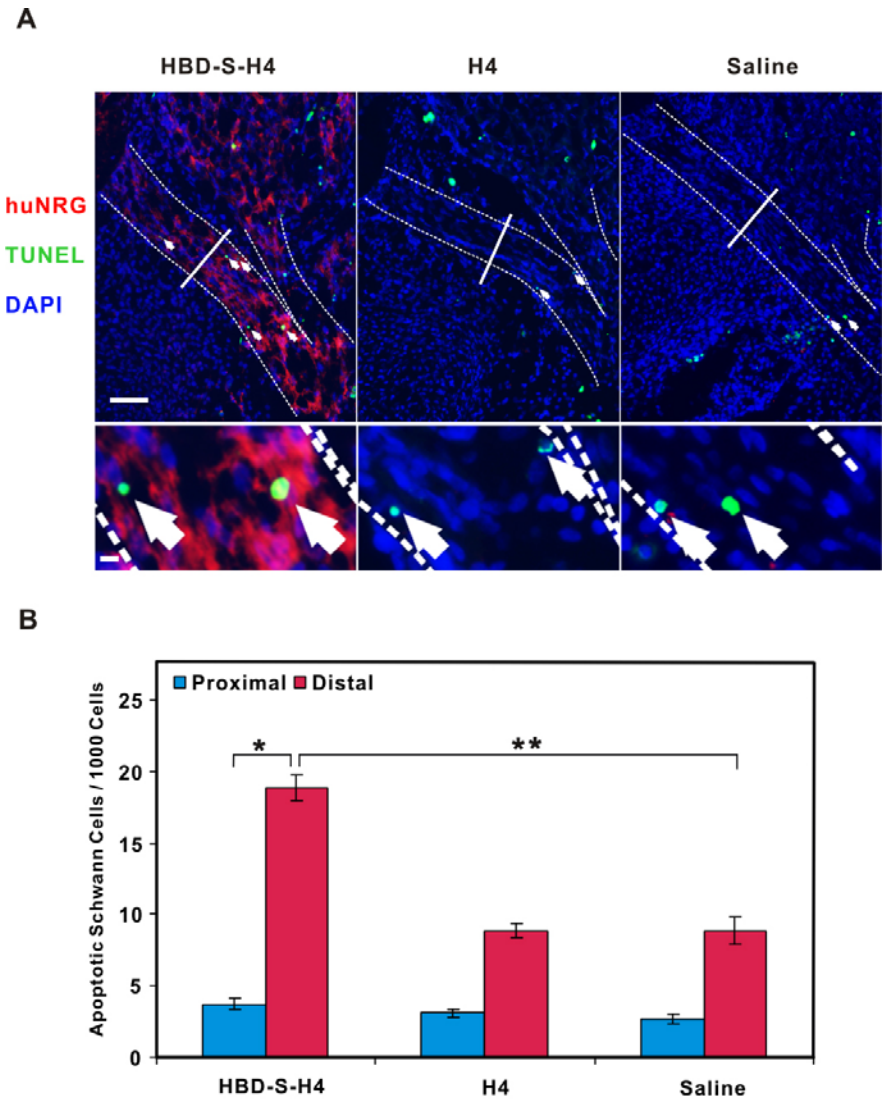


Figure 10. Transition of matrix-bound NRG1 from axons to Schwann cells.

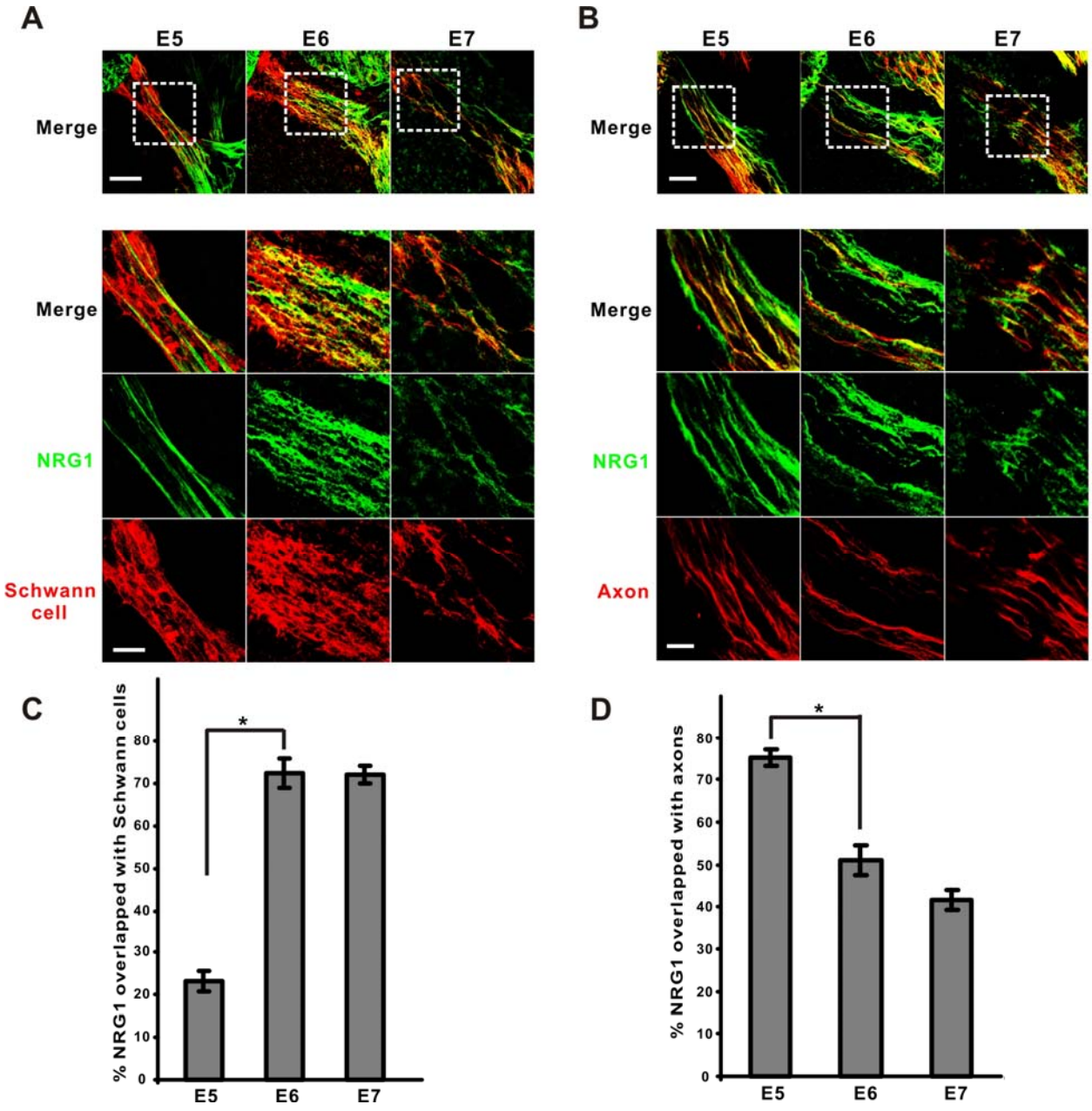


Figure 11. Release soluble NRG1 binds to heparan sulfates at axoglial interface.

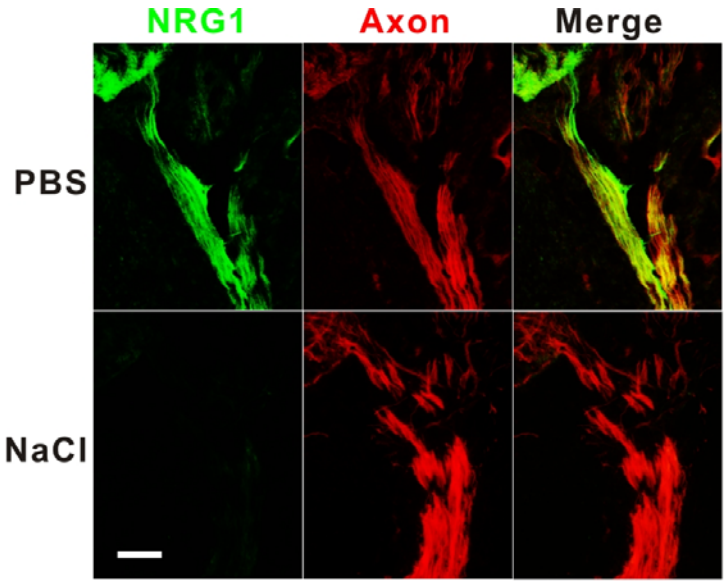


Figure 12. NRG1 signaling promotes Schwann cell survival *in vivo*.

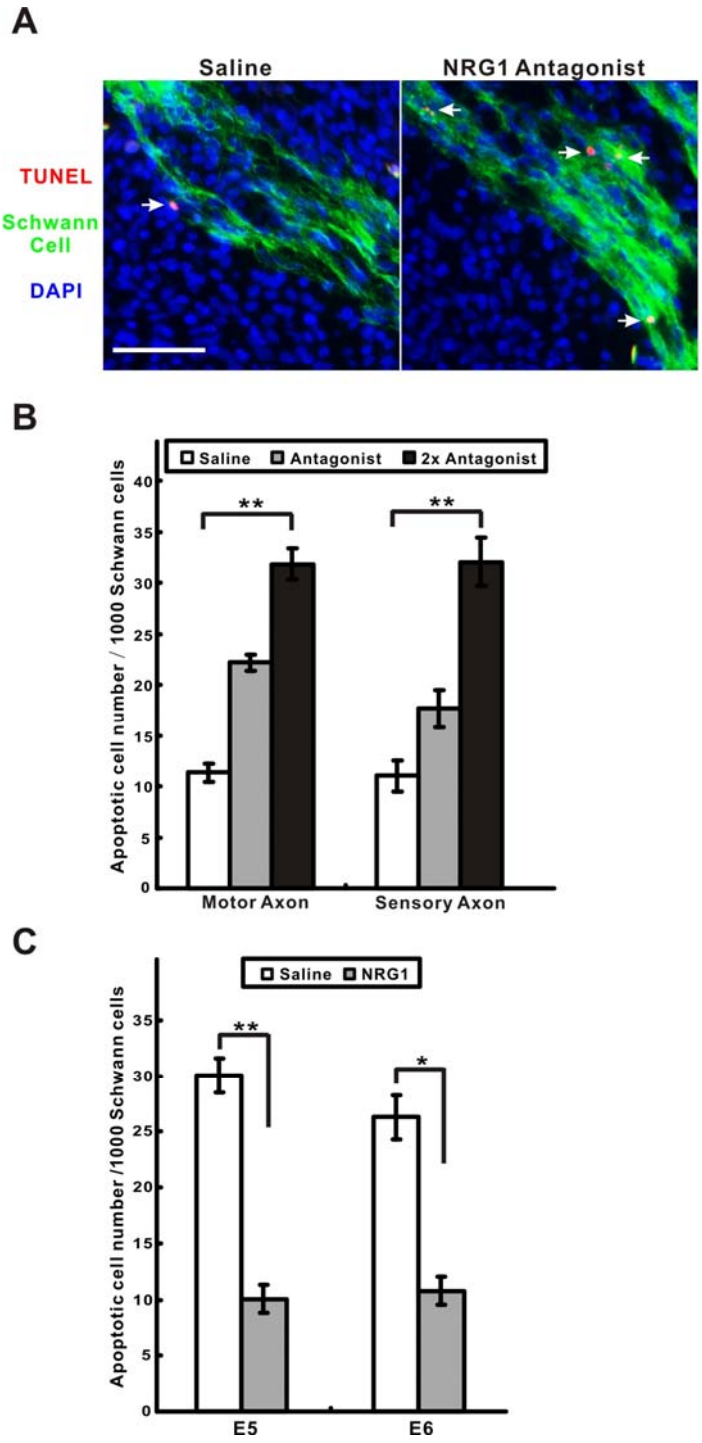


Figure 13. Chick *in ovo* electroporation can be used to modulate gene expression unilaterally in the ventral nerve roots.

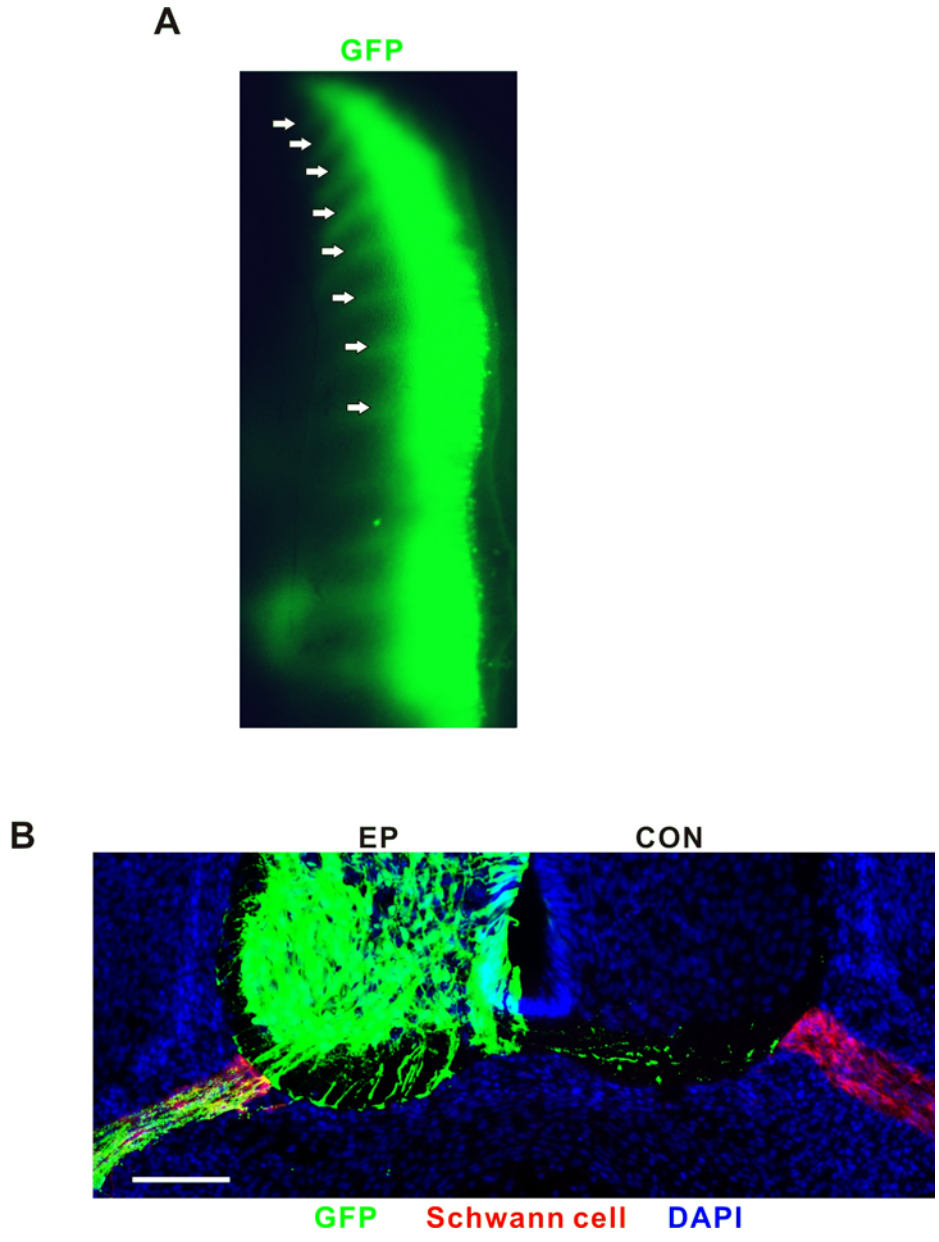


Figure 14. Specific knock-down of soluble NRG1 increases Schwann cell death.

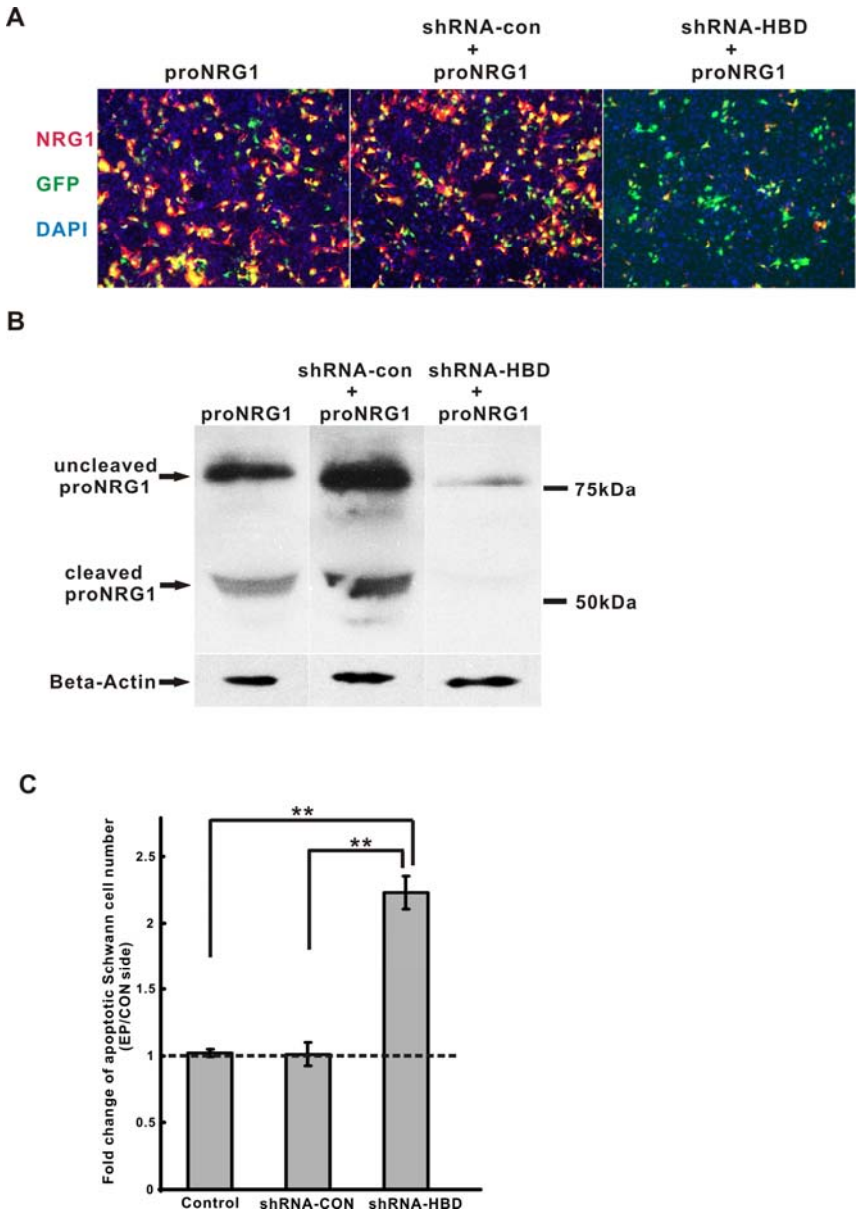


Figure 15. Over-expression of soluble NRG1 promotes Schwann cell survival.

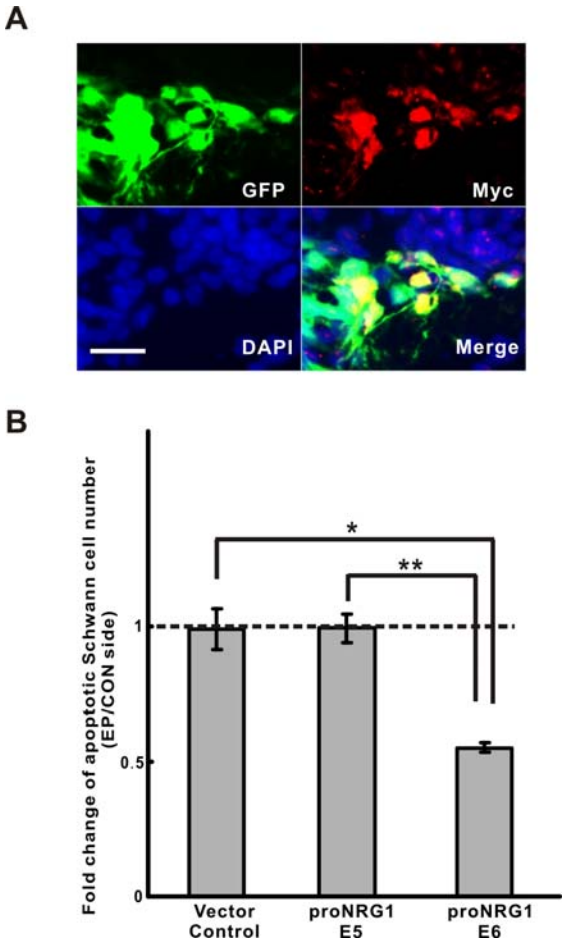


Figure 16. Schwann cell differentiation during normal development of chick embryos.

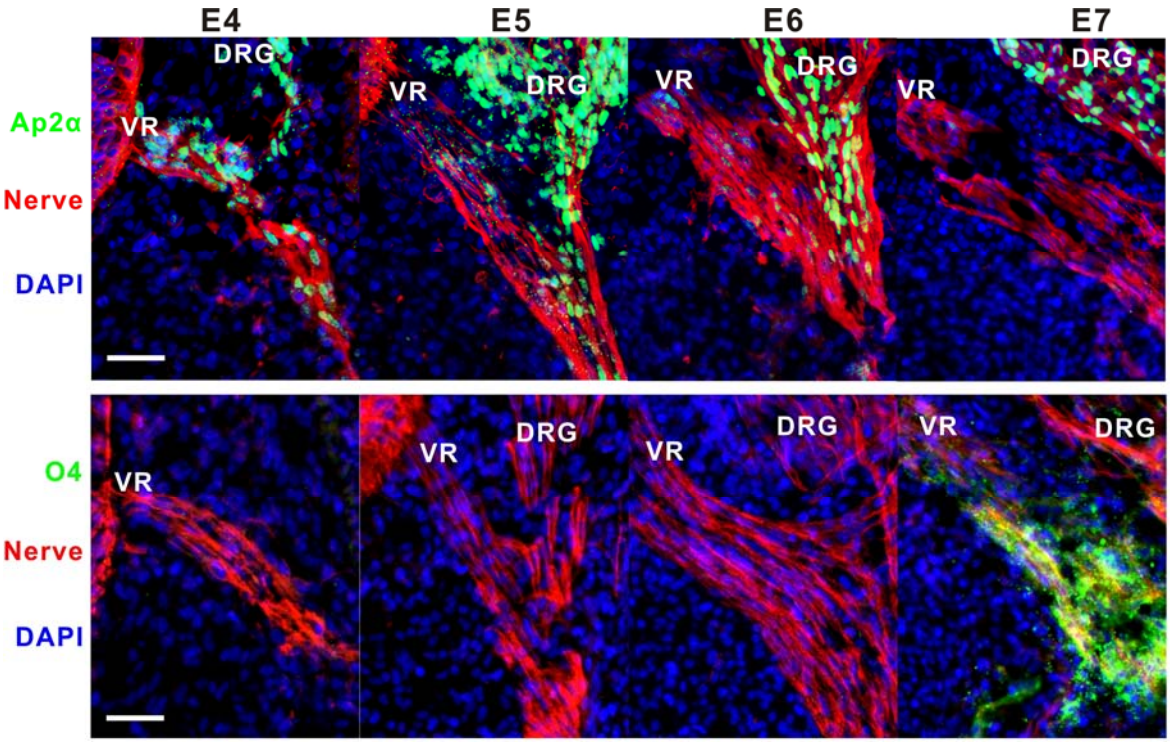


Figure 17. NRG1 signaling is required for Schwann cell differentiation.

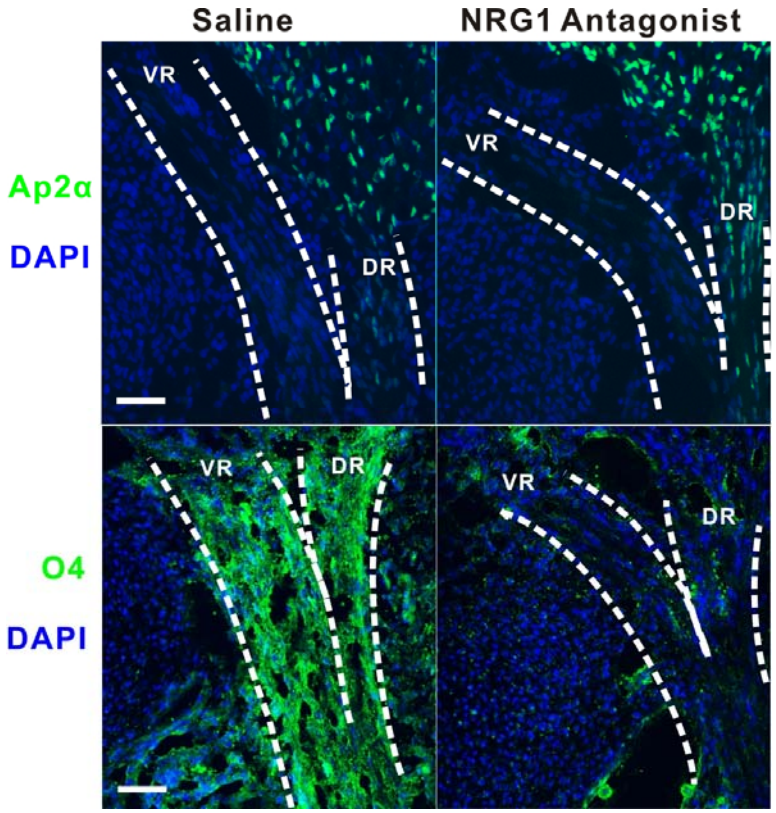


Figure 18. NRG1 signaling is not required for Schwann cell proliferation.

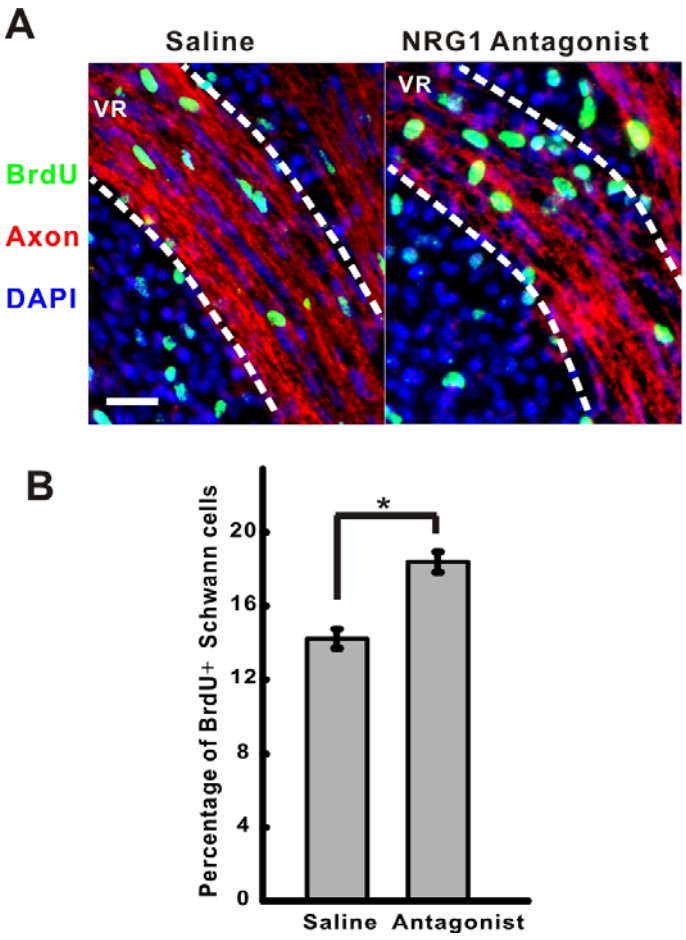


Figure 19. BDNF's receptors are exclusively localized on axons.

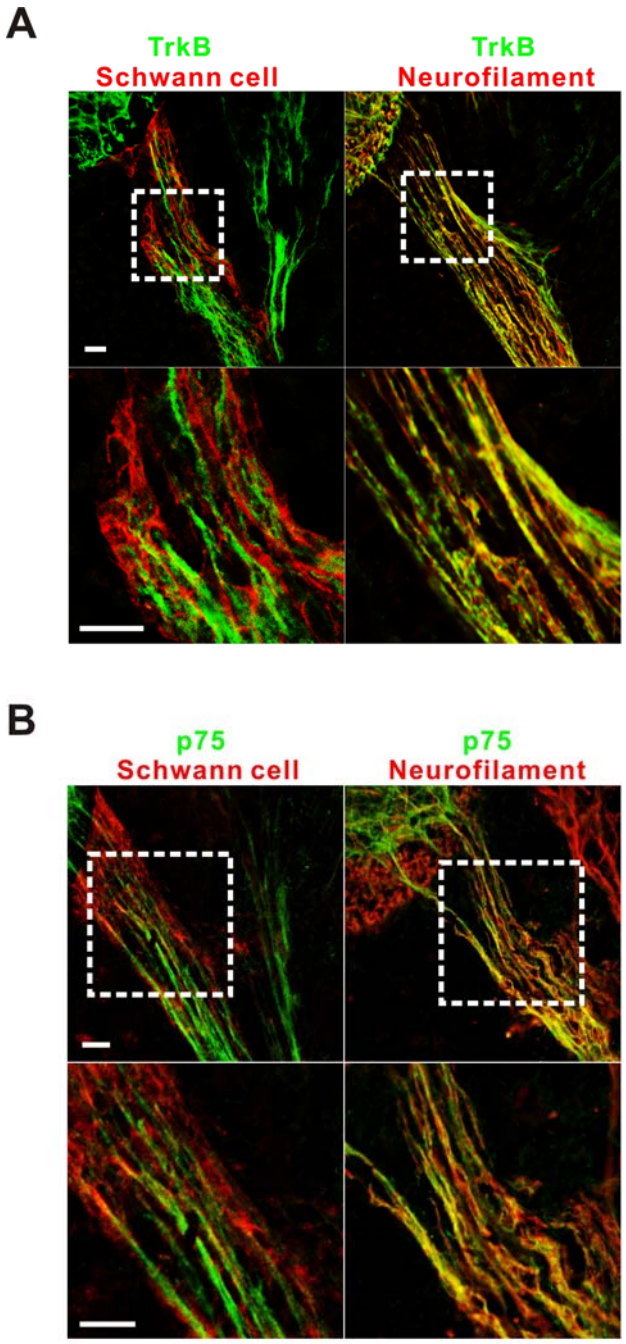


Figure 20. BDNF promotes Schwann cell survival through axon-derived soluble NRG1.

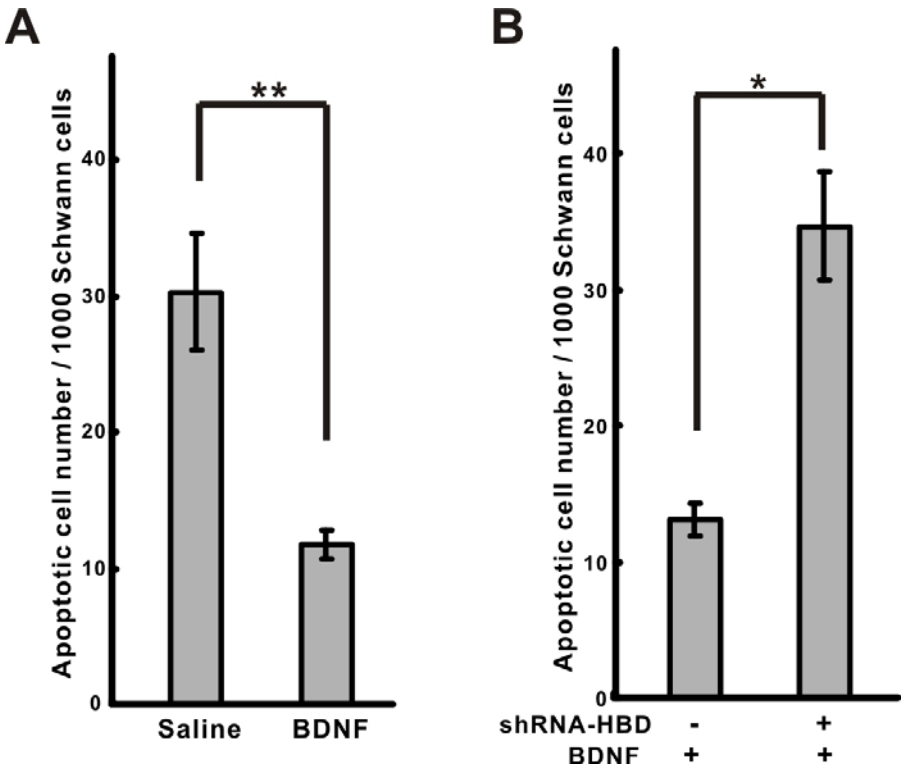


Figure 21. Axonal trkB signaling promotes Schwann cell survival through soluble NRG1.

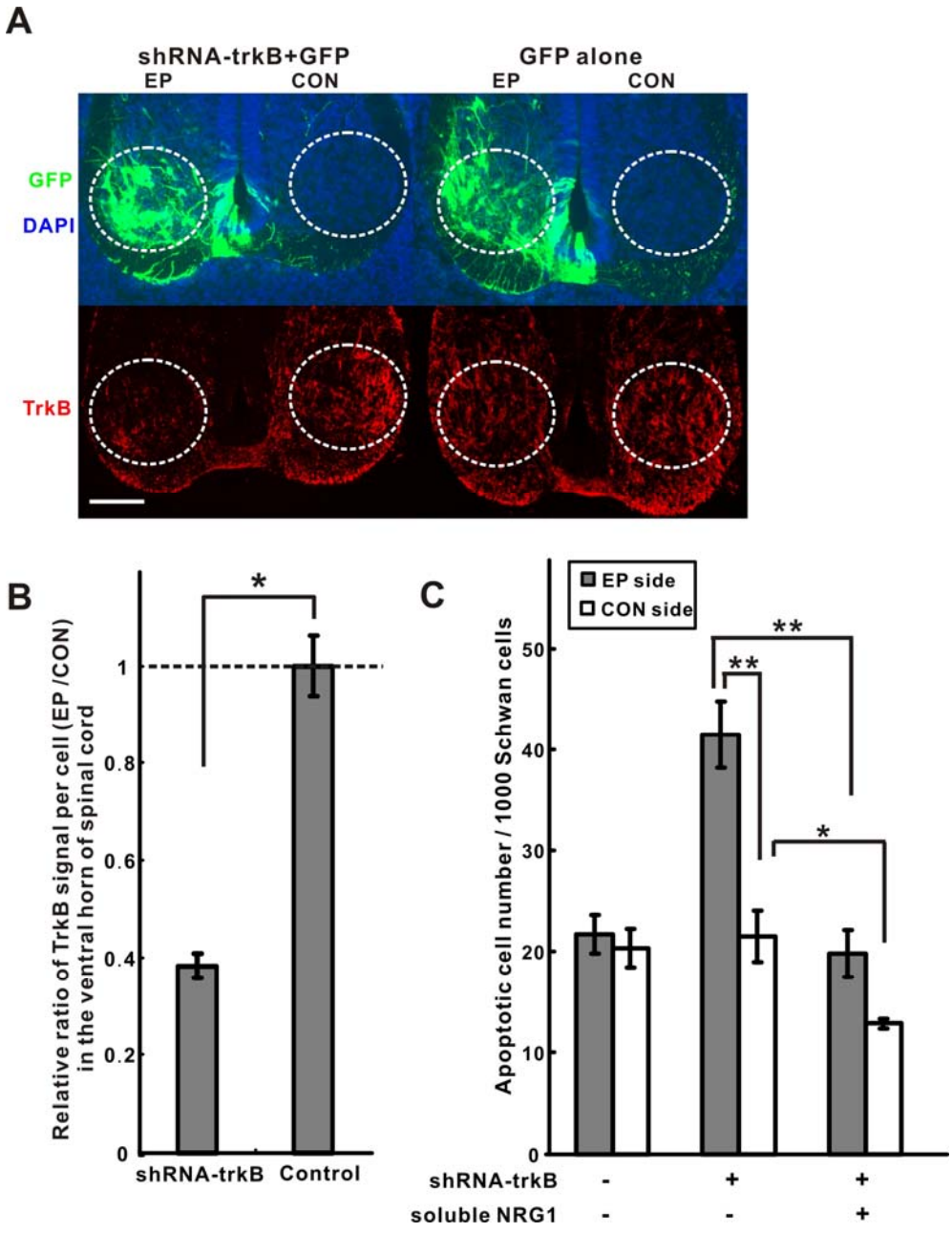


Figure 22. Schwann cell death is increased at E13.5 in TrkB knockout mice.

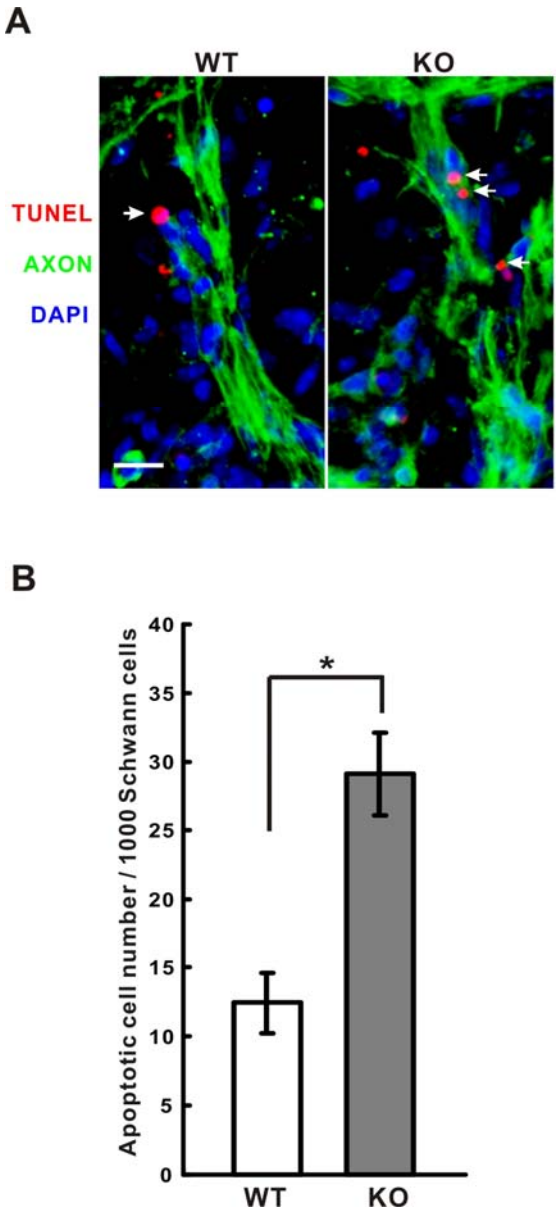


Figure 23. Over-expression of BDNF-trkB signaling does not significantly affect NRG1 expression in spinal cord.

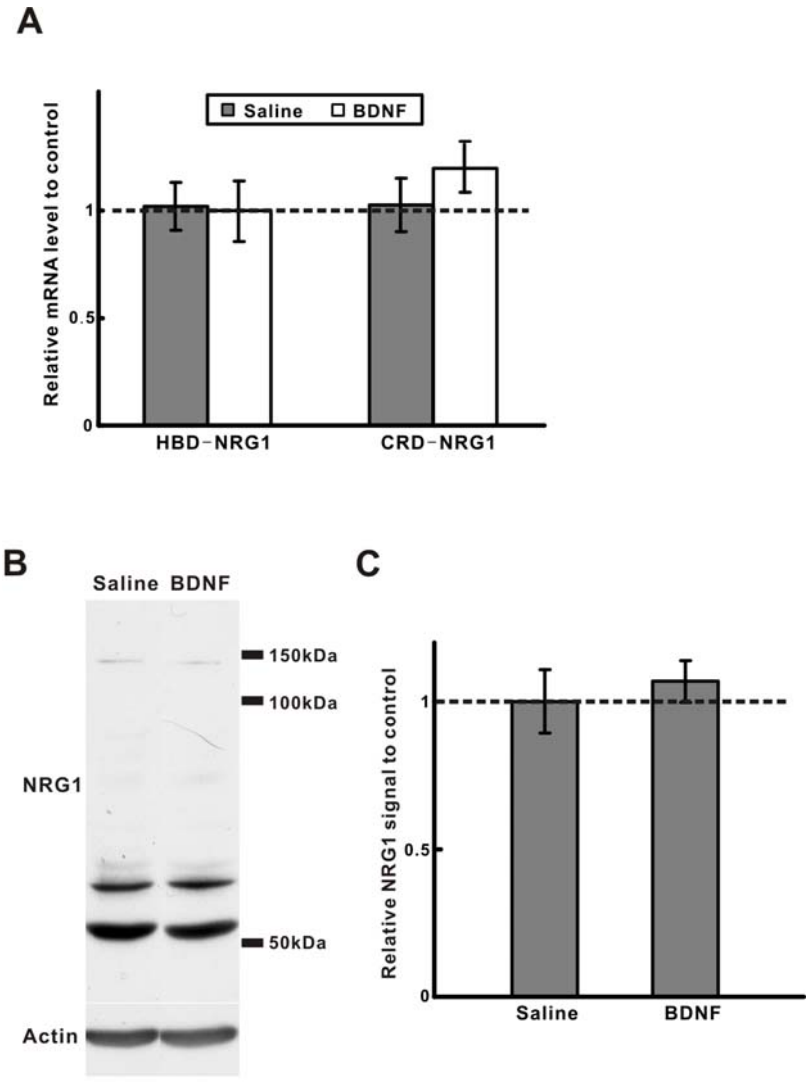


Figure 24. Knocking-down of BDNF-trkB signaling does not significantly affect NRG1 expression in spinal cord.

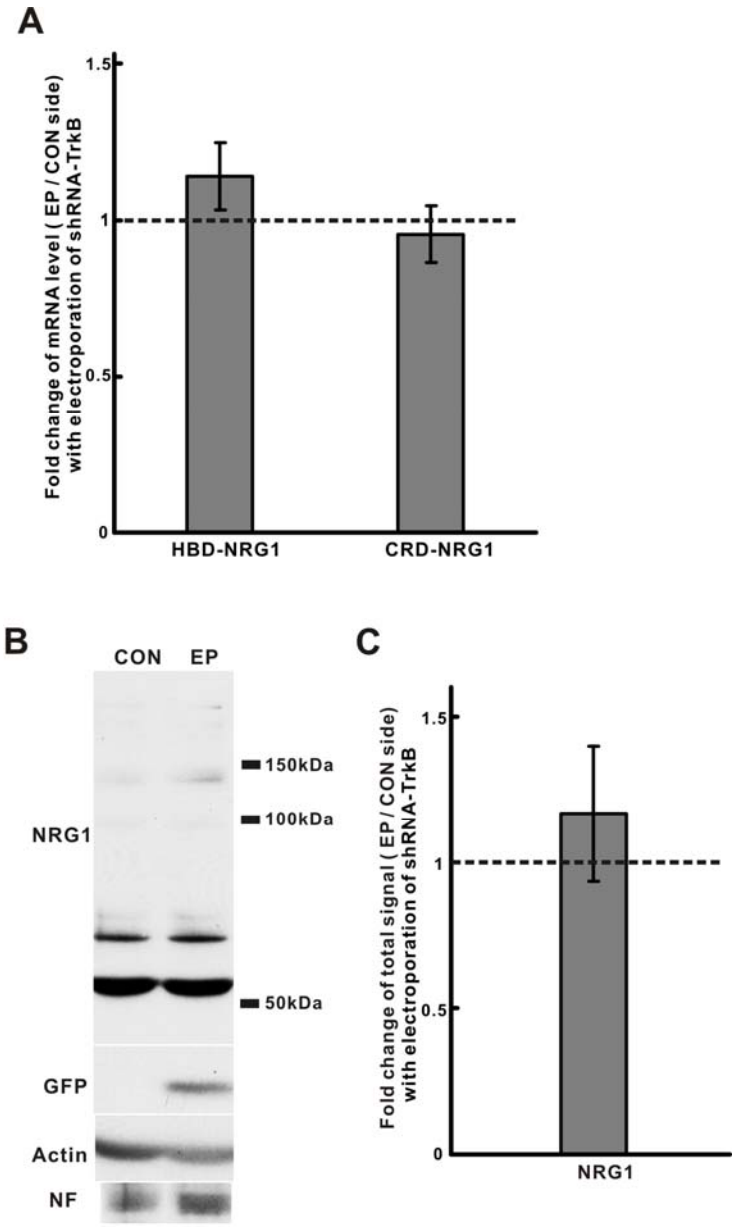
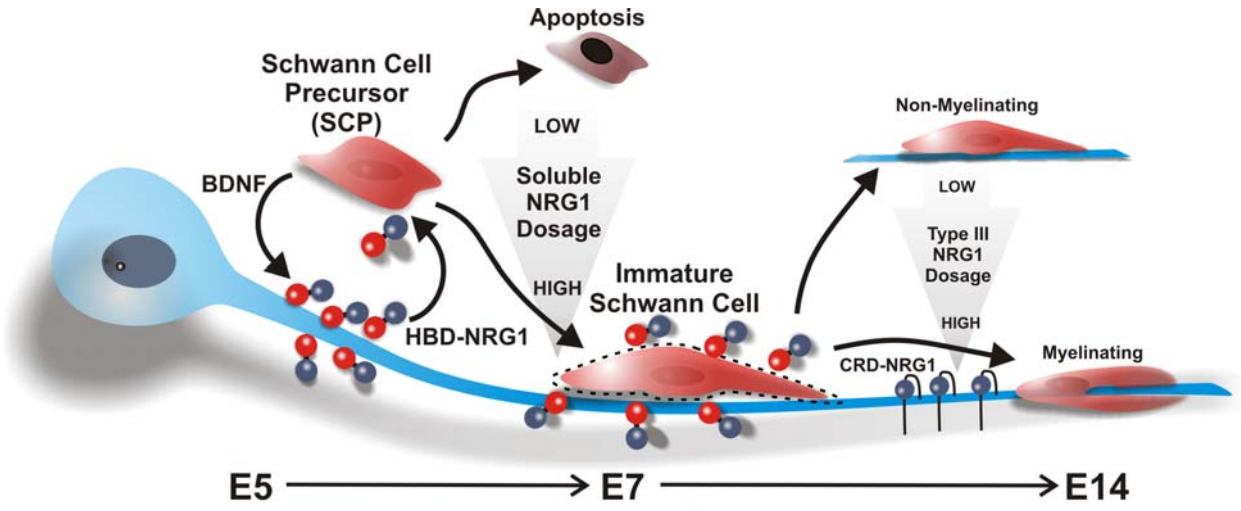


Figure 25. A stage-dependent model for axoglial NRG1 signaling.



APPENDIX B

FIGURE LEGENDS

Figure 1. Molecular structures of NRG1 isoforms. Over 30 protein isoforms are produced by *NRG1* gene, and mainly divided to two groups, soluble form and membrane-bound form. They are first expressed as trans-membrane precursors and then undergo proteolytic cleavage by specific proteinases in the extracellular matrix. Most of soluble form of NRG1 has heparin-binding domain (HBD) that is also called Ig-like domain (IG), and will be released from membrane after the cleavage. In contrast, membrane –bound form of NRG1 has cysteine-rich domain (CRD) that could work as the second trans-membrane domain and keep the protein tethered on the membrane even after the cleavage.

Figure 2. Soluble NRG1 signaling activates erbB receptors through the interaction between HBD and HSPGs. The heparin-binding domain (HBD) targets the released soluble NRG1 proteins to the cell surface that is rich in specific heparan-sulfate proteoglycans (HSPGs) in the extracellular matrix. The interaction of HBD and HSPGs not only facilitate the deposition of soluble NRG1 proteins, but also potentiate the NRG1 signaling through erbB receptor activation via the formation of hetero-/homo-dimers such as erbB2-erbB3, erbB2-erbB4 and erbB4-erbB4, on the cell membrane. Because erbB3 has inactive tyrosine kinase domain, it has to be coupled with erbB2 or erbB4 that has active kinase function so that the down-stream signaling pathways can be activated. Soluble NRG1-erbB signaling has been shown to regulate acetylcholine receptor

expression and insertion in neuro-muscular junction, glial cell development including proliferation, survival, differentiation and migration, as well as the pathogenesis of some cancer cells.

Figure 3. The HBD of NRG1 retains heparin-binding ability and potentiates an HER4 antagonist. ***A.*** Schematic diagram of secreted NRG1 and four antagonist constructs. The heparin-binding domain (HBD) was fused N-terminal or C-terminal to extracellular binding domain of HER4 receptor (H4) with or without a spacer domain(S). H4 alone was made as a control. Each was applied to a heparin column. The flow-through and successive elutions with increasing concentrations of NaCl were measured by immunoblotting with an anti-HA antibody. The HBD-S-H4 protein had the highest affinity for heparin compared to other constructs. ***B.*** Comparable amounts of each fusion protein were premixed with 50pM recombinant NRG1 protein and applied to L6 muscle cells to determine the effect of each protein on the phosphorylation of erbB receptors (p185) normalized to erbB2 levels (bottom gel). This experiment was repeated for 3 times.

Figure 4. Purified HBD-S-H4 blocks NRG1 induced erbB phosphorylation by concentrating on cell surface. ***A.*** Silver stained gel of purified HBD-S-H4 and H4 fusion proteins. The predicted molecular weight of HBD-S-H4 and H4 is around 88kd and 70kd respectively. The higher apparent molecular weight on the gel reflects that both fusion proteins are glycosylated during expression in mammalian cells. ***B.*** L6 cells were treated with either purified HBD-S-H4 or H4 as a control at 1 and 10nM for 1 hour. The

cells were washed for several times to remove any unbound fusion proteins and then challenged with 75 pM recombinant NRG1 protein. While H4 alone had no residual effects, HBD-S-H4 had sustained effects completely blocking NRG-induced erbB receptor phosphorylation (p185). This experiment was repeated for 3 times.

Figure 5. NRG1's HBD targets HBD-S-H4 to cell surfaces through heparan sulfate interactions. ***A.*** CHO-wt and CHO-pgsd677 (deficiency in heparan-sulfate synthesis) cells were incubated with biotinylated HBD-S-H4 or H4 (50 nM) with or without heparinase treatment. Compare to other conditions, HBD-S-H4 treated CHO-WT had the brightest green fluorescence intensity that appeared to concentrate in extracellular matrix between cells. The absence of heparan-sulfate in mutant cells and the heparinase treatment dramatically reduce the fluorescence to the level similar to H4 treatment. Scale bar is 50μm. ***B.*** A binding assay of protein constructs to CHO cells shows the significantly enhanced binding ability of HBD-S-H4 to wild type CHO cells (CHO-wt), but not to mutant cells with deficient heparan sulfate synthesis (CHO-pgsd677). The H4 construct did not bind to these cells. Error bars represent standard error of four experiments. ***** and ****** indicate significant differences and $p < 0.001$ and < 0.002 respectively, using a student's t-test comparing the indicated conditions. ***C.*** Increasing concentrations of biotin-conjugated HBD-S-H4, H4 or IgG were added to heparin coated 96-well plates to produce a saturation binding curve. The amount of HBD-S-H4 bound to heparin increased until the saturation of all binding sites. Data points are shown as mean $\pm$ SEM of quadruplicate wells.

Figure 6. HBD-S-H4 targets to the same regions of the developing spinal cord where NRG1 accumulates. 20μg of HBD-S-H4 was added onto the chorioallantoic membrane of embryonic chicken embryos. Tissue transverse sections at lumbar level of spinal cord at E7 show a comparison between endogenous chicken NRG1 expression (green, left) and HBD-S-H4 distribution (green, right) at low power (top). Higher power Images (bottom panel) focusing on the spinal cord show that HBD-S-H4 adhered to the same regions as endogenous NRG1 along axonal tracts in the spinal cord (arrows) and along the ventral root (arrowhead). Sections were counterstained for nuclei with DAPI (blue). Scale bars are 200μm.

Figure 7. HBD-S-H4 is concentrated in the ventral nerve area through the interaction with HSPGs. Both endogenous chicken NRG1 (chNRG, green) and HBD-S-H4 (huNRG, green) were concentrated in the ventral nerve root identified by a Schwann cell marker (red). High salt (1M NaCl for 2 hours at 37°C) treatment on sections of HBD-S-H4 removed the fluorescent signal in the ventral root. Control treatments with saline did not reveal any immunoreactivity with the same antibody in the ventral nerve root. Scale bar is 50μm.

Figure 8. Immunostaining with an antibody against His-tag present in both HBD-S-H4 and H4 constructs. Immuno-staining with an antibody against His-tag present in both HBS-D-H4 and H4 constructs shows that His-tag immunoreactivity (Histag, green) was only present in the HBD-S-H4 treated embryo and concentrated in the same ventral nerve region as endogenous chicken NRG1 (chNRG, red). Scale bar is

50μm.

Figure 9. HBD-S-H4 induces Schwann cell apoptosis in the ventral nerve root. *A.* Chicken embryos were treated with HBD-S-H4, H4, or saline from E5-E7. Increased numbers of TUNEL positive Schwann cells (green, arrows) were seen in ventral root regions (highlighted with dashed lines) that were stained positively for HBD-S-H4 (red) with the human NRG1 antibody (huNRG). Each section was also stained with DAPI to show cell nuclei (blue). The ventral root was divided equally into proximal and distal regions (solid line) showing increased HBD-S-H4 accumulation in the distal versus proximal nerve segments. Scale bar is 50μm. Bottom panel is high power images of TUNEL positive Schwann cells in each condition. Scale bar is 5μm. ***B.*** Quantification of apoptotic Schwann cells shows significantly more apoptotic Schwann cells in the distal nerve root regions with higher levels of HBD-S-H4 accumulation, compared to proximal nerve root regions. Results are expressed as mean $\pm$ SEM with n=5, 7, and 6 for each, respectively. ***** and ****** indicate significant differences and $p < 0.0001$ and <0.00005 respectively.

Figure 10. Transition of matrix-bound NRG1 from axons to Schwann cells. Representative confocal images show the co-localization of soluble NRG1 (green) and Schwann cells (P0 protein, Red) (***A***), or axons (neurofilament, Red) (***B***) between E5 and E7. Scale bar = 50μm, upper panel. Lower panel shows high power images of area from the dashed-line square, scale bar = 20μm. The percentage of pixel overlapping between NRG1 immunoreactivity and Schwann cells (***C***) (*n* = 9, 9, 5 for each stage, respectively)

or axons (***D***) (*n* = 7, 4, 4 for each stage, respectively) was measured in the ventral root (***p* < 0.0001 for E5 versus E6).

Figure 11. Release soluble NRG1 binds to heparan sulfates at axoglial interface. Matrix-bound soluble NRG1 signal (green) was dramatically reduced after high salt (1M NaCl) treatment compared to a PBS control at E6, while axon immunoreactivity (red) was intact . Scale bar = 50μm.

Figure 12. NRG1 signaling promotes Schwann cell survival *in vivo*. *A*. A significant increase in TUNEL positive cells (red, arrows) were detected in motor axons after treatment with a targeted NRG1 antagonist compared to a saline control (Schwann cells: green, DAPI stained nuclei: blue). Scale bar = 50μm. ***B.*** Dose-dependent increase in apoptotic Schwann cells treated with the antagonist in both motor and sensory axons (*n* = 7, 6, 7 for each condition, respectively; ***p* < 0.0001 for both axon regions using one-way ANOVA). ***C.*** Addition of soluble type I NRG1 rescues Schwann cells from cell death at both E5 and E6 (*n* = 4 for each stage; ***p* < 0.001, ***p* < 0.0001, for saline versus NRG1 treatment).

Figure 13. Chick *in ovo* electroporation can be used to modulate gene expression unilaterally in the ventral nerve roots. *A.* Top view of whole chick embryo after *in ovo* electroporation shows that GFP (green) is highly expressed only in one side of spinal cord, and can be transported along spinal nerves at lumbar level (arrows). ***B.*** Transverse sections of the electroporated animal shows selective expression of GFP (green) in the

ventral spinal cord of the electroporated side (EP) as well as in the exiting motor axons surrounded by Schwann cells (red) that are also shown at the contralateral side (CON) without GFP expression. DAPI stains cell nuclei (blue). Scale bar = 50μm.

Figure 14. Specific knock-down of soluble NRG1 increases Schwann cell death. *A*. Efficacy of shRNA in down-regulating soluble NRG1 in COS7 cells was measured by co-transfection of full-length NRG1 type I (proNRG1) together with a shRNA-HBD specific for NRG1's heparin-binding domain. Both no shRNA and a scrambled shRNA (shRNA-con) were used as controls. Transfection efficiency was tracked by GFP (green) expression from a third plasmid. DAPI stains cell nuclei (blue). Scale bar = 50μm. ***B.*** proNRG1 expression in COS7 cell lysates was significantly reduced with shRNA-HBD transfection showing both the un-cleaved (~75kDa) and the cleaved (cytoplasmic tail) fragment (~50kDa) of proNRG1 by immunoblot analysis. ***C.*** Increased number of apoptotic Schwann cells was seen at E7 after electroporation with shRNA-HBD compared to either shRNA-con or GFP alone (n = 7, 4, 6 for each condition, respectively; ********$p < 0.0005$, for shRNA-HBD versus control or shRNA-con).

Figure 15. Over-expression of soluble NRG1 promotes Schwann cell survival. *A*. Overexpression of soluble NRG1 (type I) in ventral spinal cords *in vivo* using a C-terminal myc-tagged proNRG1 shows myc expression (red) in GFP-positive cells (green). DAPI stains cell nuclei (blue). Scale bar = 20μm. ***B.*** Overexpression of proNRG1 in motor axons significantly promotes Schwann cell survival at E6, but not at E5 or control (pMES empty vector alone) (n = 4, 6, 4 for each condition, respectively; *******p*

< 0.005, for proNRG1 at E6 versus vector control; ***p* < 0.0005, for proNRG1 at E6 versus E5).

Figure 16. Schwann cell differentiation during normal development of chick embryos. Schwann cell precursors differentiate into immature Schwann cells between E6 and E7 as evidenced by the down-regulation of AP2α and up-regulation of O4 antigens. AP2α (green) or O4 (green) were used to label SCPs and immature Schwann cells, respectively, from E4 to E7 in the ventral root (VR) labeled by a trkB antibody (red). DRG denotes dorsal root ganglia. DAPI stains cell nuclei (blue). Scale bar = 50μm.

Figure 17. NRG1 signaling is required for Schwann cell differentiation. Treatment with the NRG1 antagonist at days E5 and E6 prevented the expression of O4 at E7 (bottom, green) in both the ventral root (VR) and the dorsal root (DR) highlighted by the dashed lines. The normal developmental loss of AP2α (top, green) expression in the ventral root (VR) was not affected by the antagonist treatment. DAPI staining shows cell nuclei (blue). Scale bar = 50μm.

Figure 18. NRG1 signaling is not required for Schwann cell proliferation. *A.* BrdU incorporation (green) in Schwann cells at E7 in the ventral root (VR, dash lines) counterstained with trkB antibody (red) is slightly increased after 2-day treatment with the NRG1 antagonist compared to a saline control. DAPI staining shows cell nuclei (blue). Scale bar = 20 μm. ***B.*** Quantification of the percentage of BrdU positive Schwann cells in motor axons at E7 shows a small increase in proliferation rate with antagonist treatment

(n = 5, 4 for each condition, respectively; **p < 0.002).

Figure 19. BDNF's receptors are exclusively localized on axons. *A, B.* Confocal microscopy of the ventral root at E5 with trkB (green) in (***A***), or p75 (green) in (***B***), together with Schwann cells (P0 protein, red, left panel) or neurofilament (red, right panel) demonstrate the axonal localization of both BDNF receptors. The bottom panels in each show high power images of corresponding areas highlighted by the dashed lines. Scale bars = 20μm.

Figure 20. BDNF promotes Schwann cell survival through axon-derived soluble NRG1. *A.* BDNF *in ovo* treatment significantly decreased Schwann cell death compared to a saline control at E5. (n = 5, 8 for saline and BDNF, respectively; **p < 0.0005). ***B.*** Knocking-down soluble NRG1 with shRNA-HBD electroporation on one side of the spinal cord abolished BDNF's survival effect seen on the opposite side without shRNA electroporation (n = 5, *p < 0.005).

Figure 21. Axonal trkB signaling promotes Schwann cell survival through soluble NRG1. ***A.*** Electroporation (EP) of a shRNA against trkB together with GFP reduced trkB staining (red) in the ventral horn (circle), relative to both the contralateral side of the spinal cord (CON) and GFP electroporation alone. DAPI staining shows cell nuclei (blue). Scale = 50μm. ***B.*** Quantification of the ratio of relative trkB fluorescence between the electroporated versus control side of the spinal cords shows that the shRNA-trkB knocked-down trkB expression by 60% per cell in the ventral horn (n = 5, 3 for each

group, respectively; ***p* < 0.005). ***C.*** While Schwann cell death at E7 significantly increased after knocking-down trkB, adding back soluble NRG1 to these embryos rescued Schwann cells on both electroporated and control sides. (*n* = 3, 7, 6 for each group, respectively; ***p* < 0.02, for CON side of shRNA-trkB versus shRNA-trkB plus NRG1; ****p* < 0.0005, for EP side of shRNA-trkB versus shRNA-trkB plus NRG1, and versus CON side of shRNA-trkB).

Figure 22. Schwann cell death is increased at E13.5 in TrkB knockout mice. *A.* TUNEL staining of motor axons (green) at E13.5 shows more TUNEL positive Schwann cells (red, arrows) in knockout (KO) compared to wild-type (WT) littermates. DAPI stains cell nuclei (blue). Scale bar = 20μm. ***B.*** Significantly increased numbers of apoptotic Schwann cells are seen in the knockout embryos. *(n* = 5 for each group; ***p* < 0.005).

Figure 23. Over-expression of BDNF-trkB signaling does not significantly affect NRG1 expression in spinal cord. *A.* qPCR showed no significant changes in spinal cord mRNA levels for both type I HBD-NRG1 and type III CRD-NRG1 at HH stage 27 after BDNF treatment (*n* = 5 for each group). ***B, C.*** NRG1 protein was also unchanged after BDNF treatment by Western blot. Quantification of the bands ranging from 50-150kDa was done by normalization to actin after reprobing (*n* = 3, for each group).

Figure 24. Knocking-down of BDNF-trkB signaling does not significantly affect NRG1 expression in spinal cord. *A.* Following electroporation with shRNA-trkB, the EP and CON side of lumbar level spinal cord were isolated based on GFP expression.

mRNA levels for both HBD-NRG1 and CRD-NRG1 were unchanged by qPCR (*n* = 5). ***B, C.*** NRG1 protein expression was also unchanged by Western blot and quantified as described above (*n* = 3) with normalization to both actin and neurofilament (NF). Reprobing with a GFP antibody shows the presence of GFP expression only in the electroporated side.

Figure 25. A stage-dependent model for axoglial NRG1 signaling. From E5-E7 in chick embryos, Schwann cell precursor (SCP) survival is dependent on a concentration gradient of soluble NRG1 that can be increased at local axon segments through axonal trkB receptor activation (the red spheres represent the heparin-binding domain, while the blue spheres are the EGF-like domain of type I/II NRG1). Those SCPs that do not receive sufficient levels of NRG1 undergo apoptosis. Those that survive require further NRG1 signaling to differentiate into immature Schwann cells. The immature Schwann cells turn on the expression of cell-surface HSPGs (dashed lines) that act as a ‘sink’ for heparin-binding forms of NRG1, that in turn provides sustained NRG1 signaling at E7. Later, the ensheathment fate (myelination) of axons at E14 requires type III CRD-NRG1.

APPENDIX C

IACUC APPROVAL

WAYNE STATE UNIVERSITY

INSTITUTIONAL ANIMAL
CARE AND USE COMMITTEE
101 E. Alexandrine St.
Detroit, MI 48201-2011
Telephone: (313) 577-1629
Fax Number: (313) 577-1941

ANIMAL WELFARE ASSURANCE # A 3310-01

PROTOCOL # A 05-02-07

Protocol Effective Period: June 15, 2007 – May 31, 2010

Year 2 Annual Review Date: June 1, 2008
Year 3 Annual Review Date: June 1, 2009

TO: Dr. Jeffrey A. Loeb
Department of Neurology
3122 Elliman Clinical Research Building

FROM: Lisa Anne Polin, Ph.D.
Chairperson
Institutional Animal Care and Use Committee

SUBJECT: Approval of Protocol # A 05-02-07

"Axoglial Communication Through Regulated Release of Neuregulin (NMSS)"

DATE: June 1, 2009

The Annual Review of your animal research protocol and any applicable grant applications has been conducted and approved by the Wayne State University Institutional Animal Care and Use Committee (IACUC). The species and number of animals approved for the duration of this protocol are listed below.

Species	Strain	Qty.	Cat.
CHICKENS	Eggs (0-19 days), Fertilized, Specific Pathogen Free	10000	C
RATS	Female, timed pregnant, Sprague-Dawley	200	C
MICE	Female, timed pregnant, Balb/c	200	C
MICE	Male/Female, 0-2 years, GDNF+/-	27	D
MICE	Male/Female, 0-2 years, TRK-B+/-	20	D
MICE	Male/Female, 0-2 years, TRK-B+/-, GDNF +/- heterozygotes	30	D
MICE	Male/Female, GDNF+/-	1500	D
MICE	Male/Female, TRK-B+/-	1500	D
MICE	Harvested Embryos, Balb/c	1800	C
RATS	Harvested Embryos, Sprague-Dawley	1800	C

Species Amendments	Strain	Qty.	Cat.
MICE	B6SJL-Tg(SOD1*G93A), male and female, adult	20	B
MICE	B6SJL-Tg(SOD1*G93A), male and female, adult	80	C
MICE	B6SJL-Tg(SOD1*G93A), male and female, adult	80	D
MICE	wild type, male and female, adult	20	B
MICE	wild type, male and female, adult	80	C
MICE	wild type, male and female, adult	80	D
RATS	NTac:SD-Tg(SOD1G93A), male and female, adult	20	B
RATS	NTac:SD-Tg(SOD1G93A), male and female, adult	80	C
RATS	NTac:SD-Tg(SOD1G93A), male and female, adult	80	D
RATS	wild type, male and female, adult	20	B
RATS	wild type, male and female, adult	80	C
RATS	wild type, male and female, adult	80	D
MICE	B6SJL-Tg(SOD1*G93A), male and female, adult	500	D
MICE	wild type, male and female, adult	500	D
RATS	NTac:SD-Tg(SOD1G93A), male and female, adult	500	D

WAYNE STATE UNIVERSITY

INSTITUTIONAL ANIMAL
CARE AND USE COMMITTEE
101 E. Alexandrine St.
Detroit, MI 48201-2011
Telephone: (313) 577-1629
Fax Number: (313) 577-1941

ANIMAL WELFARE ASSURANCE # A 3310-01

PROTOCOL # A 04-02-10

Protocol Effective Period: July 28, 2010 – April 30, 2013

TO: Dr. Jeffrey A. Loeb
Department of Neurology
3122 Elliman Clinical Research Building

FROM: Lisa Anne Polin, Ph.D. Lisa Anne Polin
Chairperson
Institutional Animal Care and Use Committee

SUBJECT: Approval of Protocol # A 04-02-10

"Axoglial Communication Through Regulated Release of Neuregulin"

DATE: July 29, 2010

Your animal research protocol has been reviewed by the Wayne State University Institutional Animal Care and Use Committee, and given final approval for the period effective **July 28, 2010** through **April 30, 2013**. The listed source of funding for the protocol is **NIH**. The species and number of animals approved for the duration of this protocol are listed below.

Species	Strain	Qty.	USDA Cat.
CHICKENS	Embryos (eggs 0-19 days old) whitehorn "Fertilized, Specific Pathogen free"	6000	C
MICE	B6SJL-Tg(SOD1*G93A), male, female, adult	20	B
MICE	B6SJL-Tg(SOD1*G93A), male, female, adult	80	C
RATS	NTac:SD-Tg(SOD1G93A) male, female, adult	20	B
RATS	NTac:SD-Tg(SOD1G93A) male, female, adult	80	C
MICE	B6SJL, wild, male, female, adult	20	B
MICE	B6SJL, wild, male, female, adult	80	C
RATS	NTac:SD, wild, male, female, adult	20	B
RATS	NTac:SD, wild, male, female, adult	80	C
MICE	B6.Cg-Tg(GFAP-tTA)110Pop/J adult	20	B
MICE	C57BL6 WT, male, adult	20	B
MICE	C57BL6 WT, female, adult	20	B

*To be purchased

MICE	GlyB4-C57BL6, female, 3 month	20	B
MICE	GlyB4-C57BL6, male, 3 month	20	B
MICE	Thy1.1-N, female, 1 month	20	B
MICE	Thy1.1-N, male, 1 month	20	B

**To be transferred from Columbia University, Eric Kandel, PI; protocol 525A

MICE	TrkB knockout, female, 3 month	20	B
MICE	TrkB knockout, male, 3 month	20	B

**To be transferred from WSU expiring protocol #A 05-02-07

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ABSTRACT

AXOGIAL COMMUNICATION MEDIATED BY SOLUBLE NEUREGULIN-1 AND BDNF

by

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During peripheral nervous system development, successful communication between axons and glial cells including Schwann cells in peripheral nervous system and oligodendrocytes in central nervous system, is required for the proper functions of both neurons and glia. Three types of alternatively-spliced proteins belonging to the neuregulin1 (*NRG1*) gene family of growth and differentiation factors are essential for Schwann cell survival and peripheral nerve development. While membrane-bound NRG1 forms (type III) has been strongly implicated in the regulation of myelination process at late stage of Schwann cell development, little is known about the role of soluble, heparin-binding forms of NRG1 (type I/II) in regulating early Schwann cell development *in vivo*. These forms are rapidly released from axons *in vitro* by Schwann cell-secreted neurotrophic factors, and, unlike membrane-bound forms, have a unique ability to diffuse and adhere to heparan sulfate-rich cell surfaces. We harness this natural targeting ability of soluble NRG1 to develop a novel antagonist by fusing its heparin-binding domain (HBD) to the soluble human epidermal growth factor receptor 4

(HER4). This fusion protein retains high affinity for heparin binding and to specific cell surface that express heparan sulfates resulting in a much more potent NRG1 antagonist than any other inhibitors for this molecule. *In vivo*, it is targeted to peripheral nerve segments where endogenous soluble NRG1 binds to and efficiently blocks the activity of NRG1 as a Schwann cell survival factor, leading to significant cell apoptosis in both motor and sensory axon area dose-dependently.

In this thesis work, we also show that axon-derived soluble NRG1 translocates from axonal to Schwann cell surfaces in the embryonic chick between days 5-7, corresponding to the critical period of Schwann cell survival during the normal development of peripheral nervous system. Down-regulating endogenous soluble NRG1 signaling with the targeted antagonist or shRNA via chick *in ovo* electroporation, blocks their differentiation from precursors into immature Schwann cells and increases programmed cell death, while up-regulating NRG1 rescues Schwann cells from normal-occurring apoptosis. Furthermore, exogenous BDNF also promotes Schwann cell survival through promoting the local release of axonal NRG1 by binding axonal trkB or p75 receptor. Consistently, increased Schwann cell death occurs both in trkB knock-out mice and after knocking-down axonal trkB in chick embryos, which can then be rescued with soluble NRG1. These findings suggest a localized, axoglial feedback loop through soluble NRG1 and BDNF critical for early Schwann cell survival and differentiation *in vivo*, which may not only be important for the axoglial communication, but may also be helpful in understanding nervous system diseases that involve the axoglial interface and the providing better therapeutic strategies for their treatments.

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SELECTED PUBLICATIONS

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