Agrin Can Mediate Acetylcholine Receptor Gene Expression in Muscle by Aggregation of Muscle-derived Neuregulins

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Abstract. The neural isoforms of agrin can stimulate transcription of the acetylcholine receptor (AChR) ε subunit gene in electrically active muscle fibers, as does the motor neuron upon the formation of a neuromuscular junction. It is not clear, however, whether this induction involves neuregulins (NRGs), which stimulate AChR subunit gene transcription in vitro by activating ErbB receptors. In this study, we show that agrin-induced induction of AChR ε subunit gene transcription is inhibited in cultured myotubes overexpressing an inactive mutant of the ErbB2 receptor, demonstrating involvement of the NRG/ErbB pathway in agrin-induced AChR expression. Furthermore, salt extracts from the surface of cultured myotubes induce tyrosine phosphorylation of ErbB2 receptors, indicating that muscle cells express biological NRG-like activity on their surface. We further demonstrate by RT-PCR analysis that muscle NRGs have Ig-like domains required for their immobilization at heparan sulfate proteoglycans (HSPGs) of the extracellular matrix. In extrasynaptic regions of innervated muscle fibers in vivo, ectopically expressed neural agrin induces the colocalized accumulation of AChRs, muscle-derived NRGs, and HSPGs. By using overlay and radioligand-binding assays we show that the Ig domain of NRGs bind to the HSPGs agrin and perlecan. These findings show that neural agrin can induce AChR subunit gene transcription by aggregating muscle HSPGs on the muscle fiber surface that then serve as a local sink for focal binding of muscle-derived NRGs to regulate AChR gene expression at the neuromuscular junction.

The high density accumulation of acetylcholine receptor (ACHR)1 channels at the neuromuscular junction (NMJ), required for impulse transmission across the synapse, is the result of transcriptional activation of AChR subunit genes in the subsynaptic muscle nuclei (Brenner et al., 1990; Sanes et al., 1991) and of the insertion of their gene products, the AChR channels, in the synaptic muscle membrane (for review see Sanes, 1997). The AChRs are stabilized in the subsynaptic membrane by anchoring to the cytoskeleton via an elaborate subsynaptic apparatus of highly specialized molecular composition (Fallon and Hall, 1994; Apel and Merlie, 1995; Carbone et al., 1995). Both the transcription of AChR genes and the differentiation of the subsynaptic apparatus are under the control of molecules originating from the motor neuron and associated with the synaptic portion of the muscle fiber’s basal lamina (BL) (McManus, 1990; Brenner et al., 1992; Jo and Burden, 1992).

The neural signal proposed to activate AChR gene transcription in muscle is acetylcholine receptor–inducing activity (ARIA; Martinou et al., 1991; Corfas et al., 1993; Chu et al., 1995; Fischbach and Rosen, 1997), a member of the neuregulin (NRG) family of growth and differentiation factors (Falls et al., 1993) arising in several isoforms from a single gene, nrg-1, by alternative mRNA splicing. ARIA/NRG precursors are expressed in motor neurons (Falls et al., 1993) as transmembrane glycoproteins and are cleaved near their transmembrane domains for release (for reviews see Lemke, 1996; Fischbach and Rosen, 1997). The mature form of ARIA/NRG is characterized by an Ig-like domain that binds heparin (Falls et al., 1993) and by a conserved EGF-like domain sufficient to activate receptors of the ErbB family of receptor tyrosine kinases. Neuregulin binding to ErbB receptor heterodimers induces...
their tyrosine phosphorylation and activates AChR subunit gene transcription in cultured myotubes (Si et al., 1996; Tansey et al., 1996; Altio et al., 1997).

The neural signal controlling nerve-dependent aggregation of AChR channels in the subsynaptic muscle membrane is agrin (McMahan, 1990), a multidomain heparan sulfate proteoglycan (HSPG) with binding affinities for α-dystroglycan, laminin, and heparin (for review see Denzer et al., 1996). Unlike motor neurons, muscle fibers do not synthesize agrin isoforms active in AChR aggregation (Ferns et al., 1992; Ruegg et al., 1992; Hoch et al., 1993; Gesemann et al., 1995). Agrin is associated with the synaptic BL of the NMJ (Reist et al., 1987) presumably by its binding to laminin (Denzer et al., 1997, 1998). Neuregulins are also bound to synaptic BL (Goodearl et al., 1995; Jo et al., 1995; Sandrock et al., 1995), but the mechanism of this immobilization is not known.

Recent experiments demonstrate, however, that active agrin not only causes the redistribution of cell surface AChRs in cultured myotubes and in vivo but that agrin also induces expression of the AChR ε subunit gene in the absence of nerve-derived NRGs; the expression is resistant to muscle activity as it is at normal synapses (Jones et al., 1996, 1997). In primary myotube cultures, AChR gene transcription induced by agrin depends on its binding to the culture substrate, but conspicuously, does not depend on its AChR aggregating activity (Jones et al., 1996). These findings led us to propose that agrin rather than NRGs may be the key neural factor regulating subsynaptic AChR gene transcription in the muscle fiber. If NRGs are involved in this process they may be derived from the muscle fibers, under the local control of matrix-bound agrin (Jones et al., 1996, 1997). Consistent with this hypothesis, motor neuron–specific agrin isoforms alone, upon expression in extrasynaptic regions of innervated muscle fibers, induce ectopic accumulation of the NRG receptors, ErbB2 and ErbB3 (Rimer, M., I. Cohen, T. Lomo, S.J. Burden, and U.J. McMahan, 1996. Soc. Neurosci. Abstr. 1689; Meier et al., 1997). Furthermore, muscle cells express transcripts encoding ARIA/NRG isoforms (Moscoso et al., 1995; Ng et al., 1997). However, it is not known whether muscle cell–derived NRGs are biologically active.

In this paper, we have tested the hypothesis that muscle cells are a source of functional ARIA/NRG-like biological activity that could be locally concentrated at the muscle surface by agrin to activate AChR subunit gene transcription. We found all elements required for such a process: (a) in cultured myotubes, transcription of AChR ε subunit gene induced by substrate-bound agrin is inhibited when myotubes overexpress an inactive human mutant of ErbB2, HER2KM, as is transcription induced by NRG; (b) NRG transcripts are expressed in innervated muscle and NRG-like biological activity can be extracted from cultured muscle cells; (c) NRG-like immunoreactivity colocalizes with aggregates of newly synthesized AChRs induced in nerve-free segments of innervated muscle fibers by ectopic neural agrin; (d) as proposed by Loeb and Fischbach (1995), the Ig-like domains of muscle derived NRGs mediate direct interaction with glycosaminoglycan (GAG) side chains of HSPGs. Indeed, the HSPGs agrin and perlecan, but not other glycoproteins of the postsynaptic apparatus bind NRGs in overlay and radioligand-binding assays; (e) in addition to agrin, other HSPGs are accumulated in the muscle BL by agrin, thus providing additional binding sites for the localization of NRGs. Taken together, these findings support the hypothesis that agrin regulates synapse-specific AChR gene expression by localizing muscle derived NRGs and activating ErbB receptors.

**Materials and Methods**

The term neuregulin (NRG) is used in this paper whenever we wish to address transcripts or proteins encoded by the nrg-1 gene (Fischbach and Rosen, 1997) irrespective of species or isoform. Human, rat, and chick NRG isoforms are referred to as heregulin(s) (HRGs), Neu differentiation factor (NDF), or ARIA, respectively (Lemke, 1996; Fischbach and Rosen, 1997). Products of the nrg-2 gene were not considered as they appear not to be expressed in muscle (Carraway et al., 1997; Chang et al., 1997).

**AChR ε Subunit Transcription in C2C12 Cells Overexpressing HER2 or HER2KM**

Recombinant full-length neural cAgrin <sub>AB</sub> was immobilized on 35-mm culture dishes by precoating dishes with 65 μl of 20 μg/ml laminin from EHS tumor (Sigma Chemical Co., St. Louis, MO) followed by spreading 60 μl of 5 μg/ml Agrin<sub>AB</sub> diluted in PBS, and then incubation overnight at 4°C. After rinsing, 120,000 C2C12 cells were added to each well and co-transfected with 3 μg of the AChR ε-promoter luciferase reporter construct pLCEF216ε (Jones et al., 1996), 250 ng of pCH110 (Pharmacia Biotechnology Inc., Piscataway, NJ) encoding β-galactosidase, and 250 ng of either plHER2/neu or pHER2KM (Wallisch et al., 1995) using the calcium phosphate method (Chen and Okayama, 1987) in serum-free medium (Jones et al., 1996). To exclude unspecified effects on gene regulation after HER2 and HER2KM transfection, we analyzed muscle creatine kinase (MCK) transcription using a 6.5-kbp MCK promoter fragment from pUC118MCK6.5 subcloned into pGLO2 (Promega, Heidelberg, Germany). Cells were analyzed 72 h later for luciferase activity using a Luminometer (Turner Designs, Sunnyvale, CA) and relative luciferase units were normalized to β-galactosidase expression. To test for transfection efficiency and protein expression, COS cells were transfected accordingly, proteins extracted in SDS sample buffer and analyzed on Western blots for the expression and tyrosine phosphorylation of HER2 using the antibodies described below.

**Reverse Transcriptase–PCR**

Extrasynaptic segments of innervated adult rat diaphragm were dissected and polyadenylated mRNA was isolated according to the method of Henner (1993). First strand cDNA was synthesized using Superscript reverse transcriptase (Life Technologies, Inc., Gaithersburg, MD) according to the manufacturer’s instructions with 50 ng of mRNA primed with 2 pmoles of the 5′-GGAGTAGGCCACACACACATGATGCC-3′ oligonucleotide (TM-antisense) (Microsynth, Balgach, Switzerland), which corresponds to the transmembrane (TM) region of rat NDF (Wen et al., 1992). The first-strand cDNA was then extracted with phenol/chloroform, precipitated, and then resuspended in 20 μl of water. To amplify a NDF transcript spanning the TM and Ig domains, 10 μl was amplified in a volume of 50 μl with an air thermal cycler (Idaho Technology, Idaho Falls, ID) using a cycle protocol of 94°C for 15 s, 58°C for 15 s, and 72°C for 35 s for 35 cycles and the TM-antisense primer together with an Ig-sense primer 5′-CTCTGGAGAGTATATGTGCAAAGTGATCAGC-3′ that recognizes the 3′ part of the NDF Ig-domain (Wen et al., 1992). To examine whether the resulting Ig/TM product included α or β splice isoforms and to examine the spacer insert between the Ig and EGF domains (for review see Lemke, 1996), the reaction mix was diluted 1:100 and 1 μl was amplified in a 10 μl volume using 35 cycles of 94°C for 1 s, 58°C for 1 s, and 72°C for 10 s. Primer combinations were as follows: to examine the spacer insert we used the Ig-sense primer together with the EGF-antisense (5′-GATGGCAGCTGATGACGTGTC-3′) primer. To detect α- and β-specific isoforms, either an α-specific primer 5′-CCAAAAAC-TACGTAATGGCCACACACATGATGCC-3′ or a β-specific primer 5′-TGATCCTAGTAAAAGTTCCCAACACAATCAACACACACAC-3′ was used in combination with the TM-antisense primer. PCR products were resolved on a 2% agarose gel and transferred to nylon membrane (Boehringer Mannheim GmbH, Mannheim, Germany), which was then hybridized overnight at 42°C with...
a PCR-generated NDF probe labeled with Digoxigenin-11-dUTP (Boehringer Mannheim GmbH) and spanning the Ig–TM domains, ensuring recognition of all NDF isoforms. Amplified NFD DNA was visualized using alkaline phosphatase–conjugated anti-DIG antibody and the chemiluminescence substrate CDP-Star™ (Boehringer Mannheim GmbH) according to the manufacturer’s instructions.

Agrin and Heregulin

Chick full-length agrin, cAgrin<sub>445–446</sub>, and cAgrin<sub>23–246</sub> (Denzer et al., 1995), as well as the COOH-terminal agrin fragment c95<sub>400</sub> (Gesemann et al., 1995), were purified from conditioned medium of stably transfect Hek 293 cells as described elsewhere (Gesemann et al., 1995; Denzer et al., 1997). HRG<sub>BbsI1–246</sub> cDNA encoding a full-length extracellular domain of the human orthologue of chicken ARIA (Falls et al., 1993; Jeschke et al., 1995) was subcloned into the bacterial expression vector pFLAG-1 (International Biotechnologies Inc., Cambridge, UK), and then used for transformation of Escherichia coli XL-1 blue. Expression of recombinant protein was induced with 1 mM isopropylthio-β-D-galactoside (IPTG), homogenate was enriched for inclusion bodies, and then extracted with 6 M urea followed by extensive dialysis against PBS and purification with anti-FLAG M2 affinity gel (International Biotechnologies Inc., Santa Cruz, CA) and 10 μl Protein A-Sepharose beads for 2–3 h at 4°C. As a control for nonspecific binding resulting from the presence of a His tag we subcloned the BamHI–HindIII insert of pQE30. This fragment was ligated into the BamHI–HindIII cut plasmid pQE30. This pQE30/HRG<sub>BbsI</sub> His tag at its 3′-end was blunted with BbsI, fill-in with T4 DNA polymerase, and subsequent digestion with Smal and religation. Finally, plasmid pQE30/HRG<sub>BbsI</sub>BsSl was obtained by replacing the SpeI/HincII fragment of pQE30/HRG with the corresponding restriction fragment from plasmid pEGFP/HRG<sub>AbsI</sub>bsl. Bacteria (E. coli XL1 blue) transformed with pQE30/HRG or pQE30/HRG<sub>BbsI</sub>BsSl were induced with 0.4 mM IPTG for 5 h, inclusion bodies were solubilized in 6 M urea and purified over a cation exchange column (SP Sepharose Fast Flow; Pharmacia Biotech Sevrage, Uppsala, Sweden) using HPLC equipment. The resulting HRG<sub>BbsI</sub> was dialyzed against 50 mM Tris-HCl, 150 mM NaCl, 0.1% Tween 20, and used for most overlay assays and for radiolabeling. For some experiments, this enriched HRG<sub>BbsI</sub> fraction A was further purified by Ni<sup>2+</sup>-NTA (Qiagen) affinity chromatography (HRG<sub>BbsI</sub> fraction C). HRG<sub>BbsI</sub>BsSl was purified on His-Trap affinity column in the presence of 6 M urea and stored at 4°C. As a control for nonspecific binding resulting from the presence of a His tag we subcloned the BamHI–HindIII insert of pQE16 (Qiagen) encoding dihydrofolate reductase (DHFR) cDNA ligated to a 6× His tag at its 3′-end into the BamHI–HindIII cut plasmid pQE30. This provided the control protein DHFR flanked with COOH- and NH<sub>2</sub>-terminal His tags (His-DHFR-His). The NH<sub>2</sub>-terminal His tag (Arg-Gly-Ser-[His]<sub>10</sub>) was recognized by the RGS-His antibody (Qiagen). His-DHFR-

His was extracted from bacterial inclusion bodies with 6 M urea and used without further purification.

Extraction and purification of recombinant proteins was monitored by SDS-PAGE and Coomassie brilliant blue staining. Protein concentrations of HRGγ and His-DHFR-His were determined by comparing absorptions of Coomassie-stained protein bands with calibration curves obtained with BSA as standard. His- and FLAG-tagged heregulins were analyzed further on immunoblots under standard conditions using anti-His antibodies (Qiagen) and anti-FLAG M1 antibody (International Biotechnologies Inc.) for the detection of HRGβ1<sub>177–246</sub>, or anti–FLAG M2 antibody for the detection of HRGβ1<sub>1–246</sub> combined with peroxidase-conjugated antimouse antibodies (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) and chemiluminescence detection using Super Signal™ substrate (Pierce Chemical Co., Rockford, IL). Relative concentrations of HRGβ1<sub>1–246</sub>, HRGβ1<sub>177–246</sub>, and HRGβ1<sub>BbsI</sub>BsSl were adjusted to oh, and comparable signals on immunoblots. Fig. 1 shows the heregulin isoforms used in this study and their detection by anti-His or anti-FLAG antibodies.

**Extraction of NRG-like Biological Activity from Myotubes and ErbB2 Immunoprecipitation**

Extracts from extracellular matrix (ECM) of chick brain and spinal cord cells have been shown previously to contain NRG (Loeb and Fischbach, 1995). Primary rat myotubes cultured according to Brenner et al. (1992) for 6 d and C2C12 myotubes cultured for 5–6 d in differentiation medium were used to test muscle cells for their content of NRG-like biological activity. After washing in ice-cold PBS, three to four 30-mm plates were extracted sequentially with 1 ml salt extraction solution (PBS supplemented with 1 mM MgCl<sub>2</sub>, 2.5 mM CaCl<sub>2</sub>, and NaCl to 1.0 or 1.5 M) by gentle agitation on ice. After centrifugation at 4°C, salt extracts were dialyzed against 0.5× PBS for several hours and then dialyzed against serum-free DMEM overnight at 4°C. To monitor NRG-like biological activity, fresh cultures of 6-d-old primary rat myotubes or C2C12 cells differentiated in 30-mm plates were stimulated with 1 ml of this dialyzed, conditioned salt extract for 8 min at 37°C. For comparison, cultures were either incubated with dialysis medium (DME) as unstimulated control or with dialysis medium supplemented with saturating amounts of recombinant HRGβ1<sub>177–246</sub> (stimulated control). After washing in ice-cold PBS, membrane proteins were extracted with 1 ml detergent extraction buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 5 mM EDTA, 5 mM EGTA, 30 mM NaF, 10 mM sodium-molybdate, 1 mM sodium-orthovanadate, 1% (vol/vol) Triton X-100, 5 μg/ml leupeptin, 5 μg/ml aprotinin, and 1 μM PMSF) and extracts cleared from cell debris by centrifugation at 4°C. ErbB2 was specifically immunoprecipitated with 0.1 μg/ml anti-ErbB2 antibody (C18; Santa Cruz Biotechnology Inc., Santa Cruz, CA) and 10 μl protein A-Sepharose beads for 2–3 h at 4°C. Control experiments showed that this antibody does not cross-react with either ErbB3 or ErbB4 in such detergent extracts (after washing). After washing three times with 1 ml detergent extraction buffer and once with 1 ml PBS, excess liquid was removed, and then beads were boiled in 20 μl SDS sample buffer. Samples were separated on 6% SDS-PAGE and transferred to nitrocellulose. In our gel system ErbB2 protein migrated as an ~200-kd band; this was confirmed by immunoblots with anti-ErbB2 antibodies. For detection of tyrosine phosphorylation, blots were preincubated with 3% BSA in TBS (25 mM

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**Figure 1. Summary diagram of HRG isoforms and their detection.** (A) Schematic representation of HRGs indicating the position of the Ig-like domain and EGF-like domain, as well as FLAG and His tags used for purification and detection. HRGβ1<sub>177–246</sub> representing the EGF-like domain, is derived from full-length HRGβ1<sub>1–246</sub>, whereas the Ig-like domain is contained in the HRGβ1<sub>BbsI</sub>BsSl fragment of HRGγ. (B) Immunoblot of recombinant proteins. HRGγ, HRGβ1<sub>BbsI</sub>BsSl, and the control protein His-DHFR-His were detected with anti-His tag primary antibody, HRGβ1<sub>177–246</sub> and HRGβ1<sub>BbsI</sub>BsSl were detected with anti–FLAG M2 and anti–FLAG M1 primary antibodies, respectively. In this and some of the following figures molecular weights (kD) of standard proteins are indicated.
Tris-HCl, pH 7.5, 150 mM NaCl, and 0.1% [vol/vol] Tween-20, incubated with peroxidase-conjugated anti-phosphotyrosine antibody 4G10 (1:3,000; Upstate Biotechnology Inc., Lake Placid, NY) followed by chemiluminescence detection. Densitometric analyses of Western blots were carried out using ImageQuant software and data from different experiments were combined by normalizing the results of each experiment to saturating HRG-stimulated ErbB2 phosphorylation.

**Immunoblot and Transfer Blot Overlay Assays**

Binding of heregulin was assayed for the chick agrin constructs, cAgrin7A4B8 or cAgrin1A4B8. Proteins were separated by SDS-PAGE on 3–12% gradient gels under reducing conditions and transferred to nitrocellulose (Towbin et al., 1979). Routinely we loaded 100 ng/lane of agrin for immunoblots and up to 250 ng/lane of agrin for transfer blot overlay assays. To test whether agrin proteins were efficiently transferred, blots were incubated with anti-agrin antibodies (No. 3240) against the c95 fragment of agrin (Gesemann et al., 1995), followed by peroxidase-conjugated secondary antibodies (Jackson ImmunoResearch Laboratories) and chemiluminescence detection (Pierce Chemical Co.). Transfer blot overlay assays were carried out essentially as described (Yamada et al., 1996). Briefly, unsppecific binding sites were blocked with 10 mM triethanolamine, pH 7.6, 140 mM NaCl, 1 mM CaCl2, 1 mM MgCl2, 0.01% Tween 20 (TLBB) supplemented with 5% (wt/vol) nonfat milk powder (MILTLBB) for 45 min before they were overlaid with heregulin protein diluted in TLBB for 1.5 h. In some experiments, MTLBB was supplemented with 10 mM β-mercaptoethanol to prevent hydrogen peroxide (HRG) formed in Ca2

**Solid Phase Radioligand-binding Assay**

HRGγ (fraction A; 130 μl of a 1.4 mg/ml protein solution) was iodinated using the chloramine T method (Hunter and Greenwood, 1962) according to Geisemann et al. (1995). Samples of HRGγ were separated on SDS-PAGE and analyzed by a PhosphorImager (Molecular Dynamics, Inc., Sunnyvale, CA). Microtiter plates (Becton Dickinson Co., Mountain View, CA), were coated with 1 μg/ml Ag2ADF or Ag1ADF or 10 μg/ml of c95ADF (Geisemann et al., 1995), laminin-1 prepared from mouse Engelbreth-Holm-Swarm sarcoma (Timpl et al., 1979), tenasin (Chiquet et al., 1991), fibronectin (purified from conditioned fibroblast medium, Chiquet et al., 1991), or perlecain (for review see Timpl, 1993; Timpl and Brown, 1995) diluted in 50 mM sodium bicarbonate, pH 9.6, overnight at 4°C, or perlecain (for review see Timpl, 1993; Timpl and Brown, 1995) diluted in 50 mM sodium bicarbonate, pH 9.6, overnight at 4°C. α-Dystroglycan prepared from chicken lung (Geisemann et al., 1997), followed by peroxidase-conjugated secondary antibodies (Jackson Immunoresearch Laboratories) and chemiluminescence detection (Pierce Chemical Co.). Transfer blot overlay assays were carried out essentially as described (Yamada et al., 1996). Briefly, unsppecific binding sites were blocked with 10 mM triethanolamine, pH 7.6, 140 mM NaCl, 1 mM CaCl2, 1 mM MgCl2, 0.01% Tween 20 (TLBB) supplemented with 5% (wt/vol) nonfat milk powder (MTLBB) for 45 min before they were overlaid with heregulin protein diluted in TLBB for 1.5 h. In some experiments, MTLBB was supplemented with 10 mM β-mercaptoethanol to prevent hydrogen peroxide (HRG) formed in Ca2

**Ectopic Expression of Agrin and Immunocytochemistry**

Full-length cAgrin7A4B8 or the agrin fragment cN25C95A4B8, a fusion construct of the NH2-terminal laminin-binding domain of agrin (cN25) with the c95A4B8 fragment (Gesemann et al., 1995; Denzer et al., 1997; construct

**Results**

Previous experiments demonstrated that agrin alone is sufficient to initiate transcription of the AChR ε subunit gene (Jones et al., 1996, 1997) and accumulation of adult AChR channels (Jones et al., 1997; Rimer et al., 1997) in muscle. This prompted the hypothesis that NRGs supplied by the nerve terminal are not required to control AChR gene expression. Instead, nerve-derived agrin might initiate AChR subunit gene transcription by recruitment of muscle-derived NRGs and their localization to synaptic BL (Jones et al., 1996). The experiments described below were designed to test this hypothesis.

**Blockade of the NRG/ErbB Pathway Blocks Agrin-induced AChR Gene Transcription**

If agrin indirectly increases AChR gene expression by recruiting muscle-derived NRG, thus activating the ErbB receptor signaling pathway, inhibition of ErbB receptor acti-
vation would be expected to abolish agrin-induced gene transcription. We tested this prediction by overexpressing an inactive HER2 receptor construct (HER2KM) in C2C12 myotubes and monitoring the effect on agrin-induced AChR ε subunit gene induction. HER2KM is a kinase inactive single point mutant of HER2, the human orthologue of rat ErbB2, and HER2KM is thought to form inactive heterodimers with HER3/ErbB3, thereby blocking activation of the ErbB signaling pathway in the myotubes (Wallasch et al., 1995).

An AChR ε subunit promoter luciferase reporter gene construct (Jones et al., 1996) was transiently transfected into C2C12 myoblasts cultured on a laminin substrate impregnated with recombinant full length chick Agrin7A4B8. After differentiation into myotubes, luciferase activity was increased approximately fourfold above that in control cells grown on laminin alone (see Fig. 2). A similar increase in luciferase activity was seen, when parallel cultures were treated with saturating concentrations of a fragment of recombinant heregulinβ1 (HRGβ1[177-246]), which is in the range of induction previously reported by others (Tansey et al., 1996). In contrast, cotransfection of the AChR ε-promoter luciferase reporter construct with a plasmid encoding inactive HER2KM significantly reduced the induction of luciferase activity, both that by substrate-bound agrin and that by HRGβ1, to levels not different from that in control cultures. HER2 and HER2KM acted specifically on the activation of the AChR ε subunit promoter, as no effects were observed on the levels of muscle creatine kinase (MCK) expression.

The reduction in agrin- and HRGβ1-induced luciferase activities by HER2KM was not a nonspecific effect arising from overexpression of HER2KM. First, unlike HER2KM, cotransfection of an expression plasmid encoding active, wild-type HER2 did increase luciferase activity even in the absence of agrin. This increase produced by HER2 alone may be due to the formation of autophosphorylated HER2 homodimers, as transfection of HER2 alone into COS cells produced—when compared with transfection with HER2KM—a substantial level of phosphoher2 kinase, despite the absence of ligand (data not shown; Wallasch et al., 1995). Second, expression of the two plasmids was similar, as tested in COS cells by Western blotting HER2 and HER2KM protein levels (not shown).

**Expression of Neuregulins Containing Ig and EGF Domains in Muscle**

It has been proposed by Loeb and Fischbach (1995) that immobilization of NRGs to the ECM might be via their Ig-like domains binding to HSPGs. For maximal induction of AChR gene transcription by ErbB receptor activation, β isoforms of NRGs are required (Fischbach and Rosen, 1997). We therefore examined whether NRG β isoforms are expressed in innervated adult muscle. To determine whether active NRGs containing the Ig and EGF domains are expressed in adult skeletal muscle, we isolated mRNA from the extrasynaptic region of innervated rat diaphragm. Using reverse transcriptase PCR (Fig. 3 A), NRG transcripts spanning the Ig domain through to the transmembrane domain were amplified. The resulting products (Fig. 3 B, *left panel*) were then subjected to a second round of PCR using primers which specifically amplified either α- or β-NRG isoforms or amplified the spacer insert between the immunoglobulin and EGF domain. In this way we could amplify β-specific, but not α-specific isoforms that are cotranscribed with the Ig domain (Fig. 3 B, *right panel*). Further, the calculated molecular weight of the β isoform suggested it to be β1 (148 bp) or β2 (124 bp), but not β4 (205 bp; Wen et al., 1994). This is in agreement with experiments carried out in adult chicken muscle in which the expression of β1-NRG is predominant but very little α-NRG is transcribed (Ng et al., 1997). Three PCR products were amplified using the Ig-EGF primers, which flanked the alternatively spliced spacer domain. Based upon the expected molecular weight, the predominant spacer domain isoform in nonsynaptic region of rat diaphragm is the 34-amino acid (277 bp) variant, although lower amounts of both the 17–amino acid (226 bp) and 0–amino acid (175 bp) isoforms were also amplified (Fig. 3 B, *right panel*). Taken together, these results suggest that neuregulin transcripts in extrasynaptic regions of innervated adult muscle encode a protein containing functional domains es-
Neural Agrin Induces Accumulation of NRG at Ectopic Postsynaptic Specializations

At normal neuromuscular synapses NRGs are accumulated (Jo et al., 1995), and ectopic expression of agrin in innervated muscle fibers induced accumulations of ErbB receptors (Meier et al., 1997). This prompted us to test whether agrin might induce the local accumulation of muscle-derived NRG. For this purpose, we injected innervated rat soleus muscle in vivo with expression plasmids encoding neural cAgrin7A4B8 (Jones et al., 1997). Double labeling of ectopic, agrin-induced AChR aggregates with rhodamine-α-bungarotoxin and with antibodies specific for NRGs revealed the accumulation of muscle-derived NRG at sites of ectopic AChR aggregation (Fig. 5). Antisera raised against either the extracellular or intracellular domains of NRG resulted in similar staining patterns. This indicates that membrane bound NRG precursors are accumulated at sites of AChR synthesis and aggregation in the nerve-free region of muscle fibers expressing agrin.

Binding of Neuregulin to Agrin Isoforms

Since NRGs bind to heparin (Falls et al., 1993) and agrin is a HSPG (Denzer et al., 1995; Tsen et al., 1995), we examined whether recombinant NRG isoforms directly bound to recombinant chick agrin. When tested in transfer blot overlay assays, both HRGβ1(177–246) and HRGγ, a novel NRG isoform (Schoumacher, F., H. Mueller, and U. Eppenberger, manuscript in preparation), bound to immobilized full-length neural cAgrin7A4B8, an ~400–600-kD glycoprotein (Fig. 6, A and B). These results indicate that...
HRG isoforms bind directly to immobilized full-length neural agrin and that this interaction does not require the presence of the complete EGF-like domain of HRG.

Next, we examined whether HRGγ binds to Agrin7A0B0, a muscle-specific full-length isoform that lacks AChR aggregating activity (Ferns et al., 1992, 1993; Gesemann et al., 1995), but induces AChR subunit gene expression in cultured myotubes (Jones et al., 1996). In solid phase radioligand-binding assays (Fig. 6 C), as well as in transfer blot overlay assays (Fig. 7 A) we detected binding of HRGγ to Agrin7A0B0, but only when Ca²⁺ was present (data not shown). We then asked whether NRGs bind to other, non-agrin glycoprotein components of muscle synaptic membrane, such as α-dystroglycan, or of the BL, such as perlecan, tenascin, fibronectin, and laminin-1. In overlay assays, HRGγ did not bind to immobilized α-dystroglycan purified from chicken lung, nor to laminin-1, and nidogen separated under reducing conditions on SDS-PAGE (data not shown). This was confirmed by solid phase radioligand-binding assays in which the proteins were coated in non-denatured and non-reduced configuration to microtiter wells (Fig. 6 C). Similar results were obtained for fibronectin, tenascin, and Matrigel™. 125I-HRGγ did, however, exhibit strong affinity for the muscle ECM component perlecan (Timpl, 1993). A possible explanation for the lack of binding to Matrigel™, which contains perlecan, might be the reduced accessibility of perlecan incorporated into the laminin and collagen matrix. Thus, HRGγ exhibits only a weak nonspecific binding to BSA and to non-HSPG proteins, suggesting that this low level of binding most likely reflects nonspecific interactions. Specific binding was only seen for the HSPGs agrin and perlecan. Therefore, we asked whether their binding to NRGs might be mediated by their negatively charged GAG side chains, as proposed by Loeb and Fischbach (1995). This would be consistent with the previous finding that neuregulins bind heparin, a polymer of sulfated glycosaminoglycans (Falls et al., 1993).

**The Ig-like Domain of Neuregulins Mediates Binding to the GAG Chains of Agrin**

As expected for NRG binding to agrin and perlecan via GAG chains, overlay, and radioligand-binding assays showed that binding of HRGγ to immobilized full-length agrin isoforms cAgrin7A4B8 and cAgrin7A0B0 was completely inhibited by heparin at concentrations as low as 2 μg/ml, the lowest concentration tested (data not shown). Similar inhibition was also seen for perlecan at heparin concentrations 20 μg/ml (data not shown). In addition, enzymatic digestion of Agrin 7A0B0 with heparitinase, an enzyme that cleaves GAG side chains from proteoglycans, resulted in a shift to lower apparent molecular weight, as reported earlier (Denzer et al., 1995), and in substantially reduced binding of HRGγ to cAgrin7A0B0 depleted of GAG chains (Fig. 7 A). This result was confirmed by solid phase radioligand-binding assays where treatment of cAgrin7A0B0-coated wells with heparitinase reduced binding of 125I-HRGγ to the wells ~20-fold (n = 4; data not shown), comparable to binding to immobilized BSA. Finally, if binding of NRGs to agrin were mediated by GAG chains, then NRGs should not bind to shorter COOH-terminal fragments of agrin-lacking GAG chains. Therefore, we tested binding of 125I-HRGγ to immobilized agrin-c95A0B0, a COOH-terminal fragment lacking conserved GAG side chain attachment sites (Denzer et al., 1995; Tsen et al., 1995). HRGγ clearly bound to both full-length agrin isoforms, although with different apparent affinity most likely representing different levels of glycosylation because of clonal selection of agrin-producing HEK 293 cells. Binding of 125I-HRGγ to cAgrin7A0B0, however, was comparable with nonspecific binding to BSA (Fig. 7 B).

Taken together, these experiments demonstrate that binding of HRG to agrin is restricted to a region located NH₂-terminal to the first laminin G–like domain (Gesemann et al., 1995), and is mediated by GAG side chains. They also demonstrate that the region of agrin required for HRG binding does not overlap with the region of agrin that is sufficient to cause AChR aggregation.

Indirect evidence led to the conclusion that interaction of neuregulins with components of the synaptic BL might be mediated by the Ig-like domain of NRGs (Loeb and Fischbach, 1995). To test this directly, we expressed trun-
cated HRG protein containing either the Ig-like domain, HRGΔBbsI, or the EGF-like domain, HRGβ1(177–246), and tested whether these isolated domains bound immobilized full-length agrin isoforms. Overlay assays (Fig. 7C) revealed binding of HRGΔBbsI, i.e., the Ig-like domain, but not the HRGβ1(177–246) EGF-like domain to agrin. This experiment demonstrates that the Ig-like domain of neuregulins is sufficient to account for the binding to the synaptic BL proteoglycan agrin.

**Agrin Induces the Accumulation of HSPGs at the Muscle Surface**

The experiments described so far are consistent with a mechanism whereby agrin induces AChR gene transcription by binding muscle-derived NRGs by their Ig domains to its GAG side chains. The following observations suggest, however, that neural agrin may not be the sole binding partner for muscle NRGs: (a) ectopic NRG accumulations expressed in response to neural agrin only partially overlaps with ectopic agrin deposits (see also Meier et al., 1997); and (b) neural agrin induces the accumulation of muscle-derived HSPGs on the muscle surface. This was seen in experiments in which chick agrin pcN25-C95AAB8, a fusion construct consisting of the ~25-kD laminin-binding NtA domain (Denzer et al., 1997) ligated to c95AAB8 (Gesemann et al., 1995) was injected into myofibers. This agrin construct lacks the region that carries GAG chains but is sufficient to bind to laminin and to induce the synthesis and accumulation of extrasynaptic AChRs when injected as expression plasmids into extrasynaptic regions of innervated rat muscle fibers (Fig. 8). Double labeling of pcN25-C95AAB8-injected muscle with Rh-α-BGT and antibodies specific for rat HSPGs (Eldridge et al., 1986) revealed close colocalization of ectopic AChR aggregates and HSPGs. This suggests that nerve-derived agrin isoforms expressed ectopically in innervated muscle induce the accumulation of HSPGs that can serve as local sink for the immobilization of muscle-derived NRGs. The nature of the HSPGs that are accumulated by agrin is presently unknown.

**Discussion**

We have demonstrated previously that expression of neural agrin locally induces the transcription of AChR subunit genes (Jones et al., 1996, 1997), a function classically attributed to NRGs released from motor neurons; the same sites also contain ErbB receptors (Rimer, M., I. Cohen, T. Lømo, S.J. Burden, and U.J. McMahan. 1996. Soc. Neurosci. Abstr. 1689; Meier et al., 1997). Based on these findings we proposed that synapse-specific AChR subunit gene expression by agrin is induced indirectly via muscle-derived NRGs binding to agrin-induced components of the synaptic BL (Jones et al., 1996, 1997). The present findings further support this hypothesis: the induction of AChR gene expression by agrin can be blocked by inhibiting the ErbB receptor pathway by overexpression of an inactive mutant of HER2/ErbB2. NRG-like immunoreactivity is accumulated at the sites of agrin-induced AChR gene activation and AChR accumulation. We further show that recombinant NRG isoforms that are expressed in muscle bind directly to the HSPGs agrin and perlecan but not to several other components found at the muscle basal lamina. Binding is to the GAG chains of these HSPGs and is mediated by the Ig-like domain of NRGs. Synaptic accumulation of NRGs may thus be due, at least in part, to binding to agrin itself. Other HSPGs are accumulated in the synaptic BL under the control of agrin, providing further binding sites for NRGs. Thus, agrin induces on the muscle fiber surface all components for regulating AChR gene expression according to the above model.

**Expression of NRGs in Muscle**

In the absence of NRGs from other potential sources, NRGs mediating agrin-induced AChR gene transcription in nerve-free myotube cultures must be supplied by the muscle cells themselves. Indeed, cultured muscle cells synthesize NRG-like biological activity, which can be extracted from the cell surface and induces phosphorylation of the ErbB2 receptor tyrosine kinase. An indirect action
of agrin via the accumulation of NRG derived from muscle could also explain why agrin’s action on AChR expression depends on its binding to substrate (Jones et al., 1996): substrate-bound agrin, but not soluble agrin, could locally enrich and present muscle-derived NRGs on the muscle surface, thus activating AChR gene expression.

Previous work has identified NRG transcripts in muscle, but these studies were done using non-innervated clonal cell lines (Moscoso et al., 1995) that, unlike adult muscle, express substantial amounts of AChR β subunit transcripts and adult-type AChR channels independently of innervation (Pinset et al., 1991; Shepherd and Brehm, 1994). Moreover, it has not been addressed whether active neuregulin β isoforms are colinearly transcribed with the Ig domain (Ng et al., 1997), a prerequisite for binding of muscle NRGs to HSPGs in the BL. Our study now demonstrates that not only cultured myotubes but also adult muscle fibers express NRG mRNA encoding isoforms with an Ig domain. Such transcripts were resolved both in synaptic (data not shown) and extrasynaptic regions of adult innervated muscle, as predicted earlier (Jones et al., 1997). In agreement with results obtained from SolB myotubes (Moscoso et al., 1995) or chicken muscle (Ng et al., 1997) muscle-derived NRGs probably represent NRGβ1 and NRGβ2 isoforms arising from alternative mRNA splicing at a conserved site located between the EGF domain and the transmembrane domain (Lemke, 1996; Fischbach and Rosen, 1997). NRGβ isoforms have been shown to be most potent in activation of ErbB receptor heterodimers and in the induction of AChR β subunit gene transcription (Fischbach and Rosen, 1997). These findings suggest that the protein products of the muscle-derived NRG transcripts are biologically active and that they contain domains required for binding to synaptic HSPGs.

**Requirements for NRG Immobilization in the Synaptic Basal Lamina**

The expression of NRG-like activity by cultured muscle cells suggests that (a) the transcripts resolved by reverse transcriptase–PCR in muscle are translated into biologically active NRGs, and (b) that muscle-derived NRGs can be associated with the ECM through ionic interactions, as has been proposed for ARIA/NRG released from cerebellar and spinal cord cells (Loeb and Fischbach, 1995). However, their conclusion that ARIA binds to unidentified HSPGs via the Ig domain was derived indirectly, i.e., based on the heparin inhibition of ErbB phosphorylation by recombinant NRGs. In the present work, we demonstrate the importance of the GAG chains directly by showing that binding between NRGs and agrin is inhibited by enzymatic removal of GAGs, and accordingly, that NRGs do not bind to an ~95-kD COOH-terminal agrin fragment lacking conserved GAG attachment sites. We have identified two potential binding partners at the NMJ, agrin and...
perlecan, and have specifically excluded other glycoproteins of the NMJ for NRG binding.

Of the potential NRG-binding HS PG partners identified in this paper, i.e., cAgrin7A4B8, cAgrin7A0B0, and perlecan, neither cAgrin7A0B0 nor perlecan were enriched at extrasynaptic AChR clusters induced by ectopic neural agrin in vivo (Meier et al., 1997; Moll, J., M.A. Ruegg, and H.R. Brenner, unpublished observation), and thus, are not involved in NRG accumulation at such sites. However, this does not exclude a role for cAgrin7A0B0 in NRG accumulation during synaptogenesis, as in nerve–muscle cocultures, muscle-derived agrin is focally expressed at sites of neurally induced AChR clusters (Lieth and Fallon, 1993). Perlecan, on the other hand, is expressed at high levels in non-synaptic BL throughout the muscle, and thus would not appear to contribute to synaptic localization of NRGS.

Whereas neural agrin alone can induce AChR gene expression via NRG accumulation, its GAG chains do not provide the only binding sites for NRGS at the synapse. In addition, agrin induces one other HSPG that in turn may contribute to localize NRGS to the synapse. Specifically, NRGS are also accumulated at AChR aggregates induced by a COOH-terminal fragment of full-length agrin, agrin cN25C95A4B8, a construct that lacks GAG chains. Consistent with this, deposits of full-length agrin extend further along the muscle fiber surface (Meier et al., 1997) than NRGS, which are tightly colocalized with AChR aggregates and agrin-induced HS PGs (see Figs. 5 and 8). Furthermore, CBA-1 agrinA4B10, another COOH-terminal agrin fragment lacking GAG chains, induces AChR e subunit gene transcription both in vitro and in vivo (Jones et al., 1996, 1997). Accumulation of newly synthesized HS PGs by agrin was previously shown in cultured myotubes (Wallace, 1989). Finally, focal accumulation of NRGS by agrin might be further enhanced if nerve-released agrin not only caused the accumulation of NRG-binding HS PGs but in addition, also induced NRG gene transcription from myonuclei underlying the postsynaptic membrane.

In contrast to neural agrin, muscle cAgrin7A0B0 does not induce ectopic synaptic membrane (Meier et al., 1997), yet in cultured myotubes its potency to induce AChR e subunit gene transcription is similar to that of neural agrin7A4B8. This apparent discrepancy might reflect the considerably higher levels of expression of MuSK, NRGS, and ErbB receptors in cultured myotubes over that seen in the non-synaptic segments of mature muscle (Moscoso et al., 1995; Jones, G., and H.R. Brenner, unpublished data). As a consequence, the accumulation of constitutively expressed NRGS to muscle agrin’s GAG chains and the activation of constitutively expressed ErbB receptors would be sufficient to stimulate AChR gene expression in cultured myotubes. In contrast, in adult muscle the ectopic induction and accumulation of NRG and ErbB receptors may depend on the activation of MuSK, which is phosphorylated by neural but not by muscle agrin (Glass et al., 1996).

In conclusion, our data demonstrate at sites of ectopic agrin deposits, the presence of the major molecular components required for agrin to stimulate AChR gene expression indirectly via the accumulation of muscle-derived NRGS. According to this model, agrin induces local deposits of muscle HS PGs in the muscle BL. Muscle-derived NRGS then bind with their Ig domains to accumulated HS PGs, creating a local source of this differentiation factor. NRGS would then stimulate muscle ErbB receptors that are accumulated in synaptic muscle membrane under the direction of neural agrin.

Alternative Pathways for Agrin-induced AChR Expression?

Whereas this model can explain how agrin induces AChR gene expression involving NRGS in the absence of a nerve terminal, two questions arise. First, could agrin’s action to stimulate the expression of AChR genes also be mediated directly via a cognate receptor in the muscle membrane, such as MuSK (Valenzuela et al., 1995), a muscle-specific receptor tyrosine kinase phosphorylated by agrin and mediating AChR aggregation (Glass et al., 1996)? Indeed, deletion of the MuSK gene inhibits NMJ formation including synapse-specific AChR gene transcription (De Chiara et al., 1996), and deletion of the rapsyn gene, which abolishes the synaptic aggregation of ErbB receptors, does not affect synapse-specific expression of AChR genes (Gautam et al., 1995). On the other hand, direct involvement of MuSK in agrin-induced expression of AChRs is not supported, since only neural isoforms of agrin that cluster AChRs also cause phosphorylation and activation of MuSK (Glass et al., 1996). In contrast, the ability of substrate-bound agrin isoforms to activate AChR gene expression is independent of their AChR clustering activity (Jones et al., 1996). Furthermore, AChR gene expression can be induced in cultured myotubes both by COOH- and by NH2-terminal fragments of agrin (Jones et al., 1996; and unpublished results), making binding to a specific, cognate receptor appear unlikely. The second question is whether or not (during the formation of the normal NMJ) NRGS released from motor nerve terminals are important for the expression of AChR subunit genes at the synapse, as originally proposed (Martinou et al., 1991; Falls et al., 1993). The reduction of AChR levels observed in subsynaptic membrane of mice heterozygous for a deletion of Ig(+)NRG isoforms (Sandrock et al., 1997) is consistent with both a pre- and a postsynaptic origin of BL-bound NRG. Thus, deleting expression of NRGS selectively either in the muscle fibers or in the motor neurons will be required to determine the relative roles of nerve- and muscle-derived NRGS in activating AChR gene transcription during synapse formation.
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