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Roles Of Neuregulin1 In Neuromuscular Junction Development

Jiajing Wang
Wayne State University,

ROLES OF NEUREGULIN1 IN NEUROMUSCULAR JUNCTION DEVELOPMENT

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JIAJING WANG

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Advisor Date

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DEDICATION

This work is dedicated to my parents, Bofang Wang and Liping Jin. It is their unconditional love, understanding, trust, and encouragement during all these years of study that motivates me to achieve my dream. Without their support, I would not be where I am. I owe my profound gratitude and deepest appreciation to them.

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PREFACE

Synapses are essential specialized units for communication between neuronal cells or between neuronal cells and target cells. A neuromuscular junction (NMJ) is a synapse built asymmetrically between a motor neuron and a skeletal muscle fiber, and it is associated with Schwann cells. In vertebrates, motor neurons release the neurotransmitter acetylcholine (ACh) at NMJs to convey signals to the muscle cells, which initiates a mechanism that eventually results in muscle contraction. Precisely orchestrated conversation among motor neurons, adjacent glial cells, and muscle fibers is crucial to compose coordinated NMJs. Among all the signaling molecules identified at NMJs, a family of growth and differentiation factors known as neuregulin1s (NRG1s) has been implicated to have multiple functions during neuromuscular synaptogenesis. However, its mode of function during NMJ development *in vivo* remains elusive. Meanwhile, the NRG1 gene encodes a spectrum of alternatively spliced products that have distinct expression profiles. These NRG1 isoforms play differential important roles in the development of both central nervous system (CNS) and peripheral nervous system (PNS). However, the regulatory machinery for NRG1 isoforms expression is mostly unknown.

This dissertation focuses on the developmental roles of soluble NRG1 in regulating different aspects of the NMJ, and the regulation of NRG1 expression in motor neurons by neurotrophic factors and axon-target interactions. Chapter I begins with a necessary background of the NMJ structure and its development, the NRG1 gene and the structure of NRG1 isoforms. Previous studies on the roles of NRG1 signaling at NMJs, together with neurotrophic factor signaling and their functions in gene regulation,

are briefly introduced. Finally, the rationality of the dissertation project is presented. In Chapter II, a quantitative analysis of NMJ development in chicken embryos is described. From this background, evidence from studies with both genetic modulations and novel antagonist treatment reveal that NRG1 signaling regulates acetylcholine receptor (AChR) cluster density and size in the early developmental stage. In the middle stage, it is involved in regulating pre-synaptic synaptic vesicle transport and aggregation, while in the late stage, NRG1 is more involved in fine-tuning the NMJ asymmetrical structure and in promoting peri-synaptic Schwann cell development. In Chapter III, evidence is presented to suggest that isoform-specific transcription of NRG1 can be induced by neurotrophic factors, such as brain-derived neurotrophic factor (BDNF), both *in vivo* and *in vitro*. The isoform-specific kinetics and underlying signaling pathway are investigated by further pharmacological studies. The contribution of potential cis-elements to the induction by BDNF is studied as well. In Chapter IV, discussion is provided, together with the significance of the novel understanding in the context of both normal development and a variety of neuromuscular disorders.

NRG1 plays multiple important roles in NMJ development and maintenance. Expression of NRG1 is also regulated by neurotrophic factor signaling both transcriptionally and post-translationally. Understanding distinct functions of NRG1 at NMJs during different developmental stages not only promotes more appreciation for the multi-faceted signaling pathway, but also provides the possibility for the development of therapeutics to treat neuromuscular diseases. Exploring the regulatory machinery for the feedback loop between signaling molecules, like NRG1 and BDNF, is

of great interest due to their important roles in both the CNS and PNS, in the context of both normal development and neurological pathogenesis.

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CHAPTER I

INTRODUCTION

A history of the NMJ

In the nervous system, information is propagated in networks of proper neuronal connections. A neuromuscular junction (NMJ) is the essential specialized unit for communication between a motor neuron, a target muscle cell, and surrounding Schwann cells. The study of the NMJ can be traced back to the birth of neuroscience. It has been utilized to illustrate synaptogenesis for the last century due to its large size, accessibility, and simplicity. Santiago Ramón y Cajal, the winner of the Nobel Prize in Physiology or Medicine in 1906, described some main structural features of neuromuscular synaptogenesis and regeneration (Ramón y Cajal, 1928). The first detailed description of chemical synaptic transmission in vertebrate skeletal NMJs was published by Henry Dale (Dale et al., 1936), who won the Nobel Prize in Physiology or Medicine in 1936, for discoveries relating to chemical transmission of nerve impulses. With the development of techniques like intracellular recording and electron microscopy during the 1950s, Bernard Katz and colleagues showed that the release of the neurotransmitter, acetylcholine (ACh) at the NMJ, is quantal and vesicular (Katz, 1966). Katz won the Nobel Prize in Physiology or Medicine for his discovery in 1970. With these descriptive studies as the foundation for experimental analysis, orchestrated signaling between nerve and muscle began to be appreciated (Dennis, 1981). Several candidate signaling molecules were isolated, and their bioactivities were demonstrated *in vitro* (Hall and Sanes, 1993). In the era of advanced imaging, molecular biology, and

animal genetic tools, genes coding for structural components of the NMJ and signaling molecules that mediate the interaction were molecularly cloned. The critical hypotheses generated from the previous studies were tested *in vivo* (Sanes et al., 1998). This rich history has given us an enormous understanding of the NMJ architecture, development, and function. Some of the fundamental mechanisms and signaling molecules identified at NMJs are shared by synaptogenesis in the central nervous system as well (Lai and Ip, 2003).

The architecture of the NMJ

The vertebrate NMJ is composed of the distal portion of a motor neuron axon, a muscle cell, and peri-synaptic Schwann cells. To fulfill the task of generating an appropriate muscle contraction by the chemical transmission of the electrical impulses from the nerve to the muscle, these three cell types are specialized at the junctional part with the restricted subcellular distribution of arrays of molecules, resulting in a structurally distinctive and functional NMJ. The pre-synaptic motor axon terminals have the apparatus featured to release neurotransmitter at high efficacy. Arrays of synaptic vesicles containing ACh dock at the thickened pre-synaptic membrane patches, referred as active zones, where the vesicles fuse with the membrane to release neurotransmitters. A large number of mitochondria accumulate at the nerve terminal to provide energy for ACh synthesis and release. On the other hand, cytoskeleton structures such as microtubules and neurofilaments are restricted in the pre-synaptic terminal to provide space for the mass of vesicles. Some voltage-dependent potassium

and calcium channels are associated with the active zone to facilitate the calcium-dependent neurotransmitter release. The post-synaptic fraction of the muscle fiber is specialized to respond efficiently and rapidly to the neurotransmitter from the overlying nerve terminal. The post-synaptic membrane is enriched with acetylcholine receptors (AChRs) opposite the active zone, the density of which (>10,000 receptors/μm^2) is extremely high compared to the non-synaptic area (~10 receptors/μm^2 in adults).

The post-synaptic membrane invaginates to form junctional folds beneath the nerve terminal. AChRs are concentrated at the crests and upper parts of these folds. On the vertebrate skeletal muscle fiber, ACh binds to the AChRs to open the ligand-gated sodium channels, allowing sodium influx to initiate muscle contraction. Peri-synaptic Schwann cells extend processes to wrap the axon terminal. In frogs and rodents, a typical adult NMJ is associated with three to four peri-synaptic Schwann cells. Unlike the axon-associated Schwann cells, peri-synaptic Schwann cells are specialized not to form a myelin sheath, but to act as a physical barrier to insulate the junction structure from environmental perturbations. A synaptic cleft separates the pre- and post-synaptic components of the NMJ, spanning ~50 nm with the basal lamina, a highly specialized extracellular matrix (ECM) made up of a number of molecules. The ECM is comprised of arrays of laminin, collagen, heparan sulfate proteoglycan (HSPG), and acetylcholinesterase (AChE), to facilitate cell adhesion and signaling processes. The short distance of the synaptic cleft in combination with the high concentrations of neurotransmitter promote the rapid diffusion of ACh to the opposite post-synaptic muscle membrane. AChE that is anchored on the lamina degrades acetylcholine into

acetate and choline to terminate the signal efficiently and to halt the nerve impulse. The architecture of a vertebrate NMJ is illustrated in Fig. 1.

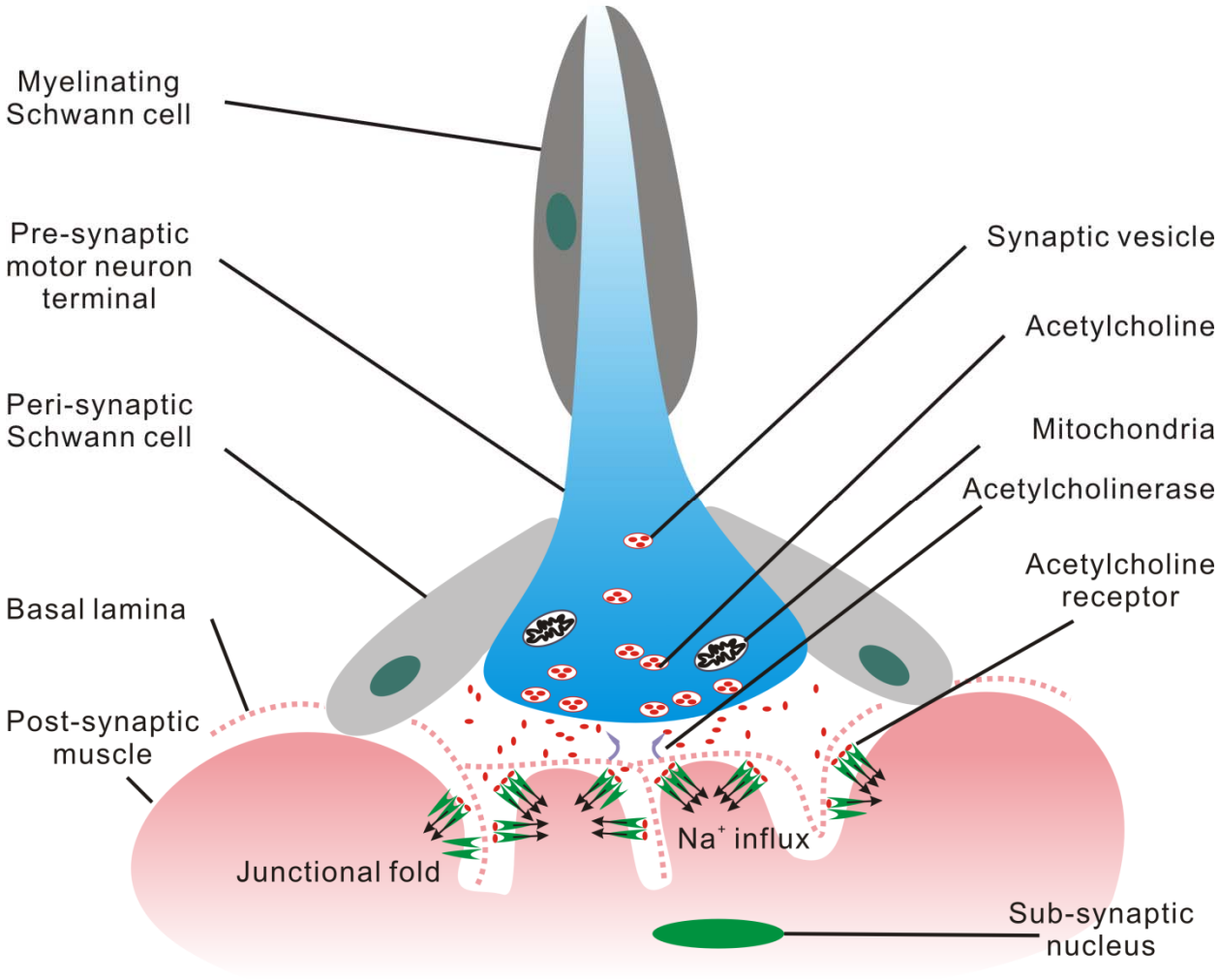

Figure1. Cellular components of a neuromuscular junction. The NMJ consists of a pre-synaptic motor neuron, a post-synaptic muscle fiber, and peri-synaptic Schwann cells. A layer of extracellular matrix separates the pre- and post-synaptic components. The post-synaptic membrane is organized into junctional folds. The action potential is propagated along the motor axon to the pre-synaptic nerve terminal. The calcium influx triggers the release of acetylcholine (ACh) from synaptic vesicles at active zones. ACh binds to the acetylcholine receptors (AChRs) concentrated on the crests of the junctional folds. Post-synaptic membrane depolarization results in the muscle fiber

action potential. Subsequently, ACh diffuses away from the receptors and undergoes breakdown by acetylcholinesterase (AChE). Peri-synaptic Schwann cells insulate the junctional structure. Sub-synaptic nuclei in muscle fibers underlying pre-synaptic motor axon endings are specialized in the transcriptional programs of proteins enriched at the NMJ. Adapted from (Sanes and Lichtman, 1999a).

Development of the NMJ

Along with Schwann cells, motor axon trunks arrive at peripheral regions of developing muscles shortly before the primary myotubes are differentiated. Later on, primary intramuscular branches diverge from the nerve trunk to enter the muscle mass vertically to the proximodistal axis of the myotubes. Secondary and tertiary ramifications are subsequently formed to innervate the skeletal muscle fibers. When motor terminals contact muscle fibers, pre-synaptic components are accumulated in the terminal apparatus, which results in morphological changes. Distinct types of muscle-derived organizing factors coordinate the NMJ formation, maturation, and maintenance by promoting nerve terminal differentiation. It has been shown that members of the fibroblast growth factor (FGF) family and extracellular matrix proteins such as laminins and collagens control discrete sequential aspects of pre-synaptic apparatus development. The expression profiles of these target-derived proteins spatiotemporally correlate with their regulatory functions (Fox et al., 2007). The bidirectional signaling between the signaling molecules of ephrins and their Eph family of receptor tyrosine kinases is involved in axon-guidance and motor neuron terminal differentiation. The ephrin-Eph signaling is frequently repulsive; therefore, motor axons expressing Eph are guided to the appropriate tissue lacking ephrin expression with surrounding

inappropriate tissue expressing ephrins (Marquardt et al., 2005). Disturbance of the expression selectivity resulted in abnormal topographic innervations and defective NMJs (Feng et al., 2000). Muscle-derived β-catenin has been suggested to serve as a retrograde signal to modulate pre-synaptic differentiation and function (Li et al., 2008). Taken together, multiple signaling pathways are mandatory to promote differentiation and positioning of pre-synaptic terminals at NMJs.

On the post-synaptic side, prior to the arrival of nerve terminals onto the muscle fibers, the expression of AChR subunits is up-regulated and AChR clusters appear to be evenly distributed along the myotubes; these initial clusters are not in direct contact with the nerve profiles (Arber et al., 2002). When innervation occurs, large AChR clusters appear in the synaptic regions while AChR clusters in the non-synaptic regions are finally eliminated. Meanwhile, by the subjunctional myonuclei, a variety of genes coding for post-synaptic proteins, such as rapsyn and dystrophin, are expressed to aggregate in the small invaginated fractions of the muscle membrane. Together with these proteins, AChRs are highly concentrated in the post-synaptic membrane and co-register with active zones in the pre-synaptic terminal to ensure robust and fast synaptic transmission (Sanes and Lichtman, 2001). Although intensively investigated, the underlying mechanisms responsible for this process are not fully understood. Traditionally, it is believed that post-synaptic differentiation is regulated by neural inputs, including both motor neuron-derived growth factors and neuronal activity. Low-density lipoprotein receptor-related protein 4 (Lrp4) interacts with muscle-specific kinase (MuSK) to form a receptor complex on the post-synaptic density. Agrin, a heparan sulfate proteoglycan derived from motor neurons, binds to the Lrp4-MuSK complex to

facilitate MuSK phosphorylation and consequent kinase activation. Activated MuSK triggers a signaling pathway that leads to post-synaptic differentiation which includes AChR clustering (Kim et al., 2008, Zhang et al., 2008). ACh, the neurotransmitter at the vertebrate NMJ, not only mediates physiological function, but also plays a regulatory role in the formation of the synapses they serve. Eliminating ACh in mice lacking the ACh synthetic enzyme, choline acetyltransferase (ChAT), results in a markedly increased number of AChR clusters which occupy a broader band width on the muscle, suggesting that ACh negatively regulates post-synaptic differentiation (Misgeld et al., 2002, Brandon et al., 2003). It has been proposed that agrin promotes AChR clustering, while ACh counteracts by destabilizing nascent post-synaptic sites, because mice lacking both ChAT and agrin reverse the NMJ deficits exhibited by mice lacking agrin alone (Misgeld et al., 2005). The balanced interactions between the neurotransmitter and the growth factors ensure that the NMJ forms correctly.

Compared to the pre-synaptic nerve terminal and post-synaptic density differentiation, developmental processes of peri-synaptic Schwann cells have attracted little attention until recently. In fact, peri-synaptic Schwann cells had not been recognized as an integral component of the NMJ until two decades ago (Araque et al., 1999). In the mouse, similar to myelinating Schwann cells, peri-synaptic Schwann cells also originate from the neural crest cells. During migration along the nerve, Schwann cell precursors differentiate to immature Schwann cells; subsequently, some of the immature Schwann cells further differentiate to non-myelinating peri-synaptic Schwann cells, probably depending on their association with the terminal axon and apposition to the junction site. In contrast to the more well-characterized axon-associated Schwann

cell development, the underlying mechanisms for peri-synaptic Schwann cell differentiation are poorly understood. Except for the roles of providing essential energy metabolites and maintaining the ionic balance of the extracellular environment, several lines of evidence have revealed that peri-synaptic Schwann cells actively interact with both pre- and post-synaptic compartments and are important for proper information processing in NMJ development (Araque et al., 1999, Auld and Robitaille, 2003a, b). These cells detect synaptic activity at NMJs and, in turn, release signal molecules that modulate neurotransmission (Jahromi et al., 1992, Reist and Smith, 1992, Robitaille, 1998, Castonguay and Robitaille, 2001).

The wrapping of nerve terminals by immature Schwann cells tightens up during pre-synaptic and post-synaptic specialization (Sugiura and Lin, 2011). Additionally, the number of peri-synaptic Schwann cells per NMJ is associated with the size of the NMJ that they cover. Neurotrophin 3 (NT-3) secreted by muscles has been shown to have regulatory roles in determining peri-synaptic Schwann cell number (Hess et al., 2007). Perturbation of peri-synaptic Schwann cells leads to retraction of nerve terminals from NMJs and reduction of pre-synaptic function, suggesting that they play important roles in maintaining NMJ integrity (Trachtenberg and Thompson, 1997, Reddy et al., 2003). Recently, it has been suggested that a new component of the NMJ, the kranocyte, a fibroblast-like cell, lies outside the synaptic basal lamina and caps the peri-synaptic Schwann cells by extending processes over the entire end-plate. They are evenly distributed in the embryonic muscle, but become restricted to the NMJ during postnatal development. However, the presence of endothelial cells at NMJs remains debatable and their function in NMJ formation remains unclear (Court et al., 2008).

Thus, during NMJ development, reciprocal signaling between motor neurons, their target muscle fibers, and peri-synaptic glial cells is important for the precise physical apposition of the tripartite complex, which further ensures reliable functional neurotransmission. Among all the different signaling molecules acting as either pro- or anti-synaptogenic signals, neureuglin1 (NRG1) is one of the important participants due to its multi-faceted function at NMJs.

Neuregulin1

The neuregulins (NRGs) are a family of growth and differentiation factors with important roles in heart and nervous system development, and pathogenesis of diseases from breast and ovarian cancers to schizophrenia (Fischbach and Rosen, 1997, Buonanno and Fischbach, 2001, Falls, 2003, Esper et al., 2006, Mei and Xiong, 2008). Although there are 4 *NRG* genes in the human genome, most research interests have focused on the first *NRG* gene discovered, *NRG1*, in part because it is abundant in the nervous system and might play a role in cancer (Carraway et al., 1997, Zhang et al., 1997, Harari et al., 1999, Esper et al., 2006). The nomenclature of NRG1 isoforms is originally based on their biological activities when they were first discovered independently, such as acetylcholine receptor inducing activity (ARIA), glial growth factor (GGF), sensory and motor neuron derived factor (SMDF), neu differentiation factor (NDF), and heregulin. Later, it was revealed that the single *NRG1* gene gives rise to at least 33 different isoforms and several long non-coding RNAs (lncRNAs) through a combination of usage of different regulatory regions and alternative splicing (Meyer and

Birchmeier, 1995, Falls, 2003, Steinthorsdottir et al., 2004, Tan et al., 2007, Mei and Xiong, 2008, Rosenbloom et al., 2013).

Most NRG1 isoforms are synthesized as transmembrane precursors (proNRG1s) and are processed by proteolytic cleavage at a juxtamembrane region mediated by various transmembrane proteases, including a disintegrin and metalloproteinase 10 (ADAM10), tumor necrosis factor-α converting enzyme (TACE/ADAM17), β-site of amyloid precursor protein cleaving enzyme (BACE), and meltrin beta (ADAM19) (Loeb et al., 1998, Shirakabe et al., 2001, Horiuchi et al., 2005, Hu et al., 2006, Willem et al., 2006, Montero et al., 2007, Yokozeki et al., 2007, La Marca et al., 2011, Luo et al., 2011). The common feature of all known NRG1 is an EGF-like domain that is necessary and sufficient for binding and activating their tyrosine kinase receptors, including homo- and hetero-dimers of ErbB2, ErbB3 and ErbB4 as shown in Fig. 3 (Loeb and Fischbach, 1995, Buonanno and Fischbach, 2001, Citri et al., 2003). Based on their domain structure, NRG1 isoforms can be categorized into six types (I-VI) which can be further divided into two groups. A majority of the soluble forms of NRG1 have been found to have a heparin binding, immunoglobulin-like domain N terminal to the EGF-like domain, referred to HBD-NRG1 (type I/II/IV/V) (Loeb and Fischbach, 1995, Mei and Xiong, 2008). This type of NRG1 releases its ectodomain through a protein kinase C-δ (PKC-δ) mediated cleavage (Esper and Loeb, 2009). CRD-NRG1 (type III) has a cysteine-rich domain that can keep it membrane-tethered after processing so that it mainly works through direct cell-cell interactions as shown in Fig. 2 (Ho et al., 1995, Yang et al., 1998, Wang and Scott, 2000, Wolpowitz et al., 2000). Type V NRG1 lacks the HBD but has a

unique N-terminal domain, the function of which is less understood (Mei and Xiong, 2008).

This dissertation will mainly focus on types I-III, because they are the most abundant isoforms in the nervous system (Liu et al., 2011). Although these NRG1 isoforms share complimentary activities in signaling, they have significant differences in both post-translational processing and temporal-spatial specific expression profiles, which are precisely regulated by intercellular communication and neuronal activity (Meyer et al., 1997, Eilam et al., 1998, Liu et al., 2011). It will be important to understand their specific functions, both independently and jointly, and where they function along different regions of the same motor axon during the development of NMJs.

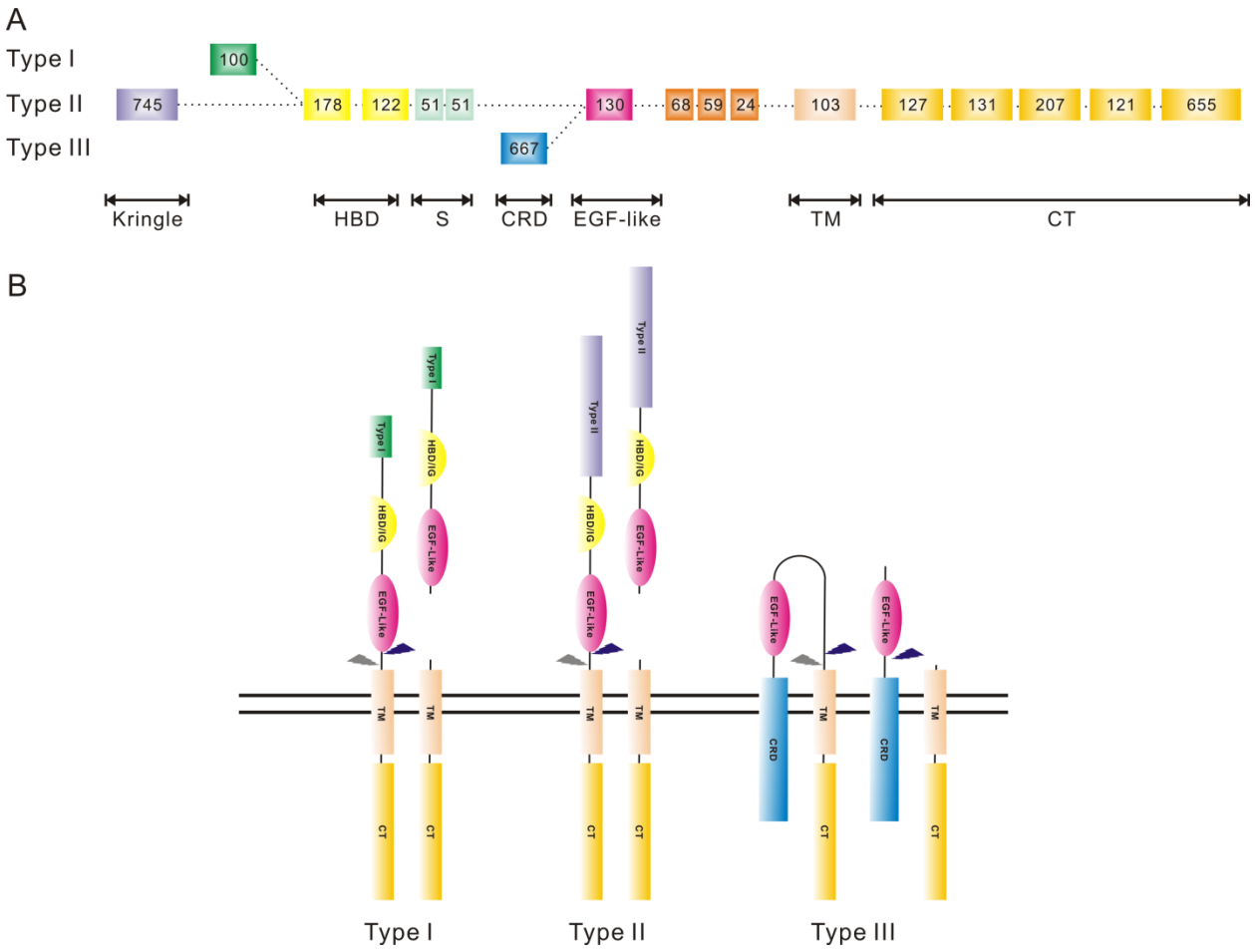

Figure2. Molecular structure of neureuglin1 isoforms. A. The *NRG1* gene encodes over 30 protein isoforms, which are classified into six types based on the N-terminal sequences. The most abundant forms in the nervous system are type I, II, and III. All types of NRG1 isoforms share an epidermal growth factor (EGF)-like domain. Types I/II have an immunoglobulin (Ig)-like heparin-binding domain (HBD) before the EGF-like domain, either with or without the spacer region (S). Type III contains a cysteine-rich domain (CRD) that is directly connected to the EGF-like domain. Variants of types of NRG1 isoforms are generated by alternative splicing in the linker regions and the C-terminal regions. Main exons are indicated by boxes with typical length of the exons in the human genome. **B.** Most NRG1 isoforms are synthesized as transmembrane precursors (pro-NRG1s) with the EGF-like domain located in the extracellular region. Proteolytic shedding produces mature NRG1 proteins that are either soluble (type I/II), or membrane-tethered (type III). Adapted from (Mei and Xiong, 2008).

The putative roles of NRG1 at the NMJ

The NRG1-ErbB ligand-receptor signaling system is perfectly positioned in NMJ development. NRG1 is highly expressed in spinal motor neurons and sensory ganglia neurons shortly after their birth and is efficiently transported along the axon to reach endplates (Loeb et al., 1999). ErbB receptors are highly expressed in corresponding targets including skin, muscle, and Schwann cells (Moscoso et al., 1995, Zhu et al., 1995). Due to the interaction between the HBD and HSPGs, HBD-NRG1 is concentrated in the extracellular matrix of the synaptic cleft and provides biologically important, sustained activation of ErbB receptors on muscle cells and peri-synaptic Schwann cells (Loeb and Fischbach, 1995, Meier et al., 1998, Loeb et al., 1999, Trinidad et al., 2000, Li and Loeb, 2001). In contrast, the function of CRD-NRG1 is confined between cellular components within a short distance, such as motor axons and myelinating Schwann cells, because the molecule remains membrane-tethered after proteolysis (Michailov et al., 2004, Taveggia et al., 2005, Nave and Salzer, 2006). The signaling from activated ErbB receptors can be conveyed through a number of pathways, including Ras-Extracellular signal regulated kinase 1/2 (Erk1/2), phosphatidylinositol-3-Kinase (PI3K)-Akt, c-JUN N-terminal kinase (JNK), focal adhesion kinase (FAK), and Calcineurin/NFAT, depending on the cellular environmental contexts (Si et al., 1996, Tansey et al., 1996, Altiok et al., 1997, Si et al., 1999, Vartanian et al., 2000, Yarden and Sliwkowski, 2001, Kao et al., 2009). Activation of these pathways initiates, alone or in concert, cellular responses, such as survival, proliferation, differentiation, and migration, in muscle targets or Schwann cells, as shown in Fig. 3. In muscle cells, NRG1 signaling elicits increased expression and

phosphorylation of particular E-twenty six (ETS) family transcription factors, such as Ets-2 and GA-binding protein (GABP)-α, that bind to an N-box on the promoter of *CHRNE*, which encodes an AChR ε subunit, to promote transcription (Sapru et al., 1998, Schaeffer et al., 1998, Sapru, 2001, Schaeffer et al., 2001). Besides the AChR ε subunit, several other post-synaptic components such as AChE and utrophin possess an N-box in their corresponding promoter regions, whose expression is also regulated by Ets transcriptional factors (de Kerchove D'Exaerde et al., 2002).

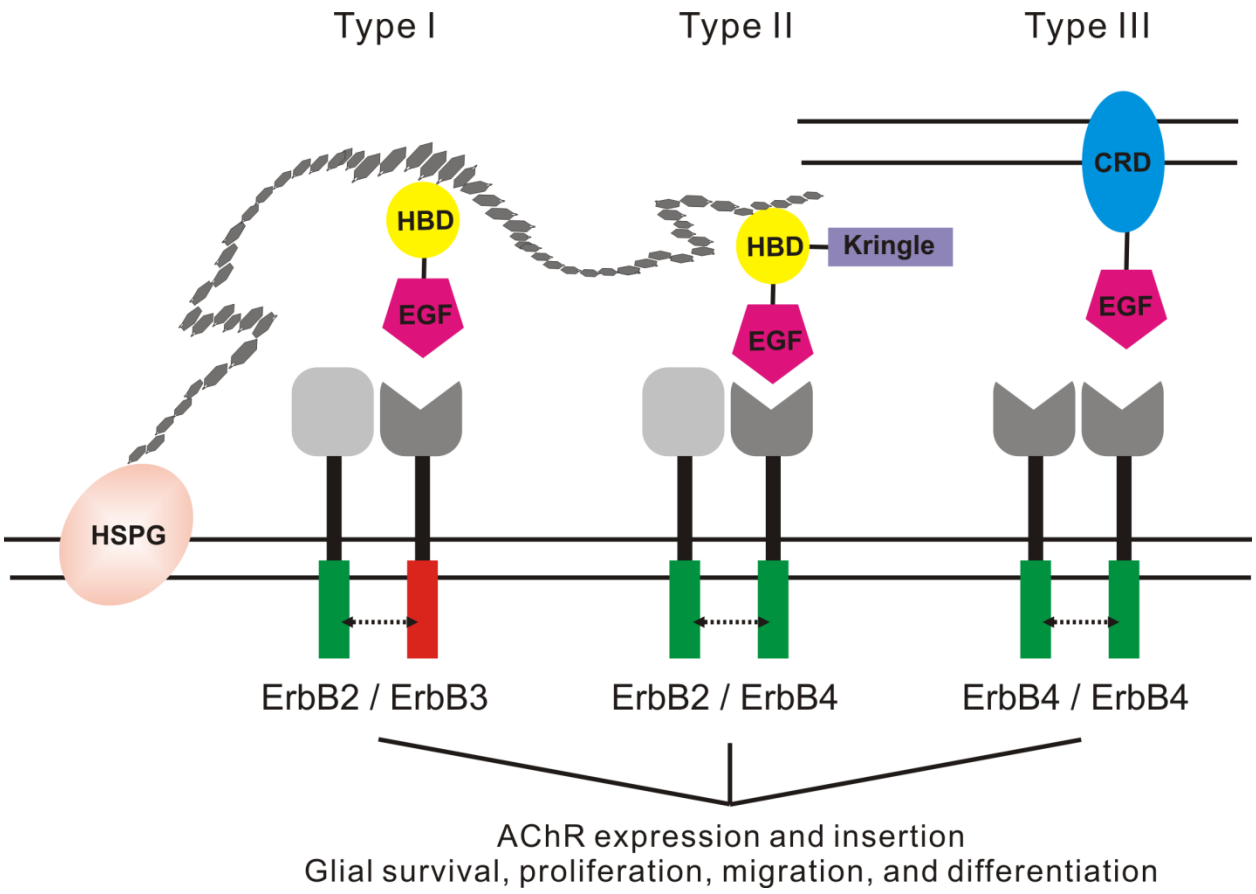

Figure3. Neuregulin1 and ErbB receptors. ErbB receptors are a family of transmembrane receptor tyrosine kinases. NRG1 binds to either ErbB3 or ErbB4. ErbB3 has an impaired tyrosine kinase domain (red box) while ErbB2 has a non-functional ligand binding site (grey box). NRG1 stimulation leads to receptor dimerization to form ErbB2-ErbB3 or ErbB2-ErbB4 heterodimers, or ErbB4-ErbB4 homodimers. Phosphorylation of the intracellular kinase domains (green box) activates downstream signaling. The heparin-binding domain (HBD) on the soluble NRG1 (type I/II) guides the molecule to be concentrated in the extracellular matrix by interacting with heparan sulfate proteoglycans (HSPGs). Type III NRG1 functions in a manner that requires cell contact because the cysteine-rich domain (CRD) anchors the molecule after proteolytic cleavage. In the peripheral nervous system, NRG1-ErbB signaling regulates acetylcholine receptor (AChR) expression and local insertion, and Schwann cell development. Adapted from (Esper et al., 2006).

Although *in vitro* studies have shown that NRG1 can induce the local insertion of new AChRs into the cell surface and can increase AChR ε subunit transcription to promote the transition from embryonic to adult forms of AChRs (Jessell et al., 1979, Falls et al., 1993), *in vivo* studies using mouse genetic models thus far have not shown a clear requirement for NRG1 signaling at NMJs. Mice with disruptions in either all NRG1 forms, the HBD forms, or the NRG1 receptors ErbB2 or ErbB4, die of cardiac defects well before the NMJ formation, while mice with disruptions of ErbB3, the CRD-NRG1 form, or mice that are rescued from their cardiac defects, survive until birth or die shortly thereafter due to a massive reduction in the number of Schwann cells in the peripheral nerve with the subsequent death of most motor and sensory neurons. In almost all cases except the CRD-NRG1 knockout mice, early embryonic NMJs appear grossly normal (Gassmann et al., 1995, Lee et al., 1995, Meyer and Birchmeier, 1995, Kramer et al., 1996, Erickson et al., 1997, Riethmacher et al., 1997, Britsch et al., 1998, Liu et al., 1998, Morris et al., 1999, Wolpowitz et al., 2000), while in CRD-NRG1 knockout mice, the loss of NMJs after the initial stage probably results from sensory nerve and motor nerve withdrawal after Schwann cells fail to develop (Wolpowitz et al., 2000). These limitations to studying mature NMJs have been partially overcome by measurements of the physiology and structure of NMJs from mice with a specific disruption of NRG1 signaling through the selective knockouts of NRG1 or its receptors in Schwann cells, motor neurons, and muscles (Escher et al., 2005, Jaworski and Burden, 2006, Kummer et al., 2006, Ponomareva et al., 2006). However, there are no major anatomical or functional deficits in the NMJ formation of any of these mice, indicating that NRG1 signaling does not have essential roles at NMJs *in vivo* despite its

presence. Nonetheless, consistent with the *in vitro* studies, HBD-NRG1 heterozygous mice have a 50% reduction in the AChR density on the post-synaptic membrane and a reduced safety factor for neuromuscular transmission, but the deficiency has been compensated by an increase of quantal content, suggesting that NRG1 can modulate the local density of AChRs (Sandrock et al., 1997, Mann et al., 2006). Selective knockout of ErbB2 and ErbB4 in muscle similarly show a reduction, though more moderate, in AChR density at NMJs (Jaworski and Burden, 2006). However, it has been reported that NRG1 signaling is involved in regulating the stability of post-synaptic AChRs at the mature NMJ by phosphorylation of α-dystrobrevin1 (Schmidt et al., 2011). For embryonic NMJ development, NRG1 can increase the size of AChR clusters by increasing the tyrosine phosphorylation of MuSK to potentiate AChR clustering (Ngo et al., 2012).

The interaction between axon-derived NRG1 and ErbB2/3 expressed on Schwann cell precursors, immature Schwann cells, and Schwann cells, is well appreciated, but roles of the signaling in the differentiation of peri-synaptic Schwann cells at NMJs are not as well understood (Nave and Salzer, 2006, Birchmeier and Nave, 2008, Ma et al., 2011). Several lines of evidence suggest that NRG1 signaling is important for peri-synaptic Schwann cell development. Neuron-derived NRG1 is required for peri-synaptic Schwann cell survival during embryonic development and becomes dispensable in adults (Trachtenberg and Thompson, 1996). Unregulated NRG1 signaling by either exogenous NRG1 protein application or ectopic expression of activated ErbB2 in Schwann cells causes excessive sprouting and migration of peri-synaptic Schwann cells, which mimics their response to muscle denervation and

subsequent reinnervation (Trachtenberg and Thompson, 1997, Hayworth et al., 2006). The data suggest that NRG1 signaling is critical for the development, maturation, and maintenance of this subpopulation of Schwann cells. However, the underlying mechanisms remain unknown.

Therefore, most of the data to date in mouse models suggest that while NRG1 is not required to build an NMJ, it may have important modulatory roles at NMJs. However, fine-tuning signaling might be more valuable in the context of human degenerative diseases. Unlike the essential signaling such as agrin-Lrp4-MuSK, the deficiency of which leads to the absence of functional NMJ formation, modulatory signals such as NRG1-ErbB are more likely to be involved in neuromuscular degenerative disease pathogenesis resulting from the accumulation of subtle changes (DeChiara et al., 1996, Gautam et al., 1996). Our laboratory has demonstrated that neuregulin1 works synergistically with agrin to promote muscle AChR expression (Li et al., 2004a) and has provided *in vivo* evidence in the chicken embryo that NRG1 release at NMJs is activity dependent and plays an important role in converting synaptic activity to structural changes that maintain NMJs (Loeb et al., 2002). The laboratory has also studied the importance of HSPG interactions and has developed a novel antagonist to specifically block NRG1 signaling (Ma et al., 2009). Thus, I have a number of tools to assess more subtle effects of NRG1 signaling in distinct developmental stages during NMJ development.

In summary, the exact functions of NRG1 in NMJ development need to be revealed. Should they prove important for normal development, the underlying mechanisms have the advantage of being potential treatments for diseases of the

nervous system that affect the neuromuscular interface such as myasthenia gravis (MG) and amyotrophic lateral sclerosis (ALS).

Target-derived Neurotrophic Factors

The developmental program of the peripheral nervous system, including axon guidance, axon-Schwann cell interaction, and the development of the NMJ, requires temporally- and spatially-orchestrated communication among motor neurons, surrounding Schwann cells, and target muscle fibers. The complex network of intercellular communication provides each cell type with distinct instructive cues, resulting in the activation of corresponding intracellular signaling pathways to support its survival and differentiation, which reinforce the supply of molecular cues to its counterparts. Motor neuron derived-NRG1 plays important roles in promoting Schwann cell development, muscle cell differentiation, and formation of the NMJ (Hippenmeyer et al., 2002, Loeb, 2003, Birchmeier and Nave, 2008). Conversely, both Schwann cells and target muscle cells provide arrays of growth and trophic factors to support the survival and function of motor neurons. Among these cues, members of neurotrophic factors are the best described. The classic neurotrophin family of trophic factors consists of nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3), and neurotrophin-4/5 (NT-4/5), which are highly homologous and conserved. They bind to the specific tropomyosin-related kinases (Trks), which include TrkA, TrkB, and TrkC, with high affinity, and the p75 neurotrophin receptor (p75NTR) with low affinity. NGF predominantly binds to TrkA, BDNF and NT-4/5 to TrkB, and NT-3 to TrkC. However, there is considerable crosstalk in the binding of neurotrophins to Trk

receptors. Each neurotrophin can interact with every member of the Trk receptor family under specific conditions, such as a high concentration of ligand (Huang and Reichardt, 2001, 2003). To increase complexity, the *TRKB* and *TRKC* genes can be alternatively spliced into multiple isoforms, including full-length and truncated isoforms. Full-length receptors bear a tyrosine kinase domain, the phosphorylation of which activates the downstream signaling cascades, whereas the truncated isoforms that lack the tyrosine kinase domain function as dominant negative receptors, though other specific cellular functions have been proposed (Biffo et al., 1995, Yacoubian and Lo, 2000, Esteban et al., 2006). Besides neurotrophins, members of other families of proteins, most notably the glial cell–derived neurotrophic factor (GDNF) family, have been shown to be produced and secreted by muscle cells to regulate the survival and function of motor neurons as well (Moore et al., 1996, Oppenheim et al., 2000, Haase et al., 2002). Homodimeric GDNF activates receptor tyrosine kinase (RET) by first binding to GDNF-family receptor-α1 (GFRα1) receptors (Airaksinen and Saarma, 2002). Together with members of the neuropoietic cytokine family, such as ciliary neurotrophic factors (CNTF), these neurotrophic factors function synergistically to promote the growth and development of neurons.

It is well-established that a series of developmental events govern the establishment of appropriate connections between neurons and targets, in which neurons are overproduced and then compete for survival factors derived from targets. The neurotrophic theory is best understood for sensory and motor neurons in the peripheral nervous system (Oppenheim, 1989). The development and survival of sensory and motor neurons are differentially dependent upon specific neurotrophic

factors. Motor neurons express high levels of TrkB and require BDNF signaling (Koliatsos et al., 1993), NT-3 signaling is critical to early sensory neuron progenitors, while mature sensory neurons require NGF by elevating the expression of TrkA (Lefcort et al., 1996, Sharma et al., 2010). Subpopulations of sensory neurons express TrkB and TrkC, and thus, depend on BDNF and NT-3, respectively (Wright and Snider, 1995, Rifkin et al., 2000). In addition to TrkB, motor neurons also express a low level of TrkC, and thus respond to NT-3 (Ip et al., 2001). Both motor and sensory neurons respond to GDNF by expressing GFRα1 and Ret (Airaksinen and Saarma, 2002) as shown in Fig. 4.

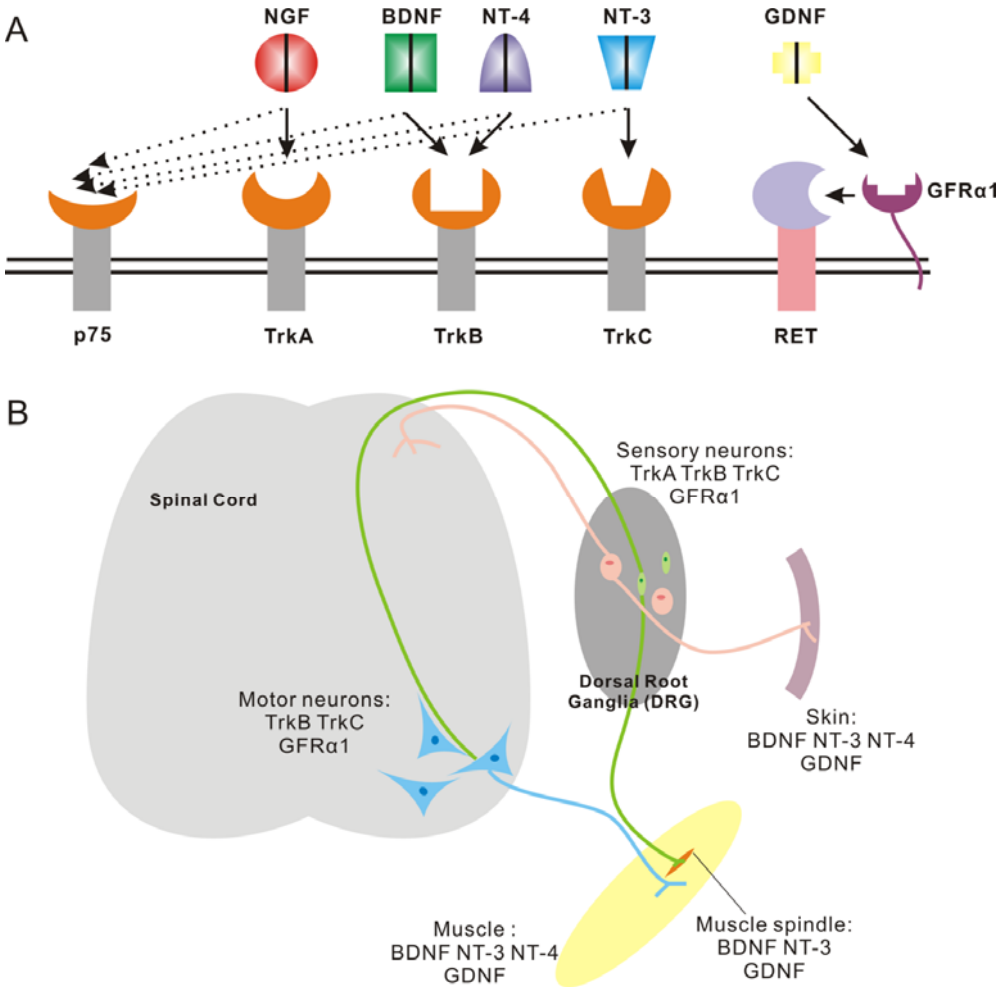

Figure4. Neurotrophic factors and their receptors. A. Nerve growth factor (NGF) binds and activates TrkA receptor tyrosine kinase; brain-derived neurotrophic factor (BDNF) and Neurotrohoin-4/5 (NT-4/5) bind TrkB. Neurotrohoin-3 (NT-3) binds TrkC with high affinity, but has low affinity for both TrkA and TrkB. Additionally, all neurotrophins bind to the p75 neurotrophin receptor (p75NTR) with low affinity. Glial cell line-derived neurotrophic factor (GDNF) binds to the soluble co-receptor GFRα1 and forms a complex with RET to convey the signal into the cell. **B.** In the peripheral nervous system, target cells, including muscle fibers and skin cells, provide a variety of neurotrophic factors to motor neurons residing in the spinal cord and sensory neurons in the dorsal root ganglion, to support their survival and differentiation. Accordingly, both neuronal types express types of Trk receptors and GFRα1 differentially. Adapted from (Segal, 2003, Harrington and Ginty, 2013).

The binding of neurotrophic factors to their receptors leads to the recruitment of proteins that interact with specific phosphotyrosine residues in the cytoplasmic domains. These interactions lead to the activation of signaling pathways, such as the Ras/MAPK, PI3k/Akt, and phospholipase C (PLC)-γ pathways; afterward, specific transcriptional factors are recruited and chromosome modifications are subsequently altered, which ultimately result in the expression of downstream genes that can be involved in neuronal survival, axonal growth, and neuronal specification. as shown in Fig. 5 (Segal, 2003, Zweifel et al., 2005). However, the downstream targets of these neurotrophic factor-dependent signals and how their dynamics contribute to the cooperative development are not fully understood.

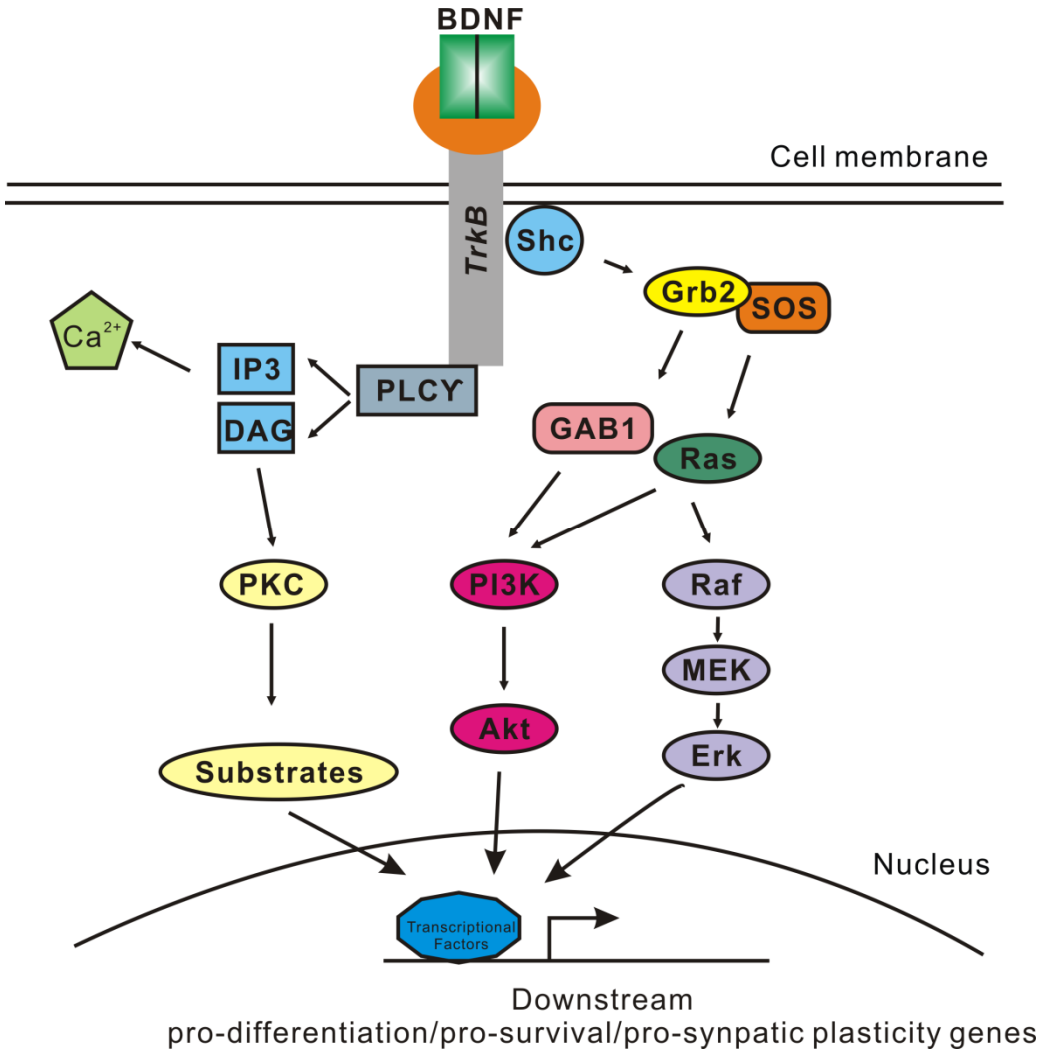

Figure5. Simplification of Trk signaling. BDNF binds and activates TrkB. Upon ligand binding, cytoplasmic tyrosine residues of the receptor are phosphorylated to generate docking sites for linker proteins, such as Shc, and PLCγ. Shc binding initiates downstream signaling cascades leading to activation of MAPK signaling cascade, which promotes neuronal differentiation; or PI3k/Akt signaling cascade, which promotes neuronal survival and growth. Phosphorylation and activation of PLCγ leads to formation of IP3 and DAG and subsequent activation of the pathways is associated with either Ca^{2+} or PKC, which promotes synaptic plasticity. The concert of ligand, receptors, signal strength and duration, and neuronal types is critical to the differential activation of the cascades. Adapted from (Segal, 2003).

It has been demonstrated that some of the neurotrophic factors can not only induce NRG1 expression in spinal cord motor neurons (Loeb and Fischbach, 1997), but also promote the rapid release of soluble NRG1 from sensory and motor neurons in a dose-dependent manner through PKC-δ activation (Esper and Loeb, 2004, 2009). Regulation of neuron-derived NRG1 expression and processing by glial cell-derived neurotrophic factors resembles a reciprocal, positive feedback loop to promote the survival and differentiation of Schwann cells (Ma et al., 2011). Similarly, at NMJs, it has been shown that NRG1 expression is regulated by neuromuscular activity and is promoted by neurotrophic factors (Loeb et al., 2002). However, little is known about the composition of NRG1 isoforms regulated by the neurotrophic factors signals in both scenarios. Together, it raises the possibility of the presence of a bi-directional mechanism mediated by neuron-derived NRG1 and target-derived neurotrophic factors at NMJs, where neurotrophic factors regulate NRG1 isoform expression to provide various types of NRG1 as growth and differentiation factors to the adjacent cells.

Here, I propose that the expression of different isoforms of NRG1 can be regulated by neurotrophic factors and axon-target interactions. The complexity of NRG1 isoform-specific regulatory machinery in response to neurotrophic factors may contribute not only to the bidirectional communication between motor neurons and muscle targets, but also to the dynamic functions of NRG1 in other regions in the nervous system and pathological conditions in various diseases.

CHAPTER II

FUNCTIONS OF NRG1 IN NEUROMUSCULAR JUNCTION DEVELOPMENT IN THE CHICKEN EMBRYO

Summary

The development of the neuromuscular junction (NMJ) is a multistep process mediated by coordinated interactions between axon terminals, target muscles, and peri-synaptic glial cells, and therefore requires reciprocal signals derived from every cell type. Neuregulin1s (NRG1s) are a group of growth and differentiation factors that are important for many aspects of nervous system development. While NRG1 is potent in inducing acetylcholine receptor (AChR) transcription and clustering *in vitro*, whether it plays any regulatory roles at NMJs *in vivo* remains controversial. Using the chicken model, we developed methods to study the effects of NRG1 signaling on NMJ development *in ovo* using sequential, quantitative measures of NMJ formation including AChR cluster size and density, pre- and post-synaptic apposition, and the alignment of peri-synaptic Schwann cells. We show that over-expression of soluble NRG1 increased AChR cluster density, but not size, in the early developmental stage, whereas blocking NRG1 activity with a targeted antagonist had little effect. In the middle stage, the NRG1 antagonist led to moderate reduction of AChR cluster size in a temporal-specific and muscle type-dependent fashion. The NRG1 antagonist resulted in an altered distribution of synaptic vesicles, suggesting that NRG1 plays roles in directing pre-synaptic apparatus assembly. In the late stage, pre- and post-synaptic apposition and peri-synaptic Schwann cell alignment were affected slightly when the antagonist was

applied. These findings suggest that, while not critical for development, NRG1 signaling can have important roles in fine-tuning multiple stages of NMJ development.

Materials and Methods

Chicken eggs and in ovo *treatment*

Fertilized chicken eggs were obtained from Michigan State University Poultry Farms and incubated in a Kuhl rocking incubator at 50% humidity. Daily treatment of the NRG1 antagonist (HBD-S-H4) on chick embryos was performed as described previously (Loeb et al., 2002, Winseck et al., 2002, Ma et al., 2009, Ma et al., 2011). In brief, 20 µg of HBD-S-H4 was 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; this was done for two consecutive days. HBD-S-H4 was prepared, purified, and tested for its ability to block NRG1 activity as described (Ma et al., 2011). Staging of chicken embryos was determined according to Hamburger-Hamilton (HH) stage series (Hamburger and Hamilton, 1951); E2.5 (Stage 15-16); E7 (stage 30-31); E8 (stage 34); E10 (stage 36); E12 (stage 38); E14 (stage 40); E16 (stage 42); E18 (stage 44).

In ovo *electroporation*

Chick type I proNRG1β1a cDNA with a c-Myc tag at the C terminus was subcloned into the pMES vector downstream from the chick β-actin promoter with IRES–EGFP (Krull, 2004). Similarly, a c-Myc tag was added at the C terminus of the coding sequence of HBD-S-H4, which was subsequently subcloned into the pMES vector. The pCAX vector expressing EGFP was used for coelectroporation with other plasmids to visualize electroporated cells (George et al., 2007). The final concentration of each plasmid was 3 µg/µl. The plasmid DNAs were electroporated unilaterally into the ventral part of the

neural tube at the lumbar level at E2.5 as described previously (Ma et al., 2009). Electrodes were placed ventrodorsal across the neural tube and pulsed for five times at 35 V for 50 ms with a square-wave pulse generator (Intracept TSS10; Intracel). Embryos were collected at time indicated, and only those with strong GFP expression were processed for additional analysis.

In situ *hybridization*

Chicken embryos were fixed in 4% paraformaldehyde in PBS at 4°C overnight, washed in PBS, equilibrated in 30% sucrose, and mounted in OCT (Tissue-Tek). Frozen sections were cut transversely at 14 µm and placed on Superfrost plus slides (VWR). Non-radioactive in *situ* hybridization was performed using Digoxigenin (Dig)-labeled RNA probes. In brief, sense and antisense Dig-labeled RNA probes were generated from linearized plasmid containing chicken NRG1 heparin binding domain, by *in vitro* transcription (Roche). Probes were hydrolyzed to optimum length of ~250 bp, and then purified on Sephadex G-50 quick spin columns for RNA purification (Roche). Sections were treated for 10 min at room temperature with 1 µg/ml proteinase K (Sigma) and acetylated with 0.25% acetic anhydride in 0.1 M triethanolamine, pH 8.0 for 15 min. After this, the sections were incubated with 2 µg/ml probes in hybridization buffer at 60°C overnight. Hybridization buffer contained 50% formamide, 0.3 M NaCl, 0.5 mg/ml yeast tRNA, 1x Denhardt's solution (Sigma), 20 mM Tris, pH 7.4, 10% Dextran Sulphate, 10 mM $NaPO_4$ and 5 mM EDTA. After hybridization, the sections were sequentially washed in 5x SSC, 50% formamide in 1x SSC, 2x SSC, and 0.2x SSC for 10, 30, 20, and 20 min at 65°C. To remove excessive cRNA hybrids, the sections were incubated with 20 µg/ml RNase A (Sigma) in TNE buffer (10 mM Tris pH

7.5, 0.5 M NaCl, 1 mM EDTA), at 37°C for 30 min before they were washed by 2x SSC. The sections then were blocked with Blocking solution (Roche) for 1 h at room temperature and incubated with sheep anti-DIG conjugated with alkaline phosphatase (AP) (1:1000) (Roche) in blocking solution at 4°C overnight. Finally, the sections were washed in washing buffer (Roche), incubated in detection buffer (Roche), and then visualized by reacting with BM purple (nitroblue-tetrazolium-chloride (NBT)/5- bromo-4-chlor-indolyl-phosphate (BCIP) ready-to-use solution) (Roche) in the dark for 2 h.

Immunofluorescence staining

Muscle whole-mounts were prepared as described previously (Dahm and Landmesser, 1988, Loeb et al., 2002). Briefly, embryos were decapitated and eviscerated. Skin and connective tissue were removed to expose the underlying muscles. Anterior latissimus dorsi (ALD) muscles were exposed and fixed *in situ* by fresh 4% paraformaldehyde in PBS for 40 min to maintain normal muscle shape. To obtain posterior iliotibialis (PITIB), thighs were fixed in 4% paraformaldehyde in PBS overnight and rinsed with PBS at room temperature; PITIBs were then dissected off all connective tissue and the sciatic trunk. Whole-mount staining was adapted from previously described (Dong et al., 2006). Briefly, muscles were incubated in 0.1 M glycine in PBS for 1 h, and rinsed with 0.5% Triton X-100 in PBS. After incubation in the blocking buffer (3% BSA, 5% goat serum, and 0.5% Triton X-100 in PBS) for 2 h at room temperature, muscles were then incubated with primary antibodies (SV2, 1:100; 3A10, 1:100; Developmental Studies Hybridoma Bank) and α-bungarotoxin conjugated with Alexa Fluor 488 (1:500, Life Technologies) in the blocking buffer overnight at 4°C. After washing three times for 1 h each in 0.5% Triton X-100 in PBS, the muscles were incubated with goat-anti-mouse

IgG conjugated with Alexa Fluor 546 (1:500, Life Technologies) for 2 h at room temperature. After three times washed for 1 h each in 0.5% Triton X-100 in PBS, muscles were rinsed with PBS, and flat-mounted in mounting medium.

For ALD after E14 and PITIB, muscles were mounted into OCT and sectioned into 20 μm sections. Frozen embryo sections were the same as used for *in situ* hybridization. After incubation with blocking solution (10% goat serum, 0.1% Triton X-100 in PBS) at room temperature for 1 h, sections were incubated with α-bungarotoxin conjugated with Alexa Fluor 488 (1:500) and primary antibodies in blocking solution overnight at 4°C, followed by incubation with the corresponding goat anti-mouse or goat anti-rabbit IgG conjugated with Alexa Fluor antibodies for visualization. Primary antibodies were used at the following dilutions: Iselt1/2 (1:10, 39.4D5), P0 (1:5; 1E8), SV2 (1:100, SV2), c-Myc (1:100, 9E10) and neurofilament (1:10; 3A10) (Developmental Studies Hybridoma Bank); GFP (1:100; ab6662, Abcam).

Imaging and quantitative analysis

Confocal stacks of images of whole mount muscle staining were acquired with a z-step of 1.05 μm using a Nikon Eclipse microscope with a D-Eclipse C1 confocal system. Twenty consecutive images were combined into a single image processed by MetaMorph image analysis software (Molecular Devices) using Maximum-Stack Arithmetic. Epifluorescent images were obtained with a Nikon Eclipse 600 epifluorescence microscope with a Princeton Instruments Micromax 5 MHz cooled CCD camera, and analyzed using MetaMorph. Briefly, the threshold was adjusted for each nonsaturated image. Regions of interest (ROIs) were selected as objects with a

nearest-neighbor filter and a size filter to α-bungarotoxin signal above threshold. The size and total grey value of AChR clusters were determined based on ROI measurement. AChR cluster size was converted from pixels into area (μm^2). Ratio of pre-synaptic and post-synaptic apposition was determined as the percentage of area positive for nerve terminal SV2 within each ROI of the same scope. Similarly, ratio of pre-synaptic and peri-synaptic apposition was determined as the percentage of area positive for Schwann cell P0. Extra-synaptic synaptic vesicle signal was determined by subtraction of terminal SV2 positive areas that were overlapped by α-bungarotoxin signal, from the total signal for synaptic vesicles. Subsequently, the percentage of extra-synaptic synaptic vesicles was calculated. To quantify the density of AChR cluster in the early stage, the dorsal muscle mass regions with adjacent GFP^+ motor axons were defined as ROI. The number of AChR clusters and total grey value of each cluster were measured within each ROI. The density was calculated after pixel-to-area conversion.

Statistical Analysis

Frequency histograms were produced in Prism 5.0 (GraphPad Software Inc.). Numerical datasets presented in histograms were tested for statistical significance by means of the nonparametric, two-sided Mann-Whitney *U*-test. For comparison between groups for *in ovo* electroporation, statistical differences were tested using one-way ANOVA followed by Dunnett's *post hoc* test. Unpaired two-tailed Student's *t*-test was used for analysis between animals with and without antagonist treatment. The median was used to represent the measurement of each section. The mean of the median from 3-5 sections was used to represent each animal. All data are presented as the mean ±

SEM with an n of at least 3 animals in each group. Statistical significance is presented as * $p<0.05$, ** $p<0.01$.

Results

Quantitative parameters for neuromuscular junction development in the chicken embryo

Although there have been a number of studies on NMJ development in the chicken embryo, few of them provided multiple quantitative measures along with development (Burden, 1977a, b, Dahm and Landmesser, 1988). Here, we developed three quantitative parameters to investigate NMJ development in the hindlimb posterior iliotibialis (PITIB) muscle, which is a focally innervated, homogeneous, fast-twitch muscle (Dahm and Landmesser, 1988). The first measure includes both AChR cluster size and density using fluorescently labeled α-bungarotoxin (BTX). The other two measures quantify the degree of apposition of motor nerve endings and peri-synaptic Schwann cells with those AChR clusters. Fig. 6 and 7 show the developmental time course for each of these 3 measures from embryonic day 7 (E7) through E18. At E7, the majority of AChR clusters are small (median size <2 μm^2) and are not yet associated with nerve terminals. AChR cluster size increases most significantly between E7 and E10 and then gradually increases further to around 15 μm^2 by E18 (Fig. 6A, 6B).

Pre- and postsynaptic apposition occurs synchronously with increased AChR cluster size at E10 to E12. Between E12 and E18 pre- and post-synaptic apposition increases at a steady rate until most AChR clusters are fully associated with axon terminals (Fig. 6A, 6C). Lastly, the peri-synaptic Schwann cells migrate into the maturing NMJ starting at E14 and continue to gradually engulf the NMJ through E18 (Fig. 7A, 7B).

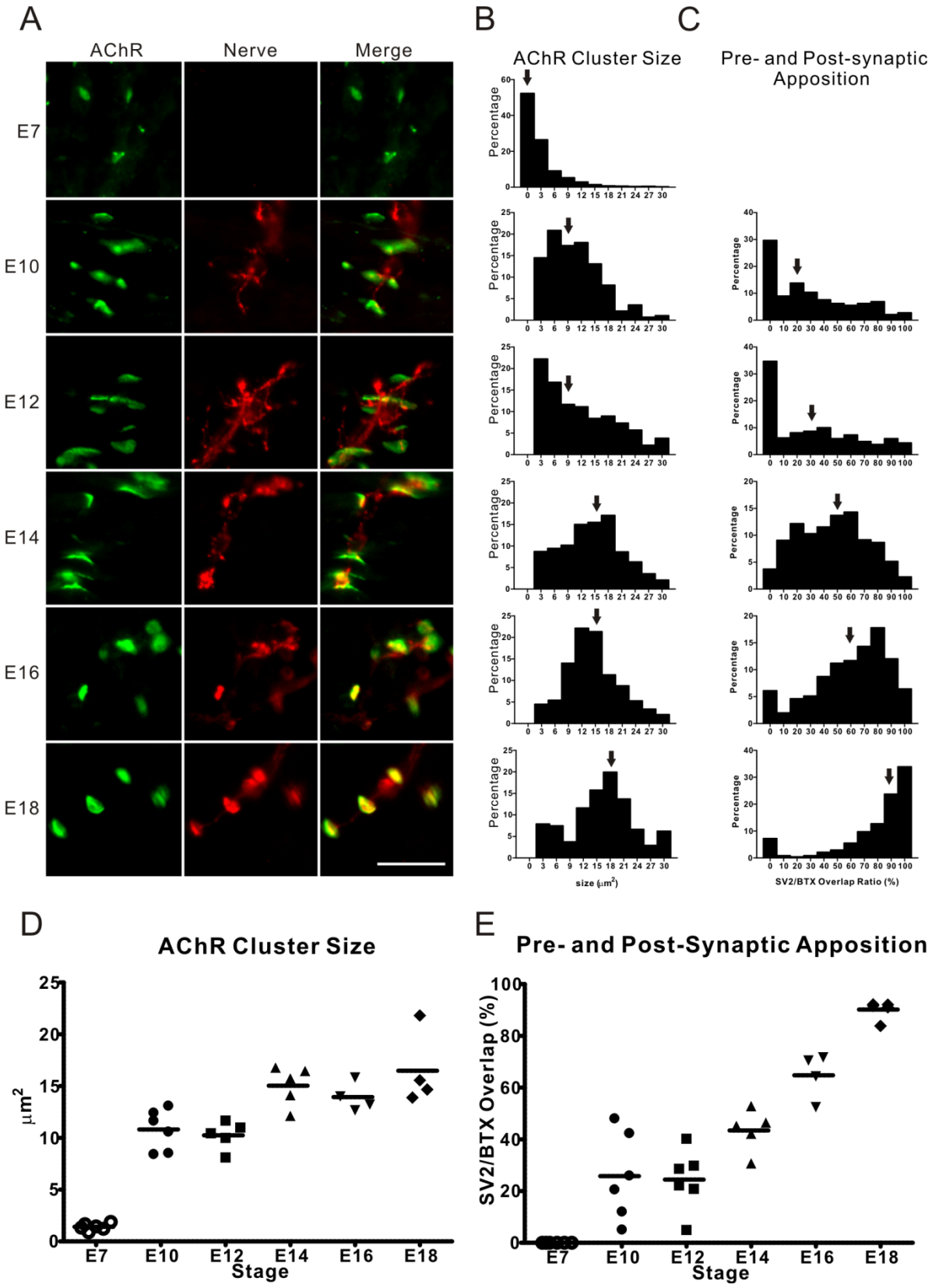

Figure6. The size of AChR clusters and the degree of pre- and post-synaptic apposition increase during NMJ development. A. Representative images of the dorsal muscle mass of the hindlimb on E7 and the PITIBs from E10 to E18 at a two-day interval, showing the growth of AChR clusters and the apposition of AChR clusters (BTX, green) and nerve terminals (SV2, red). **B, C.** Graphs show the normalized distribution (percentage of the number of observations per bin of the total number within the group) of the size of AChR clusters and the percentage of post-synaptic territory occupied by pre-synaptic motor axon endings. Data were collected from at least 200 NMJs. Arrows indicate the median occupancy. **D, E.** The medians of the two measures (n=6, 6, 6, 5, 4, 4 for each stage, respectively) are plotted against developmental stages. Scale bar: 25μm.

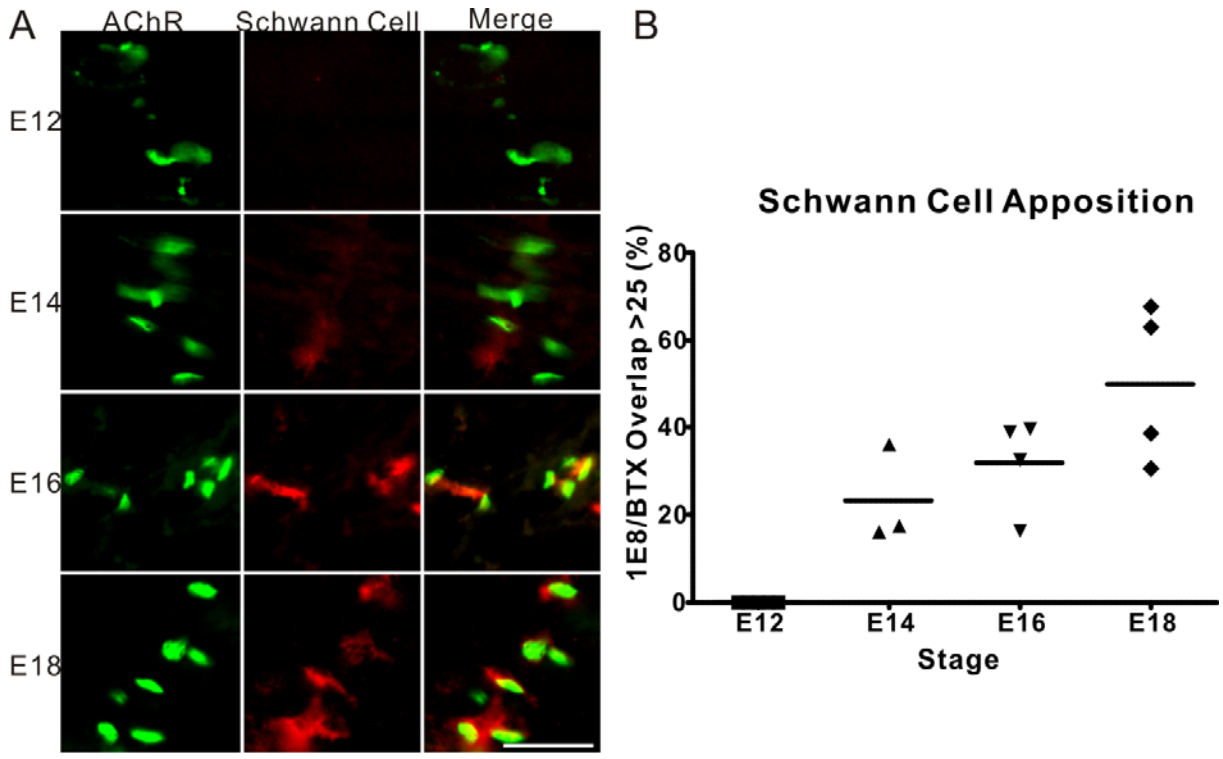

Figure7. Peri-synaptic Schwann cell development. A. Representative images of the PITIBs from E12 to E18 at a two-day interval show the development of Schwann cells (P0, red) at the neuromuscular junctional area (BTX, green). **B.** The percentage of

NMJs with >25% post-synaptic territory occupied by peri-synaptic (n=4, 3, 4, 4 for each stage, respectively) was plotted against developmental stages. Scale bar: 25μm.

Over-expression of NRG1 in motor axons increases AChR cluster density

In the chicken hindlimb, the first appearance of AChR clusters coincides with the ingrowth of nerve trunks at E4. By E7, numerous AChR clusters can be observed in a broad region on developing muscle fibers that are still not yet in direct contact with nerve endings as shown in Fig. 6A. Previous studies have shown that those AChR clusters that form contacts with nerve terminals enlarge, while those that do not receive direct nerve contact are eventually eliminated (Dahm and Landmesser, 1988). We used *in ovo* electroporation to over-express soluble NRG1 isoforms in lumbar motor neurons at E2.5 with a full-length type I proNRG1 β1a cDNA with a c-Myc tag at the C terminus, to track its distribution. This plasmid was cotransfected with a plasmid expressing GFP. Over-expression of NRG1 was confirmed at the mRNA level by *in situ* hybridization and at the protein level using anti-c-Myc antibodies on the electroporated side (Fig. 8A). Compared to controls that were electroporated with the empty vector, over-expression of NRG1 in motor neurons resulted in a significant increase in the density of AChR clusters at E7. Morphometric analysis, however, did not show a change in AChR cluster size (Fig. 8C, 8D). This suggests that, prior to nerve AChR cluster contact, NRG1 can increase the total number of AChR clusters without regulating cluster size. In contrast, reducing NRG1 signaling using the plasmid expressing the NRG1 antagonist HBD-S-H4 in motor neurons did not change AChR cluster density or size (Fig. 8C, 8D). Over-expression of HBD-S-H4 was confirmed using antibodies against the fusion protein (Fig.

8B). Together, these results suggest that while NRG1 is not required for cluster formation or cluster size regulation, the amount of NRG1 released from nerve endings can promote the global level and number of AChR clusters prior to nerve muscle contact.

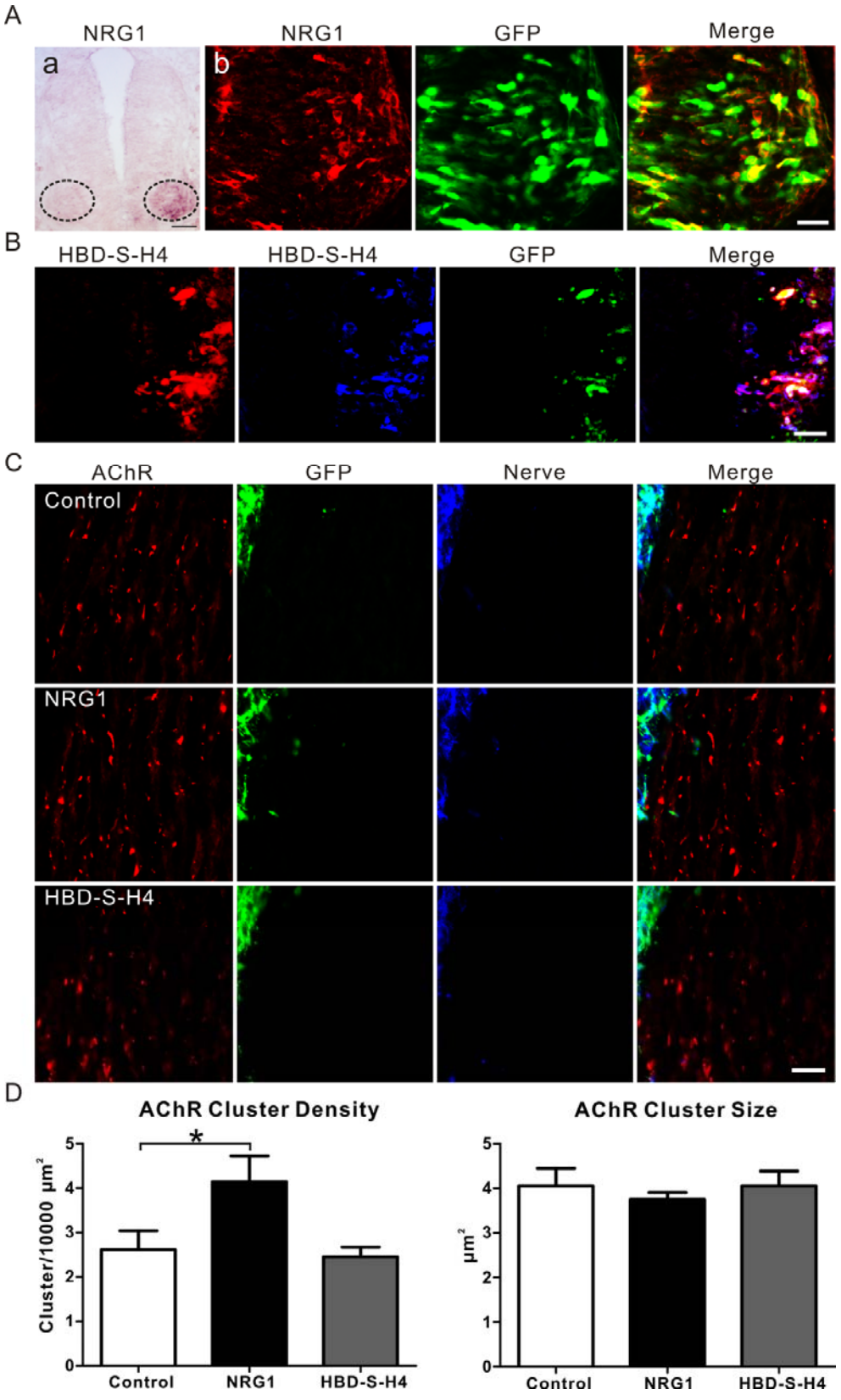

Figure8. Soluble NRG1 promotes the density of AChR clusters. A. *In situ* hybridization shows up-regulated soluble proNRG1 mRNA in the ventral spinal cord of the electroporated side but not the contralateral side. Scale bar, 50μm. Over-expression of c-Myc tagged proNRG1 protein was demonstrated by c-Myc expression (red) in GFP (green)-positive cells. **B.** Expression of HBD-S-H4 was detected by HBD expression (anti-HBD, red; ADO3, blue) in GFP (green)-positive cells. Scale bar, 25μm. **C.** Increased AChR cluster (BTX, red) density was observed at E7 after electroporation with proNRG1 (GFP, green; 3A10, blue) compared with vector alone or HBD-S-H4. **D.** Data are reported as the mean ± SEM of n = 8, 10, 8 for each condition, respectively; * $p<0.05$, one-way ANOVA followed by Bonferroni's *post hoc* test by comparison to control. No detectable change of AChR cluster size was seen in the animals with either proNRG1 over-expression or HBD-S-H4 expression.

NRG1 signaling promotes post-synaptic AChR clustering

The presence of NRG1 at developing NMJs starts early and lasts until NMJ maturation in both chickens and mice (Meyer et al., 1997, Loeb et al., 1999). To determine the function of NRG1 at NMJs in the different developmental phases after AChR clusters get into direct contact with nerve terminals, I applied NRG1 antagonist HBD-S-H4 *in ovo* to knock down NRG1 signaling. The antagonist has been shown to specifically target the heparan sulfate-rich surfaces to which endogenous NRG1 binds and to effectively block NRG1-meditated Schwann cell precursor survival and differentiation (Ma et al., 2009, Ma et al., 2011). The antagonist was added to developing embryos for two consecutive days before the NMJs were examined at every stage.

NRG1 has been postulated to increase the transcription of AChR subunit genes, such as AChRα and AChRε, from sub-synaptic muscle nuclei by activating their ErbB receptors (Brenner et al., 1990, Martinou et al., 1991, Chu et al., 1995, Moscoso et al., 1995). However, the *in vivo* physiological roles in promoting AChR expression remain contentious, especially during NMJ development. To ask whether NRG1 plays roles in post-synaptic AChR clustering, we examined AChR cluster size in two different muscle groups, the PITIB and the anterior latissimus dorsi (ALD), which is a mostly multi-innervated, slow-tonic muscle, with and without HBD-S-H4 treatment (Hess, 1967, Kwong and Gauthier, 1987). We found that administration of the antagonist resulted in a slight, yet significant reduction of AChR cluster size in E10 ALDs. Compared to the control group with the mean value of 13.3 μm^2 (201 clusters in 4 animals), the average AChR cluster size in HBD-S-H4 treated animals decreased to 11.9 μm^2 (177 clusters in 4 animals) (Fig. 9A, 9B). However, the decrease of cluster size was not observed in ALDs of any other stages (E12-E18) or in PITIBs of any stages (E10-E18). The developmental-stage-dependent requirement of NRG1 suggests that the signaling promotes AChR clustering in the early stage, and becomes dispensable when other complementary mechanisms become dominant during the late stage.

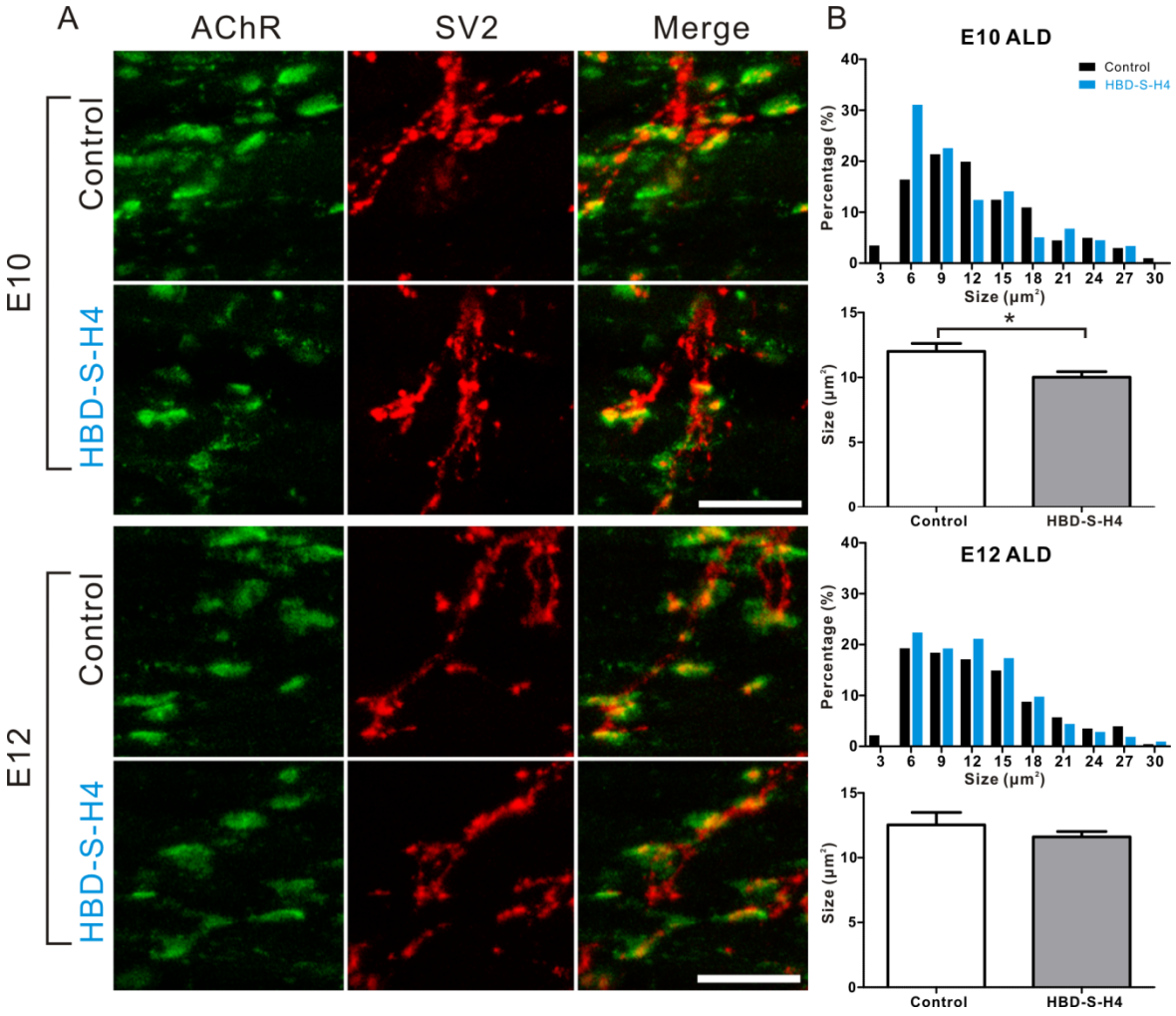

Figure9. Down-regulated NRG1 signaling decreased the size of AChR cluster in ALDs. A. Representative stacked confocal images show that treatment with HBD-S-H4 at E8 and E9 resulted in the reduction of AChR cluster (BTX, green) size in E10 ALDs. The same treatment at E10 and E11 did not affect the AChR cluster size at E12. Scale bar, 25μm. **B.** The histogram summarizes the normalized distribution (percentage of the number of observations per binned AChR cluster size of total number within the group) between Saline (control) and HBD-S-H4 treated embryos, and shows significant decrease of AChR cluster size at E10 (* p<0.05, two sided Mann-Whitney U-test), but not at E12 (p>0.05, two sided Mann-Whitney U-test). Quantification of AChR cluster size across animals (mean ± SEM) shows similar significant decrease of AChR cluster size only at E10 (* p<0.05, n=4 for each condition, unpaired two-tailed Student's t-test).

NRG1 signaling is required in pre-synaptic differentiation

To ask whether NRG1 plays roles in assembling the pre-synaptic apparatus, we examined the pre-synaptic component of NMJ in both PITIBs and ALDs. We found that, in the ALDs of the saline treated group, similar to normal development, synaptic vesicles are initially distributed throughout the axon, but become concentrated at synaptic sites when pre- and post-synaptic apposition consolidates. In the antagonist-treated muscles, the apparent major branching pattern and arborization of axon terminal did not differ detectably (Data not shown). However, by E14, synaptic vesicles were less restricted to synaptic sites in treated animals than in controls. The average percentage of extra-synaptic to total synaptic vesicle was 46.2 ± 5.1 (n=4) for control and 69.7 ± 2.9 (n=4) for treatment. The similar defect was observed at E16 as well. However, the abnormality was less striking, with the average percentage of extra-synaptic synaptic vesicle level increasing from 43.9 ± 1.7 (n=4) for control to 54.8 ± 4.2 (n=4) for treatment. In the E18 animals, the failure of clustering of synaptic vesicles to NMJ site was barely detectable. The corresponding mean values were 36.7 ± 3.4 (n=4) and 35.9 ± 5.3 (n=4) for controls and antagonist-treated animals, respectively (Fig. 10). The results suggest that NRG1 signaling is required for transporting and clustering synaptic vesicles to nerve terminals in a developmental-stage-dependent fashion. However, the stage-dependent failure of synaptic vesicle clustering was not observed in PITIB for all the stages, indicating that the pre-synaptic vesicle transport and clustering regulated by NRG1 signaling might be muscle type-specific.

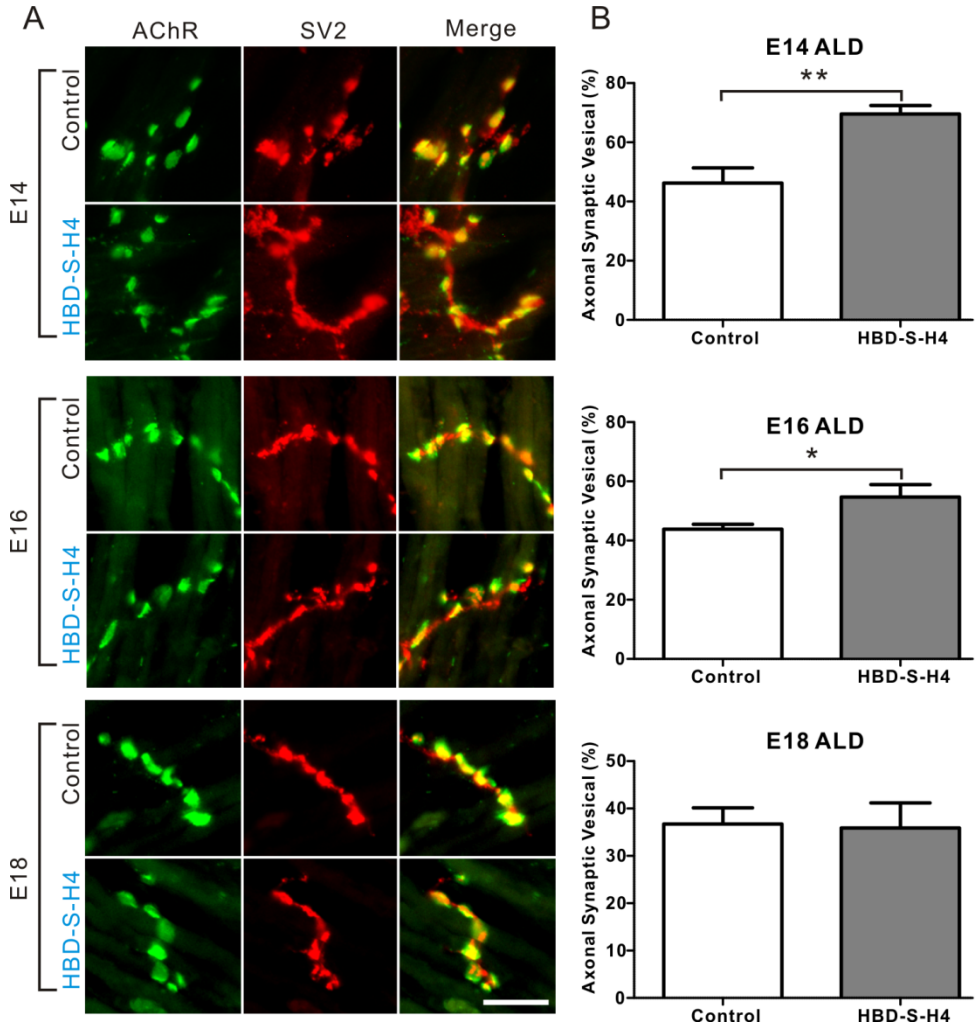

Figure10. Down-regulated NRG1 signaling affected synaptic vesicle distribution in ALDs. A. Representative images show that treatment with HBD-S-H4 at E12 and E13, or E14 and E15 resulted in more synaptic vesicle aggregation (SV2, red) in the axonal area distal to junction sites (BTX, green) in ALDs at E14 or E16, respectively. The synaptic vesicle distribution was not affected at E18 with the antagonist treatment from E16 to E17. **B.** Quantification of the percentage of synaptic vesicles failing to concentrate in nerve endings over total synaptic vesicle staining shows a significant increase of synaptic vesicles residing in the axonal area. Data are reported as the mean ± SEM of n=4 for both saline and HBD-S-H4 group, * $p<0.05$, ** $p<0.01$, unpaired two-tailed Student's *t*-test. Scale bar, 25μm.

NRG1 signaling promotes pre- and post-synaptic apposition

The fine apposition of pre-synaptic nerve terminal and post-synaptic AChR cluster is required for fast and robust neurotransmission at NMJs. Pre- and post-synaptic apposition increases steadily from E10 to E18, until most AChR clusters are fully associated with axon terminals (Fig. 6A, 6B). To ask whether NRG1 plays roles in regulating pre- and post-synaptic apposition, we examined the degree of apposition of pre-synaptic nerve endings and AChR clusters on PITIBs in the presence or absence of HBD-S-H4 treatment. The degree of association was classified into four categories: 0-25%, 25-50%, 50-75%, and 75-100%, as the percentage of AChR cluster territory occupied by nerve endings. The application of the antagonist resulted in a significant decrease of the proportion of high degree of association (75-100%) at E18. Compared to the control group with the average percentage of 70.2 ± 2.3 (n=5), the contribution of this category decreased to 55.6 ± 7.5 (n=5). The decrease of pre- and post-synaptic apposition was not observed in any other stages (E10-E16), as shown in Fig. 11. The results suggest that NRG1 signaling is involved in fine-tuning the apposition of the pre-synaptic nerve terminal and post-synaptic apparatus when the NMJ matures.

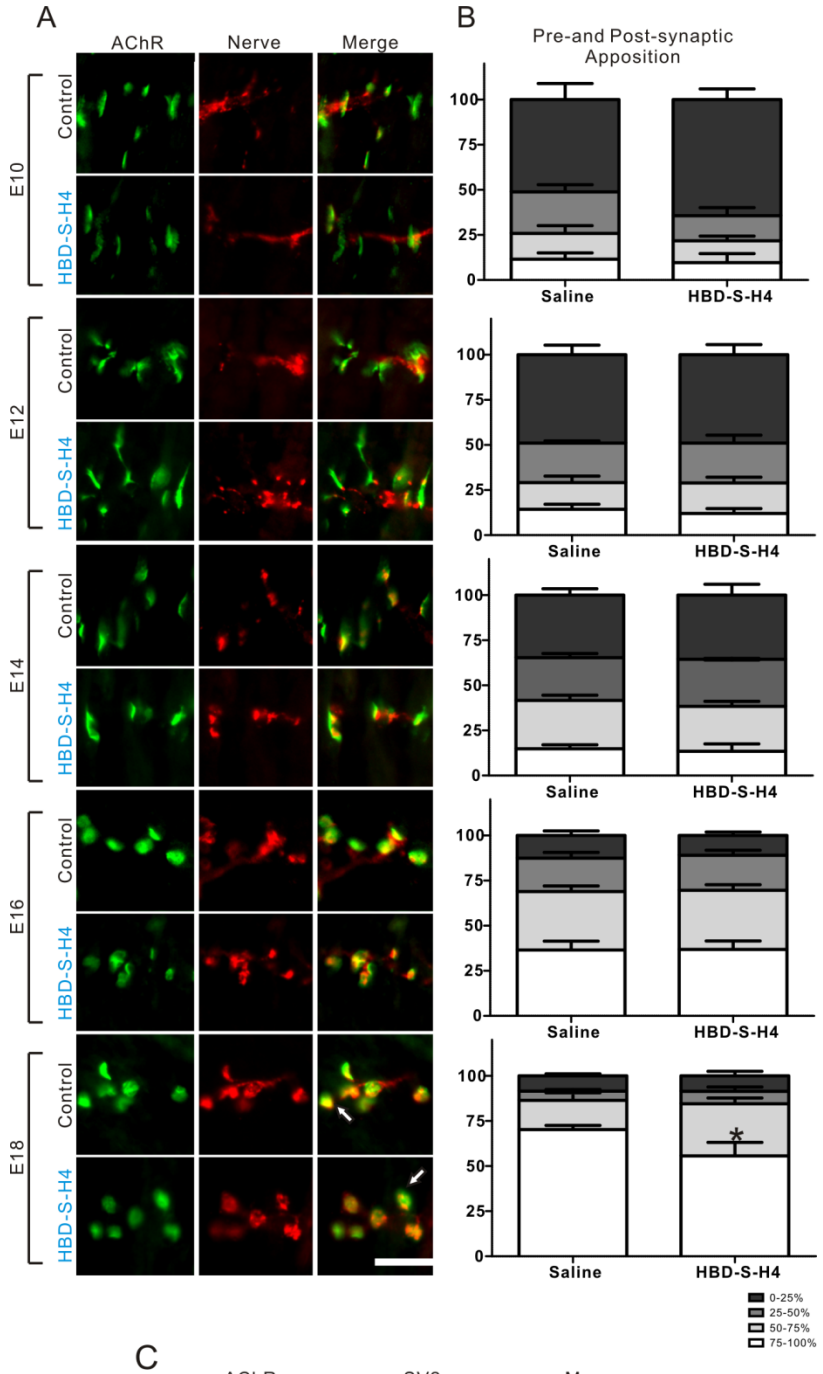

Figure11. Down-regulated NRG1 signaling decreased pre- and post-synaptic apposition in PITIBs. A. Representative images of the PITIBs from E10 to E18 with two-day interval with or without HBD-S-H4 treatment show that the antagonist applied at E16 and E17 resulted in less degree of post-synaptic AChR cluster (BTX, green) territory association with nerve terminal (SV2, red) in PITIBs at E18. **B.** The association was classified into four categories: 0-25%, 25-50%, 50-75%, and 75-100%. Quantification of the percentage of each category shows significant decrease of the proportion of the NMJs that have high degree of association (75-100%). Data are reported as the mean ± SEM of n=5 for saline and HBD-S-H4 group, two-way ANOVA; * $p<0.05$. The effect was not observed in all the earlier stages. **C.** Bottom panel shows zoomed-in images of the arrowed NMJ in the image of E18. Scale bar, 25μm.

NRG1 signaling promotes peri-synaptic Schwann cell development

Peri-synaptic Schwann cells wrap the junctional site of both motor axon terminals and post-synaptic apparatuses. This ideal location allows them to be actively involved in NMJ formation, maintenance of structural integrity, and signal transduction (Auld and Robitaille, 2003b). In contrast to axon-associated Schwann cell development, the underlying mechanisms for peri-synaptic Schwann cell differentiation are poorly understood. To determine if modulation of NRG1 signaling is involved in the development of the subtype of Schwann cells, we examined the alignment of Schwann cells and AChR clusters in the absence or presence of HBD-S-H4. The treatment with the antagonist significantly decreased the degree of Schwann cells apposed to AChR clusters at E14, E16, and E18, compared to the saline treatment. The mean value of percentage of AChR cluster area associated with Schwann cells decreased from approximately 15 with Saline to 11 with HBD-S-H4 at E14 ($p<0.0001$, 584 AChR clusters across three animals (n=3), and 680 AChR clusters (n=3), two sided Mann-

Whitney *U*-test). Similar decreases were observed in E16 (27.66 versus 19.18, $p<0.0001$, 489 AChR clusters (n=4), and 463 AChR clusters (n=4), respectively, two sided Mann-Whitney *U*-test) and E18 (38.87 versus 29.96, $p<0.0001$, 627 AChR clusters (n=4), and 571 AChR clusters (n=4), respectively, two sided Mann-Whitney *U*-test) as well, suggesting that NRG1 is involved in promoting peri-synaptic Schwann cell development, as shown in Fig. 12.

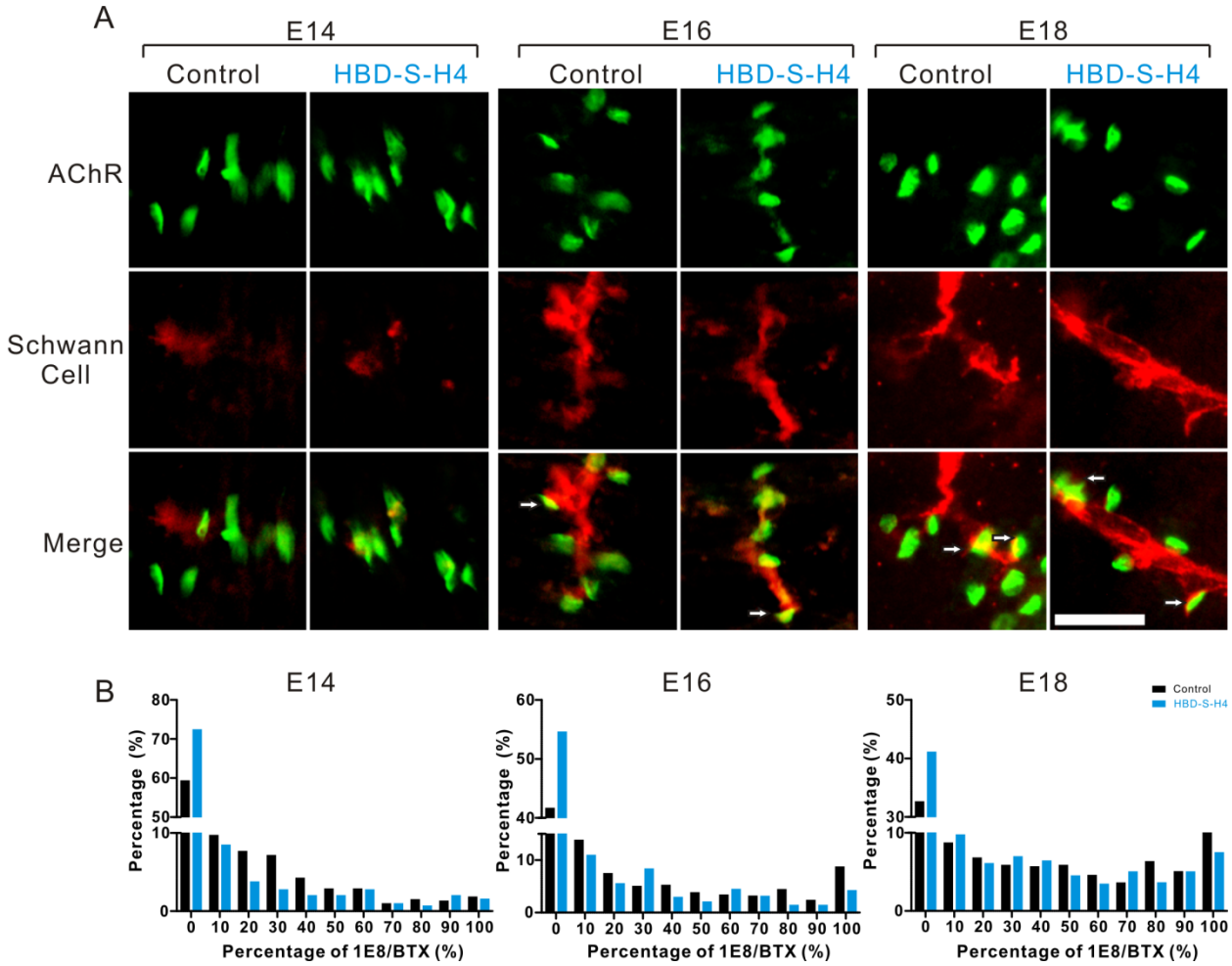

Figure12. Down-regulated NRG1 signaling affected peri-synaptic Schwann cell development in PITIBs. A. Representative images show that treatment with HBD-S-H4 resulted in less degree of post-synaptic AChR cluster (BTX, green) territory association with Schwann cells (P0, red) in PITIBs at E14, E16, and E18. **B.** The histogram summarizes the normalized distribution (percentage of the number of observations per bin of total number within the group) between Saline (control) and HBD-S-H4 treated embryos and shows significant decrease of the association between AChR cluster territory and Schwann cell signals (E14, E16, E18, $p<0.0001$, two sided Mann-Whitney *U*-test). Scale bar, 25μm.

Discussion

Motor neurons and muscle targets are connected by NMJs, where the communication is conveyed between the nervous system and the muscular systems. NMJ development is a multi-stage process including an early stage, defined as motor axon extension toward targets to the initial axon-target contact; a middle stage, when pre-synaptic, post-synaptic, and peri-synaptic components differentiate to form mature synapses; and a late stage, which includes synapse elimination and life-long maintenance (Sanes and Lichtman, 1999a). The accomplishment of this developmental process is dependent on a variety of organizing molecules at the site of the NMJs, including NRG1.

Here, we used three parameters to quantitatively measure NMJ development in chicken embryos. We proposed that the more each parameter changes between adjacent stages, the more dynamic NMJs are with respect to the components involved. The size of AChR clusters represents the net combination of AChR expression and turnover. The measure showed that there are two phases of steep increase for AChR cluster size: E7-E10 and E12-E14 are the most dynamic stages in AChR synthesis and/or receptor degradation. The two phases of AChR cluster size growth coincide with the stages of NMJ development. E7-E10 represents the initiation of innervation, as the early stage transitions to the middle stage. Accordingly, the major intramuscular branches have formed while the detailed arborization pattern has not been fully established. The nerve terminals are close to the AChR clusters while pre- and post-synaptic appositions have not been consolidated. The shorter distance facilitates soluble nerve-derived factors to function more locally. E12-E14 coincides with pre- and

post-synaptic differentiation, when the growth cones have transformed into pre-synaptic terminals specialized for neurotransmitter release with the accumulation of synaptic vesicles and vesicle-associated proteins. Post-synaptically, the clustering of AChR is likely to be strengthened by organizing factors directly from nerve terminals. The degree of pre- and post-synaptic apposition indicates the level of differentiation of both pre- and post synaptic structures. The steady increase from E12 to E18 corresponds to the middle to late stages of NMJ development. The peri-synaptic Schwann cells start to engulf and cap the maturing NMJs from at E14 through E18.

NRG1 isoforms are produced as trans-membrane precursors, some of which undergo proteolytic cleavage to become soluble proteins (Loeb, 2003, Mei and Xiong, 2008). At the chicken NMJ, the accumulation of NRG1 immunoreactivity can be only detected in the synaptic basal lamina after E16, concurrent with the concentration of HSPGs (Loeb et al., 1999). Therefore, it is likely that, before being immobilized by HSPGs, released forms of NRG1 mainly function at distant sites by diffusion, such as the developing hindlimb muscles at E7, and the ALD muscle from E8-E10. In these areas at an early developmental stage, NRG1 promotes either AChR cluster density or AChR cluster size, depending on the muscle type. However, when pre- and post-synaptic appositions start to develop approximately from E10, other stronger post-synaptic organizing factors, such as agrin, take over to dominate the clustering of AChR, leaving the function of NRG1 dispensable afterward. NRG1 then transitions to a secondary modulatory signal for AChR expression or clustering. When concentrated at basal lamina from E16 by the interaction between HBD and HSPGs, NRG1 can fine-tune other events at NMJs, such as pre- and post-synaptic appositions and peri-

synaptic Schwann cell development. The modes of function of NRG1 at NMJs are illustrated in Fig. 13.

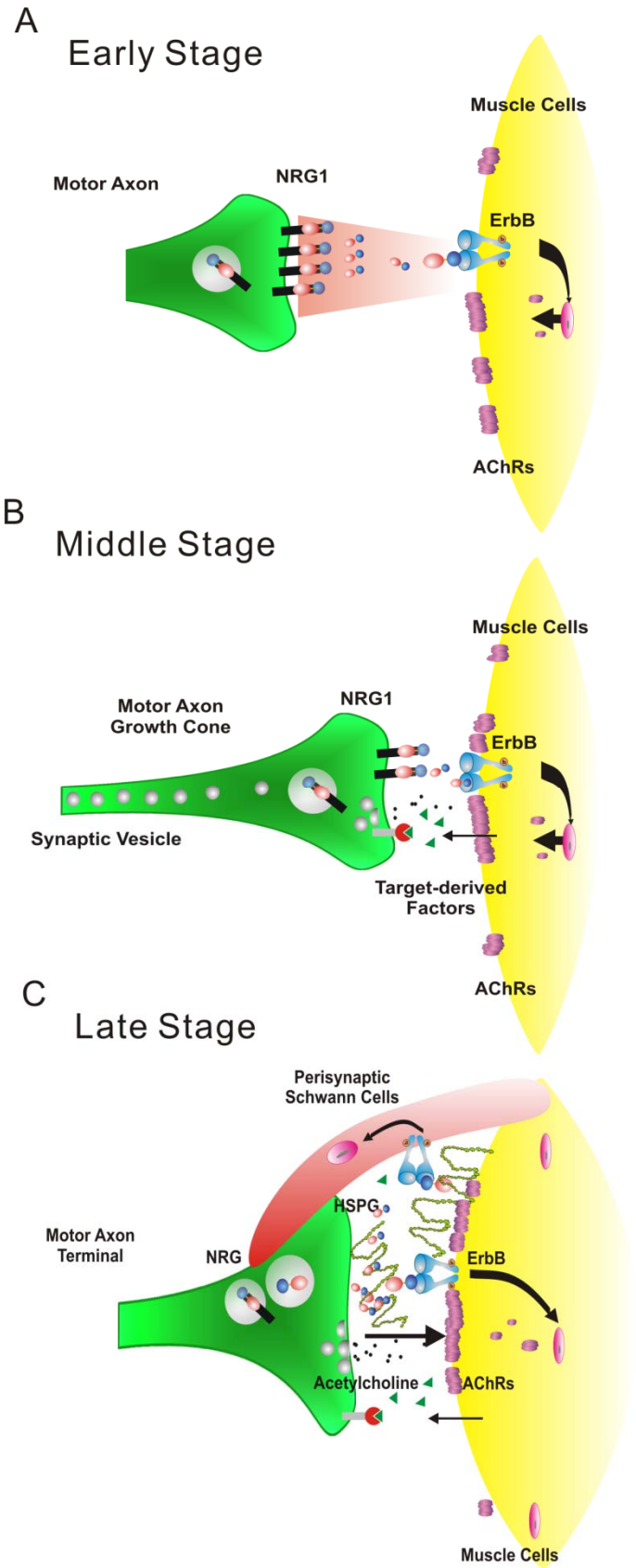

Figure13. A stage-dependent model of NRG1 signaling functions in NMJ development. A. During the early stage when motor axons extend toward targets for the initial axon-target contact, released soluble NRG1 proteins function as diffusible growth and differentiation factors to induce AChR cluster density in the hindlimb muscle mass and to increase cluster size in the ALD muscle. **B.** When pre-synaptic and post-synaptic components differentiate at newly formed NMJs in the middle stage, NRG1 supports the survival and further differentiation of the post-synaptic muscle cells which provide the motor axons with pre-synaptic organizers for the proper transport and aggregation of synaptic vesicles. **C.** In the middle-late stage, peri-synaptic Schwann cell development is dependent on neuron-derived NRG1. Both peri-synaptic Schwann cells and muscle targets express HSPGs on their cell surface to interact with soluble NRG1 to potentiate NRG1 signaling. Potential reciprocal signaling between nerve terminals and muscle cells mediates the fine alignment of pre- and post-synaptic appositions.

It is quite surprising to find that down-regulation of NRG1 signaling by antagonist treatment resulted in pre-synaptic defects in synaptic vesicle transport and terminal clustering at certain stages. Because the expression of NRG1 ErbB receptors is barely detectable in either motor neurons or in extending axons during the middle stage of NMJ development (unpublished data), it is likely that these defects arise indirectly through changes in Schwann cells or muscle cells. Muscle-derived fibroblast growth factors (FGFs) of the 7/10/22 subfamily have been reported to regulate synaptic vesicle transport and clustering as nerve terminals form (Fox et al., 2007).

It will be interesting to examine the expression level of FGF ligands and the activation of corresponding FGF receptors when NRG1 signaling is down-regulated. The potential indirect involvement of NRG1 in pre-synaptic apparatus assembly suggests the existence of reciprocal signaling between motor neurons and muscle

targets, where motor neurons release growth factors like NRG1 to support the expression of muscle-derived growth factors that play important roles in the regulation of pre-synaptic motor axon differentiation. A similar signaling network, mediated by NRG1 and neurotrophic factors such as BDNF, has been suggested to regulate Schwann cell development (Ma et al., 2011). Moreover, as shown in the results, the antagonist led to a decrease of pre- and post-synaptic apposition when the NMJs became mature, suggesting that NRG1 might also calibrate the specialization of pre-synaptic machinery in the late stage as the final fine-tuning step. The decrease of the apposition seems to at least partially result from more 'puncta'-like synaptic vesicle signals in antagonist-treated animals, suggesting that the distribution of the vesicles within the pre-synaptic apparatus might be altered when NRG1 signaling was down-regulated in the late stage. However, it is not clear if the underlying mechanisms are the same as in the synaptic transport failure.

Here, we found that over-expression of NRG1 resulted in the increase of AChR cluster density, without regulating AChR cluster size. However, Ngo *et al*. reported that exogenous NRG1 increased the size of developing AChR clusters when injected into muscles of embryonic mice (2011). The conditions differ in several ways. First, we used a different system to address this issue: they used embryonic mouse sternomastoid muscle while we assayed the AChR cluster in embryonic chick thigh muscle. Considering the difference of NMJ development among different species, NRG1 might play distinct roles in regulating the AChR cluster formation. Secondly, they assayed AChR cluster four hours after the exogenous application of NRG1 while we used *in ovo* electroporation to achieve over-expression and assayed the effects about four days

later. Therefore, their effects might be due to acute NRG1 stimulation onto developing myotubes while our result was closer to continuous NRG1 over-expression during the whole critical period for motor neurons to develop and innervate into limbs. Finally, they applied recombinant NRG1-β1 to muscle cells directly, while we used the full-length type I NRG1 coding sequence which gave us the advantage of utilizing an endogenous regulatory mechanism. Moreover, when NRG1 signaling was down-regulated by the application of antagonist, we found that AChR cluster size decreased slightly in ALD muscle but not in PITIBs, suggesting that NRG1s are actually involved in the regulation of AChR cluster size in our system. To add a new layer of complexity, ALD is mainly a multi-innervated slow-tonic muscle which is rare in mammalian species, suggesting the muscle-type-dependent function of NRG1s might be different in mammalian.

Despite the difference between this dissertation and the report by Ngo *et al*., both studies show that during the early development stage, NRG1 plays important roles in regulating AChR cluster formation. Schmidt *et al.* recently reported that loss of neuromuscular NRG1-ErbB signaling destabilized anchoring of AChRs in the postsynaptic muscle membrane in adult animals (2011). Taken together, these new *in vivo* results support the notion that NRG1 signaling has multiple functions during NMJ development and maintenance.

NRG1 signal plays important roles in Schwann cell migration, maturation, and myelination (Birchmeier and Nave, 2008, Ma et al., 2011), whereas the function of NRG1 in peri-synaptic Schwann cell development remains mostly unknown, mainly because the subpopulation does not express distinct molecular markers. We used antibody 1E8 targeting P0 protein to label peri-synaptic Schwann cells and calculated

the alignment between peri-synaptic Schwann cells and post-synaptic AChR clusters. In mice, it is believed that non-myelinating Schwann cell express low levels of P0 protein (Sanes and Lichtman, 1999b). However, a study in the frog also showed that peri-synaptic Schwann cells express enough P0 protein for immunostaining, suggesting that there is variability in different aspects of NMJ development across species (Georgiou and Charlton, 1999). The result showed that some of the P0 signal was associated with post-synaptic AChR clusters, indicating that pre-synaptic Schwann cells were actually labeled. Although we have found that the development of peri-synaptic Schwann cells was disrupted when NRG1 signaling was down-regulated, the specific underlying mechanism remains to be determined in future studies.

In summary, the data suggest that NRG1 has different roles in distinct aspects of NMJ development by inducing AChR cluster density and size in the early stage, regulating pre-synaptic synaptic vesicle recruitment in the middle stages, maintaining the pre- and post-synaptic apposition in the late stage, and promoting peri-synaptic Schwann cell development.

CHAPTER III

EXPRESSION OF NEUREGULIN1 ISOFORMS IS REGULATED BY NEUROTROPHIC FACTORS AND AXON-TARGET INTERACTIONS

Summary

The neuregulin1s (NRG1s) are a family of alternatively spliced growth and differentiation factors that play important roles in many aspects of nervous system development and in disease. In motor neurons, NRG1 expression at the axon-Schwann cell and neuromuscular junctions is regulated by synaptic activity and neurotrophic factors; however, little is known about the mechanisms that control NRG1 isoform-specific transcription, critical for the specific regulation of membrane-bound and secreted isoforms. Here we show that NRG1 expression of both types of isoforms increases in motor neurons that have extended axons in the chick embryo. Limb bud ablation before the time of motor axon outgrowth prevents the induction of *NRG1* mRNA, suggesting that the limb bud supplies factors that promote *NRG1* mRNA expression. Consistently, NRG1 induction can be rescued by adding back exogenous neurotrophic factors, including brain-derived neurotrophic factor (BDNF) and glial-cell-line-derived neurotrophic factor (GDNF), but not nerve growth factor (NGF). As a means to explore the molecular basis for *NRG1* transcriptional regulation, we measured the effects of neurotrophic factors on mRNA levels of the three major mammalian alternatively-spliced forms of *NRG1* in rat embryonic ventral spinal cord cultures. BDNF induced a rapid and transient increase in type I and III *NRG1* mRNA that peaked at 4 h and then returned to baseline. No effects were seen on type II *NRG1* mRNA. Specific inhibitors that blocked MAPK or PI3K signaling prevented this induction. While the

global transcription inhibitor Actinomycin D blocked BDNF-induced *NRG1* gene induction, BDNF had no effects on mRNA degradation, suggesting that transcriptional activation, rather than message stability, drives BDNF-induced *NRG1* mRNA induction. Consistently, BDNF was able to activate transcription of a reporter construct containing 700bp of the 5' regulatory region flanking the transcription start site of type I NRG1. Protein synthesis is also required for type I *NRG1* mRNA transcription, because the translation inhibitor cycloheximide produced a super-induction of type I, but not type III *NRG1* mRNA, possibly through a mechanism involving sustained activation of MAPK and PI3K. These results reveal the existence of highly responsive, transient transcriptional regulatory mechanisms that differentially modulate NRG1 isoform expression as a function of extracellular and intracellular signaling cascades mediated by neurotrophic factors and axon-target interactions. Understanding these mechanisms will be important for elucidating the role of NRG1 in both development and in pathological disorders of the nervous system.

Materials and Methods

Chick eggs, unilateral limb ablation and in ovo *treatment*

Fertilized chicken eggs were obtained from Michigan State University Poultry Farms and incubated in a Kuhl rocking incubator at 50% humidity. Limb buds were removed unilaterally on E3 and embryos were returned to the incubator until the time indicated. In some experiments, recombinant BDNF, GDNF (Amgen), or NGF (Life Technologies) were added on E4 and E5 as described previously (Loeb et al., 2002, Ma et al., 2009). In brief, 1 µg/embryo/day of BDNF, GDNF, or NGF was prepared in saline containing 0.2% BSA, and added onto the chorioallantoic membrane for two consecutive days. Embryos were collected at indicated times and processed for total RNA, or *in situ* hybridization combined with immunofluorescence staining as described below. Staging of chick embryos was determined according to the Hamburger-Hamilton (HH) stage series (Hamburger and Hamilton, 1951): E3 (stage 18-19); E4 (stage 23-24); E5 (stage 26-27); E6 (stage 28-29).

Immunofluorescence staining and in situ *hybridization*

Embryos were fixed in 4% paraformaldehyde in PBS at 4°C overnight, washed in PBS, equilibrated in 30% sucrose in PBS, and sectioned into 20 µm sections. Neuregulin1 was labeled using 1310 antibodies against the proNRG1 precursor cytoplasmic tail (Loeb et al., 1999). RT-97 (1:50) and Islet1/2 (1:50) were obtained from the Developmental Studies Hybridoma Bank at the University of Iowa, to label neurofilament and spinal cord motor neurons, respectively. Sections were incubated with antibodies in blocking solution (10% normal goat serum, 0.1% Triton X-100 in PBS)

overnight at 4°C, followed by incubation with the corresponding goat anti-mouse or anti-rabbit Alexa Fluor antibodies (1:500; Life Technologies) for visualization. Radioactive *in situ* hybridization was performed using ^{35}S-labeled RNA probes as previously described (Beaumont et al., 2012). In brief, sense and antisense ^{35}S-labeled RNA probes were generated from linearized full-length chicken proARIA cDNA clones by *in vitro* transcription (Life Technologies). Probes were purified on NuClean R50 Sephadex columns (Shelton Scientific). Tissues were hybridized at 52°C overnight, followed by washing, and dehydrated in ethanol. Slides were then dipped in photographic emulsion (Kodak NTB), dried, and exposed for 7 days at 4°C.

Imaging and quantitative analysis.

Digital images were captured using a Nikon Eclipse E600 microscope with a Princeton Instruments Micromax cooled CCD digital camera. Signal intensity was quantified using MetaMorph image analysis software (Molecular Devices). In the spinal cord, the lateral portions of the lateral motor column on each section were first defined as regions of interests (ROI) based on weaker Islet-1/2 signal on the most lateral part of the ventral spinal cord. The intensity of *in situ* hybridization signals were measured using total pixel grey value above signal threshold within each ROI. The ratio of operated/control side was calculated by dividing average grey value of the operated side by the counterpart of the control side within the same section.

Primary spinal cord motor neuron culture

Primary spinal cord motor neurons were cultured as described previously (Loeb and Fischbach, 1997). Briefly, ventral spinal cords were dissected from timed-pregnant

Sprague Dawley rat embryos (Harlan). Cultures of ventral horn neurons were prepared from the anterior two-thirds spinal cords of embryonic day 15 (E15) rat embryos and were dissociated in HBSS containing 0.1% trypsin. Cells were placed on laminin and poly-D-lysine-coated 24-well plates at density of 200,000 cells/cm^2 in Leibovitz's L-15 Glutamax Medium supplemented with N-2 supplement, MEM vitamin solution, penicillin/streptomycin (Life Technologies), 6 mM $NaHCO_3$, 6 µg/ml chick E11 pectoral muscle extract, and 54 µg/ml imidazole. Cultures were maintained at 37°C with 5% CO_2. At day *in vitro* 3 (DIV3), cultures were treated with BDNF, GDNF, or NT-3 accordingly for indicated time periods. In some experiments, inhibitors were added 30 min before neurotrophic factors. At the end of stimulation, cells were washed with cold PBS and subjected to total RNA or protein extraction, as described below.

RNA isolation and RT-qPCR

Five- to six-somite-long segments of lumbar spinal cords were harvested from unilateral limb-ablated chick embryos at E6 and then separated from the midline for RNA extraction. Total RNA from both tissue and cell cultures was isolated using an RNeasy mini kit (Qiagen). Equal amounts of total RNAs were reverse transcribed using oligo-dT by the Superscript First-Standart Synthesis System (Life Technologies). Chick NRG1 transcripts were detected as previously described (Ma et al., 2011). Rat type I and type II NRG1 were measured by Rn00580917_m1 and Rn01482172_m1, respectively; type III NRG1 were detected by: forward primer 5′-TCCTAAACTTTCCACATCGACATC; reverse primer 5′-TCTCATAAAGTGCGCGGAG; and Taqman probe 6FAM-ACGACTGGGACCAGC. Rat GAPDH was detected by Rn99999916_s1 for normalization (Life Technologies). Levels of isoforms of *NRG1* mRNA were first

normalized by GAPDH, whose levels were revealed in the same reactions and then normalized by control. Quantitative PCR data were collected from at least three biological replicates, and ΔΔCt was used for calculations.

Protein isolation and immunoblotting

Total protein from cultured ventral spinal cord neurons was extracted using RIPA lysis and extraction buffer containing 25 mM Tris, pH 7.6, 150 mM NaCl, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS, and Halt Protease and Phosphatase Inhibitor Cocktail (Thermo Scientific). Equal amount of protein samples were loaded for immunoblotting using antibodies from Cell Signaling at the following dilutions: Phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204) (1:1000, #4370), p44/42 MAPK (Erk1/2) (1:1000, #4695); Phospho-p38 MAPK (Thr180/Tyr182) (1:1000, #4511), p38 MAPK(1:1000, #8690); Phospho-Akt (Ser473) (1:1000, #4060), Akt (pan) (1:1000, #4685). SuperSignal West Pico Chemiluminescent Substrate (Thermo Scientific) was used for signal detection. Blots were first probed with antibodies against phospho-proteins, and then stripped for reprobing with antibodies against pan-proteins.

Dual luciferase reporter assay

5′ flanking regions of type I and III NRG1s were amplified by PCR from rat genomic DNA using forward primer 5'-TGCTTAGGAAGAAGCTAGAGTGAGT, and reverse primer 5'-TCAGACATCTCGCCGAAGATGA for type I; forward primer 5'-TAAGAGTCCTGTGAGTGAAC, and reverse primer 5'-ATGTCTGGGGAATAAATCTC for type III. They were subcloned into pGL3B vector upstream to the firefly luciferase gene, and verified by sequencing. Dissociated ventral spinal cord cells were

cotransfected by electroporation (Neon Transfection System, Life Technologies) with type I promoter-Luc or type III promoter-Luc together with thymidine kinase promoter-Renilla luciferase reporter plasmid (pRL-TK) at ratio of 50:1. 12 hours after transfection, cells were treated with BDNF, NGF or the combination of BDNF and NGF as indicated for 24 h before lysis. Firefly and Renilla luciferase activities were assayed using Dual-Luciferase Reporter Assay System (Promega) using a Fluoroskan Ascent microplate luminometer (Thermo Scientific). Firefly luciferase activity was normalized to Renilla luciferase activity to reduce inter-experiment sample variation.

Statistical analysis

A paired two-tailed Student's *t*-test was used to analyze differences between two treatment groups. Statistical differences for multiple group comparisons were calculated by one-way ANOVA followed by Dunnett's *post hoc* test. For comparisons of multiple groups with different treatments, two-way ANOVA followed by Bonferroni's *post hoc* test by comparison to control was used. Normalized values were averaged and reported as the mean ± SEM of at least three independent experiments. Statistical significance is presented as * $p<0.05$, ** $p<0.01$, *** $p<0.001$.

Results

Axon-target interactions regulate NRG1 mRNA expression

It has been observed that NRG1 protein and mRNA increases in spinal motor neurons following their birth and migration towards the lateral portion of the developing spinal cord in chicken embryos (Loeb et al., 1999). ProNRG1 expression is at the highest level in those motor neurons (homeodomain motor neuron marker Islet-1/2^{+}) that have completed their migration and have extended their axons into the surrounding mesoderm (Fig. 14). Consistent with the protein level, increase of *NRG1* mRNA in lateral motor column was observed during development (Unpublished data performed by Dr. Vaagn Zakarian). This observation suggests that peripheral mesoderm provides extrinsic factors to coordinate NRG1 expression.

To examine the effect of axon-target interactions on NRG1 expression, unilateral hind limb bud ablation was performed *in ovo* at E2.5, prior to axon outgrowth into the limb bud (Tosney and Landmesser, 1985) (Fig. 15A, 15B). With this model, motor axons spiral into a ball in the absence of a target to innervate. After limb bud ablation, but before the period of programmed cell death that starts at E6, *NRG1* mRNA levels did not increase on the ablated side as they do on the control side in the lateral portion of the lateral motor column (LMC_L) that normally innervates the dorsal limb bud (Fig. 15C, 15D). The weakly positive Islet-1/2 marker is used to label the lateral LMC, which shows no reduction of motor neuron numbers even after ablation (Unpublished data performed by Abdelkrim Hmadcha). This marker was used in double labeling radioactive *in situ* experiments in Fig. 15C to quantify the *in situ* signal. The results provide evidence that

target-derived factors were necessary for directing the expression of NRG1. To characterize the composition of NRG1 isoforms in the operated animals, RT-qPCR using isoform-specific primers was performed. Compared to the control side, both IG-NRG1 (type I/II) and CRD-NRG1 (type III) were significantly reduced on the ipsilateral side, suggesting that axon-target interactions are important to induce both of the major NRG1 isoform classes (Fig. 15E).

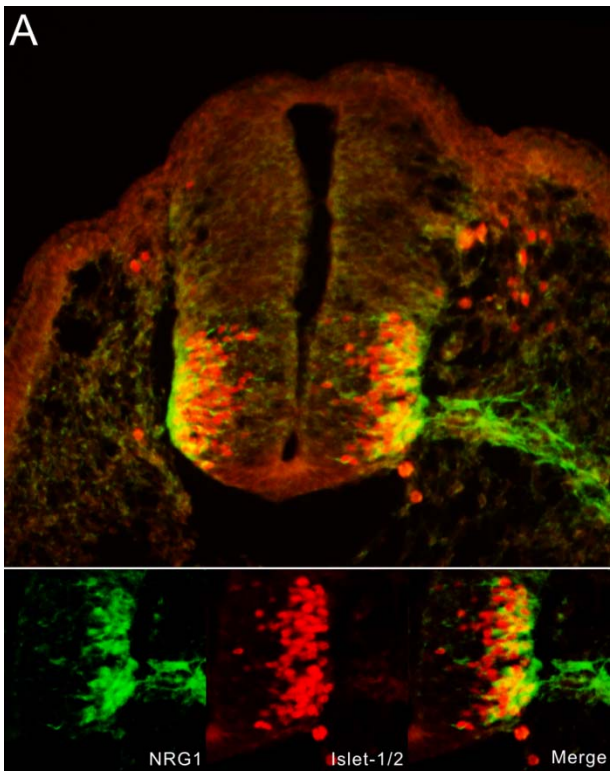

Figure14. ProNRG1 expression in motor neurons and their axons. A. An HH stage 18 chicken spinal cord was labeled with 1310 (green) and Islet-1/2 (red), showing that motor neurons (Islet-1/2^{+}) located laterally expressed a high level of NRG1 (1310). In contrast, medial motor neurons had little NRG1 expression at this stage. **B.** Zoomed-in images of right side ventral spinal cord area. (Unpublished data performed by Abdelkrim Hmadcha and Vaagn Zakarian).

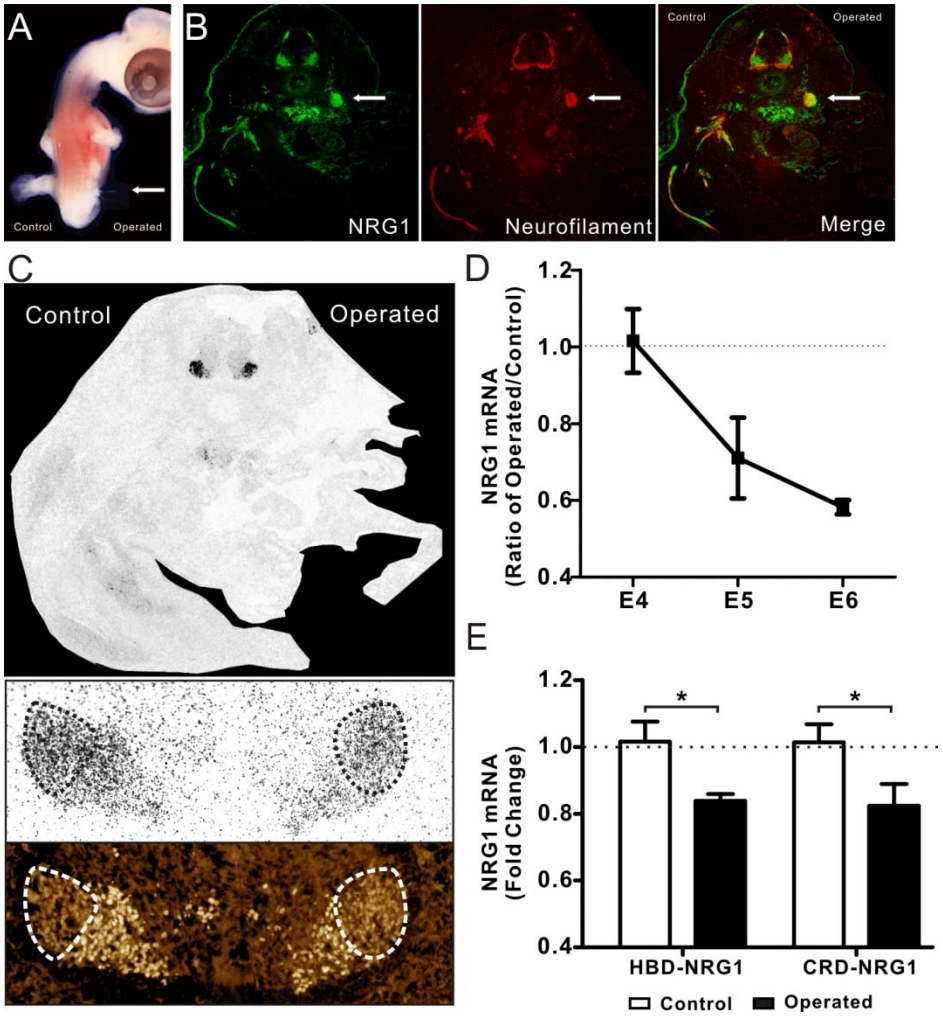

Figure15. Motor neurons that fail to contact their targets express lower *NRG1* mRNA levels. A. Removal of the apical ectodermal ridge at E2.5 results in a unilateral limbless chicken embryo at E6. **B.** Sections through the lumbar level show motor axons (RT-97, red) expressing NRG1 (1310, green) rolled into a ball (arrow), while contralateral axons innervate the limb normally. (Unpublished data performed by Vaagn Zakarian). **C.** *NRG1* mRNA levels were quantified using a double-labeling with immunofluorescence using islet-1/2 antibodies followed by radioactive *in situ* hybridization with a pan-NRG1 probe. *In situ* hybridization signals were quantified from the LMC_L (ROI determined by weaker Islet-1/2 immunoreactivity in the most lateral part of the ventral horn) and the ratio of *NRG1* mRNA expression on the operated/control side for each embryo was determined from E4-E6. (Unpublished data performed by Abdelkrim Hmadcha and Vaagn Zakarian). **D.** Limb bud ablation led to reduced NRG1

levels the LMC_L at E5 and E6. (Unpublished data performed by Abdelkrim Hmadcha and Vaagn Zakarian). **E.** In order to determine which NRG1 isoforms were responsible for this reduction, control and ablated sides of the ventral lumbar spinal cord were isolated and HBD-NRG1 (type I/II) and CRD-NRG1 (type III) were quantified qPCR (n=10) after normalization to GAPDH for each animal. Both of these showed reduced mRNA levels on the ablated side. * $p<0.05$, paired two-tailed Student's *t*-test.

Neurotrophic factors restore NRG1 mRNA reduction in motor neurons lacking targets.

A lack of neurotrophic support is one possible explanation for the failure of *NRG1* mRNA induction following limb bud ablation. Developing muscles provide a range of neurotrophic factors that support motor neuron survival and neuromuscular junction development (Levi-Montalcini and Calissano, 1979, Henderson et al., 1993, Henderson et al., 1994). These factors have distinct expression profiles at different developmental stages. Therefore, we asked whether exogenous BDNF, GDNF, or NGF could rescue *NRG1* mRNA expression after unilateral limb ablation. This was determined by measuring the ratio of *NRG1* mRNA levels in the LMC_L on the operated versus control sides of the spinal cord at E6 with or without addition of these factors at E4 (Fig. 16A) (Unpublished data performed by Dr. Abdelkrim Hmadcha). While both BDNF and GDNF maintained normal *NRG1* mRNA levels in motor neurons that lack targets, NGF failed to rescue expression (Fig. 16A, 16B). This is consistent with their known actions, because muscle- and Schwann cell-derived BDNF and GDNF have been shown to be potent survival factors for motor neurons (Yan et al., 1992, Henderson et al., 1994), whereas NGF and its receptors have little effect on motor system development (Funakoshi et al., 1993, Ip et al., 2001).

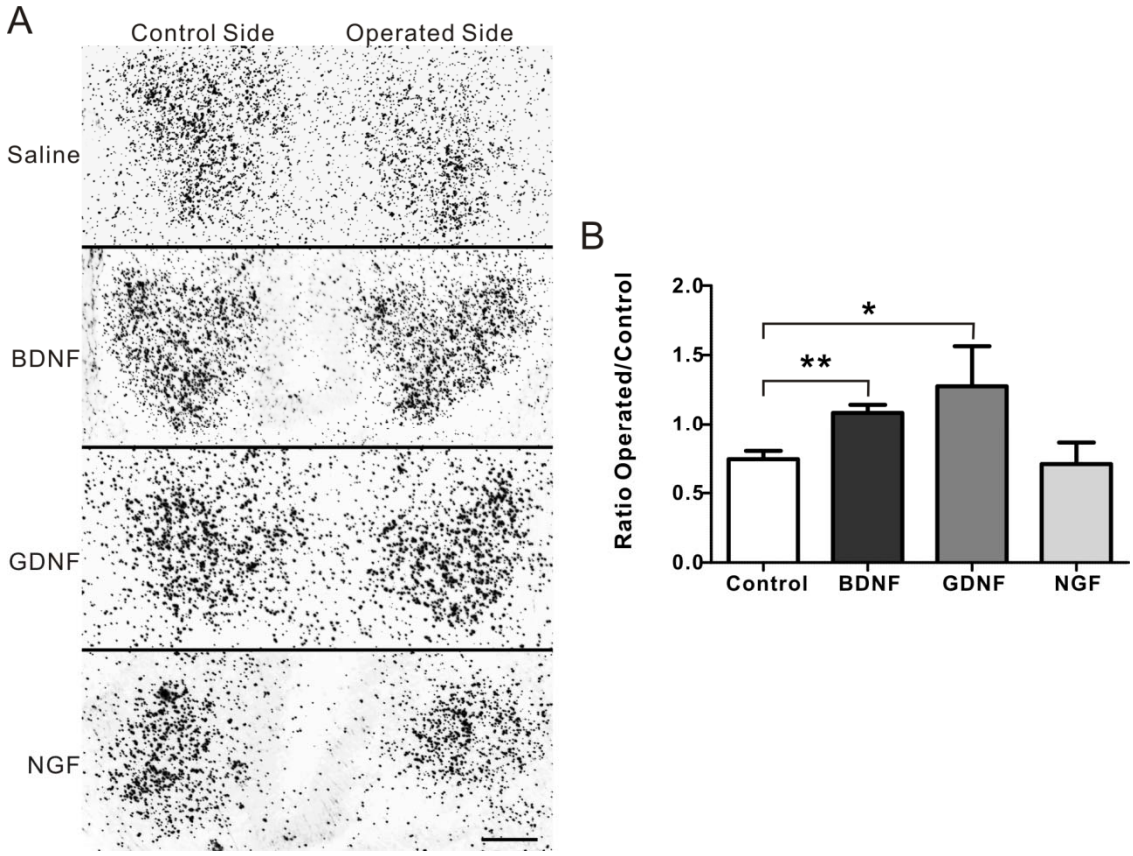

Figure16. NRG1 expression can be rescued by BDNF or GDNF after limb bud ablation. A. Representative images are shown for *in situ* hybridization from the ventral spinal cords of limb-bud ablated chick embryos treated with Saline, BDNF, GDNF, or NGF using a pan-NRG1 probe. **B.** Quantitative analysis using the ratio of NRG1 in operated/control sides showed that BDNF and GDNF, but not NGF, rescued the decrease of *NRG1* mRNA on the ablated side. Data are reported as the mean ± SEM of n=10, 11, 3, 5, for Control, BDNF, GDNF, NGF, respectively, * $p<0.05$, ** $p<0.01$, one-way ANOVA followed by Bonferroni's *post hoc* test by comparison to control. Scale bar, 50µm (Unpublished data performed by Abdelkrim Hmadcha).

Type I and type III NRG1 mRNAs are rapidly and transiently up-regulated by neurotrophic factors in mammalian motor neuron cultures.

To further address the mechanism by which neurotrophic factors regulate *NRG1* mRNA expression, we utilized an established rat embryonic ventral spinal cord motor neuron culture system where we previously have shown rapid (within 4 hours) effects of BDNF and GDNF on *NRG1* mRNA levels (Loeb et al., 1999). Using isoform-specific qPCR, we found that type I *NRG1* mRNA was induced by both BDNF and GDNF, whereas type III *NRG1* mRNA was induced by BDNF only to a smaller extent (Fig. 17A). No significant change was observed in type II *NRG1* mRNA levels in response to either neurotrophic factor (Fig. 17A). Next, to examine whether the transcriptional kinetics of the three isoforms could be regulated by neurotrophic factors, we chose BDNF, for its greater effects on transcription induction in the previous experiment, to stimulate cells for different period of time and measured corresponding isoform mRNA levels. The kinetics of mRNA induction for type I *NRG1* mRNA peaked at 4 h, but declined rapidly by 6 h, and returned to baseline 8 h after BDNF application. Type III NRG1 transcripts were also induced at 4 h; however, it was not reduced at 6 h, but returned to baseline levels by 8 h. These kinetic patterns were seen consistently on multiple experiments for type I (n=3) and type III (n=3) (Fig. 17B). These results demonstrate that both type I and type III, but not type II, *NRG1* mRNA levels are rapidly and transiently induced with BDNF stimulation, but with distinct temporal profiles, suggesting both common and unique regulatory mechanisms.

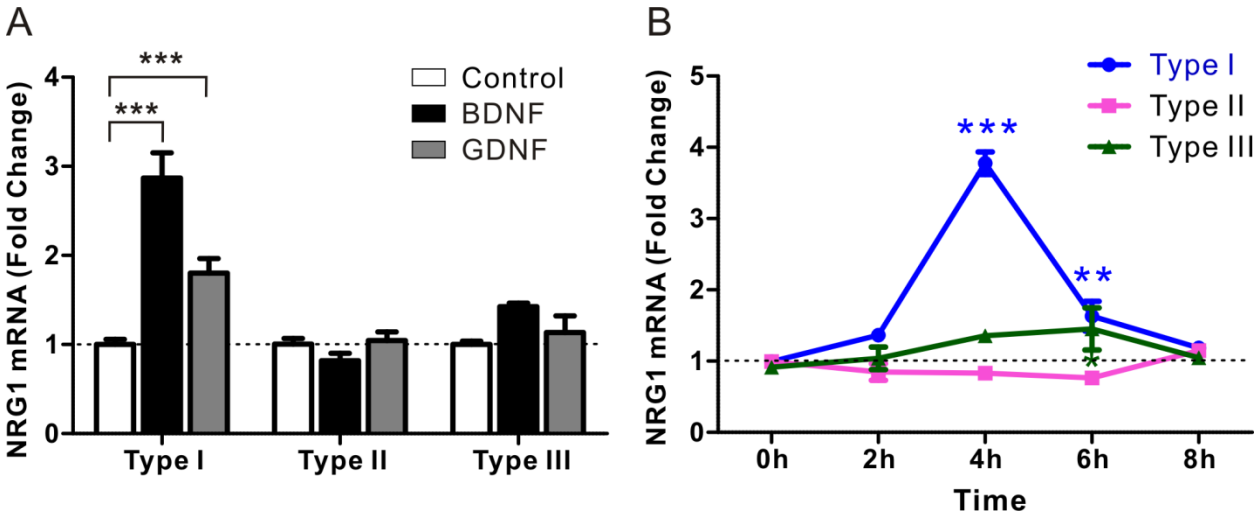

Figure17. Differential regulation of *NRG1* isoform mRNA levels by neurotrophic factors in rat embryonic ventral spinal cord cultures. A. Disassociated E15 rat embryonic ventral spinal cords were cultured for three days and then treated with and without BDNF (100 ng/ml) or GDNF (100 ng/ml) for 4 h. BDNF and GDNF induced type I greater than type III, but not type II *NRG1* mRNA using isoform-specific qPCR. **B.** A time course revealed that BDNF (100 ng/ml) induces type I *NRG1* mRNA maximally and transiently at 4 h. Type III is induced less at 4 h, but stays up longer than type I. Type II was unaffected by BDNF. Data reported are the mean ± SEM of at least three independent experiments. * $p<0.05$, ** $p<0.01$, *** $p<0.001$, two-way ANOVA followed by Bonferroni's *post hoc* test by comparison to control (A), or 0h (B).

TrkB, MAPK and PI3K signaling are required for BDNF-induced NRG1 expression

To explore the signaling pathways involved in the regulation of type I and type III *NRG1* mRNA by BDNF, we pretreated cultured cells with specific inhibitors prior to BDNF application. BDNF binds to either the TrkB tyrosine kinase or the low-affinity nerve growth factor receptor p75 and is known to lead to the activation of MAPK, phosphotidylinositol-3 kinase (PI3K) and phospholipase C-γ (PLC-γ) (Segal, 2003). Inhibition of TrkB activation by K-252a blocked NRG1 type I and type III mRNA

induction by BDNF at 4 h (Fig. 18A, 18B). Furthermore, inhibition of either MAPK by the MEK inhibitor PD98059 or U0126, or PI3K by LY294002 or Wortmannin, blocked *NRG1* mRNA induction by BDNF (Fig. 18C, 18D). These findings suggest that TrkB, MAPK, and PI3K signaling pathways are all involved in *NRG1* type I and III mRNA induction.

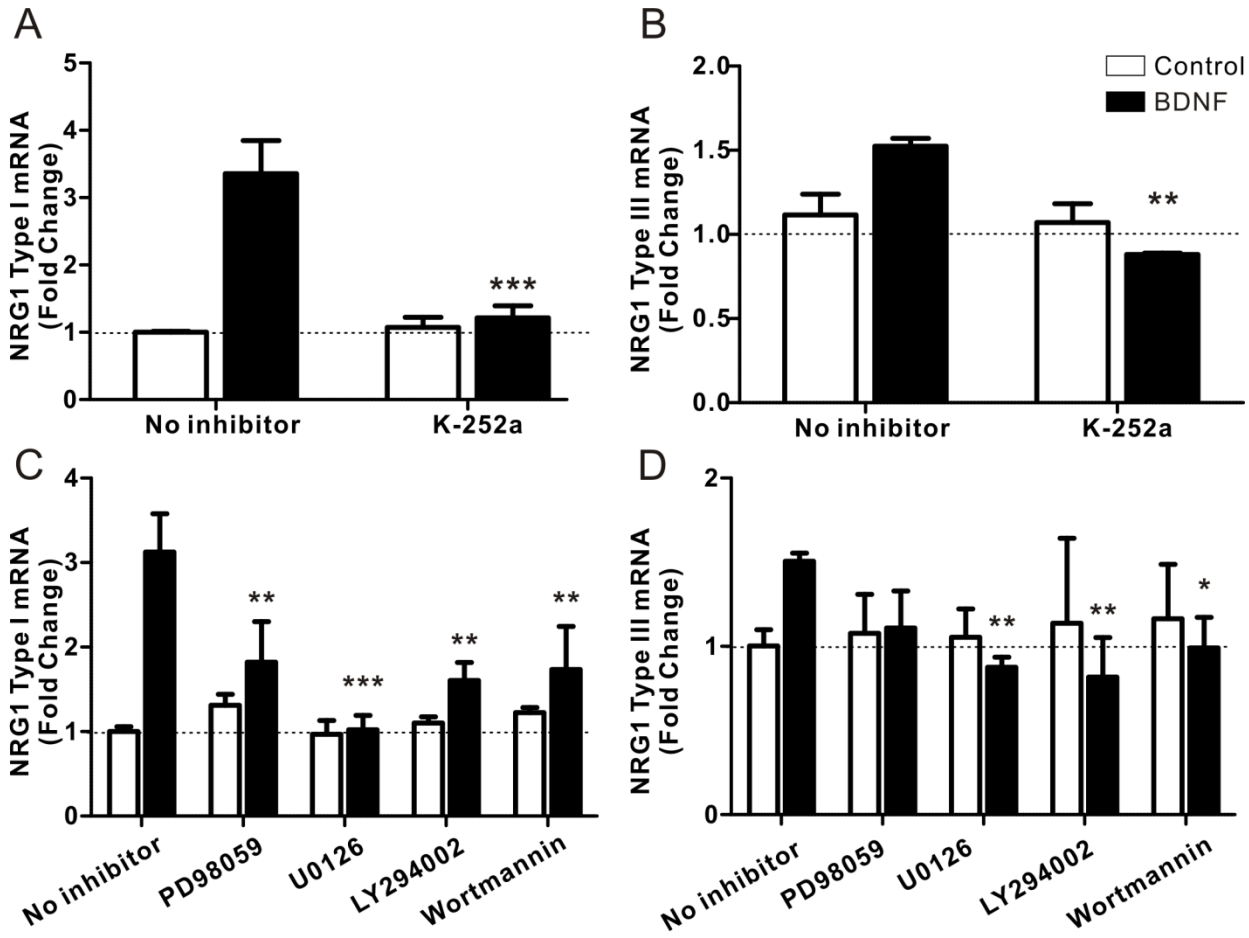

Figure18. Trk receptor, MEK, and PI3K inhibitors block the effect of BDNF on *NRG1* mRNA. Rat embryonic ventral spinal cord cultures were exposed to K-252a (200 nM) **(A, B)** or PD98059 (10 μM), U0126 (10 μM), LY294002 (50 μM), or Wortmannin (200 nM) **(C, D)** for 30 min prior to adding BDNF (100 ng/ml) for 4 h. The inhibitor pretreatment resulted in the abrogation of BDNF induction on both type I and type III NRG1 compared to the diluents alone (no inhibitor). Data are reported as the mean ± SEM of at least three independent experiments. *** $p<0.001$, two-way ANOVA followed by Bonferroni's *post hoc* test by comparison to control.

BDNF-induced NRG1 induction requires new transcription

The increase in mRNA levels could have resulted from either an increased rate of transcription, mRNA stabilization, or both. To distinguish between these possibilities,

we measured mRNA levels after BDNF-treatment in the presence or absence of the transcription inhibitor Actinomycin D (ActD). Pretreatment with ActD for 30 minutes completely prevented *NRG1* mRNA induction by BDNF, demonstrating that ongoing gene transcription is necessary for BDNF-evoked type I and III *NRG1* mRNA up-regulation (Fig. 19A). To address whether the turnover rate of *NRG1* mRNA is altered by BDNF, cultures were treated with ActD in the presence and absence of BDNF and mRNA levels were measured as a function of time. Interestingly, type I *NRG1* mRNA was relatively short-lived, with a half-life of about 1h, whereas type III *NRG1* mRNA was more stable, with a half-life of about 2h (Fig. 19B). Because the presence or absence of BDNF had no clear effects on the turnover ratio of either type I or III *NRG1* mRNA, these findings suggest the most important effect of BDNF is on transcriptional activation rather than on mRNA stability.

Given the importance of transcription for BDNF induction, we next investigated the effects of BDNF on the 5' cis-elements upstream to type I and III *NRG1* transcripts in rat embryonic ventral spinal cord cultures. Firefly luciferase constructs were prepared containing the 5' promoter regions flanking the transcription start sites for rat type I (-649 to +5) and type III (-1343 to +31) NRG1 (Fig. 20A). Each of these constructs was co-electroporated with the control vector pRL-TK in order to normalize the NRG1 induced luciferase activity with Renilla luciferase activity. After 24h, BDNF induced the type I promoter-driven luciferase (Fig. 20B). These findings suggest that cis-acting elements contribute to type I NRG1 transcription induced by BDNF.

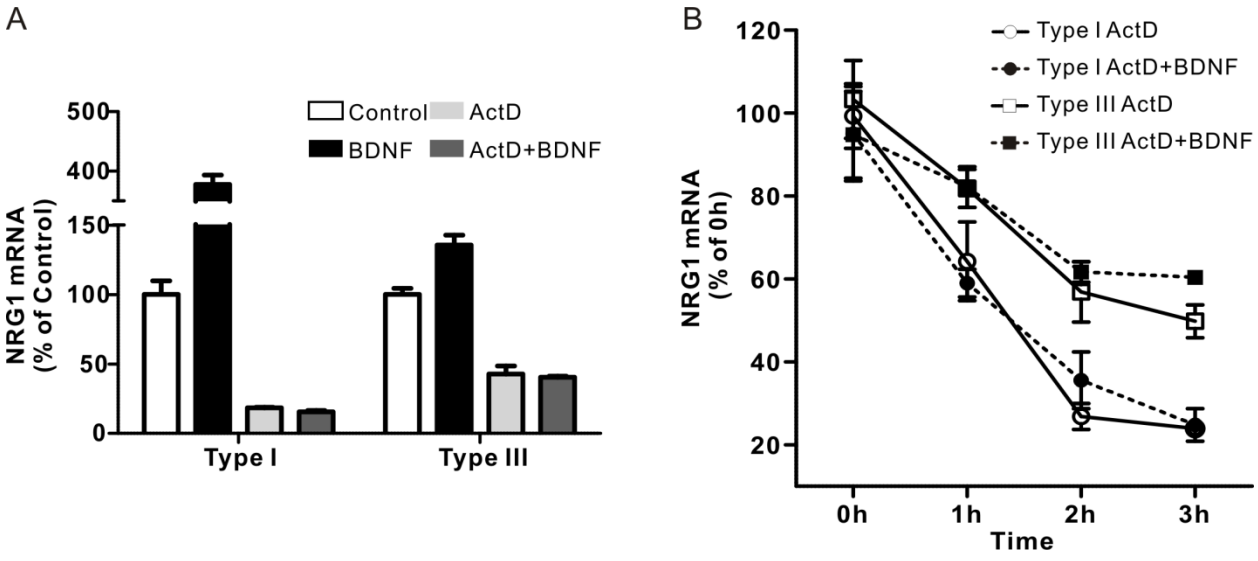

Figure19. BDNF promotes transcription but has no effect on mRNA stability. A. Rat embryonic ventral spinal cords were treated with or without actinomycin D (ActD, 2 μg/ml) for 30 minutes before BDNF (100 ng/ml) stimulation, and harvested at 4h. **B.** A time course of NRG1 decay with or without BDNF/ActD shows that BDNF does not change the rate of mRNA degradation rate for both type I and III isoforms. Decay curves were fitted to a single-phase decay curve to calculate a $t_{1/2}$=62 min (R^2 = 0.93) for type I with ActD and $t_{1/2}$=67 min (R^2 = 0.92) for ActD + BDNF. In contrast, type III NRG1 had a $t_{1/2}$=120 min (R^2 = 0.92) for ActD and $t_{1/2}$=111 min (R^2 = 0.85) for ActD + BDNF treatment.

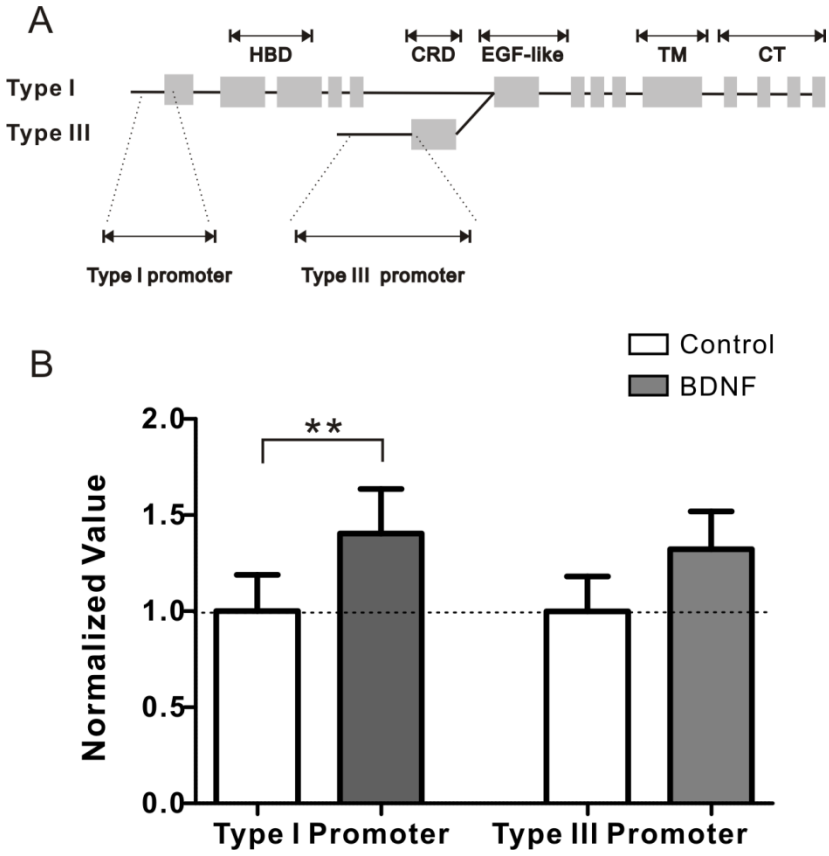

Figure20. The 5′-flanking region of type I NRG1 is sufficient for BDNF induction of transcription. **A.** 5' promoter regions for type I and type III NRG1 were cloned into a luciferase reporter plasmid as indicated. **B.** Disassociated rat embryonic ventral spinal cords were electroporated with each construct together with the pRL-TK control vector. 12 h after electroporation, cells were treated with or without BDNF (100 ng/ml) for 24 h before luciferase activity was measured. BDNF treatment induced the type I promoter-driven luciferase activity significantly, whereas there was only a non-significant increase using the type III promoter. Transcriptional activities were shown by fold change compared to control, after normalization for transfection efficiency by the activity of Renilla luciferase. Data are reported as the mean ± SEM of at least twelve biological replicates, ** $p< 0.01$, paired two-tailed Student's *t*-test.

Protein synthesis is required to maintain type I NRG1 mRNA transcription

The transient nature of *NRG1* mRNA induction by BDNF could have important biological implications. The mechanism by which NRG1 transcription is precisely shut off after 4-6 hours following BDNF exposure was assessed using the protein synthesis inhibitor cycloheximide (CHX) in cultured ventral spinal cord neurons with and without BDNF treatment. Remarkably, CHX treatment prevented the down-regulation of type I *NRG1* after 4 h of BDNF treatment, and instead led to a further almost 10-fold induction. Even in the absence of BDNF treatment, CHX produced a steady increase of type I *NRG1* mRNA, suggesting an additional regulatory mechanism that promotes *NRG1* type I mRNA induction, independent of BDNF (Fig. 21A). In contrast, CHX had no effects on type III NRG1 expression (Fig. 21B).

This 'superinduction' of type I *NRG1* could either be due to increased gene transcription or reduced degradation. However, the kinetics of type I *NRG1* mRNA stability were unchanged in the presence of CHX, suggesting that the effect is again related to increased transcription (Fig. 21C). In light of the requirement for both MAPK and PI3K signaling from Fig. 5, we asked whether CHX affected the activation of both MAPK and PI3K signaling after BDNF stimulation in the presence and absence of CHX (Fig. 21D). In contrast to a rapid, transient induction of pERK, p38, and pAKT (peaked at 30 min), BDNF treatment in the presence of CHX produced a more sustained, long term activation of all of these signaling intermediates with the greatest effect on pERK. These results suggest that protein synthesis is required to down-regulate long-term signaling effects of BDNF, with the greatest effect on MAPK signaling. These results also show additional differences in the transcriptional regulatory mechanisms between

type I and type III NRG1 forms because there was no effect of blocking protein synthesis on type III forms.

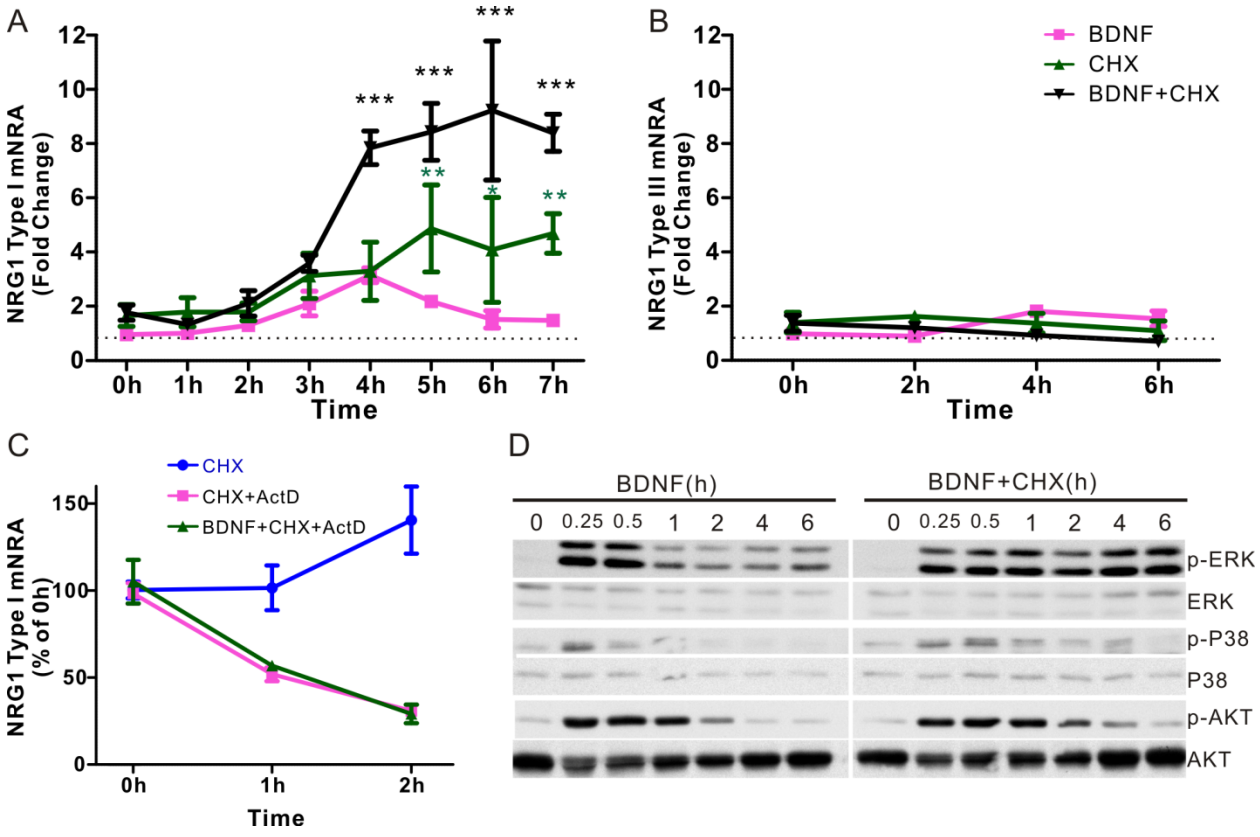

Figure21. Protein synthesis is required for the down-regulating *NRG1* mRNA after BDNF-induced stimulation. **A.** Rat embryonic ventral spinal cord cultures were pretreated with or without cycloheximide (CHX, 20 µg/ml) for 30 minutes before BDNF (100 ng/ml) stimulation. NRG1 type I mRNA levels were elevated significantly by CHX alone and super-induced with both CHX and BDNF. **B.** CHX had no clear effect on type III NRG1 expression. **C.** While CHX alone led to increased mRNA levels, CHX +ActD with or without BDNF had no effect on the rate of decay of type I *NRG1* mRNA. Data are reported as the mean ± SEM of at least three independent experiments. * $p<0.05$, ** $p<0.01$, *** $p<0.001$, two-way ANOVA followed by Bonferroni's *post hoc* test by

comparison to 0 h. **D.** CHX prevented the normal reduction in ERK, p38 and AKT signaling after BDNF treatment of rat embryonic ventral spinal cord cultures.

Discussion

A reciprocal axon-target feedback loop mediated by NRG1 and neurotrophic factors

Both neuron-derived NRG1 and target-derived neurotrophic factors have been implicated in neuromuscular system development. The functions of these mediators rely not only on the dynamic distribution of their receptors, but also on the temporal and spatial expression profile of the ligands. This diversity allows a multi-directional crosstalk between cells that can be both temporally and spatially restricted, depending on the need biologically.

Here we have focused on extracellular signaling mechanisms that underlie axon-target interactions in the developing spinal cord and limb bud. We show that axon-target interactions promote NRG1 expression in spinal motor neurons and that this effect can be replicated by target-derived neurotrophic factors, such as BDNF and GDNF. *In vitro*, we show that BDNF induces a rapid, yet transient up-regulation of *NRG1* mRNA that operates through *de novo* transcription and is mediated by TrkB-MAPK and TrkB-PI3k signaling pathways as well as by distinct 5' regulatory cis-elements. The rapid (4 h) nature of the induction may be important to increase transcription in only those axons that make appropriate contact with their target. The transient nature of the induction (sharply falls after 4 h) requires protein synthesis and is associated with a rapid shut down of the TrkB signaling cascades. Biologically, this could be important to limit NRG1 expression in axons that do not have sustained contact with their targets. For secreted (type I) forms of NRG1, in addition to these transcriptional mechanisms, we have previously shown that BDNF can work post-translationally to promote the local release

of NRG1 from axons via a mechanism that requires protein kinase C-δ (Esper and Loeb, 2004, 2009).

In addition to survival, target-derived neurotrophic factors have been implicated in supporting motor neuron function by eliciting transcriptional and translational changes (Chowdary et al., 2012). In the present study, deprivation of target-derived neurotrophic factors by unilateral removal of developing limb buds prior to the period of programmed cell death did not lead to programmed cell death, but did lead to changes in two out of the three major spliced forms of NRG1. How neurotrophic factor signaling in axons is relayed to motor neuron nuclei is not entirely clear, but has been extensively studied (Segal, 2003, Zweifel et al., 2005). Neurotrophic factor signaling pathways can also differ depending on the cellular localization of their receptors (Chowdary et al., 2012). For example, retrograde NGF-TrkA signaling on axons results in Erk5 activation to mediate NGF pro-survival signaling in dorsal root ganglion cells, while activation of TrkA in the cell bodies of these neurons uses Erk1/2 activation (Watson et al., 2001). Another level of complexity is that there are a number of seemingly redundant neurotrophic factors that can converge on common pathways. Here, we found that both BDNF and GDNF could rescue NRG1 expression after limb bud ablation while NGF failed. Although this is consistent with the expression of profiles of their receptors, the crosstalk and downstream differences in signaling pathways from each factor adds additional complexity to this communication 'language.'

The complexity of NRG1 gene structure and isoform-specific expression

The human *NRG1* gene is one of the longest and most complex genes in the genome, and is located on chromosome 8p12 and spans over 1 Mbp. Recent studies

from the ENCODE project have shown regions within this gene that have DNase hypersensitivity implicating the presence of cis-regulatory elements (Rosenbloom et al., 2013). One of the highest DNase-sensitive clusters is located -1-3 kb to the transcription start site of type I NRG1. Consistently, this enrichment region has high density of H3K27Ac and H3K4Me3 histones and transcriptional factor binding sites, determined by ChIP-seq analysis. All of these markers suggest that the ~4-5 kb DNA fragment might be an important regulatory region for type I NRG1 expression (Rosenbloom et al., 2013). Consistently, we showed that BDNF can induce transcription of a portion of this cis-element. It would be worthwhile to investigate if this region, especially within the first intron that has not been analyzed previously (Frensing et al., 2008, Liu et al., 2011), is responsible for the binding of regulatory factors induced by neurotrophic factor signaling.

Alternative splicing produces a daunting array of NRG1 proteins that have been actively investigated over the years (Falls, 2003). In the current study we focused on three major protein forms that have been shown to have important differences in how and where they signal. For example, type I and II forms both produce secreted proteins that have heparin-binding domains, but type II has an additional N-terminal Kringle domain of unclear function. Despite the similarity, we found that BDNF had no effect on type II NRG1 transcription, but affected both types I and III. Type III NRG1 isoforms are unique in that they have a second membrane-spanning region thought to activate its receptors through direct cell-cell interactions (Wang et al., 2001). While BDNF induced both type I and type III NRG1 forms by 4 h after administration, there were differences in both the magnitude of the effect and the kinetics. Type III NRG1 induction by BDNF

was lower, but with a longer time course (over 6 h), and was more slowly degraded than type I NRG1. In addition, the BDNF effect on the 5' cis-regulatory type III elements was less strong than for type I NRG1. Finally, the mechanism requiring protein synthesis that shuts off type I NRG1 expression does not occur for type III forms, because cycloheximide had no effects on type III NRG1. Taken together, each splice form has transcriptional and post-transcriptional regulatory machinery that can fine-tune its expression, leading to a dynamic spatially and temporally unique pattern of NRG1 signaling in motor neurons.

Wider roles for NRG1-BDNF reciprocal signaling in the nervous system

Both NRG1, neurotrophic factors, and their respective receptor systems have been shown to play critical roles in many aspects of nervous system development and in disease. For example, both signaling pathways are involved in long-term potentiation (LTP) in the hippocampus; however, studies have shown opposite effects with BDNF facilitating and NRG1 suppressing LTP (Patterson et al., 1996, Huang et al., 2000, Chen et al., 2010). The expression of BDNF and NRG1 are tightly regulated by neuronal activity both at the neuromuscular junction (Loeb et al., 2002) and in the CNS (Flavell and Greenberg, 2008, Liu et al., 2011). However, the link between these two signaling pathways in the CNS has not been investigated. Thus, similar regulatory machinery might exist in the CNS as we have found peripherally here. Models of epilepsy have also been used to show activity dependence. Activity-dependent transcription of BDNF has been shown to promote epileptogenesis (He et al., 2004) and *NRG1* mRNA increases in the hippocampus after a single electrical stimulation-induced seizure (Tan et al., 2012). Finally, NRG1 has recently been implicated in models of

chronic pain after nerve injury (Calvo et al., 2010) and in both human tissues and an animal model of ALS through activation of microglia (Song et al., 2012). Understanding extracellular signaling through neurotrophic factors as well as the intracellular signaling cascades that promote NRG1 expression could therefore be used to develop therapeutic targets for these disorders.

CHAPTER IV

CONCLUSIONS AND SIGNIFICANCE

The body of work in this dissertation investigates the role of neuregulin1 (NRG1) in the development of the neuromuscular junction (NMJ) and develops an understanding of the molecular mechanisms by which differential expression of NRG1 isoforms are regulated by neurotrophic factors and axon-target interactions. In Chapter II, a quantitative morphometric analysis of NMJ development was developed, which took into account synaptic size and density, together with the apposition of pre- and post-synaptic components and the alignment of peri-synaptic Schwann cells. On this background, data were presented suggesting that NRG1 promotes acetylcholine receptor (AChR) cluster density and size in the early stage. While in the later developmental stages, NRG1 signaling is involved in the processes of synaptic vesicle accumulation at nerve terminals, pre- and post-synaptic apposition, and peri-synaptic development. Data described in this chapter suggests that NRG1 plays distinct roles in multiple aspects of NMJ development in different developmental stages. In Chapter III, several lines of evidence were provided suggesting that expression of NRG1 isoforms in spinal cord motor neurons is regulated by target-derived neurotrophic factors such as brain-derived neurotrophic factor (BDNF) and glial-cell-line-derived neurotrophic factor (GDNF). The observation was expanded further, where data were presented that the transcription of type I NRG1 can be induced by neurotrophic factors in a temporal specific manner and type III can also be induced to a smaller extent. Further, it was demonstrated that the induction is mediated by both TrkB-PI3K and TrkB-MAPK signaling, mainly through transcription activation. Data were presented suggesting that

a 5' regulatory region flanking to the transcription start site is required for BDNF-induced type I NRG1 expression. Finally, it was shown that protein synthesis is necessary for type I NRG1 induction but not type III. Cycloheximide, a protein synthesis inhibitor super-induced type I NRG1 transcription, at least partially, by maintaining the activation of intercellular signaling pathways. These results suggested that expression of NRG1 isoforms in spinal cord motor neurons is regulated by neurotrophic factors and axon-target interactions through distinct molecular mechanisms, which can contribute to their versatile expression profiles and functions at NMJs.

The data presented in this dissertation are consistent with the idea that NRG1 plays modulatory roles in NMJ development. Moreover, this is the first *in vivo* developmental study showing that NRG1 plays multiple roles at NMJs along with the different developmental stages, including post-synaptic AChR clustering in the early stage; synaptic vesicle transport and localization in the middle stage; pre- and post-synaptic apposition, and peri-synaptic Schwann cell development in the late stage. The data presented here also reveal that spinal cord motor neuron-derived NRG1 and target-derived neurotrophic factors form a reciprocal system for axon-target communication, by which neurotrophic factors regulate different isoforms of NRG1 expression to account for their distinct roles in neuromuscular system development. Aside from significantly adding to our understanding of endogenous roles of NRG1 in regulating NMJ formation and function, and how the isoforms are regulated by neurotrophic factors during development in the peripheral nervous system (PNS), the findings here may have important implications in understanding and treating human

diseases which involve the PNS and the muscular system. The mechanisms in the PNS could be potentially translated to the central nervous system (CNS) disorders as well.

Isoforms of NRG1 are essential for everything from heart development to glia differentiation (Esper et al., 2006). At NMJs, it has been suggested that they promote the expression of AChR subunits and other post-synaptic components based on the observations *in vitro* (Brenner et al., 1990). However, the expression of these genes was barely reduced and the NMJs form on schedule when NRG1 signaling is disrupted in the neuromuscular system (Escher et al., 2005). NRG1 appears to be non-essential for NMJ development, since most of the NMJ defects in animals with disrupted NRG1 signaling are modest, transient, and can be compensated. These findings lead to an updated hypothesis that the family of growth factors plays modulatory, and maybe secondary, roles in modifying NMJ structure. The work from the dissertation has found that NRG1 has multiple functions at NMJs during development, and, consistent with the previous studies, their roles lean toward subtle and fine-tuning mechanisms, compared to the other dictating molecular signals such as Agrin-Lrp4-MuSK. As for their mode of functions, it has been shown that NRG1 can work synergistically with Agrin to promote post-synaptic AChR expression and clustering by potentiating MuSK phosphorylation (Li et al., 2004a, Ngo et al., 2012). Consistently, it has also been suggested that Erbin can bind to ErbB2 and MuSK to form a Erbin-ErbB2-MuSK complex at the synaptic site to mediate the induction of AChR expression and clustering, and myelination (Tao et al., 2009, Simeone et al., 2010). Thus, NRG1 can serve as a second-order regulator upon Agrin to direct post-synaptic differentiation. Besides, NRG1 can regulate NMJ maintenance alone by stabilizing the post-synaptic apparatus via phosphorylation of α-

dystrobrevin (Schmidt et al., 2011). Therefore, NRG1-ErbB can either work alone or function as a regulator to other signals at NMJs, depending on the developmental stages and extracellular or intracellular signals in the environment.

Minor factors like NRG1 can have major impacts, especially in the dynamic and slow developmental processes that require resilient signals that are subject to adjustment by activity or micro-environmental change. Pronounced plasticity occurs during postnatal pruning and maintenance with constant functional and morphological remodeling, despite overall dimensions remaining stable (Wilson and Deschenes, 2005, Tapia et al., 2012). Remodeling of the NMJ can also be amplified with alterations in neuromuscular activity such as exercise training (Valdez et al., 2010). On the other hand, the functions of the NMJ gradually deteriorate during the natural process of aging (Courtney and Steinbach, 1981, Balice-Gordon, 1997); moreover, substantial reconfiguration of NMJs are elicited in various neuromuscular diseases such as amyotrophic lateral sclerosis (ALS) (Schaefer et al., 2005, Dupuis and Loeffler, 2009). In these situations, a dominant NMJ organizer like Agrin-Lrp4-MuSK signaling might not be optimal, and thus convergence of the fine-tuning signal like NRG1-ErbB becomes important to adjust the necessary amount of signaling conveyed into post-synaptic muscle cells, or to maintain the dynamics of cellular components. It is therefore of great therapeutic interest to characterize the aging- or disease-related alterations of NRG1-ErbB, and to examine the consequences of manipulating the signaling in neuromuscular system diseases.

NRG1 isoforms are produced as trans-membrane precursors and undergo proteolytic shedding (Falls, 2003, Loeb, 2003, Mei and Xiong, 2008). At chicken NMJs, the accumulation of soluble NRG1 at the synaptic basal lamina coincides with the concentration of heparin sulfate proteoglycans (HSPGs) (Loeb et al., 1999). Therefore, modes of function of NRG1 can be categorized into three types: 1) diffusion as soluble factors in the absence of HSPGs; 2) limited diffusion to cell surface by the interaction between heparin binding domain (HBD) and HSPGs; and 3) cell-cell contact of membrane-bound CRD forms. Before being immobilized by HSPGs, it is reasonable to speculate that released forms of NRG1 mainly function at distant areas by diffusion, and that subsequent gene expression in the target cells bearing the receptors depends on the concentration gradient and the dynamic of the gradient. Similar to some other diffusible morphogens, soluble NRG1 proteins are expressed in the early embryo. One possibility is that the concentration gradient forms early during embryonic development and is retained within the developing limbs to promote myoblast survival and differentiation, as during the early stage of NMJ development discussed in Chapter II. Of note, however, is the fact that several factors are required for NRG1 expression and release, such as retrograde signals from developing muscle target as discussed in Chapter III and the activation of PKC-δ signaling within the cell body and axons (Esper and Loeb, 2009). It remains unclear at which developmental stage the endogenous soluble NRG1 gradient can be achieved. Live examination of the distribution of soluble NRG1 from an early developmental stage through the later ones will provide insights to this issue. This currently remains a technical challenge, which is shared by all the research on soluble growth and differentiation factors.

The HBD of most soluble NRG1 is unique with a stand-alone C2 immunoglobulin domain that interacts with HSPGs (Ma et al., 2009). HSPGs serve as low-affinity targeting system to guide and accumulate the more specific NRG1-ErbB interaction to exert the biological functions. Thus, the second mode of NRG1 depends on the tissue-, temporal-, and spatial-specific expression pattern of HSPGs. The antagonist used in Chapter II is developed by fusing the HBD to extracellular domain of HER4 receptor that has been shown to block NRG1-ErbB signaling efficiently both *in vivo* and *in vitro* (Ma et al., 2009). However, the delivery method of the antagonist to chicken embryo is limited as discussed in Chapter II. It is also worthwhile to develop modifications of the antagonist with a traceable tag for accurate localization *in vivo*. Type III NRG1 remains membrane-bound after proteolytic cleavage, which makes it ideally positioned to function between contacting cells such as axons and myelinating Schwann cells.

It is interesting that the high concentration of NRG1 activates Erk signaling to inhibit Schwann cell myelination, while low concentration switches to the activation of PI3K/Akt that promotes myelination, suggesting a dosage-dependent differential activation of the downstream signaling pathways in Schwann cells (Syed et al., 2010). It would be interesting to examine if the concentration gradient of soluble NRG1 *in vivo* results in similar differential signaling pathway activation. Moreover, the roles of HSPGs in the concentration-dependent mode of function can be addressed by using HSPG-deficient cells or animals. It has been shown that the EGF-like domain of type III NRG1s can be liberated further after the first cleavage, resulting in type III NRG1 functioning both in a cell-cell contact manner, and in a paracrine manner, though the proportion of type III NRG1 undergoing further process remains unknown (Luo et al., 2011, Fleck et

al., 2013). Taken together, how the cooperative dynamics of different modes of function, distinct NRG1 isoforms, unique HSPGs in extracellular matrix, and specific cellular context account for NRG1 functions *in vivo* remains unclear and needs more investigation in the future. The complexity emphasizes the importance of understanding more about the mechanisms by which how isoforms of NRG1 are differentially expressed and regulated.

Human *NRG1* is located on chromosome 8p12, spanning about 1.2 Mbp in length. It has more than 20 exons and large introns, giving rise to over 30 protein isoforms and some long non-coding RNAs, which makes it one of the largest and the most complex genes in human genome (Rosenbloom et al., 2013). Both recruitment of multiple promoters and regulatory regions and usage of alternative splicing contribute the distinct spatial- and temporal-specific expression profiles of different isoforms. It was identified as one of the susceptibility genes of schizophrenia in various populations with most of the schizophrenia-associated SNPs in 5′ and 3′ flanking regions (Stefansson et al., 2002, Stefansson et al., 2003, Li et al., 2004b, Munafo et al., 2006). Type I NRG1 was increased in the hippocampus of schizophrenic patients, suggesting that the association between NRG1 transcription and schizophrenia is in an isoform-dependent fashion (Law et al., 2006). It has been found recently that Wallerian degeneration induced *de novo* type I NRG1 expression in Schwann cells to function as a paracrine signal (Stassart et al., 2013). The transcription of type I NRG1 was significantly increased by neuronal activity as well (Liu et al., 2011). Type I NRG1 was unregulated in both patients and animal models of amyotrophic lateral sclerosis (ALS) (Song et al., 2012). Outside the nervous system, type I NRG1 was specifically activated to mediate

hyperglycemic memory effects in breast cancer (Park et al., 2012). Additionally, work from the dissertation showed that neurotrophic factors can induce the transcription of type I NRG1 in a temporal-specific manner in mammalian ventral spinal cord cultures, whose kinetics closely resembled the ones of some activity-dependent genes (Sato et al., 2001). Taken together, it appears that this isoform is the most dynamic and subject to transcriptional regulation in various circumstances. Thus it is of great interest to understand more about the isoform-specific regulatory machinery. It has been shown that there is a putative enhancer region which is responsible for regulating type I NRG1 expression in breast cancer pathogenesis (Park et al., 2012). It will be interesting to examine if the enhancer functions similarly in the nervous system. Moreover, other DNase-sensitive clusters are enriched at ~-1k to ~+3k of type I NRG1, which has high density of H3K27Ac histone mark, H3K4Me3 histone mark, and transcriptional factor binding, all of which suggest that it is a putative regulatory region. As a portion of the potential regulatory region, the cis-element of type I tested in Chapter III was found essential for BDNF induction of type I transcription. It would be worthwhile to investigate if the whole region, especially the region within the first intron, is responsible for the binding of regulatory machinery and subsequent transcriptional induction. Furthermore, transcriptional factor binding assayed by ENCODE revealed that new transcriptional factors such as YY-1 and CTCF are present in the potential regulatory regions, which have not been associated with neurotrophic factor signaling before. Whether these transcriptional factors are new players in type I NRG1 expression requires further investigation.

Unlike in the CNS, damaged neurons in the PNS have an intrinsic ability to regenerate. In Wallerian degeneration following peripheral axotomy, axons regenerate as long as the neuronal cell body is intact and no underlying disease process is present. During peripheral nerve repair, NRG1 is known to be important for Schwann cells to alter the transcriptome, de-differentiate, proliferate, and secrete arrays of neurotrophic factors (Carroll et al., 1997, Fricker and Bennett, 2011, Fricker et al., 2011). Wallerian degeneration induced *de novo* type I NRG1 expression in Schwann cells themselves (Stassart et al., 2013). Highly inducible type I NRG1 from both axons and Schwann cells can function in concert to promote Schwann cell differentiation and remyelination. Considering that the injury induction of NRG1 is isoform-specific as well, it is of interest to understand if the underlying mechanisms are shared by the induction by neurotrophic factors. Interestingly, axon-derived NRG1 signaling seemed to inhibit NRG1 expression in Schwann cell during normal development (Stassart et al., 2013). This adds more complexity onto the reciprocal signaling between axons and Schwann cells mediated by NRG1 and neurotrophic factors. Considering that NRG1-ErbB signaling has been implicated in multiple aspects of skeletal muscle development and metabolism (Florini et al., 1996, Kim et al., 1999, Suarez et al., 2001, Hippenmeyer et al., 2002, Jacobson et al., 2004). It would be of interest to examine if disturbance to the neuromuscular system would trigger similar *de novo* expression in muscle cells in an isoform-dependent manner, and if axon-derived NRG1 inhibits NRG1 expression in muscles during development as well. The potential findings can shed light on therapeutic development to a variety of muscular diseases. During development, neurons provide NRG1 to support proliferation and differentiation of the glial and target cells; at the same

time, NRG1 signal inhibits the production of the same molecule in these cells. Glial and target cells secrete neurotrophic factors to promote neuronal survival and axon projection, and to stimulate rapid proteolytic processes of NRG1, resulting in the formation of a positive feedback loop. When perturbation such as peripheral injury occurs, the lack of NRG1 supply from neurons might induce the counterparts to produce the growth and differentiation factors, together with the production of the upstream regulator such as neurotrophic factors. The network resembles a natural robust system which requires more understanding in the future.

Both NRG1-ErbB and neurotrophic factors-Trk signaling have been shown to play critical roles in various and overlapping aspects in the CNS. Brain-derived neurotrophic factor (BDNF) facilitates hippocampus long-term potentiation (LTP), while NRG1-ErbB4 regulates the same process negatively (Patterson et al., 1996, Huang et al., 2000, Chen et al., 2010). It has been demonstrated that the expression of both BDNF and NRG1 isoforms were tightly regulated by neuronal activity (Flavell and Greenberg, 2008, Liu et al., 2011). The detailed link between these two signals in the central nervous system has not been investigated yet. It is reasonable to speculate that similar reciprocal signaling might exist in the CNS as well. Of note is the fact that activity-dependent transcription of BDNF has been shown to promote epileptogenesis (He et al., 2004), while *NRG1* mRNA increases in the hippocampus after a single electrical stimulation-induced seizure (Tan et al., 2012). BDNF might contribute to the induction of NRG1 signaling, which may serve as an endogenous negative-feedback signal to repress the neuronal excitation by activating inhibitory interneurons that bear the receptor of ErbB4 (Janssen et al., 2012). This scenario suggests that similar

reciprocal signaling might function distinctively depending on the environmental context. Recent study has also showed that NRG1 signaling is aberrant in pain after peripheral nerve injury and in the motor neuron disease ALS, both of which suggest that NRG1 signaling is involved in microgliosis (Calvo et al., 2010, Song et al., 2012). In both scenarios, damaged neurons are surrounded by pro-inflammatory cytokines and chemokines. It would be interesting to examine if there exists reciprocal signaling between NRG1 and these soluble factors which can mediate the function of inflammatory system. Therefore, fully understanding of the molecular interaction of NRG1 and neurotrophic factors, especially *in vivo*, is important to understand development of the nervous system and to develop more effective treatment for neurological diseases.

REFERENCES

Airaksinen MS, Saarma M (2002) The GDNF family: signalling, biological functions and therapeutic value. Nat Rev Neurosci 3:383-394.

Altiok N, Altiok S, Changeux JP (1997) Heregulin-stimulated acetylcholine receptor gene expression in muscle: requirement for MAP kinase and evidence for a parallel inhibitory pathway independent of electrical activity. EMBO Journal 16:717-725.

Araque A, Parpura V, Sanzgiri RP, Haydon PG (1999) Tripartite synapses: glia, the unacknowledged partner. Trends Neurosci 22:208-215.

Arber S, Burden SJ, Harris AJ (2002) Patterning of skeletal muscle. Curr Opin Neurobiol 12:100-103.

Auld DS, Robitaille R (2003a) Glial cells and neurotransmission: an inclusive view of synaptic function. Neuron 40:389-400.

Auld DS, Robitaille R (2003b) Perisynaptic Schwann cells at the neuromuscular junction: nerve- and activity-dependent contributions to synaptic efficacy, plasticity, and reinnervation. The Neuroscientist : a review journal bringing neurobiology, neurology and psychiatry 9:144-157.

Balice-Gordon RJ (1997) Age-related changes in neuromuscular innervation. Muscle & nerve Supplement 5:S83-87.

Beaumont TL, Yao B, Shah A, Kapatos G, Loeb JA (2012) Layer-specific CREB target gene induction in human neocortical epilepsy. J Neurosci 32:14389-14401.

Biffo S, Offenhauser N, Carter BD, Barde YA (1995) Selective binding and internalisation by truncated receptors restrict the availability of BDNF during development. Development 121:2461-2470.

Birchmeier C, Nave KA (2008) Neuregulin-1, a key axonal signal that drives Schwann cell growth and differentiation. Glia 56:1491-1497.

Brandon EP, Lin W, D'Amour KA, Pizzo DP, Dominguez B, Sugiura Y, Thode S, Ko CP, Thal LJ, Gage FH, Lee KF (2003) Aberrant patterning of neuromuscular synapses in choline acetyltransferase-deficient mice. J Neurosci 23:539-549.

Brenner HR, Witzemann V, Sakmann B (1990) Imprinting of acetylcholine receptor messenger RNA accumulation in mammalian neuromuscular synapses. Nature 344:544-547.

Britsch S, Li L, Kirchhoff S, Theuring F, Brinkmann V, Birchmeier C, Riethmacher D (1998) The ErbB2 and ErbB3 receptors and their ligand, neuregulin-1, are essential for development of the sympathetic nervous system. Genes Dev 12:1825-1836.

Buonanno A, Fischbach GD (2001) Neuregulin and ErbB receptor signaling pathways in the nervous system. Curr Opin Neurobiol 11:287-296.

Burden S (1977a) Acetylcholine receptors at the neuromuscular junction: developmental change in receptor turnover. Developmental biology 61:79-85.

Burden S (1977b) Development of the neuromuscular junction in the chick embryo: the number, distribution, and stability of acetylcholine receptors. Dev Biol 57:317-329.

Calvo M, Zhu N, Tsantoulas C, Ma Z, Grist J, Loeb JA, Bennett DL (2010) Neuregulin-ErbB signaling promotes microglial proliferation and chemotaxis contributing to microgliosis and pain after peripheral nerve injury. J Neurosci 30:5437-5450.

Carraway KL, 3rd, Weber JL, Unger MJ, Ledesma J, Yu N, Gassmann M, Lai C (1997) Neuregulin-2, a new ligand of ErbB3/ErbB4-receptor tyrosine kinases. Nature 387:512-516.

Carroll SL, Miller ML, Frohnert PW, Kim SS, Corbett JA (1997) Expression of neuregulins and their putative receptors, ErbB2 and ErbB3, is induced during Wallerian degeneration. J Neurosci 17:1642-1659.

Castonguay A, Robitaille R (2001) Differential regulation of transmitter release by presynaptic and glial Ca2+ internal stores at the neuromuscular synapse. J Neurosci 21:1911-1922.

Chen YJ, Zhang M, Yin DM, Wen L, Ting A, Wang P, Lu YS, Zhu XH, Li SJ, Wu CY, Wang XM, Lai C, Xiong WC, Mei L, Gao TM (2010) ErbB4 in parvalbumin-positive interneurons is critical for neuregulin 1 regulation of long-term potentiation. Proc Natl Acad Sci U S A 107:21818-21823.

Chowdary PD, Che DL, Cui B (2012) Neurotrophin signaling via long-distance axonal transport. Annual review of physical chemistry 63:571-594.

Chu GC, Moscoso LM, Sliwkowski MX, Merlie JP (1995) Regulation of the acetylcholine receptor epsilon subunit gene by recombinant ARIA: an in vitro model for transynaptic gene regulation. Neuron 14:329-339.

Citri A, Skaria KB, Yarden Y (2003) The deaf and the dumb: the biology of ErbB-2 and ErbB-3. Experimental cell research 284:54-65.

Court FA, Gillingwater TH, Melrose S, Sherman DL, Greenshields KN, Morton AJ, Harris JB, Willison HJ, Ribchester RR (2008) Identity, developmental restriction and reactivity of extralaminar cells capping mammalian neuromuscular junctions. J Cell Sci 121:3901-3911.

Courtney J, Steinbach JH (1981) Age changes in neuromuscular junction morphology and acetylcholine receptor distribution on rat skeletal muscle fibres. The Journal of physiology 320:435-447.

Dahm LM, Landmesser LT (1988) The regulation of intramuscular nerve branching during normal development and following activity blockade. Dev Biol 130:621-644.

Dale HH, Feldberg W, Vogt M (1936) Release of acetylcholine at voluntary motor nerve endings. The Journal of physiology 86:353-380.

de Kerchove D'Exaerde A, Cartaud J, Ravel-Chapuis A, Seroz T, Pasteau F, Angus LM, Jasmin BJ, Changeux JP, Schaeffer L (2002) Expression of mutant Ets protein at the neuromuscular synapse causes alterations in morphology and gene expression. EMBO reports 3:1075-1081.

DeChiara TM, Bowen DC, Valenzuela DM, Simmons MV, Poueymirou WT, Thomas S, Kinetz E, Compton DL, Rojas E, Park JS, Smith C, DiStefano PS, Glass DJ, Burden SJ, Yancopoulos GD (1996) The receptor tyrosine kinase MuSK is required for neuromuscular junction formation in vivo. Cell 85:501-512.

Dennis MJ (1981) Development of the neuromuscular junction: inductive interactions between cells. Annu Rev Neurosci 4:43-68.

Dong XP, Li XM, Gao TM, Zhang EE, Feng GS, Xiong WC, Mei L (2006) Shp2 is dispensable in the formation and maintenance of the neuromuscular junction. Neuro-Signals 15:53-63.

Dupuis L, Loeffler JP (2009) Neuromuscular junction destruction during amyotrophic lateral sclerosis: insights from transgenic models. Current opinion in pharmacology 9:341-346.

Eilam R, Pinkas-Kramarski R, Ratzkin BJ, Segal M, Yarden Y (1998) Activity-dependent regulation of Neu differentiation factor/neuregulin expression in rat brain. Proc Natl Acad Sci U S A 95:1888-1893.

Erickson SL, O'Shea KS, Ghaboosi N, Loverro L, Frantz G, Bauer M, Lu LH, Moore MW (1997) ErbB3 is required for normal cerebellar and cardiac development: a comparison with ErbB2-and heregulin-deficient mice. Development 124:4999-5011.

Escher P, Lacazette E, Courtet M, Blindenbacher A, Landmann L, Bezakova G, Lloyd KC, Mueller U, Brenner HR (2005) Synapses form in skeletal muscles lacking neuregulin receptors. Science 308:1920-1923.

Esper RM, Loeb JA (2004) Rapid axoglial signaling mediated by neuregulin and neurotrophic factors. J Neurosci 24:6218-6227.

Esper RM, Loeb JA (2009) Neurotrophins induce neuregulin release through protein kinase Cdelta activation. J Biol Chem 284:26251-26260.

Esper RM, Pankonin MS, Loeb JA (2006) Neuregulins: versatile growth and differentiation factors in nervous system development and human disease. Brain research reviews 51:161-175.

Esteban PF, Yoon HY, Becker J, Dorsey SG, Caprari P, Palko ME, Coppola V, Saragovi HU, Randazzo PA, Tessarollo L (2006) A kinase-deficient TrkC receptor isoform activates Arf6-Rac1 signaling through the scaffold protein tamalin. J Cell Biol 173:291-299.

Falls DL (2003) Neuregulins: functions, forms, and signaling strategies. Exp Cell Res 284:14-30.

Falls DL, Rosen KM, Corfas G, Lane WS, Fischbach GD (1993) ARIA, a protein that stimulates acetylcholine receptor synthesis, is a member of the neu ligand family. Cell 72:801-815.

Feng G, Laskowski MB, Feldheim DA, Wang H, Lewis R, Frisen J, Flanagan JG, Sanes JR (2000) Roles for ephrins in positionally selective synaptogenesis between motor neurons and muscle fibers. Neuron 25:295-306.

Fischbach GD, Rosen KM (1997) ARIA: a neuromuscular junction neuregulin. Annu Rev Neurosci 20:429-458.

Flavell SW, Greenberg ME (2008) Signaling mechanisms linking neuronal activity to gene expression and plasticity of the nervous system. Annu Rev Neurosci 31:563-590.

Fleck D, van Bebber F, Colombo A, Galante C, Schwenk BM, Rabe L, Hampel H, Novak B, Kremmer E, Tahirovic S, Edbauer D, Lichtenthaler SF, Schmid B, Willem M, Haass C (2013) Dual Cleavage of Neuregulin 1 Type III by BACE1 and ADAM17 Liberates Its EGF-Like Domain and Allows Paracrine Signaling. J Neurosci 33:7856-7869.

Florini JR, Samuel DS, Ewton DZ, Kirk C, Sklar RM (1996) Stimulation of myogenic differentiation by a neuregulin, glial growth factor 2. Are neuregulins the long-sought muscle trophic factors secreted by nerves? J Biol Chem 271:12699-12702.

Fox MA, Sanes JR, Borza DB, Eswarakumar VP, Fassler R, Hudson BG, John SW, Ninomiya Y, Pedchenko V, Pfaff SL, Rheault MN, Sado Y, Segal Y, Werle MJ, Umemori H (2007) Distinct target-derived signals organize formation, maturation, and maintenance of motor nerve terminals. Cell 129:179-193.

Frensing T, Kaltschmidt C, Schmitt-John T (2008) Characterization of a neuregulin-1 gene promoter: positive regulation of type I isoforms by NF-kappaB. Biochim Biophys Acta 1779:139-144.

Fricker FR, Bennett DL (2011) The role of neuregulin-1 in the response to nerve injury. Future neurology 6:809-822.

Fricker FR, Lago N, Balarajah S, Tsantoulas C, Tanna S, Zhu N, Fageiry SK, Jenkins M, Garratt AN, Birchmeier C, Bennett DL (2011) Axonally derived neuregulin-1 is required for remyelination and regeneration after nerve injury in adulthood. J Neurosci 31:3225-3233.

Funakoshi H, Frisen J, Barbany G, Timmusk T, Zachrisson O, Verge VM, Persson H (1993) Differential expression of mRNAs for neurotrophins and their receptors after axotomy of the sciatic nerve. J Cell Biol 123:455-465.

Gassmann M, Casagranda F, Orioli D, Simon H, Lai C, Klein R, Lemke G (1995) Aberrant neural and cardiac development in mice lacking the ErbB4 neuregulin receptor. Nature 378:390-394.

Gautam M, Noakes PG, Moscoso L, Rupp F, Scheller RH, Merlie JP, Sanes JR (1996) Defective neuromuscular synaptogenesis in agrin-deficient mutant mice. Cell 85:525-535.

George L, Chaverra M, Todd V, Lansford R, Lefcort F (2007) Nociceptive sensory neurons derive from contralaterally migrating, fate-restricted neural crest cells. Nat Neurosci 10:1287-1293.

Georgiou J, Charlton MP (1999) Non-myelin-forming perisynaptic schwann cells express protein zero and myelin-associated glycoprotein. Glia 27:101-109.

Haase G, Dessaud E, Garces A, de Bovis B, Birling M, Filippi P, Schmalbruch H, Arber S, deLapeyriere O (2002) GDNF acts through PEA3 to regulate cell body positioning and muscle innervation of specific motor neuron pools. Neuron 35:893-905.

Hall ZW, Sanes JR (1993) Synaptic structure and development: the neuromuscular junction. Cell 72 Suppl:99-121.

Hamburger V, Hamilton HL (1951) A series of normal stages in the development of the chick embryo. J Morph 88:49-92.

Harari D, Tzahar E, Romano J, Shelly M, Pierce JH, Andrews GC, Yarden Y (1999) Neuregulin-4: a novel growth factor that acts through the ErbB-4 receptor tyrosine kinase. Oncogene 18:2681-2689.

Harrington AW, Ginty DD (2013) Long-distance retrograde neurotrophic factor signalling in neurons. Nat Rev Neurosci 14:177-187.

Hayworth CR, Moody SE, Chodosh LA, Krieg P, Rimer M, Thompson WJ (2006) Induction of neuregulin signaling in mouse schwann cells in vivo mimics responses to denervation. J Neurosci 26:6873-6884.

He XP, Kotloski R, Nef S, Luikart BW, Parada LF, McNamara JO (2004) Conditional deletion of TrkB but not BDNF prevents epileptogenesis in the kindling model. Neuron 43:31-42.

Henderson CE, Camu W, Mettling C, Gouin A, Poulsen K, Karihaloo M, Rullamas J, Evans T, McMahon SB, Armanini MP, et al. (1993) Neurotrophins promote motor neuron survival and are present in embryonic limb bud. Nature 363:266-270.

Henderson CE, Phillips HS, Pollock RA, Davies AM, Lemeulle C, Armanini M, Simmons L, Moffet B, Vandlen RA, Simpson LC, et al. (1994) GDNF: a potent survival factor for motoneurons present in peripheral nerve and muscle. Science 266:1062-1064.

Hess A (1967) The structure of vertebrate slow and twitch muscle fibers. Investigative ophthalmology 6:217-228.

Hess DM, Scott MO, Potluri S, Pitts EV, Cisterni C, Balice-Gordon RJ (2007) Localization of TrkC to Schwann cells and effects of neurotrophin-3 signaling at neuromuscular synapses. The Journal of comparative neurology 501:465-482.

Hippenmeyer S, Shneider NA, Birchmeier C, Burden SJ, Jessell TM, Arber S (2002) A role for neuregulin1 signaling in muscle spindle differentiation. Neuron 36:1035-1049.

Ho WH, Armanini MP, Nuijens A, Phillips HS, Osheroff PL (1995) Sensory and motor neuron-derived factor. A novel heregulin variant highly expressed in sensory and motor neurons. J Biol Chem 270:14523-14532.

Horiuchi K, Zhou HM, Kelly K, Manova K, Blobel CP (2005) Evaluation of the contributions of ADAMs 9, 12, 15, 17, and 19 to heart development and ectodomain shedding of neuregulins beta1 and beta2. Dev Biol 283:459-471.

Hu X, Hicks CW, He W, Wong P, Macklin WB, Trapp BD, Yan R (2006) Bace1 modulates myelination in the central and peripheral nervous system. Nat Neurosci 9:1520-1525.

Huang EJ, Reichardt LF (2001) Neurotrophins: roles in neuronal development and function. Annu Rev Neurosci 24:677-736.

Huang EJ, Reichardt LF (2003) Trk receptors: roles in neuronal signal transduction. Annual review of biochemistry 72:609-642.

Huang YZ, Won S, Ali DW, Wang Q, Tanowitz M, Du QS, Pelkey KA, Yang DJ, Xiong WC, Salter MW, Mei L (2000) Regulation of neuregulin signaling by PSD-95 interacting with ErbB4 at CNS synapses. Neuron 26:443-455.

Ip FC, Cheung J, Ip NY (2001) The expression profiles of neurotrophins and their receptors in rat and chicken tissues during development. Neurosci Lett 301:107-110.

Jacobson C, Duggan D, Fischbach G (2004) Neuregulin induces the expression of transcription factors and myosin heavy chains typical of muscle spindles in cultured human muscle. Proc Natl Acad Sci U S A 101:12218-12223.

Jahromi BS, Robitaille R, Charlton MP (1992) Transmitter release increases intracellular calcium in perisynaptic Schwann cells in situ. Neuron 8:1069-1077.

Janssen MJ, Leiva-Salcedo E, Buonanno A (2012) Neuregulin directly decreases voltage-gated sodium current in hippocampal ErbB4-expressing interneurons. J Neurosci 32:13889-13895.

Jaworski A, Burden SJ (2006) Neuromuscular synapse formation in mice lacking motor neuron- and skeletal muscle-derived Neuregulin-1. J Neurosci 26:655-661.

Jessell TM, Siegel RE, Fischbach GD (1979) Induction of acetylcholine receptors on cultured skeletal muscle by a factor extracted from brain and spinal cord. Proc Natl Acad Sci U S A 76:5397-5401.

Kao SC, Wu H, Xie J, Chang CP, Ranish JA, Graef IA, Crabtree GR (2009) Calcineurin/NFAT signaling is required for neuregulin-regulated Schwann cell differentiation. Science 323:651-654.

Katz B (1966) Nerve, muscle, and synapse. New York: McGraw-Hill.

Kim D, Chi S, Lee KH, Rhee S, Kwon YK, Chung CH, Kwon H, Kang MS (1999) Neuregulin stimulates myogenic differentiation in an autocrine manner. J Biol Chem 274:15395-15400.

Kim N, Stiegler AL, Cameron TO, Hallock PT, Gomez AM, Huang JH, Hubbard SR, Dustin ML, Burden SJ (2008) Lrp4 is a receptor for Agrin and forms a complex with MuSK. Cell 135:334-342.

Koliatsos VE, Clatterbuck RE, Winslow JW, Cayouette MH, Price DL (1993) Evidence that brain-derived neurotrophic factor is a trophic factor for motor neurons in vivo. Neuron 10:359-367.

Kramer R, Bucay N, Kane DJ, Martin LE, Tarpley JE, Theill LE (1996) Neuregulins with an Ig-like domain are essential for mouse myocardial and neuronal development. Proc Natl Acad Sci U S A 93:4833-4838.

Krull CE (2004) A primer on using in ovo electroporation to analyze gene function. Developmental dynamics : an official publication of the American Association of Anatomists 229:433-439.

Kummer TT, Misgeld T, Sanes JR (2006) Assembly of the postsynaptic membrane at the neuromuscular junction: paradigm lost. Curr Opin Neurobiol 16:74-82.

Kwong WH, Gauthier GF (1987) Neuromuscular junctions in adult and developing fast and slow muscles. The Anatomical record 219:409-419.

La Marca R, Cerri F, Horiuchi K, Bachi A, Feltri ML, Wrabetz L, Blobel CP, Quattrini A, Salzer JL, Taveggia C (2011) TACE (ADAM17) inhibits Schwann cell myelination. Nat Neurosci 14:857-865.

Lai KO, Ip NY (2003) Central synapse and neuromuscular junction: same players, different roles. Trends in genetics : TIG 19:395-402.

Law AJ, Lipska BK, Weickert CS, Hyde TM, Straub RE, Hashimoto R, Harrison PJ, Kleinman JE, Weinberger DR (2006) Neuregulin 1 transcripts are differentially expressed in schizophrenia and regulated by 5' SNPs associated with the disease. Proc Natl Acad Sci U S A 103:6747-6752.

Lee KF, Simon H, Chen H, Bates B, Hung MC, Hauser C (1995) Requirement for neuregulin receptor erbB2 in neural and cardiac development. Nature 378:394-398.

Lefcort F, Clary DO, Rusoff AC, Reichardt LF (1996) Inhibition of the NT-3 receptor TrkC, early in chick embryogenesis, results in severe reductions in multiple neuronal subpopulations in the dorsal root ganglia. J Neurosci 16:3704-3713.

Levi-Montalcini R, Calissano P (1979) The nerve-growth factor. Scientific American 240:68-77.

Li Q, Esper RM, Loeb JA (2004a) Synergistic effects of neuregulin and agrin on muscle acetylcholine receptor expression. Mol Cell Neurosci 26:558-569.

Li Q, Loeb JA (2001) Neuregulin-heparan-sulfate proteoglycan interactions produce sustained erbB receptor activation required for the induction of acetylcholine receptors in muscle. J Biol Chem 276:38068-38075.

Li T, Stefansson H, Gudfinnsson E, Cai G, Liu X, Murray RM, Steinthorsdottir V, Januel D, Gudnadottir VG, Petursson H, Ingason A, Gulcher JR, Stefansson K, Collier DA (2004b) Identification of a novel neuregulin 1 at-risk haplotype in Han schizophrenia Chinese patients, but no association with the Icelandic/Scottish risk haplotype. Molecular psychiatry 9:698-704.

Li XM, Dong XP, Luo SW, Zhang B, Lee DH, Ting AK, Neiswender H, Kim CH, Carpenter-Hyland E, Gao TM, Xiong WC, Mei L (2008) Retrograde regulation of motoneuron differentiation by muscle beta-catenin. Nature neuroscience 11:262-268.

Liu X, Bates R, Yin DM, Shen C, Wang F, Su N, Kirov SA, Luo Y, Wang JZ, Xiong WC, Mei L (2011) Specific regulation of NRG1 isoform expression by neuronal activity. J Neurosci 31:8491-8501.

Liu X, Hwang H, Cao L, Buckland M, Cunningham A, Chen J, Chien KR, Graham RM, Zhou M (1998) Domain-specific gene disruption reveals critical regulation of neuregulin signaling by its cytoplasmic tail. Proc Natl Acad Sci U S A 95:13024-13029.

Loeb JA (2003) Neuregulin: an activity-dependent synaptic modulator at the neuromuscular junction. J Neurocytol 32:649-664.

Loeb JA, Fischbach GD (1995) ARIA can be released from extracellular matrix through cleavage of a heparin-binding domain. J Cell Biol 130:127-135.

Loeb JA, Fischbach GD (1997) Neurotrophic factors increase neuregulin expression in embryonic ventral spinal cord neurons. Journal of Neuroscience 17:1416-1424.

Loeb JA, Hmadcha A, Fischbach GD, Land SJ, Zakarian VL (2002) Neuregulin expression at neuromuscular synapses is modulated by synaptic activity and neurotrophic factors. J Neurosci 22:2206-2214.

Loeb JA, Khurana TS, Robbins JT, Yee AG, Fischbach GD (1999) Expression patterns of transmembrane and released forms of neuregulin during spinal cord and neuromuscular synapse development. Development 126:781-791.

Loeb JA, Susanto ET, Fischbach GD (1998) The neuregulin precursor proARIA is processed to ARIA after expression on the cell surface by a protein kinase C-enhanced mechanism. Mol Cell Neurosci 11:77-91.

Luo X, Prior M, He W, Hu X, Tang X, Shen W, Yadav S, Kiryu-Seo S, Miller R, Trapp BD, Yan R (2011) Cleavage of neuregulin-1 by BACE1 or ADAM10 protein produces differential effects on myelination. J Biol Chem 286:23967-23974.

Ma Z, Li Q, An H, Pankonin MS, Wang J, Loeb JA (2009) Targeting HER singaling with neuregulin's heparin-binding domain. J Biol Chem.

Ma Z, Wang J, Song F, Loeb JA (2011) Critical period of axoglial signaling between neuregulin-1 and brain-derived neurotrophic factor required for early Schwann cell survival and differentiation. J Neurosci 31:9630-9640.

Mann MA, Das S, Zhang J, Wagner M, Fischbach GD (2006) Neuregulin effect on quantal content dissociated from effect on miniature endplate potential amplitude. J Neurophysiol 96:671-676.

Marquardt T, Shirasaki R, Ghosh S, Andrews SE, Carter N, Hunter T, Pfaff SL (2005) Coexpressed EphA receptors and ephrin-A ligands mediate opposing actions on growth cone navigation from distinct membrane domains. Cell 121:127-139.

Martinou JC, Falls DL, Fischbach GD, Merlie JP (1991) Acetylcholine receptor-inducing activity stimulates expression of the epsilon-subunit gene of the muscle acetylcholine receptor. Proc Natl Acad Sci U S A 88:7669-7673.

Mei L, Xiong WC (2008) Neuregulin 1 in neural development, synaptic plasticity and schizophrenia. Nat Rev Neurosci 9:437-452.

Meier T, Masciulli F, Moore C, Schoumacher F, Eppenberger U, Denzer AJ, Jones G, Brenner HR (1998) Agrin can mediate acetylcholine receptor gene expression in muscle by aggregation of muscle-derived neuregulins. J Cell Biol 141:715-726.

Meyer D, Birchmeier C (1995) Multiple essential functions of neuregulin in development. Nature 378:386-390.

Meyer D, Yamaai T, Garratt A, Riethmacher-Sonnenberg E, Kane D, Theill LE, Birchmeier C (1997) Isoform-specific expression and function of neuregulin. Development 124:3575-3586.

Michailov GV, Sereda MW, Brinkmann BG, Fischer TM, Haug B, Birchmeier C, Role L, Lai C, Schwab MH, Nave KA (2004) Axonal neuregulin-1 regulates myelin sheath thickness. Science 304:700-703.

Misgeld T, Burgess RW, Lewis RM, Cunningham JM, Lichtman JW, Sanes JR (2002) Roles of neurotransmitter in synapse formation: development of neuromuscular junctions lacking choline acetyltransferase. Neuron 36:635-648.

Misgeld T, Kummer TT, Lichtman JW, Sanes JR (2005) Agrin promotes synaptic differentiation by counteracting an inhibitory effect of neurotransmitter. Proceedings of the National Academy of Sciences of the United States of America 102:11088-11093.

Montero JC, Rodriguez-Barrueco R, Yuste L, Juanes PP, Borges J, Esparis-Ogando A, Pandiella A (2007) The extracellular linker of pro-neuregulin-alpha2c is required for efficient sorting and juxtacrine function. Mol Biol Cell 18:380-393.

Moore MW, Klein RD, Farinas I, Sauer H, Armanini M, Phillips H, Reichardt LF, Ryan AM, Carver-Moore K, Rosenthal A (1996) Renal and neuronal abnormalities in mice lacking GDNF. Nature 382:76-79.

Morris JK, Lin W, Hauser C, Marchuk Y, Getman D, Lee KF (1999) Rescue of the cardiac defect in ErbB2 mutant mice reveals essential roles of ErbB2 in peripheral nervous system development. Neuron 23:273-283.

Moscoso LM, Chu GC, Gautam M, Noakes PG, Merlie JP, Sanes JR (1995) Synapse-associated expression of an acetylcholine receptor-inducing protein, ARIA/heregulin, and its putative receptors, ErbB2 and ErbB3, in developing mammalian muscle. Dev Biol 172:158-169.

Munafo MR, Thiselton DL, Clark TG, Flint J (2006) Association of the NRG1 gene and schizophrenia: a meta-analysis. Molecular psychiatry 11:539-546.

Nave KA, Salzer JL (2006) Axonal regulation of myelination by neuregulin 1. Curr Opin Neurobiol 16:492-500.

Ngo ST, Cole RN, Sunn N, Phillips WD, Noakes PG (2012) Neuregulin-1 potentiates agrin-induced acetylcholine receptor clustering through muscle-specific kinase phosphorylation. J Cell Sci 125:1531-1543.

Oppenheim RW (1989) The neurotrophic theory and naturally occurring motoneuron death. Trends Neurosci 12:252-255.

Oppenheim RW, Houenou LJ, Parsadanian AS, Prevette D, Snider WD, Shen L (2000) Glial cell line-derived neurotrophic factor and developing mammalian motoneurons: regulation of programmed cell death among motoneuron subtypes. J Neurosci 20:5001-5011.

Park J, Sarode VR, Euhus D, Kittler R, Scherer PE (2012) Neuregulin 1-HER axis as a key mediator of hyperglycemic memory effects in breast cancer. Proc Natl Acad Sci U S A 109:21058-21063.

Patterson SL, Abel T, Deuel TA, Martin KC, Rose JC, Kandel ER (1996) Recombinant BDNF rescues deficits in basal synaptic transmission and hippocampal LTP in BDNF knockout mice. Neuron 16:1137-1145.

Ponomareva ON, Ma H, Vock VM, Ellerton EL, Moody SE, Dakour R, Chodosh LA, Rimer M (2006) Defective neuromuscular synaptogenesis in mice expressing constitutively active ErbB2 in skeletal muscle fibers. Mol Cell Neurosci 31:334-345.

Ramón y Cajal S (1928) Degeneration & regeneration of the nervous system. London: Oxford University Press.

Reddy LV, Koirala S, Sugiura Y, Herrera AA, Ko CP (2003) Glial cells maintain synaptic structure and function and promote development of the neuromuscular junction in vivo. Neuron 40:563-580.

Reist NE, Smith SJ (1992) Neurally evoked calcium transients in terminal Schwann cells at the neuromuscular junction. Proc Natl Acad Sci U S A 89:7625-7629.

Riethmacher D, Sonnenberg-Riethmacher E, Brinkmann V, Yamaai T, Lewin GR, Birchmeier C (1997) Severe neuropathies in mice with targeted mutations in the ErbB3 receptor. Nature 389:725-730.

Rifkin JT, Todd VJ, Anderson LW, Lefcort F (2000) Dynamic expression of neurotrophin receptors during sensory neuron genesis and differentiation. Dev Biol 227:465-480.

Robitaille R (1998) Modulation of synaptic efficacy and synaptic depression by glial cells at the frog neuromuscular junction. Neuron 21:847-855.

Rosenbloom KR, Sloan CA, Malladi VS, Dreszer TR, Learned K, Kirkup VM, Wong MC, Maddren M, Fang R, Heitner SG, Lee BT, Barber GP, Harte RA, Diekhans M, Long JC, Wilder SP, Zweig AS, Karolchik D, Kuhn RM, Haussler D, Kent WJ

(2013) ENCODE data in the UCSC Genome Browser: year 5 update. Nucleic acids research 41:D56-63.

Sandrock AW, Jr., Dryer SE, Rosen KM, Gozani SN, Kramer R, Theill LE, Fischbach GD (1997) Maintenance of acetylcholine receptor number by neuregulins at the neuromuscular junction in vivo. Science 276:599-603.

Sanes JR, Apel ED, Burgess RW, Emerson RB, Feng G, Gautam M, Glass D, Grady RM, Krejci E, Lichtman JW, Lu JT, Massoulie J, Miner JH, Moscoso LM, Nguyen Q, Nichol M, Noakes PG, Patton BL, Son YJ, Yancopoulos GD, Zhou H (1998) Development of the neuromuscular junction: genetic analysis in mice. Journal of physiology, Paris 92:167-172.

Sanes JR, Lichtman JW (1999a) Development of the vertebrate neuromuscular junction. Annu Rev Neurosci 22:389-442.

Sanes JR, Lichtman JW (1999b) Development of the vertebrate neuromuscular junction. Annu Rev Neurosci 22:389-342.

Sanes JR, Lichtman JW (2001) Induction, assembly, maturation and maintenance of a postsynaptic apparatus. Nat Rev Neurosci 2:791-805.

Sapru MK (2001) Neuregulin-1 regulates expression of the Ets-2 transcription factor. Life Sci 69:2663-2674.

Sapru MK, Florance SK, Kirk C, Goldman D (1998) Identification of a neuregulin and protein-tyrosine phosphatase response element in the nicotinic acetylcholine receptor epsilon subunit gene: regulatory role of an Rts transcription factor. Proc Natl Acad Sci U S A 95:1289-1294.

Sato M, Suzuki K, Nakanishi S (2001) NMDA receptor stimulation and brain-derived neurotrophic factor up-regulate homer 1a mRNA via the mitogen-activated protein kinase cascade in cultured cerebellar granule cells. J Neurosci 21:3797-3805.

Schaefer AM, Sanes JR, Lichtman JW (2005) A compensatory subpopulation of motor neurons in a mouse model of amyotrophic lateral sclerosis. J Comp Neurol 490:209-219.

Schaeffer L, de Kerchove d'Exaerde A, Changeux JP (2001) Targeting transcription to the neuromuscular synapse. Neuron 31:15-22.

Schaeffer L, Duclert N, Huchet-Dymanus M, Changeux JP (1998) Implication of a multisubunit Ets-related transcription factor in synaptic expression of the nicotinic acetylcholine receptor. EMBO J 17:3078-3090.

Schmidt N, Akaaboune M, Gajendran N, Martinez-Pena y Valenzuela I, Wakefield S, Thurnheer R, Brenner HR (2011) Neuregulin/ErbB regulate neuromuscular junction development by phosphorylation of alpha-dystrobrevin. J Cell Biol 195:1171-1184.

Segal RA (2003) Selectivity in neurotrophin signaling: theme and variations. Annu Rev Neurosci 26:299-330.

Sharma N, Deppmann CD, Harrington AW, St Hillaire C, Chen ZY, Lee FS, Ginty DD (2010) Long-distance control of synapse assembly by target-derived NGF. Neuron 67:422-434.

Shirakabe K, Wakatsuki S, Kurisaki T, Fujisawa-Sehara A (2001) Roles of Meltrin beta /ADAM19 in the processing of neuregulin. J Biol Chem 276:9352-9358.

Si J, Luo Z, Mei L (1996) Induction of acetylcholine receptor gene expression by ARIA requires activation of mitogen-activated protein kinase. J Biol Chem 271:19752-19759.

Si J, Wang Q, Mei L (1999) Essential roles of c-JUN and c-JUN N-terminal kinase (JNK) in neuregulin-increased expression of the acetylcholine receptor epsilon-subunit. J Neurosci 19:8498-8508.

Simeone L, Straubinger M, Khan MA, Nalleweg N, Cheusova T, Hashemolhosseini S (2010) Identification of Erbin interlinking MuSK and ErbB2 and its impact on acetylcholine receptor aggregation at the neuromuscular junction. J Neurosci 30:6620-6634.

Song F, Chiang P, Wang J, Ravits J, Loeb JA (2012) Aberrant neuregulin 1 signaling in amyotrophic lateral sclerosis. Journal of neuropathology and experimental neurology 71:104-115.

Stassart RM, Fledrich R, Velanac V, Brinkmann BG, Schwab MH, Meijer D, Sereda MW, Nave KA (2013) A role for Schwann cell-derived neuregulin-1 in remyelination. Nat Neurosci 16:48-54.

Stefansson H, Sarginson J, Kong A, Yates P, Steinthorsdottir V, Gudfinnsson E, Gunnarsdottir S, Walker N, Petursson H, Crombie C, Ingason A, Gulcher JR, Stefansson K, St Clair D (2003) Association of neuregulin 1 with schizophrenia confirmed in a Scottish population. Am J Hum Genet 72:83-87.

Stefansson H, Sigurdsson E, Steinthorsdottir V, Bjornsdottir S, Sigmundsson T, Ghosh S, Brynjolfsson J, Gunnarsdottir S, Ivarsson O, Chou TT, Hjaltason O, Birgisdottir B, Jonsson H, Gudnadottir VG, Gudmundsdottir E, Bjornsson A, Ingvarsson B,

Ingason A, Sigfusson S, Hardardottir H, Harvey RP, Lai D, Zhou M, Brunner D, Mutel V, Gonzalo A, Lemke G, Sainz J, Johannesson G, Andresson T, Gudbjartsson D, Manolescu A, Frigge ML, Gurney ME, Kong A, Gulcher JR, Petursson H, Stefansson K (2002) Neuregulin 1 and susceptibility to schizophrenia. Am J Hum Genet 71:877-892.

Steinthorsdottir V, Stefansson H, Ghosh S, Birgisdottir B, Bjornsdottir S, Fasquel AC, Olafsson O, Stefansson K, Gulcher JR (2004) Multiple novel transcription initiation sites for NRG1. Gene 342:97-105.

Suarez E, Bach D, Cadefau J, Palacin M, Zorzano A, Guma A (2001) A novel role of neuregulin in skeletal muscle. Neuregulin stimulates glucose uptake, glucose transporter translocation, and transporter expression in muscle cells. J Biol Chem 276:18257-18264.

Sugiura Y, Lin W (2011) Neuron-glia interactions: the roles of Schwann cells in neuromuscular synapse formation and function. Bioscience reports 31:295-302.

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

Tan GH, Liu YY, Hu XL, Yin DM, Mei L, Xiong ZQ (2012) Neuregulin 1 represses limbic epileptogenesis through ErbB4 in parvalbumin-expressing interneurons. Nat Neurosci 15:258-266.

Tan W, Wang Y, Gold B, Chen J, Dean M, Harrison PJ, Weinberger DR, Law AJ (2007) Molecular cloning of a brain-specific, developmentally regulated neuregulin 1

(NRG1) isoform and identification of a functional promoter variant associated with schizophrenia. J Biol Chem 282:24343-24351.

Tansey MG, Chu GC, Merlie JP (1996) ARIA/HRG regulates AChR epsilon subunit gene expression at the neuromuscular synapse via activation of phosphatidylinositol 3-kinase and Ras/MAPK pathway. J Cell Biol 134:465-476.

Tao Y, Dai P, Liu Y, Marchetto S, Xiong WC, Borg JP, Mei L (2009) Erbin regulates NRG1 signaling and myelination. Proc Natl Acad Sci U S A 106:9477-9482.

Tapia JC, Wylie JD, Kasthuri N, Hayworth KJ, Schalek R, Berger DR, Guatimosim C, Seung HS, Lichtman JW (2012) Pervasive synaptic branch removal in the mammalian neuromuscular system at birth. Neuron 74:816-829.

Taveggia C, Zanazzi G, Petrylak A, Yano H, Rosenbluth J, Einheber S, Xu X, Esper RM, Loeb JA, Shrager P, Chao MV, Falls DL, Role L, Salzer JL (2005) Neuregulin-1 type III determines the ensheathment fate of axons. Neuron 47:681-694.

Tosney KW, Landmesser LT (1985) Development of the major pathways for neurite outgrowth in the chick hindlimb. Dev Biol 109:193-214.

Trachtenberg JT, Thompson WJ (1996) Schwann cell apoptosis at developing neuromuscular junctions is regulated by glial growth factor. Nature 379:174-177.

Trachtenberg JT, Thompson WJ (1997) Nerve terminal withdrawal from rat neuromuscular junctions induced by neuregulin and Schwann cells. J Neurosci 17:6243-6255.

Trinidad JC, Fischbach GD, Cohen JB (2000) The Agrin/MuSK signaling pathway is spatially segregated from the neuregulin/ErbB receptor signaling pathway at the neuromuscular junction. J Neurosci 20:8762-8770.

Valdez G, Tapia JC, Kang H, Clemenson GD, Jr., Gage FH, Lichtman JW, Sanes JR (2010) Attenuation of age-related changes in mouse neuromuscular synapses by caloric restriction and exercise. Proc Natl Acad Sci U S A 107:14863-14868.

Vartanian T, Goodearl A, Lefebvre S, Park SK, Fischbach G (2000) Neuregulin induces the rapid association of focal adhesion kinase with the erbB2-erbB3 receptor complex in schwann cells. Biochem Biophys Res Commun 271:414-417.

Wang G, Scott SA (2000) The "Waiting Period" of Sensory and Motor Axons in Early Chick Hindlimb: Its Role in Axon Pathfinding and Neuronal Maturation. J Neurosci 20:5358-5366.

Wang JY, Miller SJ, Falls DL (2001) The N-terminal region of neuregulin isoforms determines the accumulation of cell surface and released neuregulin ectodomain. J Biol Chem 276:2841-2851.

Watson FL, Heerssen HM, Bhattacharyya A, Klesse L, Lin MZ, Segal RA (2001) Neurotrophins use the Erk5 pathway to mediate a retrograde survival response. Nat Neurosci 4:981-988.

Willem M, Garratt AN, Novak B, Citron M, Kaufmann S, Rittger A, Destrooper B, Saftig P, Birchmeier C, Haass C (2006) Control of Peripheral Nerve Myelination by the {beta}-Secretase BACE1. Science.

Wilson MH, Deschenes MR (2005) The neuromuscular junction: anatomical features and adaptations to various forms of increased, or decreased neuromuscular activity. The International journal of neuroscience 115:803-828.

Winseck AW, Caldero J, Ciutat D, Prevette D, Scott SA, Wang G, Esquerda JE, Oppenheim RW (2002) In vivo analysis of Schwann cell programmed cell death in the embryonic chick: Regulation by axons and glial growth factor. Journal of Neuroscience 22:4509-4521.

Wolpowitz D, Mason TB, Dietrich P, Mendelsohn M, Talmage DA, Role LW (2000) Cysteine-rich domain isoforms of the neuregulin-1 gene are required for maintenance of peripheral synapses. Neuron 25:79-91.

Wright DE, Snider WD (1995) Neurotrophin receptor mRNA expression defines distinct populations of neurons in rat dorsal root ganglia. J Comp Neurol 351:329-338.

Yacoubian TA, Lo DC (2000) Truncated and full-length TrkB receptors regulate distinct modes of dendritic growth. Nat Neurosci 3:342-349.

Yan Q, Elliott J, Snider WD (1992) Brain-derived neurotrophic factor rescues spinal motor neurons from axotomy-induced cell death. Nature 360:753-755.

Yang X, Kuo Y, Devay P, Yu C, Role L (1998) A cysteine-rich isoform of neuregulin controls the level of expression of neuronal nicotinic receptor channels during synaptogenesis. Neuron 20:255-270.

Yarden Y, Sliwkowski MX (2001) Untangling the ErbB signalling network. Nature reviews Molecular cell biology 2:127-137.

Yokozeki T, Wakatsuki S, Hatsuzawa K, Black RA, Wada I, Sehara-Fujisawa A (2007) Meltrin beta (ADAM19) mediates ectodomain shedding of Neuregulin beta1 in

the Golgi apparatus: fluorescence correlation spectroscopic observation of the dynamics of ectodomain shedding in living cells. Genes Cells 12:329-343.

Zhang B, Luo S, Wang Q, Suzuki T, Xiong WC, Mei L (2008) LRP4 serves as a coreceptor of agrin. Neuron 60:285-297.

Zhang D, Sliwkowski MX, Mark M, Frantz G, Akita R, Sun Y, Hillan K, Crowley C, Brush J, Godowski PJ (1997) Neuregulin-3 (NRG3): a novel neural tissue-enriched protein that binds and activates ErbB4. Proc Natl Acad Sci U S A 94:9562-9567.

Zhu X, Lai C, Thomas S, Burden SJ (1995) Neuregulin receptors, erbB3 and erbB4, are localized at neuromuscular synapses. EMBO Journal 14:5842-5848.

Zweifel LS, Kuruvilla R, Ginty DD (2005) Functions and mechanisms of retrograde neurotrophin signalling. Nat Rev Neurosci 6:615-625.

ABSTRACT

ROLE OF NEUREGULIN1 IN NEUROMUSCULAR JUNCTION DEVELOPMENT

by

JIAJING WANG

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Advisor: Jeffrey A. Loeb, M.D., Ph.D.

Major: Molecular Biology and Genetics

Degree: Doctor of Philosophy

Neuromuscular junction (NMJ) development is a multistep process mediated by coordinated interactions between nerve terminals, target muscles, and peri-synaptic glial cells, and thus requires reciprocal signals derived from every cell type. Neuregulin1s (NRG1s) are a family of predominantly neuronal growth and differentiation factors that are important for many aspects of nervous system development. In this thesis, both the effects of NRG1 on NMJ development and reciprocal effects of neurotrophic factors on NRG1 expression were studied as a means to define the complex regulatory communication at the NMJ. Using the chicken embryo as a model, methods were developed to study the effects of NRG1 signaling on NMJ development *in ovo* using sequential, quantitative measures of NMJ formation including AChR cluster size and density, pre- and post-synaptic apposition, and alignment of peri-synaptic Schwann cells. Over-expression of soluble NRG1 increased AChR cluster density, but not size, in the early developmental stage, whereas, blocking NRG1 activity with a

targeted antagonist had little effect. In the middle stage, the NRG1 antagonist led to a decrease of AChR cluster size in a temporal-specific and muscle type-dependent fashion. The NRG1 antagonist also altered the distribution of synaptic vesicles, suggesting NRG1 signaling can modulate the assembly of the pre-synaptic apparatus as well. In the late stage, pre- and post-synaptic apposition, and peri-synaptic Schwann cell alignment were modestly affected by the antagonist. In motor neurons, NRG1 expression at the axonal-Schwann cell and neuromuscular junctions is regulated by synaptic activity and neurotrophic factors, however, little is known about the mechanisms that control NRG1 isoform-specific transcription. Here we show that NRG1 expression in the chick embryo increases in motor neurons that have extended their axons and that limb bud ablation before motor axon outgrowth prevents this induction, suggesting a trophic role from the developing limb. Consistently, NRG1 induction after limb bud ablation can be rescued by adding back the neurotrophic factors BDNF and GDNF. Mechanistically, BDNF induces a rapid and transient increase in types I and III NRG1 I mRNAs that peak at 4 h in rat embryonic ventral spinal cord cultures. Blocking MAPK or PI3K signaling or blocking transcription with Actinomycin D blocks BDNF induced NRG1 gene induction. BDNF had no effects on mRNA degradation, suggesting that transcriptional activation rather than message stability is important. Furthermore, BDNF activates a reporter construct that includes 700bp upstream of the type I NRG1 start site. Protein synthesis is also required for type I *NRG1* mRNA transcription as cycloheximide produced a super-induction of type I, but not type III *NRG1* mRNA, possibly through a mechanism involving sustained activation of MAPK and PI3K. These findings suggest that, while not critical for development, NRG1

signaling can have important roles in fine-tuning multiple stages of NMJ development and that NRG1 isoform expression can be differentially modulated by highly responsive, transient transcriptional regulatory mechanisms mediated by neurotrophic factors and axon-target interactions. Understanding these mechanisms will be important for elucidating the role of NRG1 in both development and in pathological disorders of the nervous system.

AUTOBIOGRAPHICAL STATEMENT

JIAJING WANG

Education

2006-2013	**Ph.D.**	Major: Molecular Biology and Genetics; Wayne State University School of Medicine
2001-2005	**B.S.**	Major: Life Sciences Fudan University

Publications

Peer-Reviewed Journal Articles

- Song F, Chuang P, **Wang J**, Ravits J, Loeb JA (2012) Aberrant neuregulin1 signaling in amyotrophic lateral sclerosis. ***Journal of Neuropathology & Experimental Neurology*** 71(2):104-15

- Ma Z, **Wang J**, Song F, Loeb JA (2011) Critical period of axoglial signaling between neuregulin-1 and brain-derived neurotrophic factor required for early Schwann cell survival and differentiation. ***Journal of Neuroscience*** 31(26):9630-40.

- Ma Z, Li Q, An H, Pankonin MS, **Wang J**, Loeb JA (2009) Targeting human epidermal growth factor receptor signaling with the neuregulin's heparin-binding domain. ***Journal of Biological Chemistry*** 284(46):32108-15.

Book Chapter

- **Wang J**, Ma Z, Loeb JA (2013) Cell-specific targeting of fusion proteins through heparin-binding. In Fusion Protein Technologies for Biopharmaceuticals: Application and Challenges. ***John Wiley & Sons, Inc. Hoboken, NJ.***