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Neuregulins: Primary or secondary signals for the control of synapse-specific gene expression

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Abstract

The selective transcription of acetylcholine receptor (AChR) subunit genes in synaptic myonuclei leads to the accumulation of AChR subunit mRNAs at the neuromuscular junction (NMJ). This mechanism contributes to the concentration of AChRs at the postsynaptic sarcolemma, and its physiological significance is underscored by the cases of human congenital myasthenias caused by mutation in a cis-regulatory element of the AChRε-subunit promoter, which is necessary for its synaptic expression. The signal(s) that drives synapse-specific expression is unknown but neuregulin-1 (Nrg-1), a group of growth-factor-like polypeptides encoded by the *nrg-1* gene, has been a favorite candidate. Nrg-1 was originally thought as a nerve-derived factor, acting in parallel to pathways controlling AChR clustering at the synapse (*i.e.* agrin signaling). However, recent work suggests that Nrg-1 may actually be a muscle-derived signal that is concentrated at the NMJ, together with its receptors, by agrin and that acts as a secondary, downstream signal to enhance synapse-specific *AChR* transcription. Here, I review studies for and against Nrg-1 as a secondary signal driving synapse-specific expression at the NMJ. In addition, I briefly present new evidence that raise the possibility that Nrgs encoded by the *ngr-1*-related gene *nrg-2* might have a role controlling *AChR* expression.

Introduction

The relative simplicity and unparalleled experimental accessibility of the mammalian neuromuscular junction (NMJ) have made it a favorite model system to study synapse formation and plasticity. A hallmark feature of all chemical synapses is the high concentration of neurotransmitter receptors on the postsynaptic membrane, which ensures that neurotransmission is not limited by the availability of receptors. At the neuromuscular synapse, acetylcholine receptors (AChRs) are highly concentrated in the postsynaptic membrane, and this clustering is driven by the exchange of signals between the presynaptic cell, the motor neuron, and the postsynaptic cell, the skeletal muscle fiber.

At the molecular level, transcriptional and posttranslational mechanisms control AChR receptor expression and clustering. The posttranslational mechanisms are not the subject of this review (for recent reviews see Sanes & Litchman, 2001; Burden, 2002; Godfrey, Mei, this issue), nonetheless, these mechanisms involve the aggregation of AChRs to synaptic sites following the activation of a muscle-specific receptor tyrosine kinase (MuSK), which is present in the postsynaptic sarcolemma. Upon nerve-muscle contact, MuSK is specifically activated by neuronal isoforms of agrin, however, recent observations also indicate that MuSK can be activated in an agrin-independent fashion, leading to the formation of AChR clusters in the absence of the nerve (Jones *et al.*, 1999a; Yang *et al.*, 2000, 2001; Lin *et al.*, 2001; Sander *et al.*, 2001). Ablation of the *agrin* and *musk* genes by homologous recombination demonstrates that agrin-dependent activation of MuSK is critical for synapse formation and maintenance (Gautam *et al.*, 1996; DeChiara *et al.*, 1996; Burgess *et al.*, 1999). However, it remains unclear what the role of agrin-independent activation of MuSK is in synaptogenesis.

Muscle fibers are multinucleated cells. Synapses only cover about 0.1% of the surface in mature muscle fibers, thus, the great majority of muscle nuclei are positioned extrasynaptically, while those few positioned underneath the nerve terminal are the synaptic nuclei. Two transcriptional mechanisms regulate *AChR* expression. Acetylcholine-evoked electrical activity, which propagates along the entire length of the muscle fiber, suppresses *AChR* transcription along the fiber. This suppressive signal is counteracted at synaptic muscle nuclei by another that activates the transcription

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of *AChR* subunit genes selectively, leading to the accumulation of *AChR* mRNAs at synaptic sites. The physiological significance of this latter mechanism is underscored by the cases of human congenital myasthenias caused by mutation in a cis-regulatory element of the AChR ε-subunit promoter, which is necessary for synaptic expression of that subunit (Ohno *et al.*, 1999; Nichols *et al.*, 1999; Abicht *et al.*, 2002; Engel, this issue). These patients develop the typical myasthenic phenotype of fatigue and muscle weakness due to the low levels of *AChR* expression at the synapse. The nature of the signal(s) that drives synapse-specific expression is still unclear, however, neuregulin-1 (Nrg-1), a group of growth-factor-like polypeptides encoded by the *nrg-1* gene, is a favorite candidate (for recent reviews see Schaeffer *et al.*, 2001; Fischbach & Rosen, 1997; Falls, Loeb, this issue). Nrg-1 is produced and contributed to the NMJ by both motor neurons and muscle fibers. Although, originally thought as a nerve-derived factor, acting independently of agrin/MuSK signaling, Nrg-1 may actually be a muscle-derived signal. Muscle Nrg-1 is concentrated at the NMJ, together with its receptors, by activation of the agrin/MuSK pathway. This concentration leads to activation of *AChR* transcription at synaptic sites. Here, I review evidence that Nrg-1 driving synapse-specific gene expression at the NMJ is a signal that acts downstream of agrin/MuSK. In addition, I briefly present new evidence that raise the possibility that Nrgs encoded by the *ngr-1*-related gene *nrg-2*, have a role controlling *AChR* expression.

Neuregulins, their receptors, and downstream signaling

Work from three independent avenues of research has led to the identification of Nrg-1 as a factor critical for the proliferation, survival, migration, and differentiation of several cell types. The search for ligands for the *neu* proto-oncogene led to the cloning of *neu* differentiation factor (NDF) (Wen *et al.*, 1992) and heregulin (HRG) (Holmes *et al.*, 1992). The purification of a nerve-derived factor able to promote Schwann cell proliferation *in vitro*, led to the cloning of glial growth factor (GGF)(Marchionni *et al.*, 1993). The isolation of a brain-derived activity able to increase the number of AChRs on cultured muscle fibers led to the cloning of AChR-inducing activity (ARIA) (Falls *et al.*, 1993). cDNAs encoding NDF, HRG, GGF, and ARIA, turned out to be alternative spliced forms from a single gene, which is now known as the *nrg-1* gene.

STRUCTURE OF NRG-1S

About 15 different Nrg-1 isoforms are generated by alternative splicing (for review see Buonanno & Fischbach, 2001; Falls, this issue). All Nrg-1 isoforms have an epidermal growth factor-(EGF-) like sequence (Fig. 1), which is necessary and sufficient to account for Nrg-1's biological activities (Holmes *et al.*, 1992; Wen *et al.*, 1994). Splicing also generates two variants of the C-terminal part of the EGF-like domain resulting in Nrg-1α and Nrg-1β, which display different affinities for Nrg receptors (Wen *et al.*, 1994; Pinkas-Kramarski *et al.*, 1998; Jones *et al.*, 1999b). Amino-terminal to the EGF-like domain, Nrg-1s contain either an immunoglobulin- (Ig-) like domain (Ig-Nrg-1s) or a cysteine-rich domain (CRD-Nrg-1s). It is believed that the Ig-like domain mediates Nrg-1 binding to the extracellular matrix (Loeb & Fischbach, 1995). Carboxyl-terminal to the EGF-like domain, some Nrg-1s contain a common transmembrane (TM-) domain (TM-Nrg-1s), which is followed by an intracellular domain, which in turn can be one of the a-, b-, or c-types (Fig. 1). Other *nrg-1* mRNAs do not code for the TM domain, and depending on the sequence spliced amino-terminal to the Ig-like domain, are secreted into the extracellular medium (*e.g.* GGF), or are intracellular (Wen *et al.*, 1994). Proteolytic cleavage of TM-Nrg-1s between the EGF-like domain and the TM-domain is a major mechanism for the generation of secreted Nrg-1s (Burgess *et al.*, 1995; Han & Fischbach, 1999; Montero *et al.*, 2000). Alternative splicing yields several of these juxtamembrane domains, thus producing multiple Nrg-1αs and Nrg-1βs. Recent work suggests that CRD-domain-containing TM-Nrg-1s are synthesized as precursors with two transmembrane domains, the additional one within the carboxyl-terminal region of the CRD domain. It is proposed that cleavage downstream of the EGF-like domain results in membrane-bound rather than soluble CRD-Nrg-1s (Wang *et al.*, 2001; Falls, this issue).

NEW *nrg* GENES

Homology at the amino acid level led to the cloning of three additional Nrg genes coding for 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). Among the new Nrgs, Nrg-2 shows the highest homology to Nrg-1, sharing similar domain structure with Ig-Nrg-1s. Like Nrg-1, alternative splicing in the EGF-like domain of Nrg-2 generates Nrg-2α and Nrg-2β (Fig. 1). *nrg-3* and *nrg-4* were cloned *in silico* by searching DNA sequence databases for sequences homologous to Nrg-1. Aside from the EGF-like and TM-domains, Nrg-3 shares little similarity with Nrg-1. Nrg-4 appears very different from other Nrgs as its extracellular domain consists of just the EGF-like domain, and its intracellular domain is very short. Thus, structural similarities among the Nrgs, particularly between Nrg-1s and Nrg-2s, suggest some overlap in their potential functions; at the same time, however, structural differences also suggest unique roles for each Nrg.

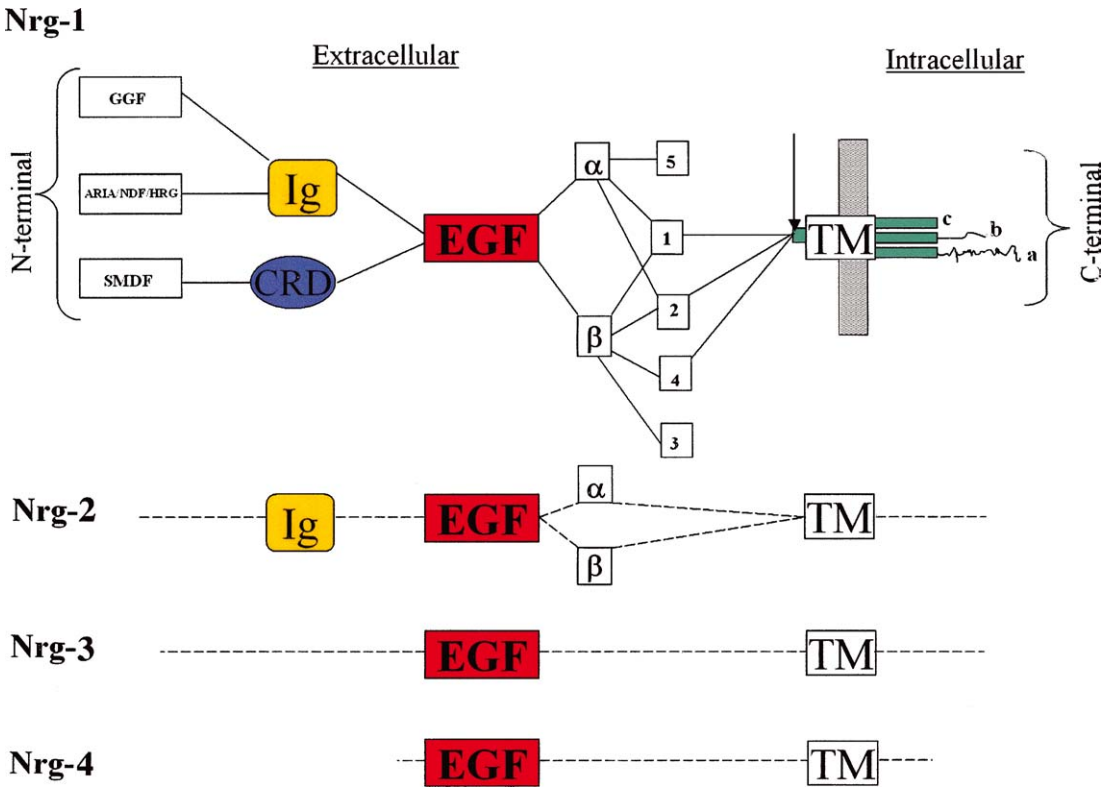

Fig. 1. Structure of Nrgs. Protein-coding regions are represented as boxes. Solid lines represent observed isoforms for Nrg-1. Arrow points to putative cleavage site for some membrane-bound Nrg-1s. Only domains with significant homology to Nrg-1 are shown for Nrg-2-4. Non-homologous regions are represented as hyphenated lines. SMDF: sensory and motor neuron-derived factor. Glycosylation-rich segments in Nrg-1s are omitted for simplicity, however, Ig-Nrg-1s containing them are sometimes referred as Type I (*e.g.* ARIA), and Ig-Nrg-1s lacking them are referred as Type II (*e.g.* GGF). CRD-Nrg-1s (*e.g.* SMDF) are Type III. For other abbreviations see text.

NRG RECEPTORS

Nrgs bind and activate members of the epidermal growth factor (EGF) receptor family of receptor tyrosine kinases known as the "ErbBs", named after the viral oncogene v-Erb-B, which is a mutated form of the EGF receptor (EGFR or ErbB1). ErbBs are also referred to in the literature as "HERs" for "human epidermal growth factor receptors". Although, ErbB1 is the prototype receptor of this family, it fails to bind Nrgs, which rather are ligands for ErbB3 and ErbB4. The fourth member of the family, ErbB2, also known as the *neu* proto-oncogene, lacks any known ligands but it is often activated as a result of receptor heterodimerization with ErbB1, ErbB3, or ErbB4. ErbB3 is also activated in a heterodimer situation as it lacks detectable tyrosine kinase activity. On the other hand, ErbB4 is endowed with both ligand (*i.e.* Nrg) binding and catalytic activity. Binding studies with soluble ErbB receptor bodies indicate that the heterodimers ErbB2/3 and ErbB2/4 serve as the high affinity receptors for Nrg-1 (Pinkas-Kramarski *et al.*, 1998; Jones *et al.*, 1999b). Nrg-2 displays higher affinity for ErbB4 than for ErbB3, and Nrg-3 and Nrg-4 appear to be selective ligands for ErbB4 (Zhang *et al.*, 1997; Harari *et al.*, 1999; Jones *et al.*, 1999b). Thus, it would seem that ErbB4 is likely to be a critical player mediating biological activities induced by the new Nrgs.

DOWNSTREAM SIGNALING IN SKELETAL MUSCLE CELLS

Phosphorylation of tyrosine residues in the cytoplasmic domains of the ErbB receptors creates binding sites for several intracellular signaling molecules, such as Shc, Grb2, and the regulatory p85 subunit of phosphoinositide 3-kinase (PI 3-kinase). Recruitment of Shc and/or Grb2 in turn, activates the mitogen-activated protein (MAP) kinase pathway, while p85 docking leads to activation of a parallel PI 3-kinase pathway. Stimulation of C2C12 mouse myotubes or primary chick myotubes with Nrg-1 activates the MAP kinase pathway (also known as the ERK kinase pathway, for extracellular-signal-regulated kinase) (Si *et al.*, 1996; Altiok *et al.*, 1997; Si & Mei, 1999), whereas on Sol8 mouse myotubes, Nrg-1 treatment also activates the PI-3 kinase pathway (Tansey *et al.*, 1996). Si and colleagues have also reported activation of c-JUN N-terminal kinase (JNK) downstream of ERK/MAP kinase activation by

Nrg-1 in C2C12 myotubes (Si *et al.*, 1999). Activation of these signaling cascades by Nrg-1 ultimately leads to induction of AChR transcription in all 3 cultured muscle cells. SHP2, a cytoplasmic tyrosine phosphatase, has been shown to negatively regulate Nrg-1-induced AChR transcription in C2C12 cells (Tanowitz *et al.*, 1999). The signaling pathways that might be activated by Nrg-2, Nrg-3, and Nrg-4 on muscle cells remain to be characterized.

AChR promoters for the δ- and ε-subunit genes contain a sequence (CCGGAA), called an N-box (Koike *et al.*, 1995; Duclert *et al.*, 1996; Sunesen & Changeux, this issue) that is critical for synapse-specific expression *in vivo* and Nrg-1-responsiviness *in vitro*. This sequence has been shown to bind a transcription factor of the Ets family of proteins, GABP (GA-binding protein) (Fromm & Burden, 1998; Sapru *et al.*, 1998; Schaeffer *et al.*, 1998). Recent work suggests that Nrg-1-induced activation of the MAP kinase pathway leads to phosphorylation of GABP (Fromm & Burden, 2001). Importantly, transgenic mice overexpressing dominant-negative GABP show reduced synaptic mRNA levels of *AChR* subunit genes and morphological alterations in synaptic structure (de Kerchove D'exaerde *et al.*, 2002). Thus, GABP is required for synapse-specific *AChR* expression. Interestingly, expression of other postsynaptic genes, most notably *musk*, is not affected in these animals, suggesting that GABP-independent mechanisms control synapse-specific expression of a separate subset of postsynaptic genes (Sunesen & Changeux, this issue).

Ip and colleagues have shown that cyclin-dependent kinase 5 (Cdk-5) and its activator, p35, form a complex with ErbB2 and ErbB3 in C2C12 myotubes. Moreover, Nrg-1 treatment induces activation of Cdk-5/p35, and blocking Cdk-5/p35 prevents Nrg-1-induced stimulation of *AChR* transcription (Fu *et al.*, 2001; Lai & Ip, this issue). It is unclear whether, and how, cross-talk occurs between the Cdk-5/p35 pathway and the other signaling cascades activated by Nrg-1 in these cells.

Neuregulin-1 at the NMJ: Role as a primary signal

Nrg-1 is thought to play at least two important roles at neuromuscular synapses. On the one hand, Nrg-1 is a survival factor for terminal Schwann cells at developing NMJs (Trachtenberg & Thompson, 1996, Kang, Tian & Thompson, this issue). Genetic evidence indicates that neuronal-derived CRD-Nrg-1 isoforms are critical primary signals for this function as Schwann cells are absent from peripheral axons in *crd-nrg-1*$^{-/-}$ mice (Wolpowitz *et al.*, 2000).

On the other hand, motor neuron-derived Ig-Nrg-1 has been the leading candidate for the signal that activates *AChR* synapse-specific transcription at the NMJ. The evidence supporting this role is as follows:

(1) Nrg-1 activates *AChR* transcription in cultured muscle cells (Jessell *et al.*, 1979; Usdin & Fischbach, 1986; Martinou *et al.*, 1991; Chu *et al.*, 1995; Jo *et al.*, 1995). Nrg-1 also upregulates the expression of Na^+ channels (Corfas & Fischbach, 1993) utrophin (Gramolini *et al.*, 1999) and possibly, acetylcholinesterase, as the *ache* gene contains a functional intronic N-box (Chan *et al.*, 1999). All of these proteins are concentrated at the postsynaptic apparatus *in vivo*.

(2) Sequences in *AChR* gene promoters that are required for Nrg-1 responsiveness in cultured myotubes (*e.g.* the N-box) are also required for synapse-specific transcription *in vivo* (Duclert *et al.*, 1993; Gundersen *et al.*, 1993; Tang *et al.*, 1994; Jo *et al.*, 1995; Koike *et al.*, 1995; Duclert *et al.*, 1996; Fromm & Burden, 1998; Schaeffer *et al.*, 1998).

(3) Nrg-1 is expressed by motor neurons (Corfas *et al.*, 1995; Meyer *et al.*, 1997; Loeb *et al.*, 1999).

(4) Nrg-1, ErbB2, ErbB3, and ErbB4 are concentrated at synaptic sites (Chu *et al.*, 1995; Jo *et al.*, 1995; Altiok *et al.*, 1995; Zhu *et al.*, 1995). Some Nrg-1 immunoreactivity is localized to the synaptic basal lamina (Jo *et al.*, 1995; Goodearl *et al.*, 1995), which has been shown to contain a signal that drives synapse-specific expression even following removal of the nerve (Goldman *et al.*, 1991; Brenner *et al.*, 1992; Jo & Burden, 1992). This presumably derives from Ig-NRG-1s bound to extracellular matrix (Loeb & Fischbach, 1995). It worth mentioning that no single study of ErbB receptor localization at NMJs has reported all three receptors (*i.e.* ErbB2, ErbB3, ErbB4) as concentrated in the postsynaptic sarcolemma. Thus, the ErbB2/3 combination has been reported by some (Chu *et al.*, 1995; Moscoso *et al.*, 1995; Altiok *et al.*, 1995) the ErbB3/4 combination by others (Zhu *et al.*, 1995), and more recently, the ErbB2/4 combination by yet another group (Trinidad *et al.*, 2000). Thus, the precise receptor combination that mediates Nrg signaling at the postsynaptic membrane remains an open question.

(5) Perhaps most compelling, heterozygous mutant mice carrying a deletion in the exons encoding the Ig-like domain of Nrg-1 have 50% fewer AChRs at their NMJs than normal mice and are mildly myasthenic (Sandrock *et al.*, 1997).

Despite all these seemingly compelling findings, evidence that it is Nrg-1 provided by the motor neuron and secreted locally at the synapse, inducing synapse-specific gene expression still lacks the level of proof now established for neuronally-provided agrin (Sanes & Lichtman, 2001). Inactivation by genetic manipulation of the *nrg-1* and *erbB* encoding genes has failed to provide compelling support for neuronal Ig-Nrg-1 as the signal that activates synapse-specific transcription. Homozygous mutant embryos lacking Ig-Nrg-1, ErbB2,

or ErbB4 die around embryonic day (E) 10.5 due to their inability to make a functional heart (Kramer *et al.*, 1996; Lee *et al.*, 1995; Gassmann *et al.*, 1995). Because NMJs don't start forming until around E13, other strategies have been used to assess the role of Nrg-1/ErbB signaling in NMJ synaptogenesis *in vivo*. One of those was the analysis of *ig-nrg-1* heterozygotes mentioned above. Although, at first glance supportive of a role for neuronal Ig-Nrg-1 in synapse-specific expression, interpretation of the reduction in synaptic AChRs in these mice is complicated by the finding that Ig-NRG-1 is the main *nrg-1* isoform produced by muscle fibers (see next section). In addition, although CRD-Nrg-1 is essential for the survival of Schwann cells during fetal development (Wolpowitz *et al.*, 2000), it is possible that Ig-Nrg-1 could be required for normal terminal Schwann cell function in the adult. Hence, the phenotype of *ig-nrg-1*$^{+/-}$ mice could indirectly result from the effect of Ig-Nrg-1 on terminal Schwann cells.

erbB3 null mice and rescued *erbB2* null mice, which express ErbB2 in the heart in an otherwise mutant animal, also show AChR clusters and selective induction of AChR transcription at synaptic sites (Riethmacher *et al.*, 1997; Morris *et al.*, 1999; Woldeyesus *et al.*, 1999; Lin *et al.*, 2000). Interpretations that reconcile the above results with the idea that neuronal Ig-Nrg-1 is critical for synapse-specific expression are plausible. Thus, although present on terminal Schwann cells at synaptic sites, ErbB3 might not localize to the postsynaptic membrane after all (Trinidad *et al.*, 2000) and ErbB2 function at the postsynaptic apparatus in ErbB2-rescued mice (Morris *et al.*, 1999; Woldeyesus *et al.*, 1999) could be compensated by ErbB4. However, Burden and colleagues showed recently that synapse-specific *AChR* expression is normal in mice in which *nrg-1* was selectively inactivated in sensory and motor neurons by Cre-mediated recombination (Yang *et al.*, 2001). Moreover, characterization of mice that fail to form NMJs because their motor neurons fail to develop axons, surprisingly showed that AChR clusters and *AChR* transcription are concentrated in the central region of embryonic muscles despite the absence of innervation (Lin *et al.*, 2001; Yang *et al.*, 2001). Thus, nerve-independent processes may actually initiate synapse-specific expression, previously thought as a nerve-dependent phenomenon.

Neuregulin-1 at the NMJ: Role as a secondary signal

Although initial studies of the expression pattern of *nrg-1* clearly indicated it is transcribed in skeletal muscle (Holmes *et al.*, 1992), it took a few years to recognize that muscle-derived Nrg-1 could play a role in NMJ synaptogenesis. Moscoso *et al.* (1995) first reported Nrg-1 immunoreactivity in synaptic and extrasynaptic regions of developing muscle fibers, using an antibody against an extracellular epitope; much of the immunoreactivity remained at denervated endplates, which suggested that either muscle fibers or terminal Schwann cells contributed Nrg-1 to the synapse. Rimer *et al.* (1998) used an antibody against an intracellular Nrg-1 epitope, and an antibody specific to Schwann cells, to show that the Nrg-1 immunoreactivity at denervated endplates was associated with muscle fibers and not with terminal Schwann cells. In normal synapses, Nrg-1 immunoreactivity, with the antibody against the cytoplasmic epitope, appeared to extend for a short distance into the postsynaptic sarcoplasm (Rimer *et al.*, 1998). Together, these results strongly suggested that muscle fibers contribute transmembrane Nrg-1 to the normal synapse. Sanes and colleagues first proposed an autocrine signaling pathway for Nrg-1, in which both the Nrg-1 ligand and its ErbB receptors would be produced by the muscle fiber, and the local synaptic transcription they induce would result from the concentration of these proteins at the synapse (Moscoso *et al.*, 1995). This idea found strong support after two groups independently showed that ectopic expression of neuronal agrin in extrasynaptic regions of adult rat muscle was sufficient to induce postsynaptic-like apparatus that displayed local synthesis of AChRs (Jones *et al.*, 1997; Meier *et al.*, 1997; Rimer *et al.*, 1997). These groups also showed that ectopic agrin clusters muscle-derived Nrg-1 and its receptors (Meier *et al.*, 1998; Rimer *et al.*, 1998). Rimer *et al.* (1998) demonstrated that muscle tissue *in vivo* produces Nrg-1βs, the same isoforms shown to have AChR-inducing activity *in vitro*, and Meier *et al.* (1998) showed that these Nrg-1s were Ig-Nrg-1s. Brenner and colleagues have performed elegant experiments that further link agrin and muscle Nrg-1 signaling. Substrate-bound agrin can induce *AChR*ε transcription on cultured myotubes (Jones *et al.*, 1996), and this activation can be blocked with ErbB2-dominant negative constructs (Meier *et al.*, 1998). Moreover, interfering with Nrg signaling by either Cre/loxP-mediated deletion of *erbB2* or overexpression of a dominant-negative ErbB4, inhibits ectopic agrin-induced AChR cluster formation in innervated fibers *in vivo* (Moore *et al.*, 2001). These findings, together with the observation that dominant-negative forms of GABP inhibit *AChR*ε transcription at agrin-induced postsynaptic-like apparatus (Briguet & Ruegg, 2000), support the notion that agrin/MuSK signaling clusters an autocrine muscle Nrg-1/Erb signaling cascade that, in turn, acts downstream as a secondary signal to induce synapse-specific *AChR* expression. Thus, agrin would be the basal lamina-associated signal that is primarily responsible for clustering muscle Nrg-1 and inducing transcription. It remains to be determined whether muscle-Nrg-1 associates with synaptic basal lamina.

Overexpression of MuSK in skeletal muscle induces ectopic AChR clusters *in vivo* that have the same properties as ectopic agrin-induced clusters, including local *AChR* transcription (Jones *et al.*, 1999a; Sander *et al.*,

2001). Hence, agrin-independent activation of MuSK leads to AChR clustering and increased local *AChR* transcription. Although yet to be shown experimentally, this activation may lead to muscle-Nrg-1/ErbB clustering as well, which in turn could account for the increased *AChR* expression. Because the pattern of AChR clusters and *AChR* transcription seen in aneural embryonic muscle is MuSK-dependent (Lin *et al.*, 2001; Yang *et al.*, 2001), muscle Nrg-1, acting downstream of MuSK, could also be implicated in establishing this pattern during early development.

Role for other neuregulins

The band of *AChR* transcription in the central portion of embryonic muscle present prior to nerve-muscle contact becomes narrower following innervation (Yang *et al.*, 2001), presumably due to nerve-derived signals that confine the boundaries of *AChR* expression to synaptic sites. Although selective inactivation of *nrg-1* in sensory and motor neurons demonstrates that neuronal Nrg-1 is not one of the refinement signals, ACh, agrin, and perhaps other factors, are likely to be such signals. Because of their structural similarity to Nrg-1, other Nrgs (Nrg-2, Nrg-3, and Nrg-4), are potential candidates for signals that might control *AChR* synapse-specific expression. We have shown that motor neurons express *nrg-2* and that Nrg-2 is concentrated at synaptic sites in skeletal muscle. Furthermore, we have found that Nrg-2, but not Nrg-3 or Nrg-4, stimulates *AChR* transcription in cultured myotubes expressing ErbB4, ErbB2, and ErbB3, but not in cells that express only ErbB2 and ErbB3 (M. Rimer, A.L. Prieto, C. Colasante, J.L. Weber, L. Fromm, M. Schwab, C. Lai, and S.J. Burden, unpublished observations). Thus, Nrg-2's activation of *AChR* expression is ErbB4-dependent. Because ErbB4 is present on the postsynaptic sarcolemma at the NMJ, (Zhu *et al.*, 1995; Trinidad *et al.*, 2000), Nrg-2 is thus a candidate for a signal that regulates *AChR* transcription at the synapse following nerve-muscle contact. Redundancy between Nrg-2 and Nrg-1 might account, at least in part, for the normal synapse-specific transcription in the *nrg-1* conditional mouse mutants.

Discussion and conclusions

Synapse-specific *AChR* expression is MuSK-dependent. While this article was under review, a new paper appeared showing that agrin activation of MuSK *in vivo* launches a signaling pathway that induces *AChR* transcription without involvement of Nrg/ErbB signaling (Lacazette *et al.*, 2003). This new pathway, however, seems to occur in parallel to a cascade in which MuSK activation leads to muscle Nrg-1/ErbB clustering and activation. Thus, the evidence presented suggests a picture in which muscle Nrg-1 is more likely to be involved in the initial induction of synapse-specific transcription than neuronal Nrg-1 (Fig. 2). Nevertheless, this does not rule out a role for neuronal Nrg-1 in the maintenance of synaptic-specific gene expression. Indeed, the potential function of neuronal Nrg-1 after birth could not be tested in the conditional *nrg-1* mutant mice as they die around birth due to the disassembly of their NMJs, likely caused by paucity of Schwann cells, which require neuronal Nrg-1 for their survival (Yang *et al.*, 2001). One such role, that could require neuronal and/or muscle Nrg-1, is directing the γ-to-ε AChR subunit switch that becomes most evident in mice during the first postnatal week. Fetal AChR is composed of $\alpha_2\beta\delta\gamma$; following nerve-muscle contact transcription of the ε-subunit gene is turned on while the γ-subunit gene is turned off, leading to the gradual replacement of the fetal receptor by the adult one composed of $\alpha_2\beta\delta\varepsilon$. The γ-to-ε switch dramatically changes the conductance properties of the channel. Interestingly, Nrg-1-induced transcriptional activation *in vitro* is larger for the ε-subunit gene than for any other *AChR* subunit gene (Martinou *et al.*, 1991).

The most compelling evidence supporting muscle Nrg-1 as a secondary signal driving synapse-specific transcription comes from the study of postsynaptic-like apparatus induced by ectopic agrin on extrasynaptic regions of adult muscles. This system has allowed the assessment of muscle Nrg-1's signaling *in vivo* in the absence of neuronal Nrg-1 and Schwann cells. Thus, the interpretation of the results is not complicated by the potential redundancy of neuronal and muscle Nrg-1, or by the requirement of Schwann cells for Nrg-1. Not withstanding these advantages, it is not unreasonable to question the similarity between the postsynaptic apparatus at normal NMJs and the one induced by ectopic agrin alone, especially in light of recent evidence suggesting that agrin-independent mechanisms may be implicated during early normal synaptogenesis. Hence, one of the most difficult challenges in the field is to devise innovative ways to test the role of muscle Nrg-1 at the synaptic site *in vivo*. These experiments must control for potential redundancy with Nrgs from other cells, and their role in Schwann cell development. In this context, it is important to investigate if indeed Ig-Nrg-1s have any role to play in normal terminal Schwann cell function.

Another pressing question concerns the exact complement of ErbB receptors localized at the postsynaptic sarcolemma. There are conflicting reports particularly on whether ErbB3, ErbB4, or both Nrg receptors are concentrated postsynaptically. One important issue connected to this relates to the downstream signaling pathways triggered by Nrg-1, and potentially by other Nrgs. So far, most of the signaling studies have been done on Sol8 or C2C12 cells, which express ErbB2 and ErbB3, but little if any ErbB4, however, muscle fibers express *erbB4* and ErbB4 protein appears concentrated in

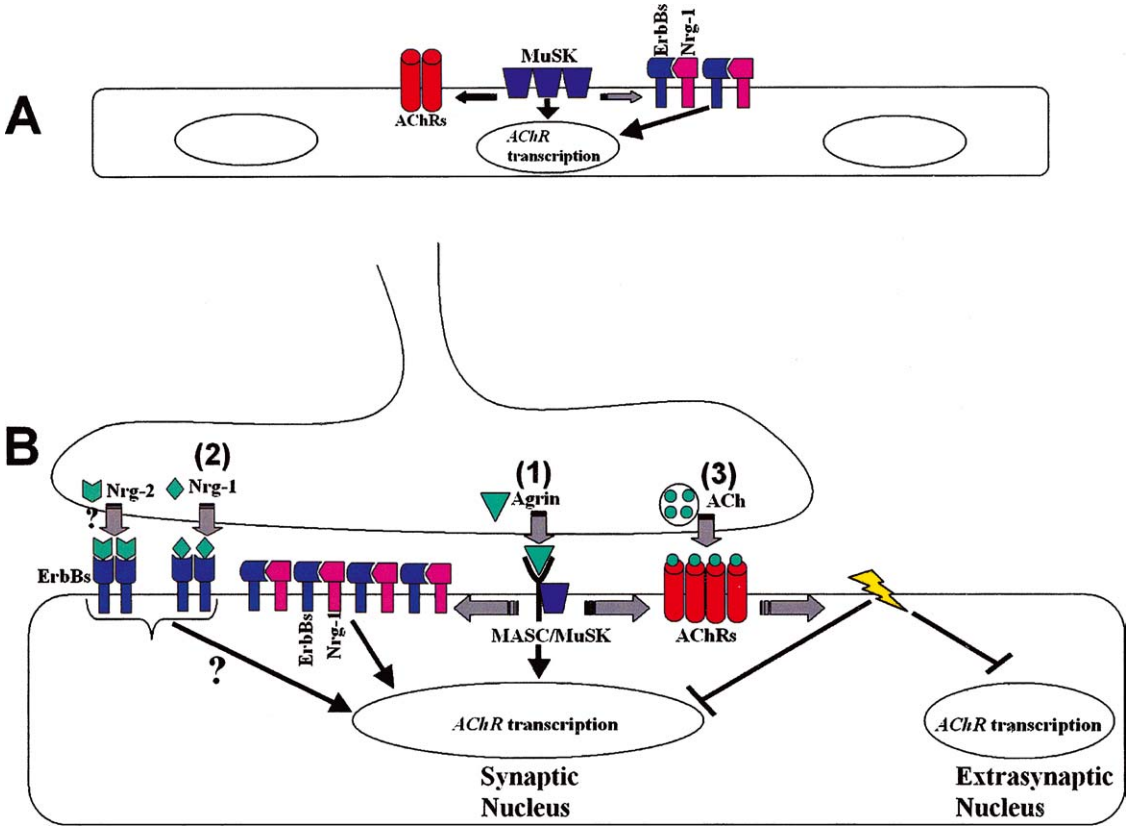

Fig. 2. Model for interactions between the agrin/MuSK and the Nrg/ErbB signaling pathways during NMJ formation. (A) Before nerve-muscle contact. MuSK expression is pre-patterned so that it is higher in the middle of the muscle fiber. Agrin-independent activation of MuSK induces clustering of AChRs and could enhance local *AChR* transcription by clustering of the muscle Nrg-1/ErbB pathway and/or by another pathway. (B) After nerve-muscle contact. (1) Neuronal agrin is released from the nerve-terminal and activates an agrin-receptor containing MuSK, which launches pathways that regulate *AChR* transcription and aggregation, respectively. Stimulation of synapse-specific transcription is achieved by inducing the clustering of muscle-Nrg-1 and its ErbB receptors and by activation of a second, Nrg-1-independent pathway. (2) Motor neurons express both Nrg-1 and Nrg-2. While there is evidence that nerve terminals release Nrg-1 into the synaptic cleft, it is unclear whether Nrg-2 is also released (indicated by the question mark). Neuronal Nrg-1 is dispensable for synapse-specific *AChR* expression before birth, and it is unclear whether it is required later. Nrg-2 is a novel candidate for a factor controlling synapse-specific transcription after nerve-muscle contact but its role *in vivo* remains to be determined. (3) ACh is released from the nerve terminal and its binding to the AChRs evokes electrical activity on the muscle fibers that represses *AChR* transcription at both synaptic and extrasynaptic nuclei. MASC: myotube-associated synaptic component, binds agrin.

the postsynaptic sarcolemma. Because the cytoplasmic domain of ErbB4 may provide docking sites for signaling molecules that are different to those on ErbB2 or ErbB3, the signaling pathways triggered by Nrgs, and the genes they ultimately regulate, could turn out quite different on muscle fibers *in vivo* than those already characterized in model cell lines. Furthermore, confirming which ErbB receptors accumulate postsynaptically is particularly relevant to evaluating a possible role *in vivo* for Nrg-2, whose AChR-inducing activity *in vitro* is ErbB4-dependent.

By and large, the characterization of synapse-specific *AChR* expression *in vivo* has relied on *in situ* hybridization methods, which provide accurate spatial and temporal detail but very little quantitative information. More quantitative analyses of gene expression are needed to really sort out the true roles of Nrgs in synapse-specific transcription. Thus, although muscle Nrg-1 appears sufficient to account for synapse-specific expression by *in situ* hybridization analysis, more quantitative studies might reveal that signaling from both neuronal and muscle Nrg-1 is necessary to reach normal levels of *AChR* transcription.

Agrin and Nrgs are widely expressed in the developing and mature CNS. Although at present there is no evidence supporting an interaction between agrin and Nrg signaling in the brain, emerging results from various labs indicate that these two signals regulate expression of CNS-specific genes (for reviews see Buonanno & Fischbach, 2001; Smith & Hilgenberg, 2002). Hence, studies of the agrin and Nrg signaling pathways at the NMJ will continue to provide significant insights into the functions of these molecules in the brain.

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