

Schwann Cell-Derived Neuregulin-2 α Can Function as a Cell-Attached Activator of Muscle Acetylcholine Receptor Expression

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ABSTRACT

Here we show that neuregulin-2 (Nrg-2) α - and β -isoforms can activate acetylcholine receptor (*AChR*) transcription as surface-attached ligands. More importantly, we demonstrate that Schwann cells that express Nrg-2 α on their cell surface, the same Nrg-2 isoform expressed by terminal Schwann cells at the neuromuscular junction, can induce *AChR* expression if brought into cell-to-cell contact with myotubes specifically expressing ErbB4. These Schwann cells, the D6P2T cell line, induce *AChR* expression apparently as well as 293T cells transfected with Nrg-2 β , the isoform with the highest *AChR*-inducing activity when presented in a soluble form. These results provide a potential role for the previously reported, paradoxical perisynaptic accumulation of Nrg-2 α , the isoform with the least *AChR*-inducing activity when presented in a soluble form. They also raise the possibility that Schwann cell-derived Nrg-2 could activate ErbB receptors on the synaptic sarcolemma and that this could account, at least in part, for the Nrg-mediated regulation of *AChR* expression. © 2006 Wiley-Liss, Inc.

INTRODUCTION

Selective transcription of acetylcholine receptor (*AChR*) subunit genes at a high rate by synaptic myonuclei at the mammalian neuromuscular junction (NMJ) is one molecular mechanism thought to contribute to the high concentration of *AChRs* at the neuromuscular synapse. However, the nature of the signals and pathways that control this process, and even the very necessity of it for the formation and maintenance of the NMJ, remain unresolved.

A traditional model that posits that synapse-specific *AChR* expression is imposed on the muscle fiber by the contacting nerve because of its release of the growth/differentiation factor neuregulin-1 (Nrg-1) (Chu et al., 1995; Falls et al., 1993; Jo et al., 1995; Sandrock et al., 1997) has been challenged by the following observations: (i) Mice, genetically engineered to lack motor nerves, show selective *AChR* transcription in the central area of their diaphragm muscles, where synapses would other-

wise form (Lin et al., 2001; Yang et al., 2001). Although this pattern could result from the preferentially central fusion of myoblasts into primary myotubes (Greer et al., 1999), it alternatively suggests that transcriptional specialization of future synaptic nuclei might be induced not by nerve contact, but as part of an intrinsic, cell-autonomous developmental program in the muscle fiber. (ii) Dispersal of the “pre-patterned” *AChR* expression in agrin-deficient mice (Gautam et al., 1996; Lin et al., 2001) as well as the apparent sufficiency of neural agrin to induce *AChR* expression, when applied to the extra-junctional regions of adult rat muscle (Jones et al., 1997), suggest that the nerve uses agrin for the maintenance and maturation of synapse-specific *AChR* transcription. (iii) The modest reduction in synaptic *AChR* density in mice with conditional ablation in skeletal muscle of the genes encoding ErbB2 and ErbB4 (Escher et al., 2005), two of the Nrg receptors expressed by muscle fibers (Moscoso et al., 1995; Trinidad et al., 2000; Zhu et al., 1995), suggests that Nrg-signaling modulates, rather than determines synaptic *AChR* expression.

A different challenge to the traditional model comes from our work demonstrating that Nrg-1 is not the only Nrg that could play a role at the NMJ (Ponomareva et al., 2005; Rimer et al., 2004). We have shown that distinct *nrg-2* isoforms are expressed by motor neurons and terminal Schwann cells and that Nrg-2 accumulates adjacent to the NMJ. Furthermore, we have shown that Nrg-2 induces *AChR* expression in cultured myotubes expressing ErbB4, that this activity is regulated by *nrg-2* RNA alternative splicing and that it requires the N-box, an enhancer essential for synapse-specific *AChR* transcription in vivo (Fromm and Burden, 1998; Koike et al., 1995). Thus, the question arises as to which Nrg, Nrg-1 or Nrg-2, accounts for the activation of ErbB

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receptors on the postsynaptic membrane that modulates *AChR* expression in vivo.

Evidence against the hypothesis that Nrg-2 is the neuregulin activating postsynaptic ErbB receptors at the NMJ includes our observation that the Nrg-2 that accumulates at the endplates is derived from terminal Schwann cells. This Nrg-2 is concentrated in the surface of the cell body and processes that faces away from the synapse (Rimer et al., 2004), and is the isoform (i.e. Nrg-2 α) with the least AChR-inducing activity when it is presented to muscle cells in soluble form (Ponomareva et al., 2005). Nevertheless, these findings do not rule out that terminal Schwann cells in vivo release large amounts of Nrg-2 α or present it to the sarcolemma in such a way that it compensates for its low potency in soluble form in vitro.

Here, we show that Nrg-2 α - and β -isoforms can activate *AChR* transcription as surface-attached ligands. More importantly, we demonstrate that Schwann cells that express Nrg-2 α on their cell surface, a characteristic of terminal Schwann cells in vivo, are capable of inducing *AChR* expression when brought into cell-to-cell contact with a myotube specifically expressing ErbB4. These results provide a potential role for the paradoxical perisynaptic accumulation of the Nrg-2 isoform with the least AChR-inducing activity in soluble form. They also raise the possibility that Schwann cell-derived Nrg-2 could activate ErbB receptors on the synaptic sarcolemma and that this could account, at least in part, for the Nrg-mediated regulation of *AChR* expression.

MATERIALS AND METHODS

Schwann Cell Culture

D6P2T cells were grown in DMEM supplemented with 10% FBS and 50 μ g/mL gentamycin. Primary Schwann cells were harvested from rat neonates and cultured as described previously (Ponomareva et al., 2005).

RNA Isolation and Reverse-Transcriptase-Polymerase Chain Reaction

Total RNA isolation and reverse-transcriptase-polymerase chain reaction (RT-PCR) was as previously described (Rimer et al., 2004). The primers for *nrg-1* were originally described by Sanes and colleagues (ARIA 5'B and ARIA 3' (Moscoso et al., 1995). The cycling parameters for *nrg-1* were identical to the ones used for *nrg-2* (Rimer et al., 2004). The primers for *gapdh* were as follows: Forward: 5'-AACTTTGGCATTGTGGAAGG-3'. Reverse: 5'-CCCTGTTGCTGTAGCCGTAT-3'. The cycling program for *gapdh* was 40 cycles with 30 s 94°C denaturation, 30 s 56°C annealing, 1 min 72°C extension. The expected product size was 472 bp. Agarose gels were stained with ethidium bromide. Pictures of gels were inverted with Adobe Photoshop 6.0, so that DNA bands appear black.

Immunostaining

A previously characterized anti-Nrg-2 rabbit polyclonal (Rimer et al., 2004) was used to stain preparations of D6P2T or primary rat Schwann cells. Briefly, for surface staining, cells washed in cold phosphate-buffered saline (PBS) and fixed in 1% paraformaldehyde in PBS at room temperature for 30 min. Nonspecific binding was blocked by incubation in 1% bovine serum albumin (BSA) in PBS for 1 h at room temperature. Anti-Nrg-2 antibody was added at 1/100 dilution in the blocking solution for 3 h at room temperature. Cells were washed in PBS at room temperature for 30 min and rhodamine-goat anti-rabbit polyclonal (Jackson ImmunoResearch, West Grove, PA) was added in blocking solution at 1/400 dilution for 1 h at room temperature. Cells were washed in PBS as mentioned earlier. Dishes were dried and coverslips were mounted with Vectashield (Vector Labs, Burlingame, CA). Triton X-100 at 0.1% was included in all solutions after fixing for whole-cell staining.

Bead-Contact AChR Expression Assay

Polystyrene 10-micron microspheres (Polysciences, Warrington, PA) were sterilized in 95% ethanol, rinsed twice with PBS, and incubated overnight at 4°C with 100 μ L of the following recombinant proteins produced in bacteria (Ponomareva et al., 2005): glutathione-S-transferase (GST, at 750–1500 μ g/mL), GST fused to the epidermal-growth factor (EGF)-like domain of Nrg-2 α (GST-Nrg-2 α , at 300–500 μ g/mL), or GST fused to the EGF-like domain of Nrg-2 β (GST-Nrg-2 β , at 75–125 μ g/mL). Beads were rinsed with PBS, and suspensions of coated beads were kept at 4°C until used. To confirm coating, the beads were stained with a rabbit serum that recognizes GST at 1/3000 dilution (Rimer et al., 2004). Rhodamine-conjugated goat anti-rabbit IgG (Jackson ImmunoResearch, West Grove, PA) at 1/3000 was used to visualize the staining. Uncoated beads were used as negative control and showed no staining (Supplementary Fig. 1). To estimate the relative amounts of protein bound to beads, pictures were taken with a 20 \times air objective (N.A. 0.50, Nikon) with the same camera gain and the same exposure time. Average gray value (i.e. intensity) per bead was measured using Methamorph (Universal Imaging). Relative to GST beads, GST-Nrg-2 β beads had higher staining (2.90 ± 0.27 -fold) while Nrg-2 α beads had lower staining (0.67 ± 0.10 -fold). One or two μ L of the suspensions were added to 35-mm dishes of ErbB2/3/4-expressing Sol8 myotubes stably transfected with an *AChR δ -human growth hormone (hGH)* fusion. Treatments were done in duplicate or triplicate per experiment. Two days later, dishes were rinsed twice with PBS and cells fixed with 2% PFA for 30 min at room temperature. Following blocking and permeabilization with 1% BSA, 0.5% Triton in PBS, myotubes were incubated with 1/400 rabbit anti-hGH (Dako, Carpinteria, CA) either overnight at 4°C or for 2–3 h at room temperature. Following extensive washing with

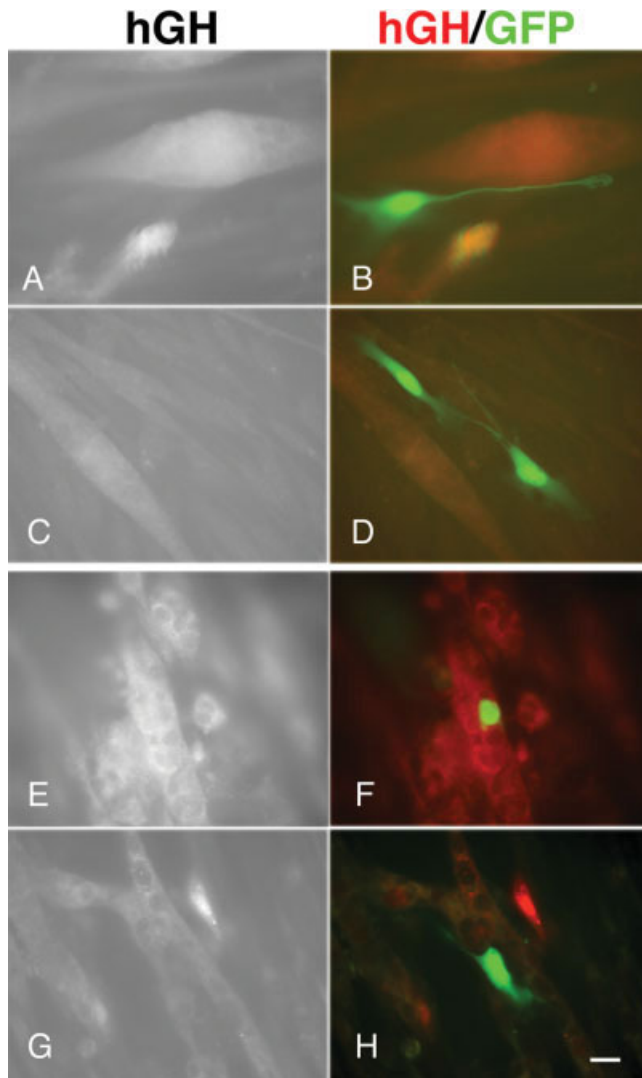


Fig. 1. Cell-contact-induced *AChR* expression assay. D6P2T or 293T cells were transfected with a plasmid encoding GFP. The following day, cells were plated at low density onto Sol8 myotubes stably transfected with a *AChR δ -hGH* fusion. Two days later, dishes were fixed and stained for hGH. GFP was visualized under FITC optics while hGH was visualized under rhodamine optics. (B, D) Representative examples of cell contacts made by D6P2T cells on myotubes expressing ErbB4. (B) One Schwann cell contacts a myotube expressing high levels of hGH (A). (D) Two D6P2T cells make contact with a myotube lacking hGH expression (C). Pictures in panels A and C were taken at the same camera gain, from fields in the same dish. (F, H) Representative examples of cell contacts made by 293T cells expressing Nrg-2 on myotubes expressing ErbB4. (F) One such cell contacts a myotube expressing very high levels of hGH (E). (H) More than one *nrg-2*-transfected 293T cells contact a myotube weakly expressing hGH (G). Pictures in panels E and G were taken at the same camera gain, from fields in the same dish. Scale bar: 20 μ m.

0.5% Triton in PBS, dishes were incubated in 1/500 rhodamine-anti-rabbit polyclonal (Jackson ImmunoResearch, West Grove, PA). After washing as above, dishes were dried at room temperature for 10 min and coverslips were mounted in Vectashield (Vector Labs, Burlingame, CA). Dishes were coded so that the observer was blind to treatments. Pictures were taken of rhodamine fluorescence using a constant camera gain and exposure time using a

40 \times oil objective. At least 20 fields/dish, were examined. Sometimes, it was necessary to view samples with transmitted light to distinguish beads from myonuclei. Contacts were scored as positive, if on a hGH-expressing myotube, or negative, if on a myotube not expressing hGH.

Cell-Contact-Mediated AChR Expression Assay

D6P2T or 293T cells were transfected with pGreen (Gibco), a green-fluorescent protein (GFP)-encoding plasmid, using Fugene (Roche) per manufacturer instructions. Alternatively, 293T cells were cotransfected with pGreen and an expression vector encoding full-length Nrg-2 β (T. Fischer, unpublished). Total amount of DNA/dish was 3 μ g. Preliminary experiments determined that the optimal ratio for transfection, the one for which every GFP+293T cell was also Nrg-2+ was 3:2 (mass to mass) pNrg-2 β to pGFP (data not shown). Sixty thousand transfected cells were plated onto 35 mm dishes harboring Sol 8 myotubes stably transfected with an *AChR δ -hGH* fusion. Two days later, dishes were fixed and stained for hGH as above. Pictures were taken with a 40 \times oil immersion objective both with FITC (for GFP) and rhodamine optics (for hGH staining). D6P2T cells were considered as contacting a myotube if any part of their somata or processes were contacting the muscle cells. Scoring was essentially as described for the bead assay above.

RESULTS

We found previously that treatment of Sol8 mouse myotubes that had been stably transfected with an *AChR δ -human growth hormone (hGH)* gene fusion, with the EGF-like domain of Nrg-2 β or Nrg-2 α fused to GST, stimulated *AChR δ* transcription measured by a radioimmunoassay that quantified the amount of hGH secreted into the medium (Ponomareva et al., 2005; Rimer et al., 2004). However, Nrg-2 α was at least 20-fold less potent than Nrg-2 β in this assay (Ponomareva et al., 2005). Sol8 myotubes that were responsive to Nrg-2 expressed the three Nrg receptors, ErbB2, ErbB3, and ErbB4, whereas Sol8 myotubes that were unresponsive to Nrg-2 lacked ErbB4 expression. ErbB4 was introduced into the cells by stable transfection because typically muscle cell lines do not express this receptor, even though muscle fibers *in vivo* do (Plowman et al., 1993; Srinivasan et al., 1998). Furthermore, ErbB4 has been reported as concentrated in the adult postsynaptic sarcolemma (Trinidad et al., 2000; Zhu et al., 1995). The Nrg-2-evoked response in ErbB4/3/2-expressing Sol8 myotubes showed specificity in that, Nrg-3 and Nrg-4 (Harari et al., 1999; Zhang et al., 1997), both selective ligands of ErbB4, failed to induce *AChR* expression in these cells.

D6P2T Cells Induce AChR Expression in an ErbB4- and Contact-Dependent Fashion

Prior work from our lab also suggested that the cellular source for the bulk of Nrg-2 at the NMJ are the ter-

minal Schwann cells (Rimer et al., 2004), and that these cells likely make Nrg-2 α , the isoform less potent in inducing *AChR* transcription in cultured myotubes when presented in soluble form (Ponomareva et al., 2005). Thus, the question arises as to whether Nrg-2 α , endogenously produced at physiological levels by a Schwann cell, could activate ErbB4 receptors on the muscle fiber surface to stimulate *AChR* expression. Terminal Schwann cell processes in vivo contact muscle fibers on the perisynaptic region (e.g. (Ogata and Yamasaki, 1984). Because all Nrg-2's characterized to date contain an extracellular immunoglobulin-like (Ig)-domain, and because the Ig-domain mediates Ig-Nrg-1's binding to extracellular matrix (Loeb and Fischbach, 1995), it is possible that Nrg-2 α localizes to the surface of terminal Schwann cells and that its effectiveness as inducer of *AChR* expression is higher as cell-attached rather than as soluble ligand.

We sought to test whether Nrg-2 α could function as inducer of *AChR* expression in a cell-attached mode. We previously found by RT-PCR analysis that D6P2T cells, a standard Schwann cell line (Bansal and Pfeiffer, 1987), synthesize Nrg-2 α but not Nrg-2 β (Ponomareva et al., 2005). We also found little, if any, Nrg-2 immunoreactivity by Western blot in D6P2T conditioned media. Such media failed to induce *AChR* transcription over control levels, even after concentration of proteins by an order of magnitude (data not shown). We decided to co-culture D6P2T cells and myotubes to assay whether these cells could induce *AChR* expression when in contact with muscle cells. To assay cell-contact-induced *AChR* expression, we plated GFP-expressing D6P2T cells at low density on top of Sol8 myotube cultures stably transfected with the *AChR δ -hGH* fusion. As explained earlier, changes in hGH levels report changes in *AChR* transcription. Following two days of co-culture and hGH staining, visualized by rhodamine-epifluorescence, we then searched for GFP-expressing D6P2T cells under FITC epifluorescence and scored whether or not a given cell was making contact with a myotube expressing detectable or undetectable levels of hGH. Although hGH is mainly released into the medium, intracellular hGH immunoreactivity accumulates in the secretory pathway, thus staining is perinuclear [Dutton et al. (1993) and Fig. 1]. If the myotube contacted by a particular D6P2T cell exhibited positive hGH staining, then that contact was scored as positively associated with high *AChR* expression (Figs. 1A,B). When D6P2T cells were plated on Sol8 myotubes that do not express ErbB4, and therefore are unresponsive to Nrg-2, about 20% of the contacts were associated with high hGH staining (Fig. 2). In contrast, when D6P2T cells were plated on Sol8 myotubes that express ErbB4, and therefore are responsive to Nrg-2, about 60% of the contacts were associated with high hGH staining (Fig. 2). Thus, there was a 3-fold increase in contacts associated with high *AChR* expression that was ErbB4-dependent. To rule out that this result was simply due to some effect of ErbB4 expression resulting in higher expression of hGH, we plated GFP-expressing 293T cells, that lack detectable expression of Nrg-2, on top of myotubes and per-

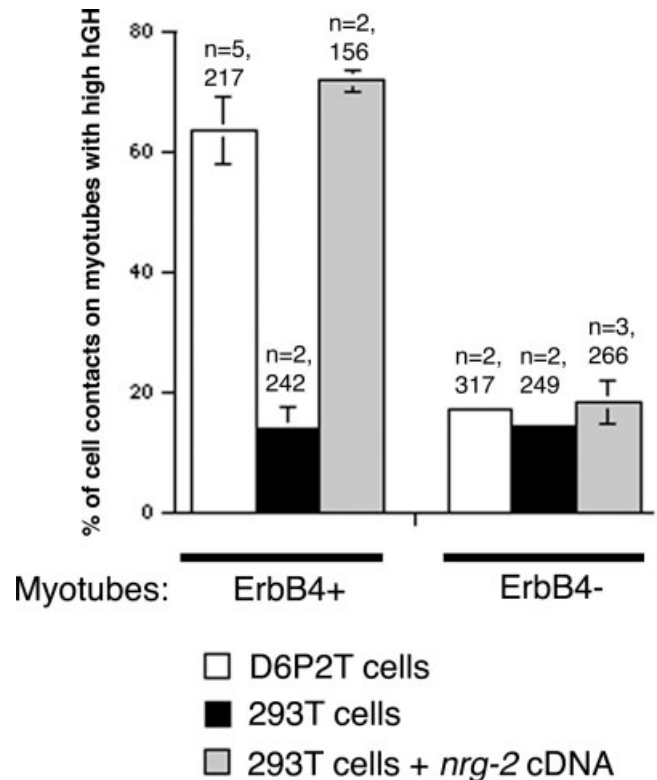


Fig. 2. Quantification of cell contacts associated with high hGH expression. GFP-expressing D6P2T, 293T, or 293T+*nrg-2 β* cDNA cells, formed contacts onto Sol8 myotubes expressing or not human ErbB4 (ErbB4+, ErbB4-, respectively). The percentage of those contacts associated with high hGH staining (Fig. 1) 2 days after co-culture is presented. About 3-fold more high-expressing hGH contacts were observed for D6P2T and 293T+*nrg-2 β* cDNA cells, when they were plated onto ErbB4+ myotubes. hGH+ contacts for 293T cells did not varied with ErbB4 expression. The number (n) of independent platings and the total number of contacts scored are indicated.

formed a similar analysis. We found no significant difference in the number of 293T contacts associated with high *AChR* expression between myotubes that expressed or lacked ErbB4 (Fig. 2). As an additional control, we transfected a full-length Nrg-2 cDNA (encoding the β -isoform) into 293T cells and plated these cells on Sol 8 myotubes expressing ErbB4 (Figs. 1E-H). 293T cells transfected with this cDNA showed Nrg-2 immunoreactivity on the surface, but released little, if any, Nrg-2 protein into the medium (data not shown). We found that the percentage of contacts by Nrg-2 β -transfected 293T cells associated with high *AChR* expression climbed to about 70% (Fig. 2). Thus, full-length, native Nrg-2 can induce contact-mediated activation of *AChR* expression.

Neuregulin-2 α Can Function as a Tethered Ligand

We next tested more directly whether Nrg-2 α could function as a tethered ligand. To this aim, we coated polystyrene beads with saturating concentrations of either

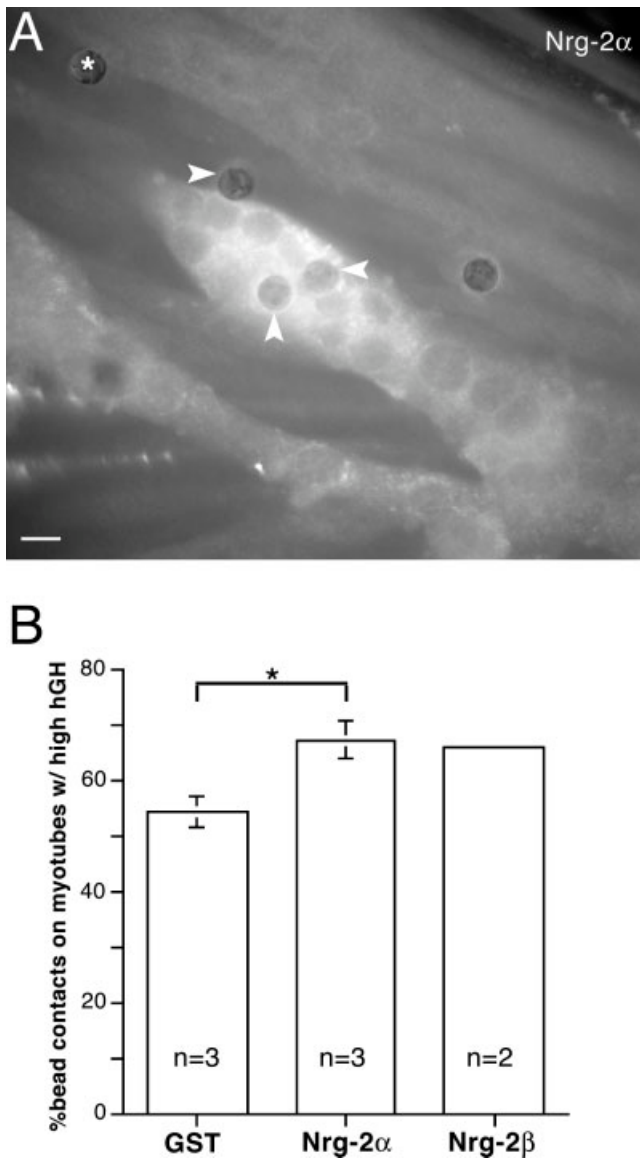


Fig. 3. Nrg-2's can function as tethered ligands. Polystyrene beads coated with GST-Nrg2 α or GST-Nrg-2 β , were added to ErbB4-expressing myotubes stably transfected with a *AChR δ -hGH* fusion. About two days later, cultures were fixed and stained for hGH as in Fig. 1A. A hGH⁺ myotube contacted by three beads coated with GST-Nrg-2 α (arrowheads) is shown in the center of the field. A hGH-myotube contacted by one bead coated with GST-Nrg-2 α (asterisk) is shown in the top left corner of the field. Scale bar: 10 μ m. (B) Quantification of the contacts. Nrg-2-coated beads had contact with a higher fraction of hGH⁺ myotubes than GST-coated beads. n, number of independent platings. *, $P = 0.038$, two-tailed t -test.

GST, or GST fused to the EGF-like domains of Nrg-2 α or Nrg-2 β . Coating was confirmed by staining the beads with antibodies specific for GST (Methods and Supplementary Fig. 1). Quantification of this staining by measuring average gray value per bead, showed that on average GST-Nrg-2 β beads displayed higher labeling than GST beads (about 3-fold), while GST-Nrg2 α beads exhibited lower labeling than GST-beads (about 0.7-fold). We then applied the coated beads to ErbB4-expressing Sol8 myotubes, stably transfected with the *AChR δ -hGH*

fusion, which are responsive to soluble Nrg-2 (Rimer et al., 2004). After 2 days, dishes were fixed and stained for hGH. To assay for *AChR* expression, we next scored whether beads contacted hGH⁺ or hGH⁻ myotubes. Figure 3A shows examples of myotubes with either abundant or undetectable hGH levels contacted by beads coated with Nrg-2 α . Figure 3B shows that, on average, beads coated with Nrg-2 were found in contact with a higher fraction of hGH-expressing myotubes than beads coated with GST. For GST-Nrg-2 α , 67.2% \pm 3.3% of the contacted myotubes expressed high levels of hGH, while for GST this fraction was 54.1% \pm 2.8%. The effect was small but reproducible and statistically significant ($P = 0.038$, $n = 3$; two-tail t -test). For GST-Nrg-2 β , the fraction of hGH-expressing myotubes contacted by beads was 65.6% \pm 0.6% ($n = 2$). Thus, even though beads coated with Nrg-2 α contained less protein than beads coated with Nrg-2 β , the former induced similar levels of *AChR* expression as the latter. We do not understand why GST-beads contact such a high fraction of hGH⁺ myotubes. This is explained, at least in part, by the fraction of myotubes with high hGH present before we apply the beads, which we estimate to be around 20% (see the earlier section and Fig. 2). In addition, it is expected that bead contacts in general cause larger surface alterations on the myotubes than cell contacts, as the former occur over a larger area while the latter are more focal. These surface alterations could somehow trigger nonspecific activation of *AChR* expression. Consistent with this interpretation, we found that about 50% of the contacts by uncoated beads, by beads coated with GST or by beads coated with the GST-Nrg-2 were on hGH⁺ myotubes when these beads were placed on myotubes unresponsive to Nrg-2 (i.e. lacking ErbB4 expression). The hGH⁺ contacts on these cells for uncoated beads: 46.3 \pm 4.5%, $n = 3$; for GST beads: 53.7% \pm 3.9%, $n = 3$; for GST-Nrg2 α beads: 51.5% \pm 0.5%, $n = 2$; and for GST-Nrg2 β beads: 55.5% \pm 0.5%, $n = 2$. Thus, despite the low signal-to-noise ratio in the assay, the results suggest that both Nrg-2 α and Nrg-2 β are capable of activating *AChR* expression as tethered ligands.

Neuregulin-2 Staining on the Surface of D6P2T Cells

We next determined whether we could detect Nrg-2 immunoreactivity on the surface of Schwann cells. We stained low-density cultures of D6P2T cells in the absence of detergent with previously characterized, affinity-purified anti-Nrg-2 antibodies, raised against an extracellular epitope (Rimer et al., 2004). We found Nrg-2 immunoreactivity on the processes and cell bodies of these cells (Figs. 4A–F). We also found surface Nrg-2 staining on neonatally derived, primary Schwann cells (data not shown). Interestingly, surface staining on D6P2T cells was not evenly distributed along the outside of the cell, and tended to concentrate on one side of the cell body, as well as in clusters along the cell processes (Figs. 4C–F). The skewed pattern of Nrg-2 staining in D6P2T cells is

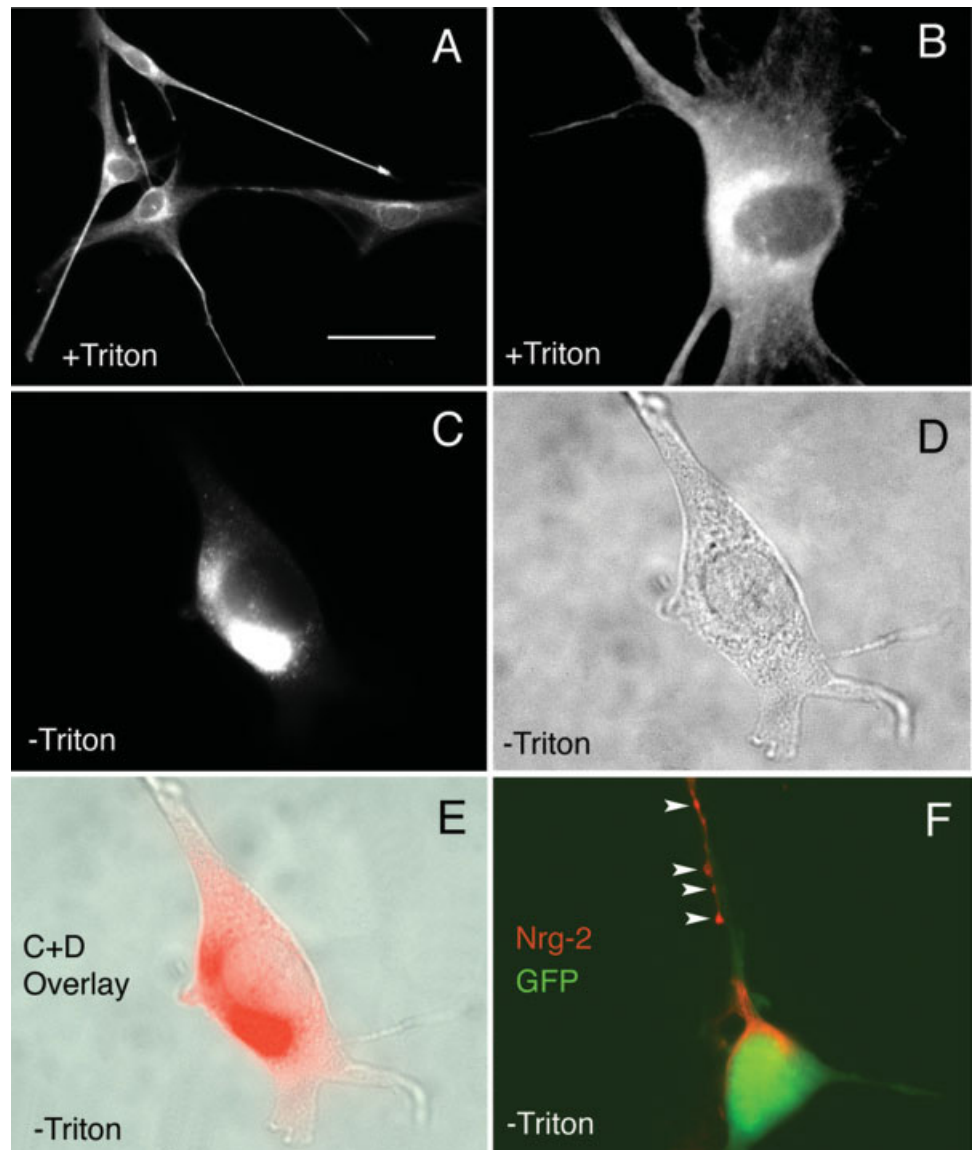


Fig. 4. Nrg-2 on the surface of D6P2T cells. D6P2T cells were fixed in 1% paraformaldehyde, and stained with affinity-purified antibodies to Nrg-2, in the absence and presence of detergent ($-$ Triton and $+$ Triton) to reveal surface, and total cell staining, respectively. (A, B) Permeabilized D6P2T cells showed staining along the entire cell body and processes, with some concentration on one side of the cell nucleus. (C–F) Nonpermeabilized D6P2T cells displayed surface staining that tended to concentrate on one side of the cell body (C and E), or that appeared as clusters along processes (arrowheads, F). (D) Phase contrast for cell in (C). Cell in (F) was transfected with a plasmid encoding GFP. Scale bar: A: 50 μ m. B–F: 20 μ m.

reminiscent of the pattern of Nrg-2 staining in terminal Schwann cells in vivo (Rimer et al., 2004), in which staining is highest on the side of the cell body away from the NMJ and on the processes. Thus, D6P2T cells localize Nrg-2 α to the surface, but appear to release very little of it into the conditioned medium, or it may be rapidly degraded after release.

D6P2T Cells Lack Transmembrane Nrg-1 Expression

The findings mentioned earlier are consistent with the notion that surface Nrg-2 α produced by the D6P2T cells induces *AChR* expression when it contacts a responsive, ErbB4-expressing Sol8 myotube. However, they do not rule out that other potential ErbB4 ligands synthesized by the D6P2T cells actually mediate this effect. The only other ErbB4 ligand known to induce

AChR expression in vitro is Nrg-1. However, the observed ErbB4 dependency suggests that Nrg-1 is not responsible, as it has been shown to induce *AChR* expression in an ErbB4-independent manner (Ponmareva et al., 2005; Rimer et al., 2004). In addition, the failure of D6P2T conditioned medium to induce *AChR* expression indicates that these cells either do not produce secreted forms of Nrg-1 or do not release them in sufficient quantity to elicit measurable effects. We used RT-PCR to simply check whether D6P2T cells express Nrg-1. We failed to detect Nrg-1 expression in D6P2T cells using primers designed to detect all Nrg-1 transmembrane isoforms (Fig. 5). As previously reported (Raabe et al., 1996), we found Nrg-1 expression (α 2 and β 4 isoforms) in primary neonatal Schwann cells (Fig. 5) using these primers. Thus, together these results strongly argue against Nrg-1 as the surface ligand mediating the contact-dependent *AChR* expression induced by the D6P2T Schwann cell line.

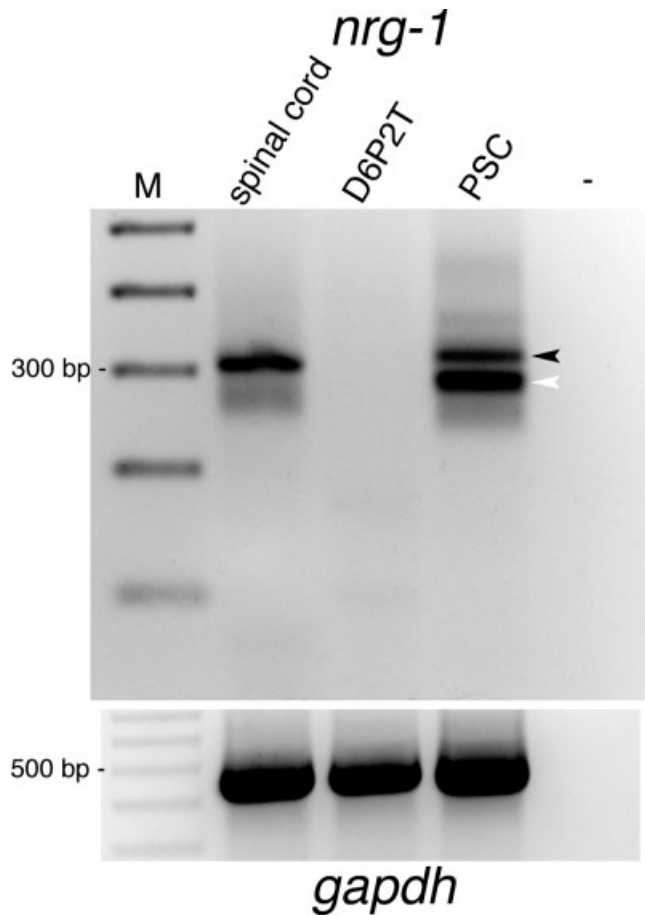


Fig. 5. Lack of *nrg-1* transcripts in D6P2T cells. Top panel: Total RNA from spinal cord, D6P2T and primary neonatal Schwann cells (PSC) was subjected to RT-PCR for *nrg-1*. No *nrg-1* transcripts were detected in D6P2T cells, while as expected, they were in spinal cord and PSC. The two PSC bands were cloned and sequenced. Black arrowhead: Nrg-1 α 2. White arrowhead: Nrg-1 β 4. Bottom panel: The integrity of the RNA was checked by RT-PCR for *gapdh*. M: 100-bp ladder. (-) H₂O control.

DISCUSSION

The main finding from this study is that D6P2T Schwann cells expressing Nrg-2 α on their surface can induce *AChR* expression if they contact ErbB4-expressing, Nrg-2-responsive Sol8 myotubes. D6P2T cells induce *AChR* expression apparently as well as 293T cells transfected with Nrg-2 β , the isoform with the highest *AChR*-inducing activity as soluble form. These results are consistent with a role for terminal Schwann cell-derived Nrg-2 α as a perisynaptic signal that boosts *AChR* expression at normal adult NMJs (see later).

We devised an assay to test whether Schwann cells with endogenous levels of Nrg-2 α on their surface, could induce *AChR* expression when in contact with myotubes. We used D6P2T and not primary Schwann cells because the latter express Nrg-1 (Fig. 5), in addition to Nrg-2 α (Ponomareva et al., 2005), whereas the former do not synthesize Nrg-1 (Fig. 5). We reasoned that if Nrg-2 were mediating contact-dependent *AChR* expression,

then the effect in myotubes would require the presence of ErbB4. Consistent with this prediction, we found 3-fold more D6P2T contacts associated with high *AChR* expression on ErbB4-expressing myotubes than on ErbB4-negative myotubes. The ~20% of cell contacts associated with high *AChR* expression on myotubes without ErbB4 presumably results from simple random contact of D6P2T, or 293T, cells with myotubes that already have high levels of hGH prior to co-culture. In addition, transfection of 293T cells, which have undetectable levels of Nrg-2, with a Nrg-2 β cDNA, also lead to ErbB4- and cell-contact-dependent increase in high *AChR* expression. Although this Nrg-2 cDNA encodes Nrg-2 β , myotubes that came into contact with beads coated with the EGF-like domain of Nrg-2 α fused to GST were also responsive, showing that Nrg-2 α could function as a tethered ligand (Fig. 3). Despite the caveat of a low signal-to-noise ratio, the bead assay suggests that Nrg-2 α might be a more potent tethered ligand than Nrg-2 β as under our conditions, they both caused similar *AChR* induction even though there were about three times more Nrg-2 β than Nrg-2 α on the beads. The results with the D6P2T and Nrg-2 β -transfected 293T cells were consistent with this interpretation, however, we did not quantify the surface Nrg-2 expression in these cells. Thus, we conclude that D6P2T cells can induce cell-contact-dependent *AChR* expression on ErbB4-expressing myotubes and that Nrg-2 α is the most likely candidate mediating this effect. The fact that not every contact by D6P2T cells was associated with high *AChR* expression may be explained by the finding that Nrg-2 is not evenly distributed along the surface of these cells (Fig. 4). These data raise the possibility that Nrg-2 α from terminal Schwann cells might activate synaptic ErbB4 on the muscle fiber in vivo. Consistent with this possibility, there is anatomical evidence that these cells contact the muscle fiber basal lamina in the perisynaptic region [e.g. Ogata and Yamasaki (1984)].

Motor neurons synthesize Nrg-2 β , an isoform 20 times more potent than Nrg-2 α in inducing *AChR* transcription in vitro as a soluble ligand (Ponomareva et al., 2005). Although, this might make Nrg-2 β a better candidate to stimulate postsynaptic ErbB receptors than Nrg-2 α , we lack evidence of its presence at the endplate (Ponomareva et al., 2005; Rimer et al., 2004). This pattern of localization is consistent with findings by others that in central neurons Nrg-2 is targeted to dendrites and not to axons (Longart et al., 2004).

Agrin/MuSk signaling appears now as the indispensable signaling cascade used by the nerve to maintain the high rate of *AChR* transcription at the synapse. However, the *AChR* density of most agrin-induced ectopic *AChR* clusters in adult muscle is lower than that at NMJs of the same muscles (Escher et al., 2005; Rimer et al., 1997). Although, alternative interpretations are plausible [see Escher et al. (2005)], this disparity raises the possibility that signaling pathways other than agrin/MuSk, perhaps derived from motor neurons and/or terminal Schwann cells, the two synaptic cell types absent in the ectopic agrin preparation, contribute to boost

AChR density to levels found at normal adult endplates. Such a role may be played by Nrg-signaling in adult muscles. In agreement with this possibility, we have recently shown that expression of a constitutively active ErbB2 receptor specifically in young adult muscle led to increased *AChR* expression. This was not so if expression of the constitutively active ErbB2 occurred in neonates or embryos. In fact, the latter led to synaptic disassembly (Ponomareva et al., 2006). These results suggest that the effects of activating Nrg-signaling in skeletal muscle are highly dependent on the state of differentiation of the muscle fiber or the state of maturation of the NMJ.

Recent characterization of ErbB- and Nrg-1-deficient mice (Escher et al., 2005; Jaworski and Burden, 2006; Leu et al., 2003), clearly demonstrate that effects on *AChR* expression of these manipulations must be analyzed quantitatively given the modulatory role of Nrg-signaling. Thus, it will be important to carefully evaluate the Nrg-2-deficient mice (Britto et al., 2004), which are viable and fertile, before a more compelling conclusion can be made concerning the role of this factor in synaptic *AChR* expression in vivo.

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