

Mini-Review

Neuregulins at the Neuromuscular Synapse: Past, Present, and Future

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At the developing vertebrate neuromuscular junction, neuregulins are growth/differentiation factors essential for terminal Schwann cell survival. Neuregulins have also been thought as the critical signals responsible for the increased transcription of acetylcholine receptor subunit genes at the neuromuscular synapse. This latter role is now highly controversial. This article reviews the evidence that has shaped the views of the neuregulins and how these views have been challenged. The most recent experiments indicate that neuregulin signaling to postsynaptic muscle fibers may modulate, rather than determine, acetylcholine receptor expression at the neuromuscular junction. Based on findings from my lab and those of others, I propose that this modulation might involve novel posttranscriptional molecular mechanisms. Finally, I also suggest that neuregulin signaling may have an important role to play in mediating the response of adult terminal Schwann cells to denervation. © 2007 Wiley-Liss, Inc.

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Like synapses in the central nervous system (CNS), the vertebrate neuromuscular junction (NMJ) is now considered as a “tripartite synapse,” consisting of a presynaptic nerve terminal provided by a motoneuron, a postsynaptic apparatus assembled on a skeletal muscle fiber, and terminal (or perisynaptic) Schwann cells (tSCs), non-myelinating glia that wrap the nerve terminal. In the now classical view, the postsynaptic apparatus at the NMJ, and more specifically, the aggregation of its acetylcholine receptors (AChRs), is induced by motor neuron-derived signals. Neuronal agrin, a proteoglycan that localizes to the synaptic basal lamina (McMahan, 1990), is responsible for the clustering of diffusely distributed AChRs at the sarcolemma. The central role of neural agrin in neuromuscular synaptogenesis now seems indisputable. There is, however, ongoing controversy about whether agrin is a true inducer of de novo postsynaptic specializations or a factor required only for their maturation and maintenance (Kummer et al., 2006). This review focuses on neuregulins (Nrgs), growth/dif-

ferentiation factors essential for tSC survival that have been considered the signals responsible for the increased transcription of *AChR* subunit genes at the neuromuscular synapse.

PAST (SIMPLER TIMES)

After the sorting of endplate-rich and endplate-poor muscle regions by hand-dissection, Merlie and Sanes (1985) first showed that *AChR* mRNA is highly concentrated in the synaptic region. This finding was later extended using in situ hybridization by Fontaine et al. (1988). Transgenic mice in which regulatory regions from AChR subunit genes were fused to reporter genes (Sanes et al., 1991; Simon et al., 1992), showed that the concentration of *AChR* transcripts was due to increased transcription and not to decreased degradation. These results suggested that synapse-specific *AChR* transcription is one mechanism that contributes to the high concentration of receptors at the NMJ.

The search for neuronal factors that stimulate AChR synthesis revealed two molecules that quickly monopolized the attention of the field: calcitonin gene-related peptide (CGRP) and AChR-inducing activity (ARIA). Both factors were shown to induce AChR synthesis on application to cultured myotubes (Jessell et al., 1979; Fontaine et al., 1986; Usdin and Fischbach, 1986). CGRP lost some interest after mice deficient for one of two CGRP-encoding genes (α CGRP) displayed normal synapse-specific *AChR* transcription (Lu et al., 1999). Although β CGRP could compensate for the lack of α CGRP in α CGRP^{-/-} mice, β CGRP immunoreactivity is undetectable at adult NMJs and it is only present

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in about 10% of developing endplates (Schutz et al., 2004).

Molecular cloning of ARIA showed that it is encoded by the neuregulin-1 gene (*nrg-1*) (Falls et al., 1993). It turned out that alternative splicing of *nrg-1* also encodes glial growth factor (GGF) (Marchionni et al., 1993), a nerve-derived protein able to induce Schwann cell proliferation in culture, and heregulin (HRG) and *neu* differentiation factor (NDF), proteins able to activate the *neu* proto-oncogene (Holmes et al., 1992; Wen et al., 1992). The term neuregulin-1 has been adopted ever since to refer to ARIA, GGF, HRG, or NDF. Homology and in silico cloning showed three additional Nrg genes in the mammalian genome: *nrg-2* (Busfield et al., 1997; Carraway et al., 1997; Chang et al., 1997; Higashiyama et al., 1997), *nrg-3* (Zhang et al., 1997), and *nrg-4* (Harari et al., 1999). All Nrgs share an extracellular epidermal growth factor (EGF)-like domain, which is necessary and sufficient for biologic activity. Alternative splicing and differential promoter usage generate multiple *nrg-1* isoforms. Structurally, rodent Nrg-1s can be classified into two classes depending on whether they carry one of two alternative domains N-terminal to the EGF-like domain. Those isoforms harboring an immunoglobulin (Ig)-like domain are referred as Ig-Nrg-1s, whereas those carrying a cysteine-rich (CRD), transmembrane domain are known as CRD-Nrg-1s. The latter are also referred as Type III Nrgs, whereas the former belong to Types I and II. The presence of a kringle domain distinguishes Type II from Type I Ig-Nrg-1. Most Nrg-1s are synthesized as transmembrane precursors that are proteolytically processed at the plasma membrane to generate the mature forms. Processing of the Ig-Nrg-1 precursors produces soluble ligands suitable for paracrine signaling, whereas processing of the CRD-Nrg-1 precursors generates transmembrane ligands suitable for juxtacrine signaling (Falls, 2003a). Additional Nrg-1 Types IV–VI have been described recently in humans (Steinthorsdottir et al., 2004). All but Type VI are Ig-Nrgs.

Nrgs activate receptor tyrosine kinases of the EGF receptor (ErbB1) family. They do not bind or activate ErbB1 itself. These receptors are known as ErbB2 (*neu*), ErbB3, and ErbB4. ErbB4 binds to Nrgs and is endowed with catalytic activity, whereas ErbB3 binds to Nrgs but lacks tyrosine kinase activity. ErbB2 possesses catalytic activity but is unable to bind any Nrg. Hence, ErbB4 is the only receptor that can be Nrg-activated as homodimer, whereas ErbB2 and ErbB3 are Nrg-activated only as heterodimers with other ErbBs (not including ErbB1). ErbB4 is also the preferred binding receptor for Nrg-2, -3, and -4. Although tSCs only express ErbB2 and ErbB3, muscle fibers express all three Nrg receptors. The ErbB2/4 heterodimer is the most likely functional receptor at the synaptic sarcolemma (Trinidad et al., 2000), although this is somewhat controversial (Rimer, 2003).

In contrast to the situation with CGRP, during the late 1990s a series of in vivo studies provided compelling

evidence for important roles for Nrg-1 at the NMJ. First, Trachtenberg and Thompson (1996) showed that soluble Nrg-1 (GGF) could rescue denervation-induced apoptosis of tSCs in neonatal rats. These results suggested that Nrg-1 was a survival factor for developing tSCs. Second, targeting replacement by homologous recombination of either the EGF- or Ig-like domains in mouse *nrg-1* yielded *nrg-1*^{-/-} embryos that died at embryonic day (E) 10.5 of heart defects, much earlier than when NMJs start forming (Meyer and Birchmeier, 1995; Kramer et al., 1996). However, study of *Ig-Nrg-1*^{+/-} mice, which are viable and fertile, showed that their NMJs had about 50% fewer AChRs than synapses in littermate controls (Sandrock et al., 1997). These results suggested that Ig-Nrg-1 was essential for the maintenance of high AChR density at the NMJ.

CHALLENGES (ROCKING THE BOAT)

Further manipulations of the Nrg-1/ErbB signaling pathway in vivo further supported the idea that Nrg-1 is an essential factor for the survival of tSCs in particular, but also for the development of the entire Schwann cell lineage (Garratt et al., 2000a). For example, targeted deletion of CRD-Nrg-1s, which are not required for heart development, led to peripheral nerves lacking Schwann cells, suggesting that these membrane-tethered isoforms account for Nrg-1 function in Schwann cell development (Wolpowitz et al., 2000). A similar Schwann cell phenotype was observed in mice with a conditional *nrg-1* mutation in motor and sensory neurons (Yang et al., 2001), and in mice expressing ErbB2 selectively in the heart in an otherwise *erbB2*^{-/-} background (Morris et al., 1999; Woldeyesus et al., 1999). The severity of the Schwann cell phenotype contrasted with the observation that in all these mice *AChR* synapse-specific transcription was marginally affected. Thus, these results challenged the necessity of Nrg-1-signaling in mediating *AChR* synapse-specific expression.

Additional challenges to the hypothesis that neuronal Ig-Nrg-1 is essential for synaptic *AChR* transcription emerged. First, it was found that skeletal muscle fibers synthesize and contribute Ig-Nrg-1 to the synapse (Moscoso et al., 1995; Meier et al., 1998; Rimer et al., 1998). Second, neural agrin was shown to induce the clustering of muscle-derived Nrg-1 and its ErbB receptors in vivo (Meier et al., 1998; Rimer et al., 1998), and to activate *AChR* gene transcription in an ErbB2-dependent fashion (Jones et al., 1997; Meier et al., 1998). These results suggested that Ig-Nrg-1 could be involved in synaptic *AChR* transcription not so much as a primary, nerve-derived signal but rather, as a secondary, muscle-derived signal downstream of agrin. Third, motoneurons and tSCs were found to express *nrg-2* and Nrg-2 immunoreactivity was observed perisynaptically (Rimer et al., 2004). Moreover, like Nrg-1, Nrg-2 is endowed with ARIA activity in vitro (Rimer et al., 2004; Ponomareva et al., 2005). This activity depends on the expression of ErbB4 by the myotubes, is regu-

lated by alternative splicing of *nrg-2*, and requires the N-box, an enhancer necessary for *AChR* synapse-specific transcription *in vivo*. Thus, *Nrg-1* is not the only Nrg that could play a role at the NMJ.

PRESENT (SORTING THINGS OUT...PERHAPS)

Despite the questions and complications raised by the above findings, it was still possible to explain the mild postsynaptic phenotype of most of the loss-of-function studies. Thus, muscle expression of Ig-Nrg-1 and its localization at the synapse could account for the mild postsynaptic phenotype of the neuronal conditional *nrg-1* mutant (Yang et al., 2001). Using Cre/loxP recombination, Jaworski and Burden (2006) inactivated *nrg-1* in motoneurons, muscle fibers, and both cell types and found in all cases that AChRs remained clustered at synapses at similar densities as in control mice. Although neuronal and neuronal/muscular conditional *nrg-1* mutants died at birth (due to lack of Schwann cells), muscle mutants were viable and fertile (Jaworski and Burden, 2006). These results showed that muscle-derived *Nrg-1* is dispensable not only for the formation of the NMJ but also for its maintenance in the adult. Expression of *Nrg-2* by motoneurons or Schwann cells could also compensate for lack of *Nrg-1*. In neuronal conditional *nrg-1* mutants synaptic contacts form initially but the absence of Schwann cells leads to motoneuron death and axonal withdrawal from the diaphragm by E18.5. Thus, one would have to assume that for neural *Nrg-2* to act it would have to be deposited in the synaptic basal lamina before the axons vacate the muscle. Only Ig-Nrg-2 have been described so far. The Ig-like domain has been implicated in binding to extracellular matrix for *Nrg-1* (Loeb and Fischbach, 1995), however, neuronal *Nrg-2* immunoreactivity is undetectable at the NMJ with the current antibodies available (Rimer et al., 2004). Thus, although rather unlikely, this possibility remains open. The bulk of *Nrg-2* at the NMJ seems tSC-derived (Rimer et al., 2004), and could compensate, at least in part, for the lack of muscle *Nrg-1*. However, the *Nrg-2* synthesized by tSCs, *Nrg-2 α* , is the isoform with the least AChR-inducing activity *in vitro* when presented to myotubes as a soluble ligand (Ponomareva et al., 2005). As tSCs contact muscle fibers perisynaptically (Ogata and Yamasaki, 1984), it is possible that tSCs *in vivo* release large amounts of *Nrg-2 α* or present it to the sarcolemma in such a way that compensates for its low potency in soluble form *in vitro*. Ponomareva et al. (2006a) showed recently that Schwann cells that express endogenous levels of *Nrg-2* on their cell surface can stimulate *AChR* transcription in an ErbB4-dependent fashion when they form cell-to-cell contacts with myotubes in culture. These results provide a potential role for the paradoxical perisynaptic accumulation of *Nrg-2 α* . They also raise the possibility that Schwann cell-derived *Nrg-2* could activate ErbB receptors on the synaptic sar-

colemma and that this could account, at least in part, for the *Nrg*-mediated regulation of *AChR* expression.

Targeted inactivation of *nrg-2* yields mice that are viable and fertile (Britto et al., 2004). However, the functional NMJs of these mice are yet to be analyzed using quantitative methods that measure the levels of *AChR* expression. Although these mice indicate that *nrg-2* is dispensable for NMJ formation and maintenance, they do not rule out a potential modulatory role for *Nrg-2* at the neuromuscular synapse.

Whether mediated by *Nrg-1*, *Nrg-2*, or both, *Nrg*-signaling to the postsynaptic muscle fiber would require the ErbB receptors. Skeletal muscle expresses all three *Nrg* receptors and, depending on the study, all three of them have been reported as concentrated in the postsynaptic apparatus (Rimer, 2003). A caveat of previous loss-of-function studies in which only one of three ErbB receptors was inactivated genetically was that the remaining two could take over and compensate for its absence in skeletal muscle. Escher et al. (2005) recently generated mice in which both *erbB2* and *erbB4* were inactivated selectively in skeletal muscle using Cre/loxP recombination (Escher et al., 2005). These mice survive to adulthood, give rise to progeny, and have a very modest postsynaptic phenotype: a 10% reduction in AChR density and a 20–30% reduction in synaptic *AChR* mRNA. In addition, ectopic synapses as well as ectopic postsynaptic-like apparatus were induced by transplanted foreign nerves and ectopic neural agrin, respectively, in conditional *erbB2^{-/-}*; *erbB4^{-/-}* mice. These results strongly suggest that postsynaptic *Nrg*-signaling is dispensable for both the formation and maintenance of the postsynaptic apparatus at the NMJ. They also suggest that *Nrg*-signaling still modulates synaptic *AChR* expression. The discrepancy between these results and those of Sandrock et al. (1997) has been ascribed to impaired tSC function in *Ig-Nrg-1^{+/-}* mice. However, direct evidence for this effect remains lacking and CRD-*Nrg-1s*, rather than Ig-*Nrg-1s*, seem to mediate *Nrg-1* effects on Schwann cells in general. Moreover, more recent estimations of AChR density in *Ig-Nrg-1^{+/-}* mice by the Fischbach lab, using miniature endplate potential (MEPP) amplitude as a measure, suggest that the effects on synaptic *AChR* expression may be more modest and complex than inferred previously. Thus, unlike previously reported (Sandrock et al., 1997), no difference in MEPP amplitude between adult *Ig-Nrg-1^{+/-}* and control wild-type mice was found in the new study. However, a 20–25% reduction in MEPP amplitude was observed in 1-week old *Ig-Nrg-1^{+/-}* neonates relative to controls (Mann et al., 2006).

FUTURE

Postsynaptic Role

Trinidad and Cohen (2004) reported that application of the EGF-domain of *Nrg-1* to C2C12 myotubes inhibited spontaneous and agrin-induced AChR clustering. Increased AChR cluster disassembly seems to

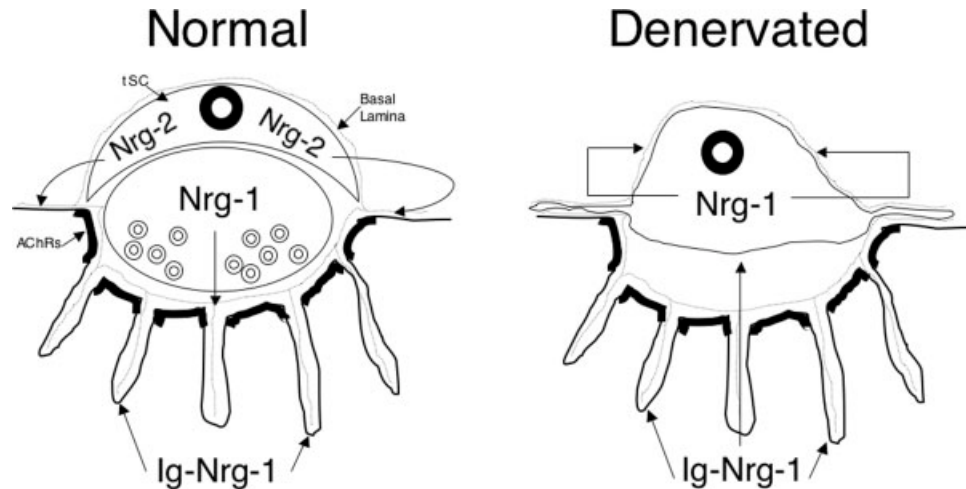


Fig. 1. Nrgs at the adult NMJ and their potential roles. At the normal NMJ (**left**), tSCs contribute Nrg-2, whereas motoneurons and muscle fibers contribute Nrg-1. Muscle fibers produce Ig-Nrg-1 only. All arrows indicate Nrg activation of ErbB receptors. At the back end of the arrow is the source cell for the Nrg. Arrowhead indicates the receiving cell of the signaling, which expresses ErbB receptors. Nrgs activate ErbB receptors on the muscle membrane and

this would lead to AChR internalization, and secondarily to increased *AChR* transcription (not depicted for simplicity). At the denervated endplate (**right**), tSCs would be activated (represented by morphologic changes in tSC) by a combination of the action of muscle Ig-Nrg-1 or of their own Nrg-1. Nrg-2 is undetectable in tSCs of denervated endplates (Rimer et al., 2004).

underlie this effect. In addition, AChRs were endocytosed into caveolae (Trinidad and Cohen, 2004). Expression of constitutively active ErbB2 (caErbB2) in vitro seems more effective than Nrg-1 application in blocking both spontaneous and agrin-induced AChR clustering (Ponomareva et al., 2006b). More interestingly, selective muscle expression of caErbB2 during development in vivo leads to an agrin-deficient-like phenotype characterized by loss of postsynaptic apparatus, extensive axonal sprouting and diffused *AChR* transcription along the entire muscle fiber (Ponomareva et al., 2006b). These results raise the intriguing possibility that the primary role of Nrg-signaling on the postsynaptic side of the NMJ might be its ability to regulate the clustering and trafficking to the surface of AChRs. Consistent with this possibility, two groups have shown recently that acute application of Nrg-1 EGF-like domain induces endocytosis of AMPA and NMDA receptors at excitatory synapses in neuron cultures and tissue slices from hippocampus and prefrontal cortex, respectively (Gu et al., 2005; Kwon et al., 2005). There is no particular reason to think that AChR trafficking at the NMJ may be exclusively regulated by Nrg-signaling. Thus, this might explain the mild postsynaptic phenotype of the conditional *erbB2*^{-/-}; *erbB4*^{-/-} mice (Escher et al., 2005). In addition, it is unclear whether these mice have been examined with this idea in mind. Methods to study AChR dynamics in vivo have been described (Akaaboune et al., 2002) and could be applied to study AChR trafficking in Nrg-signaling mutants. The ability to induce AChR cluster disassembly or surface AChR internalization might be important for the remodeling of the synapse during synaptic maturation.

Role in tSC Activation

Application of Nrg-1 to normal rat NMJs, whose tSCs do not undergo programmed cell death, resulted in Schwann cell proliferation, migration, process growth, and nerve sprouting. Neonates that received Nrg-1 also suffered a severe disruption of NMJs, evidenced by loss of AChR clusters, and muscle denervation (Trachtenberg and Thompson, 1997). Because upon denervation tSCs generate cellular processes upon which regenerating axons sprout, and because *nrg-1* expression is induced in Schwann cells along damaged axons (Carroll et al., 1997), these results suggested that an autocrine Nrg-1 signaling cascade could mediate the response of tSCs to denervation. However, as ErbB receptors are present in both tSC and muscle membranes, and perhaps at the nerve terminal as well (Pearson and Carroll, 2004), it is possible that the above effects of Nrg-1 on tSCs were not due directly to Nrg-1 signaling at the tSCs themselves. Hayworth et al. (2006) expressed caErbB2 selectively at SCs, including tSCs, and found that this manipulation, meant to mimic activation of Nrg signaling, was sufficient to induce tSC proliferation, migration, process outgrowth, and nerve sprouting. Expression of caErbB2 in neonatal tSCs after denervation was also able to rescue denervation-induced tSC apoptosis (Hayworth et al., 2006). Endplates where caErbB2 was induced in tSCs showed no loss of AChR aggregates, suggesting that activation of Nrg-signaling in tSCs fails to account for the loss of AChR clusters and muscle denervation observed after Nrg-1 application (Trachtenberg and Thompson, 1997). This effect requires activation of Nrg-signaling in muscle fibers as suggested by the results of Ponomareva et al. (2006b). Together these results are

TABLE I. Summary of NMJ Phenotypes in the Most Recent Nrg/ErbB Loss- and Gain-of-Function Mice^a

Study	Genotype	Phenotype
Britto et al., 2004	<i>nrg-2</i> ^{-/-}	Mice reach adulthood and are fertile Detailed NMJ characterization lacking
Escher et al., 2005	Muscle-specific, conditional <i>erbB2</i> ^{-/-} ; <i>erbB4</i> ^{-/-}	Mice reach adulthood and are fertile Normal tSCs and nerve terminals 10% reduction in AChR density measured by miniature endplate current (MEPC) and α -bungarotoxin (BTX) fluorescence intensity 20–30% reduction in <i>AChR</i> mRNA by real-time, quantitative PCR Normal AChR γ -to- ϵ subunit switch Agrin and foreign nerve-induced ectopic postsynaptic apparatus and synapses, respectively
Jaworski and Burden, 2006	Motor and sensory neuron, muscle, or both (double), conditional <i>nrg-1</i> ^{-/-}	Neuronal and double KO mice stillborn due to lack of SC Muscle-selective KO mice reach adulthood and are fertile AChR density normal in all three mice by α -BTX fluorescence intensity Normal <i>AChR</i> ϵ expression by in situ hybridization in muscle conditional mice
Mann et al., 2006	<i>lg-Nrg-1</i> ^{+/-}	AChR density re-evaluated using miniature endplate potential (MEPP) amplitude. Normal MEPP amplitude in adults. A 20–25% MEPP amplitude reduction in P7 neonates
Ponomareva et al., 2006b	Muscle-specific, time-controlled expression of constitutively active <i>ErbB2</i> (caErbB2)	Embryonic expression led to AChR cluster loss, <i>AChR</i> α mRNA diffused expression and extensive axonal sprouting In cultured myotubes, caErbB2 inhibited induction and maintenance of AChR clusters after agrin treatment In P30 mice, increased AChR α and AChR ϵ expression in muscles with highest caErbB2 levels
Hayworth et al., 2006	Schwann cell, time-controlled expression of caErbB2	Migration, proliferation and process extension of tSCs at innervated endplates Nerve terminal (axonal) sprouting AChR clusters were not lost Rescue of denervation-induced tSC cell apoptosis

^aSummarized NMJ phenotypes of other mice reported before 2004 (Falls, 2003b).

consistent with the notion that an autocrine Nrg-1 signaling plays an important role in mediating tSC responses to denervation (Fig. 1). However, this hypothesis needs further testing in adult animals in which Nrg-signaling is inactivated using conditional approaches to overcome the lethality during development resulting from lack of Schwann cells. Interestingly, such animals may already exist but their tSC phenotype after denervation has not been studied (Garratt et al., 2000b; Chen et al., 2003; Atanasoski et al., 2006). Alternatively, the Nrg that activates tSCs could come from the muscle fiber (Fig. 1). Testing of this possibility is also feasible due to the availability of muscle-selective, conditional *nrg-1*^{-/-} mice (Jaworski and Burden, 2006).

CONCLUSIONS

Table I summarizes the genotype and phenotype of the most recent Nrg/ErbB loss- and gain-of-function mice

covered in this review. Although the weight of the evidence supports strongly a critical role for Nrg-signaling in tSC development and survival, the weight of the evidence no longer supports the notion that Nrg-signaling to muscle fibers has an essential role in controlling synapse-specific *AChR* transcription. In the future, it will be important to investigate whether Nrg-signaling has a modulatory role in AChR trafficking. These studies will have implications for its role at CNS synapses. In addition, it will also be critical to test whether Nrg-signaling is involved in tSC responses to denervation as this will have implications for regeneration.

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