

## STIMULATION OF ACETYLCHOLINE RECEPTOR TRANSCRIPTION BY NEUREGULIN-2 REQUIRES AN N-BOX RESPONSE ELEMENT AND IS REGULATED BY ALTERNATIVE SPLICING

O. N. PONOMAREVA,<sup>a</sup> H. MA,<sup>a,b1</sup> R. DAKOUR,<sup>a1</sup>  
T. D. RAABE,<sup>d</sup> C. LAI<sup>e</sup> AND M. RIMER<sup>a,b,c\*</sup>

<sup>a</sup>Section of Neurobiology, University of Texas at Austin, Austin, TX 78712, USA

<sup>b</sup>Institute for Neuroscience, University of Texas at Austin, Austin, TX 78713, USA

<sup>c</sup>Institute for Cellular and Molecular Biology, University of Texas at Austin, Austin, TX 78712, USA

<sup>d</sup>Department of Biological Sciences, St. Mary's University, San Antonio, TX 78228, USA

<sup>e</sup>Department of Neuropharmacology, Scripps Research Institute, 10550 North Torrey Pines Road, La Jolla, CA 92037, USA

**Abstract**—The neuregulin (Nrg) family of growth/differentiation factors is encoded by at least four genes in the mammalian genome: *nrg-1*, *nrg-2*, *nrg-3* and *nrg-4*. *Nrg-1* and *Nrg-2* share the highest homology within the family, and the primary RNA transcripts from their encoding genes are subjected to extensive alternative splicing. Although little is known about the biological function of *Nrg-2-4*, their structural similarity with *Nrg-1* suggests that they could account for some of the activities presently attributed to *Nrg-1*. Thus, at the neuromuscular junction *Nrg-1* has been a favored candidate for the signal that activates selective acetylcholine receptor (AChR) transcription in synaptic myonuclei. However, we have recently shown that like *Nrg-1*, *Nrg-2* can also activate *AChR* transcription in cultured myotubes and accumulates at the synaptic site. Synapse-specific and *Nrg-1*-induced *AChR* transcription require an enhancer sequence, the N-box, which is also mutated in some patients with congenital myasthenia gravis. Here, we show that *Nrg-2*-induced *AChR* transcription requires an N-box motif and is regulated by alternative splicing. We also show that unique *Nrg-2* isoforms are differentially distributed between spinal cord and skeletal muscle, the tissues that harbor the cellular components of the neuromuscular synapse. © 2005 IBRO. Published by Elsevier Ltd. All rights reserved.

**Key words:** neuromuscular junction, synaptogenesis, neuregulin, acetylcholine receptor.

<sup>1</sup> Present address: Northern California Institute for Research and Education, San Francisco, CA 94121, USA (H. Ma); Texas Tech University Medical School, Lubbock, TX 79430, USA (R. Dakour).

\*Correspondence to: M. Rimer, University of Texas at Austin, Section of Neurobiology, 1 University Station C0920, Austin, TX 78712-0248, USA. Tel: +1-512-471-1747; fax: +1-512-471-9651.

E-mail address: rimer@mail.utexas.edu (M. Rimer).

**Abbreviations:** ACh, acetylcholine; AChR, acetylcholine receptor; CMV, cytomegalovirus; DMEM, Dulbecco's Modified Eagle's Medium; EDTA, ethylenediamine tetra-acetic acid; EGF, epidermal growth factor; E, embryonic day; GST, glutathione-S-transferase; hGH, human growth hormone; Ig, immunoglobulin; IRES, internal ribosomal entry site; NMJ, neuromuscular junction; Nrg, neuregulin; PBS, phosphate-buffered saline; RT-PCR, reverse-transcriptase–polymerase chain reaction.

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Acetylcholine receptors (AChRs) become concentrated at the mammalian neuromuscular junction (NMJ) via post-translational and transcriptional mechanisms (for reviews see (Sanes and Lichtman, 2001; Burden, 2002)). Several lines of evidence indicate that these signaling pathways are driven by neuronal forms of the proteoglycan agrin, and by the neurotransmitter acetylcholine (ACh). Thus, at the posttranslational level, agrin activates the muscle-specific receptor tyrosine kinase MuSK, and this, in turn, causes clustering of AChRs to synaptic sites via an uncharacterized mechanism that involves the peripheral membrane protein rapsyn. At the transcriptional level, ACh-evoked electrical muscle activity causes suppression of *AChR* transcription in all the muscle fiber's nuclei except in the synaptic nuclei, those myonuclei positioned directly beneath the ACh-releasing nerve terminal. In these, another nerve-dependent signal selectively stimulates the transcription of AChR subunit genes, causing the accumulation of *AChR* mRNA at the synapse (for review see Rimer, 2003). Synapse-specific *AChR* transcription requires an enhancer sequence, the N-box motif, present in the promoter and/or intronic regions of the *AChR* subunit and other postsynaptic genes including those encoding utrophin and acetylcholinesterase. Mutation of the N-box abolishes synapse-specific expression *in vivo*, and stimulation of *AChR* transcription in cultured myotubes by factors such as neuregulin-1 (*Nrg-1*). Furthermore, there are reports of human congenital myasthenias in which the N-box in the AChR $\epsilon$ -subunit promoter is mutated (for review see Engel et al., 2003). These patients show reduced *AChR* expression further supporting the N-box as a critical element in the regulation of *AChR* expression *in vivo*.

Deletion of the *agrin* and *MuSK* genes by homologous recombination (DeChiara et al., 1996; Gautam et al., 1996), and induction of an electrical activity-resistant postsynaptic-like apparatus by ectopic application of neural agrin (Cohen et al., 1997; Jones et al., 1997), or constitutively active MuSK (Sander et al., 2001), indicate that the agrin/MuSK pathway is an important part of the signal that activates synapse-specific *AChR* transcription *in vivo*. MuSK activation appears to launch two parallel signaling cascades that lead to increased *AChR* transcription (Lacazette et al., 2003). One pathway would cause stimulation of *AChR* expression by clustering of muscle-derived *Nrg-1* and its ErbB receptors, while the other pathway would operate in a *Nrg*-independent fashion. It is unclear whether these two pathways are redundant or complementary.

Other observations suggest that additional signaling cascades may also regulate synaptic *AChR* transcription,

particularly in the adult NMJ. Thus, the density of AChRs at the postsynaptic apparatus induced by ectopic agrin is lower than the AChR density at NMJs in the same muscle (Cohen et al., 1997; Rimer et al., 1997; Lacazette et al., 2003), suggesting that other signals, perhaps from cellular components absent in the ectopic agrin preparation, i.e. terminal Schwann cells and motor neurons, account for the difference. Consistent with this possibility, mice heterozygous for a mutation in the immunoglobulin (Ig)-like domain of Nrg-1 are mildly myasthenic, as they possess about half their normal complement of AChRs in their NMJs, suggesting that Nrg-1 is necessary to maintain high density of AChRs at normal synapses (Sandrock et al., 1997). Nonetheless, nerve-derived Nrg-1 seems a dispensable signal in this situation as mice with conditional ablation of *nrg-1* in sensory and motor neurons display normal synapse-specific AChR expression (Yang et al., 2001).

Homology cloning has uncovered three additional *nrg-1*-related genes: *nrg-2*, *nrg-3* and *nrg-4*, whose biological functions remain unknown, but which could account for some of the activities presently ascribed to Nrg-1 (for review see Rimer, 2003). We have recently shown that Nrg-2 is a suitable candidate for a signal that regulates AChR expression at the adult neuromuscular synapse (Rimer et al., 2004). These studies revealed that Nrg-2 is expressed by motor neurons and terminal Schwann cells, accumulates adjacent to NMJs, and is endowed with AChR-inducing activity when assayed on cultured myotubes expressing the Nrg receptor ErbB4. Here, we show that the ability of Nrg-2 to stimulate AChR transcription requires the N-box motif, and is regulated by alternative splicing affecting the domain of the molecule essential for binding and activating its cognate receptors, i.e. the epidermal growth factor- (EGF-) like domain. We also demonstrate that isoforms of the Nrg-2 EGF-like domain are expressed in a tissue-specific manner in spinal cord and skeletal muscle.

## EXPERIMENTAL PROCEDURES

### Generation of muscle cell lines expressing ErbB4, with and without mutated N-box

We first cloned full-length human ErbB4 cDNA into the pRESHyg2 vector (BD Biosciences Clontech, Palo Alt, CA, USA). Expression in pRESHyg2 is driven by the cytomegalovirus (CMV) promoter. The vector also contains an internal ribosomal entry site (IRES) downstream from the multiple cloning site followed by the hygromycin B phosphotransferase gene. ErbB4 was excised from pTZ19R, a kind gift of S. J. Burden, by *Xba*I digestion, and was ligated into *Nhe*I-cut pRESHyg2. Clones with the correct orientation were selected by digestion with *Pst*I. ErbB2/3-expressing Sol8 cell lines harboring AChR $\delta$ -human growth hormone (hGH) gene fusions, having wild-type or mutated N-boxes (Fromm and Burden, 1998), a kind gift of L. Fromm, were transfected with the CMV-ErbB4-IRES-hygromycin vector using Superfect according to manufacturer's instructions (Qiagen, San Diego, CA, USA). Single clones of stably-transfected myoblasts were selected by growth in medium containing 250 ng/ml hygromycin (A.G. Scientific, San Diego, CA, USA). Single clones were expanded in growth medium containing 170 ng/ml hygromycin and induced to differentiate into myotubes as previously described (Rimer et al., 2004). Clones that failed to differentiate properly were discarded

for further study and the others were analyzed for ErbB4 expression (below).

### Western blotting

To check for ErbB4 expression myoblast clones were differentiated into myotubes (Rimer et al., 2004) and extracted with 50 mM Tris pH 7.5, 150 mM NaCl, 0.5 mM ethylenediamine tetra-acetic acid (EDTA), 0.5% NP-40, 1% Triton X-100, 10% protease inhibitor cocktail (P-8340, Sigma, St. Louis, MO, USA). Western blotting was done as previously described (Rimer et al., 2004). Membranes were probed with an anti-ErbB4 antibody (sc-283, Santa Cruz Biotechnology, Santa Cruz, CA, USA) at 1/1000, and with HRP-conjugated anti-rabbit IgG secondary (Jackson ImmunoResearch, West Grove, PA, USA) at 1/3000. Following stripping of the blots, membranes were re-probed with an anti-tubulin antibody (T-9026, Sigma, St. Louis, MO, USA) at 1/4000. Bands were visualized by chemiluminescence using manufacturer's instructions (Western Lighting Reagent Plus, Perkin Elmer, Boston, MA, USA).

### hGH assays

Myoblasts were grown and differentiated into myotubes as previously described (Rimer et al., 2004), and cells were treated with recombinant Nrgs for 48 h. The amount of hGH secreted into the conditioned media was measured using a double antibody hGH radioimmunoassay following manufacturer's instructions (KGHD1, Diagnostic Products Corporation).

### Animals

For expression studies, Sprague–Dawley rats were used throughout and were obtained from a local colony maintained at the University of Texas–Austin Animal Resource Center. Procedures for animal killing and tissue collection were approved by the University of Texas Institutional Animal Care and Use Committee. Every attempt was made to minimize animal suffering and to limit the number of animals used.

### Schwann cell culture

Primary Schwann cells were obtained from the sciatic nerve of 2 day old rat pups using the method of Brockes et al. (1979). Following dissociation with 0.4% collagenase (Crescent Chemical, Islandia, NY, USA) for 2 h at 37 °C, nerves were triturated with a Pasteur pipette and plated in low glucose Dulbecco's Modified Eagle's Medium (DMEM; Gibco Laboratories, Inc., Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS, Hyclone, Logan, UT, USA), 3.7 g/l NaHCO<sub>3</sub>, 100 units/ml penicillin, and 100  $\mu$ g/ml streptomycin. Fibroblasts were eliminated by cycling the cultures with cytosine arabinoside (10  $\mu$ M, Sigma) every 2–3 days followed by 6 h without cytosine arabinoside until the cultures were 99% pure.

D6P2T cells were grown in DMEM supplemented with 10% FBS and 50  $\mu$ g/ml gentamycin.

### RNA isolation and reverse-transcriptase–polymerase chain reaction (RT-PCR)

Total RNA isolation and RT-PCR for *nrg-2* was as previously described (Rimer et al., 2004). Before *Sau*3A1 digestion, PCR products were purified using a MinElute PCR purification kit (Qiagen, San Diego, CA, USA) following the manufacturer's instructions. *Sau*3A1 was purchased from New England Biolabs, Beverly, MA, USA. Acrylamide and agarose gels were stained with ethidium bromide. Pictures of gels were inverted with Adobe Photoshop 6.0, so that DNA bands appear black.

## Production of recombinant proteins in bacteria

Recombinant Nrg proteins were constructed by expressing the EGF-like domains of Nrg-1 $\beta$ , Nrg-2 $\alpha$ , or Nrg-2 $\beta$  as C-terminal glutathione-S-transferase (GST) fusion proteins. Briefly, the EGF-like domain regions were amplified and inserted into the *Eco*RI/*Bam*H1 sites of pGEX-6P-2 (Amersham Pharmacia Biotech, Piscataway, NJ, USA). Recombinant plasmids were introduced into either Origami or Origami B competent cells (Novagen, Madison, WI, USA), which support disulfide bond formation required for biologically active EGF-like domains. Expression was induced with isopropyl  $\beta$ -D-thiogalactoside (IPTG) (0.04 mM) for 2 h at 25 °C prior to harvest. Frozen bacterial pellets were lysed using BugBuster (Novagen, Madison, WI, USA) supplemented with 200  $\mu$ M 4-(2-Aminoethyl)benzenesulphonyl fluoride (AEBSF) and Benzonase (Novagen, Madison, WI, USA 17 units/ml). GST fusion proteins were bound to Glutathione Sepharose 4B (Amersham Pharmacia, Piscataway, NJ, USA) and washed five times with cold (4 °C) PBS/0.2% Tween-20/0.2% Triton X-100/10 mM EDTA followed by five washes with phosphate-buffered saline (PBS), 1.0% Triton X-114 at 4 °C to remove bacterial endotoxins and five rinses with PBS to remove excess Triton X-114 prior to three rounds of dialysis in PBS (4 °C). Factor concentrations were estimated by comparative Coomassie staining.

The Nrg-1 $\beta$  EGF-like domain cassette has the sequence: "TSHLIKCAEKEKTFVNGGECFMVKDLSNPSRYLCKCPNEFTGDRCQNYVMASFYKHLGIEFMEAEELYQK\*."

The Nrg-2 $\alpha$  EGF-like domain cassette has the sequence: "SGHARKCNETAKSYCVNGGVCYYIEGINQLSCKCPNGFFGQRCKLEKPLRLLYMPDPKQEAELYQK\*."

The Nrg-2 $\beta$  EGF-like domain cassette has the sequence: "SGHARKCNETAKSYCVNGGVCYYIEGINQLSCKCPVGYTGDRQQFAMVNFSKHLGFELKEAEELYQK\*."

## RESULTS

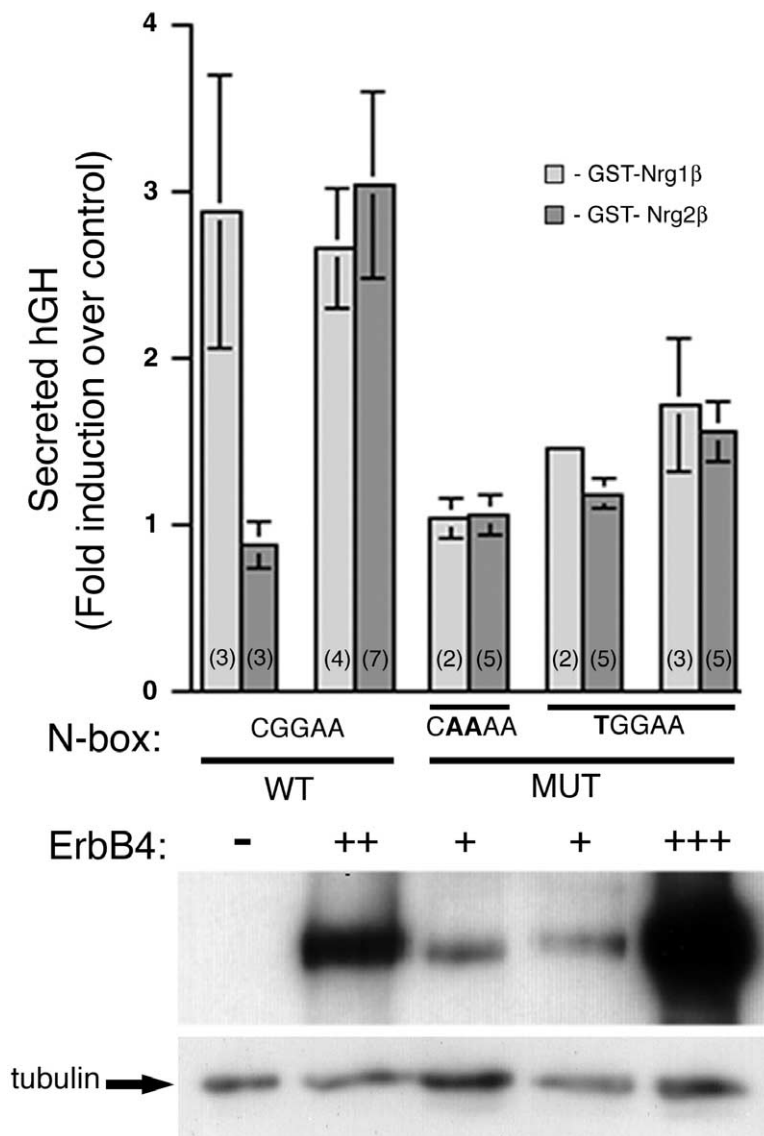
### The N-box response element is required for Nrg-2-induced AChR transcription

In a previous study, we treated Sol8 myotubes that had been stably transfected with a *AChR $\delta$ -hGH* gene fusion, with the EGF-like domain of Nrg-2 $\beta$  fused to GST, and measured the amount of hGH secreted into the conditioned medium by radioimmunoassay. We found that GST-Nrg-2 $\beta$  stimulated the production of hGH, the reporter for *AChR $\delta$  promoter-mediated* transcription, in cells that expressed the three Nrg receptors, ErbB2, 3 and 4, but not in cells that expressed only ErbB2 and 3 (Rimer et al., 2004). 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). To test whether the N-box response element is required for Nrg-2-induced *AChR* transcription, we generated new ErbB4-expressing, stable Sol8 cells lines bearing either the wild-type or mutant forms of the N-box of the *AChR $\delta$*  promoter region fused to hGH. The N-box mutations tested alter nucleotides that are known to be critical for the binding of GABP, a transcription factor of the Ets family that has been shown to interact with the N-box (Fromm and Burden, 1998), and also to be required for synapse-specific expression of *AChR* genes *in vivo* (De Kerchove D'Exaerde et al., 2002). We present studies for four cell clones, each of which expresses ErbB4 (Fig. 1). One clone contains a wild-type N-box (CGGAA), one has an N-box with two mutated

nucleotides (CAAAA, referred below as double-mutant), and the other two have an N-box with one mutated nucleotide (TGGAA, single-mutant). The latter two clones differ in their level of ErbB4 expression (Fig. 1). In addition to these four single cell lines, we also examined pools of cells from the parental cell line that do not express ErbB4 and that harbor a wild-type N-box. We treated these cells with a saturating concentration of bacterially produced GST-Nrg-2 $\beta$  or GST-Nrg1 $\beta$  (500 ng/ml or  $\sim$ 14 nM) for 48 h and determined the effect on *AChR* transcription by measuring levels of hGH in the medium. Fig. 1 shows that the parental cell line, which does not express ErbB4 (Fig. 1, Western blot, lower panel), only responded to GST-Nrg1 $\beta$  ( $2.87 \pm 0.82$ -fold induction) but not to GST-Nrg-2 $\beta$ . As previously reported for a different cell line, the cells that expressed ErbB4 in conjunction with the wild-type N-box were now capable of responding to GST-Nrg-2 $\beta$  ( $3.03 \pm 0.55$ -fold) as well as to GST-Nrg-1 $\beta$  ( $2.64 \pm 0.36$ -fold). The N-box is required for Nrg-1-induced *AChR* transcription in ErbB2/3-expressing myotubes (Koike et al., 1995; Fromm and Burden, 1998), and consistent with the observation that the response to Nrg-1 $\beta$  in ErbB2/3/4-expressing myotubes is not changed by the expression of ErbB4 ((Rimer et al., 2004), Fig. 1), cell lines with mutated N-boxes showed a dramatic decrease in their response to GST-Nrg-1 $\beta$  (histogram, Fig. 1). Thus, the response to GST-Nrg-1 $\beta$  was  $1.03 \pm 0.13$ -fold for the double-mutant,  $1.44 \pm 0.04$ - and  $1.7 \pm 0.4$ -fold, for the single-mutants, in cells that expressed moderate and high levels of ErbB4, respectively (Western blot, Fig. 1, lower panel). Thus, the N-box motif is required for Nrg-1-induced *AChR* transcription in ErbB4/3/2-expressing cells as it is in ErbB3/2-expressing cells (Fromm and Burden, 1998). Nrg-2-induced *AChR* transcription was also dramatically reduced in cells with mutant N-boxes. The response to GST-Nrg-2 $\beta$  was  $1.05 \pm 0.13$ -fold for the double-mutant, and  $1.17 \pm 0.09$ - and  $1.55 \pm 0.18$ -fold for the single-mutants, with moderate and high levels of ErbB4, respectively. Thus, these results demonstrate that the N-box enhancer is necessary for Nrg-2-induced *AChR* transcription.

### Distinct EGF-like domain Nrg-2 isoforms in spinal cord, sciatic nerve, and skeletal muscle

Molecular cloning of Nrg-2 shows that multiple isoforms are produced by alternative RNA splicing. In particular, two groups of isoforms are generated, NRG-2 $\alpha$ 's and NRG-2 $\beta$ 's, which contain alternative sequences for the C-terminal region of EGF-like domain. The  $\alpha$ - or  $\beta$ -exons encode the last two of the six cysteine residues present in the EGF-like domain. Like Nrg-1's EGF-like domain, Nrg-2's is necessary and sufficient to activate ErbB2, 3 and 4. We characterized the Nrg-2 EGF-like isoforms in the tissues that harbor the cellular components of the NMJ by RT-PCR and nucleotide sequence analysis of subcloned amplified products. Total RNA was prepared from spinal cord, sciatic nerve, and skeletal muscle of adult rat, and RT-PCR was performed using primers flanking the EGF-like domain (Fig. 2A). The expected products of about 400 bp were obtained for each sample and their identities were estab-

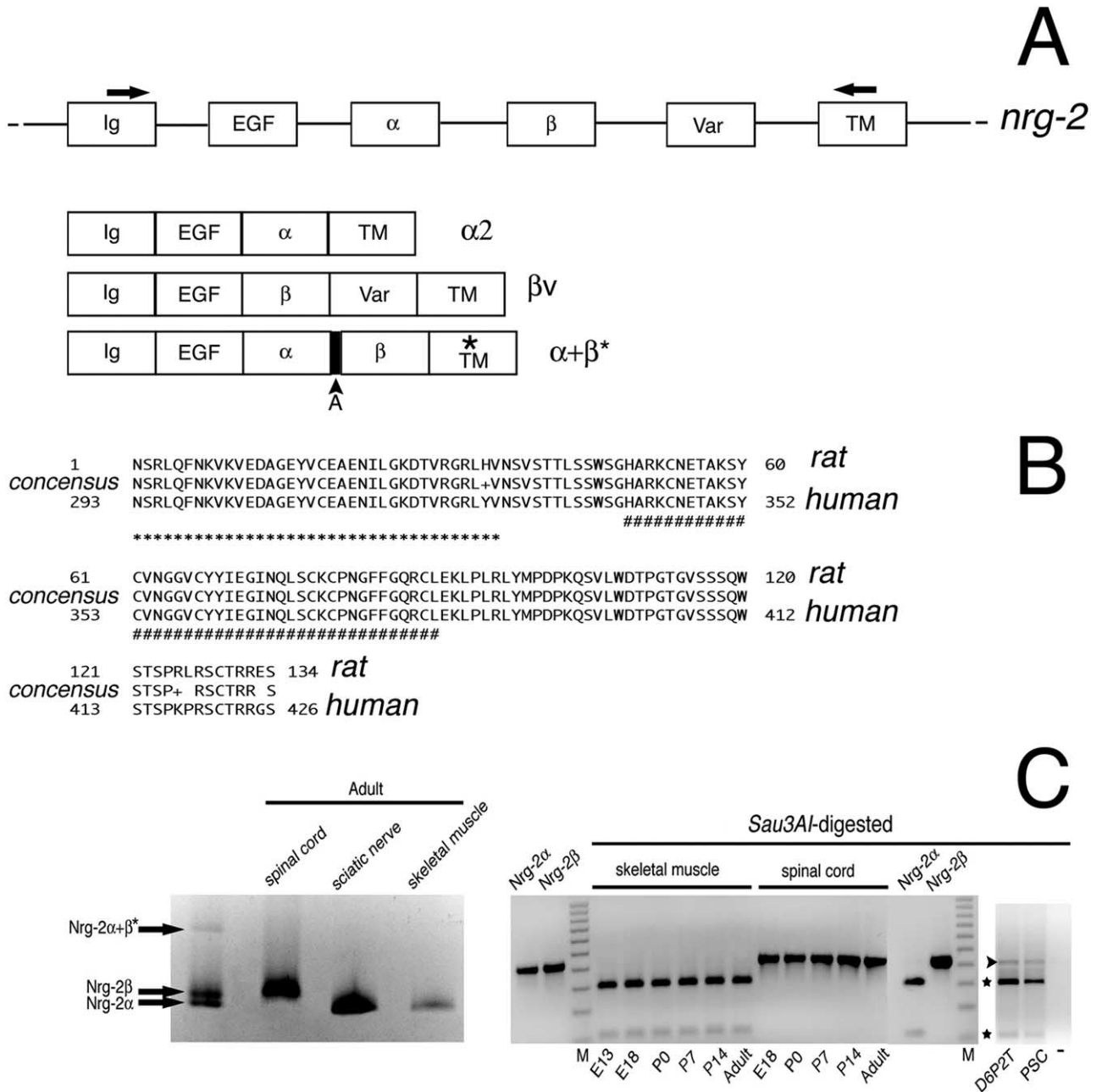


**Fig. 1.** Nrg-2-induced *AChR* transcription requires the N-box motif. Sol8 myotubes, stably transfected with an *AChR $\delta$*  subunit-hGH fusion with a wild-type (WT) or mutant N-box (MUT, bases in bold), and expressing different levels of ErbB4 (Western blot in lower panel), were treated with 500 ng/ml GST-Nrg-1 $\beta$  or GST-Nrg-2 $\beta$ . Forty eight hours later, the amount of hGH in the culture medium was measured by radioimmunoassay. In the absence of ErbB4 (indicated by (-) sign below the first two bars in the histogram, first lane in top immunoblot), myotubes of the parental cell line responded only to Nrg-1 $\beta$  (light-gray bar) but not to Nrg-2 $\beta$  (dark-gray bar). In the presence of ErbB4 (++) sign, second lane top immunoblot), and a wild-type N-box, the response to both Nrg-1 $\beta$  and Nrg-2 $\beta$  was similar. Irrespective of the levels of ErbB4 expression (+, moderate; +++, high), determined by Western blot (third through fifth lanes top immunoblot), mutations in the N-box drastically reduced the response to both Nrg-1 $\beta$  and Nrg-2 $\beta$ . The mean  $\pm$  S.E.M. of the induction relative to untreated control is given; *n*=number in parenthesis in each bar. Protein loading of Western blot was checked by re-probing membrane for tubulin (bottom immunoblot).

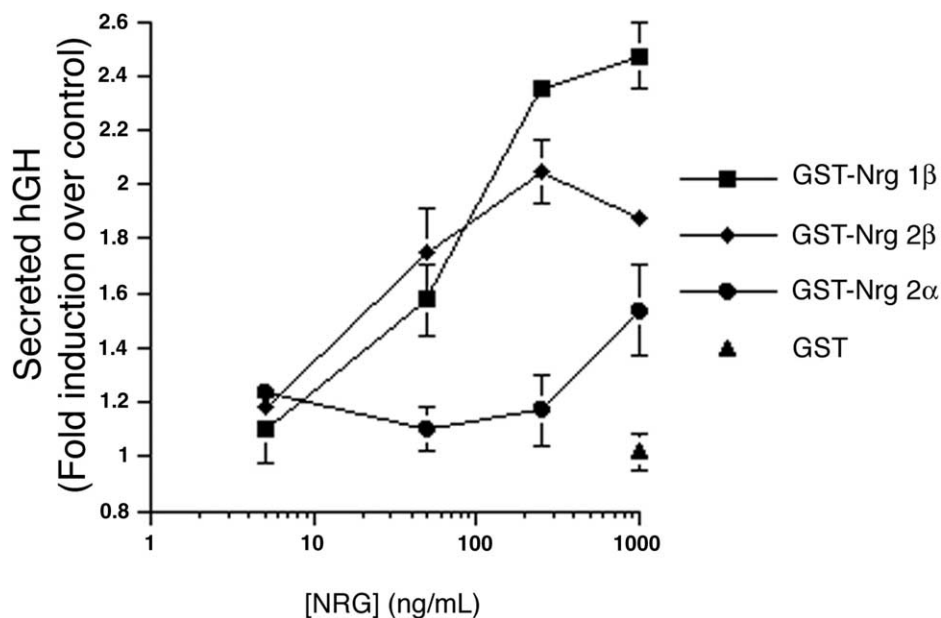
lished following cloning and nucleotide sequencing of random clones. Fig. 2A shows a cartoon of the three Nrg-2 EGF-like forms found in these tissues. Two Nrg-2 $\alpha$ 's,  $\alpha$ 2 (Yamada et al., 2000), and an isoform similar to human isoform five that we call  $\alpha + \beta^*$ , which has not been described in rodents previously, were found in sciatic nerve and skeletal muscle but not in spinal cord samples. In the latter tissue, only the  $\beta$  isoform (Yamada et al., 2000) was found. In the  $\alpha + \beta^*$  isoform, there is an additional "A" residue between the  $\alpha$ - and  $\beta$ -exons that creates a frame-shift that introduces a stop codon in the transmembrane

exon. The predicted protein product from this transcript lacks the hydrophobic membrane segment and cytoplasmic tail. Fig. 2B shows an amino acid alignment between the human Nrg-2 isoform five and the rat  $\alpha + \beta^*$  form.

The nucleotide sequence data derived from subcloned fragments suggested a tissue-specific distribution of Nrg-2 EGF-like isoforms between spinal cord and peripheral tissues: Nrg-2 $\beta$  was found in the former whereas Nrg-2 $\alpha$  was detected in the latter. To further support this conclusion, we ran the PCR products in a 6% denaturing acrylamide gel and used as size standards the amplified products from



**Fig. 2.** Tissue-specific distribution of Nrg-2 EGF-like isoforms. (A) Top: Cartoon of the *nrg-2* gene in the region amplified by RT-PCR. Arrows: approximate location of PCR primers. Boxes: exonic sequences; lines: intronic sequences. EGF, EGF-like domain, which includes the  $\alpha$ - and  $\beta$ -exons; Ig, Ig-like domain; TM, transmembrane domain; Var, variable region. Bottom: Cartoon of the three partial *nrg-2* cDNAs found by nucleotide sequencing in spinal cord ( $\beta v$  isoform), sciatic nerve and skeletal muscle ( $\alpha 2$  and  $\alpha + \beta^*$  isoforms) of adult rat. The black box in the  $\alpha + \beta^*$  isoform represents a frameshift introduced by the extra A, which also introduces a stop codon in the TM domain exon (\*). (B) Amino acid alignment between the rat  $\alpha + \beta^*$  isoform (top) and human isoform 5 (bottom, accession number: NP\_053587.1). The consensus sequence is in the middle. (+) Conservative substitution; gap: non-conservative substitution. (\*) Ig-like domain amino acids; (#) EGF-like domain amino acids. (C) Left panel: *nrg-2* isoforms in adult tissues. Amplified *nrg-2* products from RT-PCR of adult rat spinal cord, sciatic nerve and skeletal muscle, were separated in a 6% denaturing acrylamide gel. PCR products from cloned templates for  $\alpha 2$ ,  $\beta v$  and  $\alpha + \beta^*$  isoforms were used as size markers. Nrg-2 cDNAs from spinal cord co-migrated with Nrg-2 $\beta$ , whereas Nrg-2 cDNAs from sciatic nerve and skeletal muscle co-migrated with Nrg-2 $\alpha$ . Nrg-2 $\alpha + \beta^*$  was not detectable here in any of the samples. Right panel: *nrg-2* isoforms during development. Nrg-2 $\alpha$  and Nrg-2 $\beta$  PCR products are very similar in size (around 400 bp) when separated in a 2% agarose gel. Digestion of Nrg-2 $\alpha$  with *Sau3AI* yields two fragments of 107 and 307 bp (indicated by start symbols), due to a unique *Sau3AI* site. Nrg-2 $\beta$  PCR product lacks *Sau3AI* sites, and remains uncut by this enzyme. Total RNA was isolated from E13 through adult spinal cord and skeletal muscle, and RT-PCR for *nrg-2* was performed as above. PCR products were purified, digested with *Sau3AI* and separated in a 2% agarose gel. *Sau3AI*-sensitive bands were only detected in the skeletal muscle samples, while *Sau3AI*-insensitive bands were only detected in spinal cord samples. *Sau3AI*-sensitive (i.e. Nrg-2 $\alpha$ ) bands were also detected from RNA isolated from D6P2T and primary Schwann cells (PSC). Arrowhead: Cloning and sequencing of these bands showed them to be undigested Nrg-2 $\alpha$  and not Nrg-2 $\beta$ . M: 100-bp ladder. (-): H<sub>2</sub>O control.



**Fig. 3.** Differential AChR-inducing activity of Nrg-2 $\alpha$  and Nrg-2 $\beta$ . ErbB2/3/4-expressing Sol8 myotubes, stably transfected with an *AChR $\delta$  subunit-hGH* fusion, were treated with different concentrations of GST-Nrg-2 $\beta$  or GST-Nrg-2 $\alpha$ . Forty-eight hours later, the amount of hGH in the culture medium was measured by radioimmunoassay. Both Nrg-2 $\alpha$  and Nrg-2 $\beta$  caused activation of *AChR* transcription, however, 20-fold more Nrg-2 $\alpha$  was required to approach the same levels of induction reached with Nrg-2 $\beta$ . GST-Nrg-1 $\beta$  and GST were used here as positive and negative controls, respectively. The mean  $\pm$  S.E.M. of the induction relative to untreated control is given;  $n=3$  for each concentration, for each factor.

a PCR reaction in which the template was a mix of plasmids encoding each  $\alpha 2$ ,  $\beta v$ , and  $\alpha + \beta^*$  isoforms. We found that in spinal cord the Nrg-2 PCR band co-migrated with the  $\beta v$  band, while in sciatic nerve and skeletal muscle it co-migrated with the  $\alpha 2$  band (Fig. 2C, left panel). The  $\alpha + \beta^*$  isoform, which we identified by sequencing individual subclones, was not detected in this PCR analysis suggesting that it was a very rare isoform. Thus the  $\beta v$  form is primarily expressed in the adult spinal cord, while  $\alpha 2$  predominates in skeletal muscle.

We checked whether this differential distribution of isoforms was also detected during development. We isolated RNA from spinal cord and skeletal muscle from embryonic day 13 (E13) through adult, and performed RT-PCR for Nrg-2 as described above. We used a different assay to distinguish the  $\alpha 2$  and  $\beta v$  PCR bands, which only differ by 10 bp in size. We took advantage of the presence of a single *Sau3AI* restriction site within the  $\alpha 2$  PCR product that was absent in the  $\beta v$  PCR band to discriminate between these two products. Digestion of the  $\alpha 2$  PCR product with *Sau3AI* generates two fragments of about 300 and 100 bp, while it leaves uncleaved the  $\beta v$  PCR product (Fig. 2C, right panel). Following *Sau3AI* digestion, we ran the products in a 2% agarose gel and found that at all developmental stages examined, which encompass the entire period of NMJ formation and maturation, *Sau3AI*-sensitive products (i.e. Nrg-2 $\alpha$ ) were only detected in skeletal muscle, while *Sau3AI*-insensitive products (i.e. Nrg-2 $\beta$ ) were found only in spinal cord (Fig. 2C, right panel).

*In situ* hybridization and antibody staining of adult skeletal muscle has previously demonstrated that the main

source of Nrg-2 is the terminal Schwann cell at the NMJ (Rimer et al., 2004). The present data suggest that terminal Schwann cells express Nrg-2 $\alpha$  isoforms. Consistent with this notion, we found that both the Schwann cell line D6P2T, which we have previously shown expresses Nrg-2 mRNA and protein (Rimer et al., 2004), and primary Schwann cells prepared from neonatal sciatic nerve, express  $\alpha 2$  isoforms (Fig. 2C, right panel). The 11 randomly selected clones derived from these cells all had the  $\alpha 2$  isoform nucleotide sequence (data not shown).

#### Alternative splicing modulates the AChR-inducing activity of Nrg-2

Given the differential distribution of Nrg-2 EGF-like isoforms between spinal cord and skeletal muscle, and the known difference in affinity for ErbB2/4 heterodimers between Nrg-2 $\beta$  and Nrg-2 $\alpha$ , we sought to determine whether these two isoforms had differential AChR-inducing activity. For these experiments we used the same muscle cell line described previously by Rimer et al. (2004), in which ErbB4 expression is under the control of the myosin light chain regulatory sequences. We treated these ErbB4/3/2-expressing myotubes with different concentrations of either GST-Nrg-2 $\alpha$  or GST-Nrg-2 $\beta$  and measured their effects on *AChR* transcription by the indirect hGH reporter assay as in Fig. 1. Fig. 3 shows that GST-Nrg-2 $\beta$  induced dose-dependent *AChR* transcription with a maximal response of  $2.1 \pm 0.12$ -fold over control at 250 ng/ml (mean  $\pm$  S.E.M.). This induction is slightly smaller than that elicited by GST-Nrg-1 $\beta$  at the same concentration ( $2.4 \pm 0.05$ -fold, Fig. 3). Fig. 3 also shows that the response

to Nrg-2 $\beta$ , as previously reported (Rimer et al., 2004), approached saturation at around 50 ng/ml. This corresponds to about 1 nM, which is the concentration at which ErbB receptor phosphorylation also reaches saturation (Chu et al., 1995). At 250 ng/ml, GST-Nrg-2 $\alpha$  induced a  $1.2 \pm 0.13$ -fold induction that was not statistically different from the negative control (GST alone at 1000 ng/ml,  $P=0.36$ , Fig. 3). When the concentration of GST-Nrg-2 $\alpha$  was raised to 1000 ng/ml (i.e.  $\sim 28$  nM), it induced *AChR* transcription at levels ( $1.54 \pm 0.17$ -fold) statistically similar to those induced by GST-Nrg-2 $\beta$  at 50 ng/ml ( $1.75 \pm 0.17$ -fold;  $P=0.43$ ), but significantly different from the GST negative control ( $P=0.05$ ). Thus, these data show that Nrg-2 $\alpha$  has modest *AChR*-inducing activity, but it is at least 20-fold less potent than Nrg-2 $\beta$ 's. We also tested the *AChR*-inducing activity of another Nrg-2 $\alpha$ , the Don-1-like isoform (Busfield et al., 1997), in which the splicing of the  $\alpha$ - and  $\beta$ -exons generates the same amino acid sequence in this region as found in the  $\alpha + \beta^*$  isoform described above (Fig. 2B). This isoform induced a maximal response of  $1.48 \pm 0.24$ -fold over control ( $n=3$ ) at 1000 ng/ml, which was very similar to Nrg-2 $\alpha$ . This was anticipated as the EGF-like domain of the Don-1-like and  $\alpha 2$ -isoforms is the same (Fig. 2). Thus, alternative splicing of the C-terminal part of the EGF-like domain of Nrg-2 regulates the *AChR*-inducing activity of this family of growth factors.

## DISCUSSION

Here, we demonstrate that Nrg-2-mediated stimulation of *AChR* transcription *in vitro* requires the N-box enhancer sequence. We also demonstrate that there is a tissue-specific distribution of Nrg-2 EGF-like isoforms that have distinct *AChR*-inducing activities. Nrg-2 $\beta$ , which is only present in motor and other neurons in the spinal cord, is about 20-fold more potent than Nrg-2 $\alpha$ , which is present in terminal Schwann cells in skeletal muscle.

As synapse-specific *AChR* expression requires the N-box *in vivo* (Koike et al., 1995; Fromm and Burden, 1998), the finding that Nrg-1-induced *AChR* transcription in cultured myotubes also requires this *cis*-regulatory motif (Fromm and Burden, 1998) is a compelling piece of evidence that Nrg-1 is the signal that mediates synapse-specific *AChR* expression. We have now shown that Nrg-2-induced *AChR* transcription also exhibits this requirement, and this finding is consistent with Nrg-2 playing a role in synapse-specific expression *in vivo*. This result also raises the possibility that Nrg-1 and Nrg-2 may serve as redundant signals that use the same intracellular signaling pathway to activate *AChR* transcription, although Nrg-2-induced *AChR* transcription requires ErbB4 while Nrg-1-induced *AChR* transcription does not. Consistent with this possibility, like Nrg-1, Nrg-2 can also induce *AChR $\epsilon$*  transcription in ErbB2/3/4-expressing myotubes (Elaine L. Elertson and M.R., unpublished observations).

We find a differential distribution of Nrg-2 EGF-like isoforms between spinal cord and skeletal muscle that is maintained throughout development. Nrg-2 $\beta$  ( $\beta v$  isoform) was found exclusively in the neuron-containing spinal cord.

Our previous *in situ* hybridization and antibody staining data showed that in the spinal cord, Nrg-2 is expressed by motor neurons and other neurons, but not glia (Rimer et al., 2004). Therefore, we conclude that it is the Nrg-2 $\beta$  isoform that is expressed by neurons in the spinal cord. This result, together with the finding that Nrg-2 $\beta$  is also present in rat brain, but not in other tissues (Yamada et al., 2000), suggests that Nrg-2 $\beta$  is the neuron-specific Nrg-2 EGF-like isoform. Unlike Yamada and colleagues, who detected Nrg-2 $\alpha$  in all rat tissues they examined (Yamada et al., 2000), including brain and skeletal muscle, we failed to detect this isoform in the spinal cord. We did find Nrg-2 $\alpha$  ( $\alpha 2$ ,  $\alpha + \beta^*$  isoforms) in sciatic nerve and skeletal muscle, where it is the predominant EGF-like isoform. Previously, our mRNA and protein expression data had demonstrated that the main source of Nrg-2 in skeletal muscle is the terminal Schwann cell (Rimer et al., 2004), and here, we report that both a Schwann cell line, and primary Schwann cells in culture, express only Nrg-2 $\alpha$ . Thus, we conclude that terminal Schwann cells at the NMJ synthesize Nrg-2 $\alpha$  as well. We cannot rule out that other cells in the muscle tissue, including muscle fibers themselves, express levels of Nrg-2 below the limits of detection of assays used here and in our previous work (Rimer et al., 2004). It is also likely that the cellular source of Nrg-2 $\alpha$  in the sciatic nerve is non-myelinating Schwann cells, which resemble terminal Schwann cells in their general phenotype. The differential distribution of Nrg-2 EGF-like isoforms between the spinal cord, sciatic nerve and skeletal muscle, suggests specific roles for these proteins in neurons and Schwann cells, and contrasts with the findings for Nrg-1 EGF-like isoforms. In particular, Nrg-1 $\beta$  isoforms have been detected in motor neurons in spinal cord (e.g. Falls et al., 1993), Schwann cells in denervated sciatic nerve and primary cultures (Raabe et al., 1996; Carroll et al., 1997), and in skeletal muscle (Meier et al., 1998; Rimer et al., 1998).

We found that both Nrg-2 $\alpha$  and Nrg-2 $\beta$  exhibit *AChR* inducing activity, however, Nrg-2 $\beta$  is at least 20-fold more potent than Nrg-2 $\alpha$ . This is consistent with the finding that Nrg-2 $\beta$  displays much higher affinity for ErbB2/4 than Nrg-2 $\alpha$  (Jones et al., 1999). A similar difference in *AChR*-inducing activity has been reported for Nrg-1 $\beta$  and Nrg-1 $\alpha$  isoforms (Busfield et al., 1997), and similarly, differential affinity for their cognate ErbB receptor (ErbB2/3 in this case), appears to account for this finding. The higher potency of Nrg-2 $\beta$  in inducing *AChR* transcription is consistent with the notion that low levels of it, sufficient to activate *AChR* expression yet undetectable by existing antibodies (Rimer et al., 2004), might be present at the NMJ. Any Nrg-2 $\beta$  at the NMJ would have to be derived from motor neurons, which we have previously shown synthesize *nrg-2* mRNA and protein (Rimer et al., 2004). However, we now show that the Nrg-2 that is detectable at the NMJ associated with terminal Schwann cells is Nrg-2 $\alpha$ , the EGF-like isoform with the least potency in inducing *AChR* transcription. It is unknown if terminal Schwann cells release large amounts of Nrg-2 $\alpha$  *in vivo*, however, D6P2T cells, like HEK 293T cells transfected with a Nrg-2 cDNA

(Longart et al., 2004), do not seem to secrete significant amounts of Nrg-2 (data not shown).

Because recent immunological data indicate that ErbB2/4, the receptor with the highest affinity for Nrg-2, seems to mediate Nrg signaling on the synaptic sarcolemma (Trinidad et al., 2000), one attractive feature of Nrg-2 that distinguishes it from Nrg-1 as a signal for regulating *AChR* expression at the NMJ is that its *AChR*-inducing activity is ErbB4-dependent. In addition, Nrg-2 is expressed by motor neurons and terminal Schwann cells and its *AChR*-inducing activity is N-box-dependent. On the other hand, Nrg-2 accumulates at the synapse adjacent to the terminal Schwann cell surface that faces away from the endplate (Rimer et al., 2004). This Nrg-2 protein is Nrg-2 $\alpha$ , the least potent isoform inducing *AChR* transcription *in vitro*. Moreover, despite our best efforts with our own and other's antibodies (Longart et al., 2004), we have been unable to show that the more potent Nrg-2 $\beta$  isoform accumulates at nerve terminals. Thus, there is as much evidence supporting the notion that Nrg-2 regulates *AChR* expression at the NMJ as there is evidence inconsistent with this idea.

ErbB4<sup>-/-</sup> mice that die at midgestation due to failure to assemble a functional heart (Gassmann et al., 1995), have been rescued by overexpression of an ErbB4 transgene solely in the heart. The rescued mice survived into adulthood, and preliminary analysis of their NMJs failed to find major defects (Tidcombe et al., 2003). However, this analysis did not include a quantitative assessment of the *AChR* levels at synapses, and without it, it is premature to rule out a role for Nrg-2 in regulating *AChR* expression at NMJs *in vivo*. A quantitative analysis is important here, because, as shown by the ectopic expression of agrin (e.g. Rimer et al., 1997), motor neurons need only release agrin to establish synapse-specific *AChR* expression. Other gene products derived from motor neurons and/or terminal Schwann cells, such as Nrg-1 or Nrg-2, might be required later to achieve the higher levels of transcription observed at normal NMJs. Thus, interruption of Nrg-2/ErbB4 signaling by genetic means might not yield a lethal reduction in *AChR* levels at the synapse.

Britto et al. (2004) recently reported that mice deficient for Nrg-2 lack an embryonic phenotype but do show perinatal growth retardation and impaired reproductive capacity. These investigators did not examine NMJs in these mice. However, as these animals are viable their NMJs are functional. Given the redundancy suggested by our data between Nrg-1's and Nrg-2's *AChR*-inducing activities *in vitro*, it is plausible that one could compensate for the other *in vivo*.

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