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Neuregulin receptors, erbB3 and erbB4, are localized at neuromuscular synapses

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Neuregulin (NRG) is concentrated at synaptic sites and stimulates expression of acetylcholine receptor (AChR) genes in muscle cells grown in cell culture. These results raise the possibility that NRG is a synaptic signal that activates AChR gene expression in synaptic nuclei. Stimulation of NRG receptors, erbB3 and erbB4 initiates oligomerization between these receptors or between these receptors and other members of the epidermal growth factor (EGF) receptor family, resulting in stimulation of their associated tyrosine kinase activities. To determine which erbBs might mediate synapse-specific gene expression, we used antibodies against each erbB to study their expression in rodent skeletal muscle by immunohistochemistry. We show that erbB2, erbB3 and erbB4 are concentrated at synaptic sites in adult skeletal muscle. ErbB3 and erbB4 remain concentrated at synaptic sites following denervation, indicating that erbB3 and erbB4 are expressed in the postsynaptic membrane. In addition, we show that expression of NRG and erbBs, like AChR gene expression, increases at synaptic sites during postnatal development. The localization of erbB3 and erbB4 at synaptic sites is consistent with the idea that a NRG-stimulated signaling pathway is important for synapse-specific gene expression.

Keywords: acetylcholine receptor/skeletal muscle/synapse formation

Introduction

Acetylcholine receptors (AChRs) are highly concentrated in the postsynaptic membrane at neuromuscular synapses. A high density of AChRs ensures that the postsynaptic response to acetylcholine is sufficient to initiate an action potential, the mechanisms that mediate AChR clustering at synapses are critical for synaptic function (Hall and Sanes, 1993). There is good evidence that two signaling molecules, agrin and neuregulin (NRG), mediate the clustering of AChRs at neuromuscular synapses (Falls *et al.*, 1993; Burden *et al.*, 1995; Bowe and Fallon, 1995; Chu *et al.*, 1995b).

Agrin, which is synthesized by motor neurons and deposited in the synaptic basal lamina, stimulates a redistribution of pre-existing AChRs to the synaptic site (McMahan, 1990). Little is known about the agrin-stimulated signaling pathway, although there is evidence that dystroglycan, a component of the dystrophin-associated complex, is a receptor for agrin (Fallon and Hall, 1994). NRG, like agrin, is synthesized by motor neurons (Marchionni *et al.*, 1993; Corfas *et al.*, 1995) and is deposited in the synaptic basal lamina (Jo *et al.*, 1995), where it is thought to act as a signal that activates AChR gene expression selectively in myofiber nuclei located near the synaptic site (Chu *et al.*, 1995a; Jo *et al.*, 1995). Consistent with this idea, NRG increases the rate of AChR transcription in muscle cells grown in cell culture (Harris *et al.*, 1988) and the *cis*-acting sequences for NRG-stimulated AChR gene expression and for synapse-specific expression are contained in the same 5′ flanking region of AChR subunit genes (Chu *et al.*, 1995a; Jo *et al.*, 1995). Thus, two signaling pathways, one which controls the distribution of AChRs by post-translational mechanisms and the other which regulates AChR expression by transcriptional mechanisms, are thought to mediate clustering of AChRs at synaptic sites.

NRG was purified on the basis of its ability to stimulate phosphorylation of erbB2/neu and was initially termed neu differentiation factor (NDF). Subsequently, it became clear that NDF, glial growth factor (GGF) and an AChR-inducing activity (ARIA) are encoded by the same gene (Mudge, 1993). Because of their potential role in neuronal development, as well as their ability to stimulate phosphorylation of erbB2/neu, the various members of the NDF/GGF/ARIA family have been dubbed the NRGs (Marchionni *et al.*, 1993). The NRG gene encodes a large number of alternatively spliced transcripts, most of which encode integral membrane proteins containing an extracellular epidermal growth factor (EGF)-like domain. Soluble forms of NRG are produced by post-translational processing of the membrane-bound isoforms or are encoded by alternative splicing. Soluble NRG retains the EGF-like domain, which alone is sufficient to cause tyrosine phosphorylation of NRG receptors (see below) and to stimulate cellular responses (Peles and Yarden, 1993; Carraway and Burden, 1995).

Although NRG stimulates tyrosine phosphorylation of erbB2/neu, NRG is not a direct ligand for erbB2/neu. Two other members of the EGF receptor family, erbB3 and erbB4, are NRG receptors (Plowman *et al.*, 1993; Carraway *et al.*, 1994; Tzahar *et al.*, 1994). Signaling through erbB4 is thought to be mediated by ligand-stimulated homo-oligomerization of erbB4 or hetero-oligomerization of erbB4 with either erbB2 or erbB3 (Plowman *et al.*, 1993; Riese *et al.*, 1995). Since erbB3 has little, if any, tyrosine kinase activity, signaling through erbB3 is thought to require ligand-stimulated association of erbB3 with another member of the EGF receptor family

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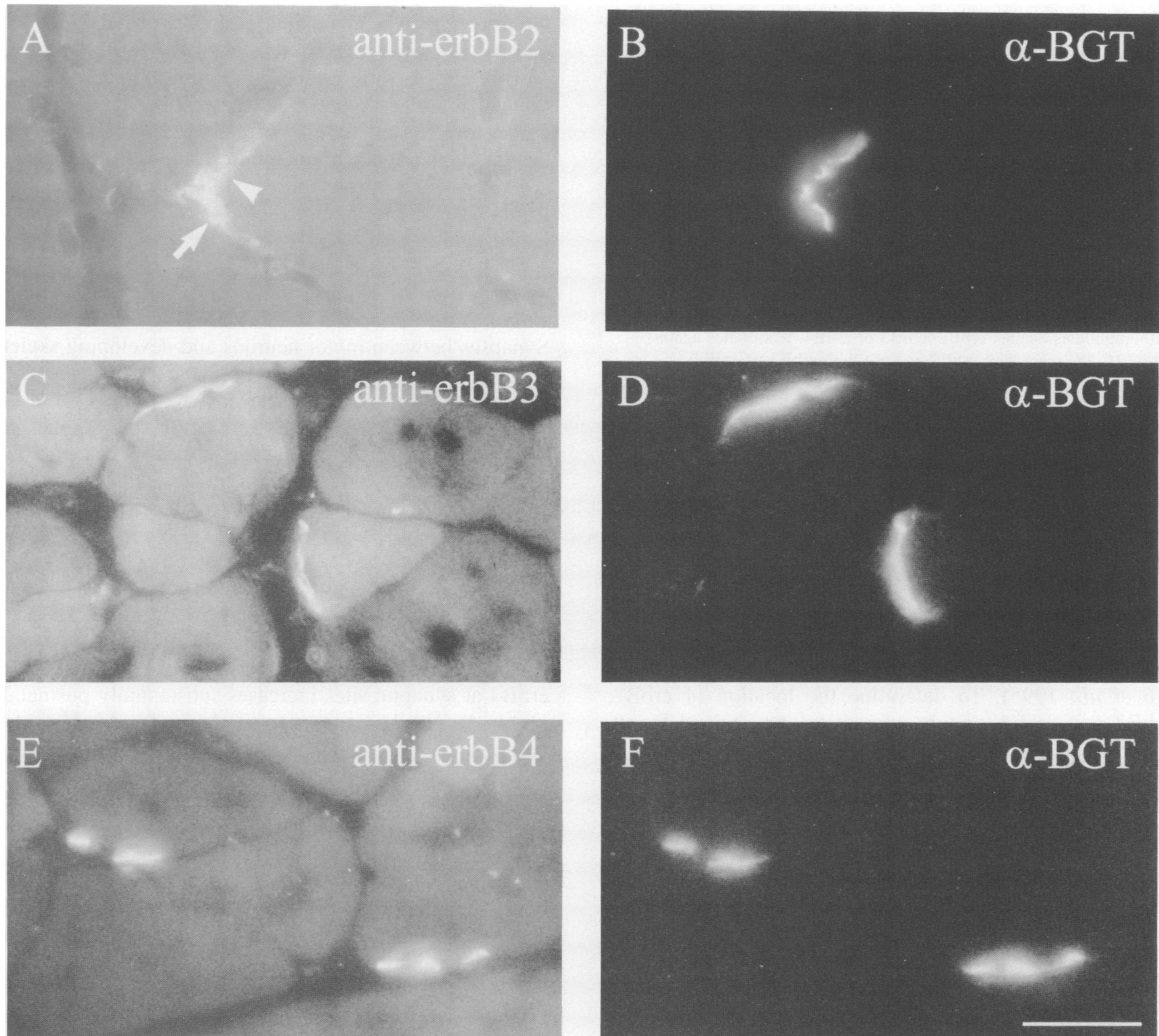

Fig. 1. ErbB2, erbB3 and erbB4 are concentrated at neuromuscular synapses. Frozen sections of skeletal muscle were stained with antibodies against erbB2 (**A**), erbB3 (**C**) or erbB4 (**E**) and with TMR-α-BGT (**B**, **D** and **F**), which marks synaptic sites. ErbB2 (A and B), erbB3 (C and D) and erbB4 (E and F) are concentrated at synaptic sites. ErbB3 (C and D) and erbB4 (E and F) staining is co-localized precisely with TMR-α-BGT staining. In contrast, erbB2 (A and B) staining extends beyond the TMR-α-BGT stained postsynaptic membrane and is evident in the myofiber cytoplasm near the synaptic nuclei (arrowhead) and near the Schwann cells that cap nerve terminals (arrow). Synaptic staining was reduced or absent following pre-incubation of the antibodies with the appropriate peptides (see Materials and methods). The bar = 30 μm.

(Carraway and Cantley, 1994; Sliwkowski *et al.*, 1994). Because the different erbBs share only limited sequence identity (10–30%) in their carboxy-terminal tail regions, which are sites of tyrosine phosphorylation (Peles and Yarden, 1993), different pairs of EGF receptor family members could potentially activate different signaling pathways (Carraway and Cantley, 1994). Thus, the repertoire and level of NRG receptor expression are likely to be critical determinants in specifying the response to NRG.

We have shown previously that C2 muscle cells respond to NRG and express erbB3 but not erbB4 mRNA, indicating that NRG-mediated signaling in C2 cells is initiated by erbB3 (Jo *et al.*, 1995). Nevertheless, both erbB3 and erbB4 mRNAs are expressed in adult skeletal muscle tissue, raising the possibility that erbB3 and/or erbB4 could act as NRG receptors in skeletal muscle cells. Here, we study expression of erbB2, erbB3 and erbB4 mRNAs in muscle cell lines, in primary muscle cells grown in cell culture and at synapses. We show that erbB2 and erbB3 mRNAs are expressed in C2 and L6 muscle cell lines and in primary muscle cell cultures. Although erbB4 mRNA is expressed poorly, if at all, in these muscle cell lines, erbB4 mRNA is expressed in primary muscle cell cultures and in normal adult skeletal muscle. Importantly, erbB3 and erbB4 proteins are highly concentrated at neuromuscular synapses. These data are consistent with the idea that a NRG-stimulated pathway mediates signaling at neuromuscular synapses.

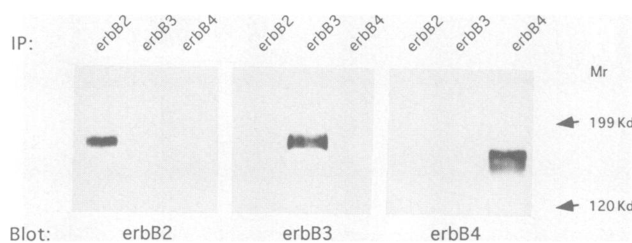

Fig. 2. Antibodies against erbB2, erbB3 or erbB4 are specific for the appropriate EGF receptor family member. Western blots of immunoprecipitated (IP) erbB2, erbB3 or erbB4 were probed (Blot) with antibodies against erbB2, erbB3 or erbB4. Antibodies against erbB2 (C-18) react with erbB2 and not with erbB3 or erbB4; antibodies against erbB3 (C-17) react with erbB3 and not with erbB2 or erbB4; antibodies against erbB4 (#616) react with erbB4 and not with erbB2 or erbB3. ErbB2 and erbB3 were immunoprecipitated from C2 myotubes and erbB4 was immunoprecipitated from High Five cells expressing erbB4. The positions of protein standards are indicated with arrows.

Results

ErbB2, erbB3 and erbB4 are concentrated at neuromuscular synapses

We showed previously that erbB2, erbB3 and erbB4 mRNAs are expressed in innervated adult skeletal muscle (Jo *et al.*, 1995). To determine the location of erbB2, erbB3 and erbB4 protein expression, we stained frozen sections of innervated, adult skeletal muscle with antibodies that are specific for erbB2, erbB3 or erbB4. Figure 1 shows that erbB2, erbB3 and erbB4 are concentrated at synaptic sites, marked by α-bungarotoxin (α-BGT) staining. ErbB3 and erbB4 staining appears to be coincident with α-BGT staining, whereas erbB2 staining extends beyond the postsynaptic membrane that is labelled by α-BGT (Figure 1). Indeed, erbB2 staining is evident in the myofiber cytoplasm near the synaptic nuclei and near the Schwann cells that cap nerve terminals (Figure 1).

Several lines of evidence indicate that antibody staining is specific. First, the antibodies were produced against peptide sequences that are unique to erbB2, erbB3 or erbB4. Second, two different antibodies against erbB3 and three different antibodies against erbB4 stain synaptic sites (see Materials and methods). Third, synaptic staining is reduced by pre-incubation with the appropriate peptides. Fourth, antibodies to each erbB react with the appropriate erbB and not with the other erbBs (Figure 2).

ErbB3 and erbB4 staining persists at synaptic sites following denervation, consistent with the idea that erbB3 and erbB4 are concentrated in the postsynaptic membrane (Figure 3). In contrast, denervation causes a marked reduction in erbB2 staining at synaptic sites. The non-identical pattern of erbB2 and α-BGT staining at synaptic sites (Figure 1), together with the decrease in erbB2 staining following denervation (Figure 3), suggests that much of the erbB2 staining at synaptic sites is associated with nerve terminals. Our data, however, does not exclude the possibility that some erbB2 staining at synaptic sites is associated with the postsynaptic membrane and/or in Schwann cells (Morrissey *et al.*, 1995). Moreover, it is possible that the level of erbB2 expression in the postsynaptic membrane and/or in Schwann cells is dependent upon innervation.

We did not detect erbB2, erbB3 or erbB4 by immunofluorescence in non-synaptic regions of adult skeletal muscle (Figures 1 and 3). Nevertheless, erbB2, erbB3 and erbB4 mRNAs are present in non-synaptic regions of skeletal muscle (Figure 4), consistent with the idea that erbB2, erbB3 and erbB4 proteins are expressed at low levels in non-synaptic membrane. Indeed, we detected erbB3 protein by immunoprecipitation in lysates prepared from synapse-free regions of skeletal muscle (unpublished data).

Appearance of NRG, erbB2, erbB3 and erbB4 at developing synapses

Synapses between motor neurons and developing skeletal myotubes form as early as E15 and AChRs begin to cluster at synapses within the next few hours (Hall and Sanes, 1993). The earliest time that AChR genes are selectively transcribed in synaptic nuclei has not been determined but synapse-specific expression of the AChR α, β and δ subunit genes are detectable by birth (Simon *et al.*, 1992; Piette *et al.*, 1993). Nevertheless, AChR gene expression has not peaked at birth, since synaptic expression of the AChR δ subunit gene appears less in neonatal than in adult myofibers (Simon *et al.*, 1992).

Like the progressive increase in AChR δ subunit expression following birth, expression of NRG, erbB2, erbB3 and erbB4 at synaptic sites increases substantially postnatally (Table I). ErbB4 expression is detectable by birth (see below) and expression increases during the next few weeks (Table I). NRG, erbB2 and erbB3 are first detectable at synapses between 1 and 2 weeks after birth and the intensity of antibody staining increases during the next few weeks (Table I). It is not clear whether NRG, erbB2 and erbB3 are truly absent from synapses in neonatal rats, or whether the apparent lack of NRG, erbB2 and erbB3 staining is owing to a low level of expression that is not detectable with our antibodies.

Although three different antibodies against erbB4 stain synaptic sites in adult muscle, only two of these antibodies (#616 and #618) stain synaptic sites in neonatal mice (Table I). A third antibody (#622) against erbB4, which stains adult synapses as intensely as antibody #618, first stains synaptic sites 1–2 weeks after birth. Thus, different epitopes in erbB4 are detected at synapses at different times during development. These results raise the possibility that alternative splicing and/or post-translational modification of erbB4 occurs during the first 2 weeks after birth.

Expression of erbB2, erbB3 and erbB4 mRNAs in muscle cell lines and primary muscle cultures

We showed previously that C2 myotubes express erbB2 and erbB3 but not erbB4 mRNA (Jo *et al.*, 1995). To determine whether erbB4 mRNA expression is absent from all muscle cells grown in cell culture, we measured the abundance of erbB2, erbB3 and erbB4 mRNAs in L6 muscle cells and in primary rat myotubes. Figure 5 shows that erbB4 mRNA is expressed at very low levels (~50-fold less than erbB3) in L6 myotubes and at moderate levels (~5-fold less than erbB3) in primary muscle cultures. Thus, unlike synapses, which express erbB3 and erbB4, C2 and L6 muscle cell lines express erbB3 but undetectable or extremely low levels of erbB4. Primary rat myotubes, however, express moderate levels of erbB4 in addition to erbB3 mRNA.

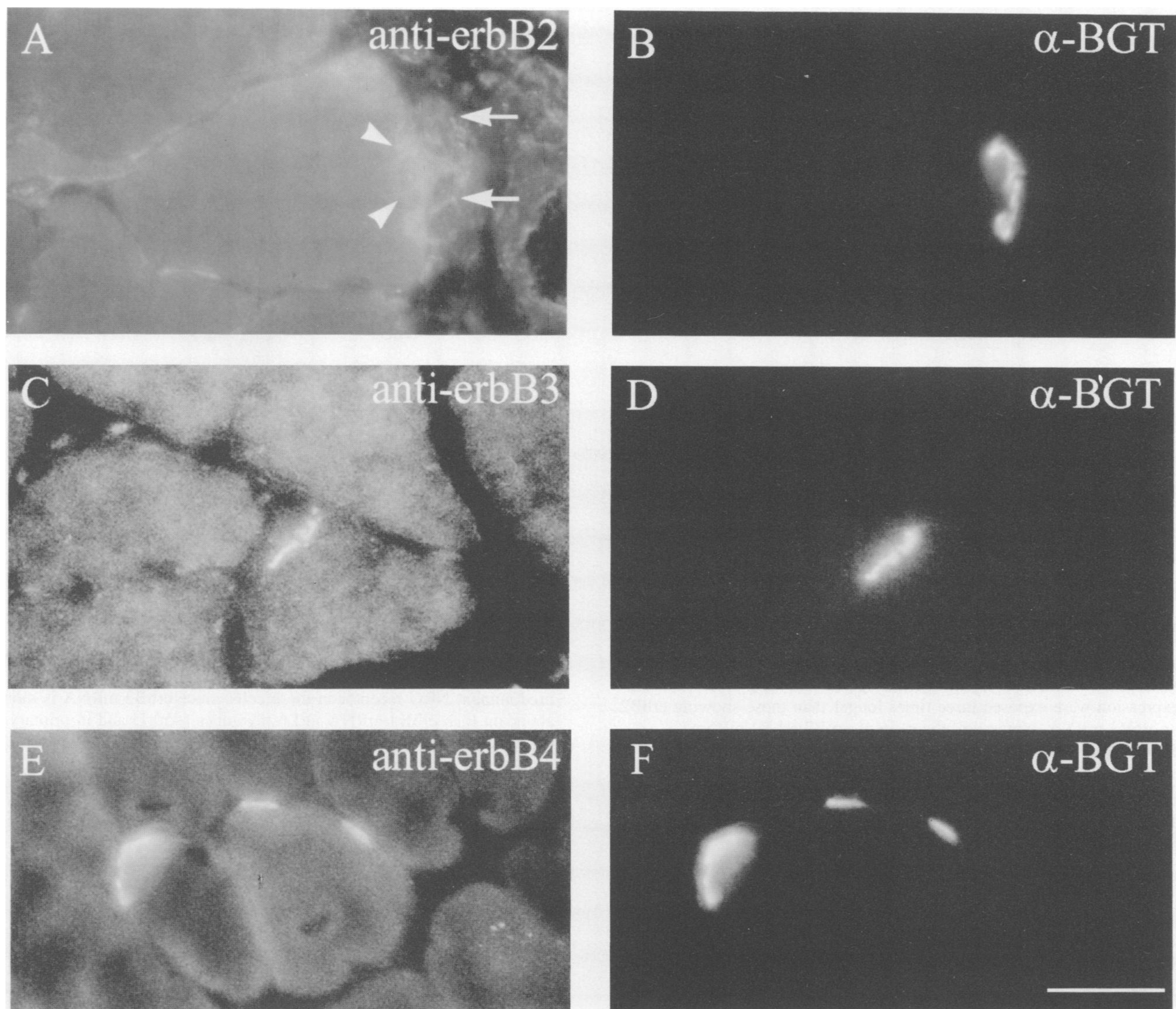

Fig. 3. ErbB3 and erbB4 are concentrated at denervated synaptic sites. Frozen sections of skeletal muscle, which was denervated for 4 days, were stained with antibodies against erbB2 (**A**), erbB3 (**C**) or erbB4 (**E**) and with TMR-α-BGT (**B**, **D** and **F**), which marks synaptic sites. ErbB3 (C and D) and erbB4 (E and F) remain concentrated at synaptic sites following denervation and subsequent degeneration of nerve terminals. In contrast, erbB2 (A and B) staining at synaptic sites is markedly reduced following denervation. Like erbB2 staining in normal muscle, erbB2 staining at denervated synaptic sites is evident in the myofiber cytoplasm near the synaptic nuclei (arrowheads) and near the Schwann cells that cap nerve terminals (arrows). Similar results were obtained in muscle that was denervated for 10 days. The bar = 30 μm.

Discussion

NRG is concentrated at neuromuscular synapses and can activate AChR gene expression in muscle cells grown in cell culture (Chu *et al.*, 1995a; Jo *et al.*, 1995). Here, we show that NRG receptors erbB3 and erbB4 are concentrated at neuromuscular synapses. Since erbB3 and erbB4 expression is co-localized precisely with AChRs and because their expression is maintained following denervation, our data are consistent with the idea that erbB3 and erbB4 are expressed in the postsynaptic membrane. We find that erbB2, which can oligomerize with erbB3 or erbB4, is concentrated at synaptic sites but our data do not allow us to determine whether erbB2 is expressed in nerve terminals, the postsynaptic membrane and/or Schwann cells. Expression of erbB2 within myofibers near synaptic nuclei, however, suggests that some of the erbB2 at synaptic sites is indeed expressed in the postsynaptic membrane. The co-localization of NRG, erbB3, erbB4 and possibly erbB2 at synapses supports the idea that NRG is a signal that regulates synaptic differentiation and suggests that accumulation of erbBs at the synapse leads to an increase in NRG signaling and an increase in AChR gene expression.

NRG can stimulate homo-oligomerization of erbB4 or hetero-oligomerization of erbB4 with erbB2 or erbB3 (Carraway and Cantley, 1994; Riese *et al.*, 1995). In addition, NRG can stimulate hetero-oligomerization of erbB3 with erbB2 or erbB4 (Carraway and Cantley, 1994; Riese *et al.*, 1995). Thus, NRG has the potential to stimulate the formation of several different erbB combinations (e.g. erbB3/erbB2, erbB3/erbB4, erbB4/erbB2 and erbB4/erbB4) at synapses. Because of differences in the amino acid sequence surrounding tyrosine residues in different erbBs, it has been suggested that different SH2

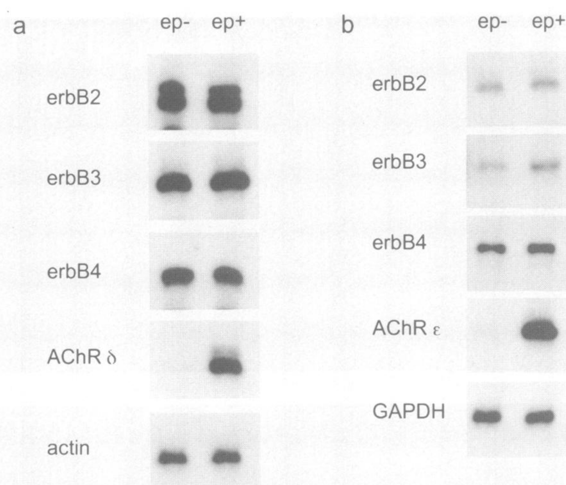

Fig. 4. ErbB2, erbB3 and erbB4 mRNAs are expressed in synaptic and non-synaptic regions of skeletal muscle. The level of mRNA encoding erbB2, erbB3, erbB4, AChR δ subunit, AChR ε subunit, actin and GAPDH in dissected synapse-free (ep–) and synapse-enriched (ep+) regions of mouse (**a**) or rat (**b**) diaphragm muscle was measured by an RNase protection assay. The abundance of erbB2, erbB3, erbB4, actin and GAPDH mRNAs is similar in synapse-free and synapse-enriched regions. The level of AChR δ and ε subunit mRNAs is higher in synapse-enriched than synapse-free regions (Merlie and Sanes, 1985; Witzemann *et al.*, 1987). The autoradiograms showing erbB4 mRNA expression were exposed three times longer than those showing erbB2 or erbB3 mRNA expression; the ratio of erbB3:erbB4 mRNA expression is ~5:1 in mouse muscle and 1:1 in rat muscle.

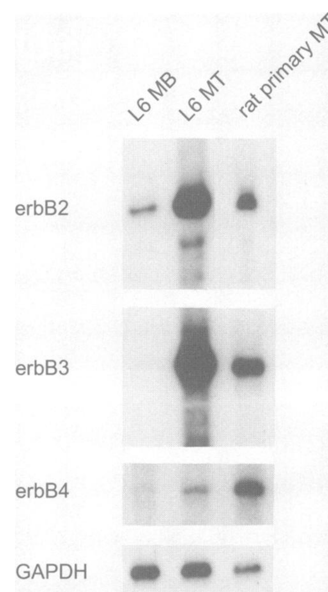

Fig. 5. ErbB2, erbB3 and erbB4 mRNAs are expressed in L6 myotubes and in primary muscle cell cultures. The level of erbB2, erbB3, erbB4 and GAPDH mRNAs was measured by an RNase protection assay. Each erbB mRNA is expressed in L6 myotubes (L6 MT) and in cultures of primary rat myotubes. ErbB3 is the predominant NRG receptor in these cells, since erbB3 mRNA is more abundant than erbB4 mRNA in L6 myotubes (~50:1) and in primary myotubes (~5:1). Expression of erbB3 mRNA is induced during differentiation of L6 cells; likewise, erbB2 and erbB4 mRNA expression is greater in L6 myotubes than in L6 myoblasts (L6 MB). The autoradiograms showing erbB4 mRNA expression were exposed longer than those showing erbB2 and erbB3 mRNA expression.

Table I. Expression of NRG, erbB3 and erbB4 at synaptic sites during development

	Day					Adult
	1	5	8	10	14	
NRG	–	–	–/+	+	++	+++
erbB2 (C-18)	–	n.d.	–	n.d.	+	++
erbB3 (C-17)	–	+	+	++	+++	+++
erbB4 (#616)	+	++	n.d.	+++	+++	++++
erbB4 (#618)	+	++	n.d.	+++	+++	+++
erbB4 (#622)	–	–	n.d.	n.d.	++	+++

NRG, erbB2 and erbB3 are first apparent at synaptic sites in rat muscle between 1 and 2 weeks after birth and their expression level at synapses increases during the next several weeks. Antibodies against erbB4 (#616 and #618) stain synapses in newborn mice and the intensity of antibody staining increases during the next several weeks. Antibody #622, which also reacts with erbB4, does not stain synaptic sites in newborn mice; staining is first detectable 2 weeks after birth. Because the intensity of staining at adult synapses is similar with antibodies #618 and C-17, the lack of erbB3 staining at synapses in newborn rats is not simply owing to greater sensitivity of the erbB4 antibodies. –: no staining; –/+: weak staining at ~20% of synaptic sites; +, ++, +++, ++++: positive staining of increasing intensity; n.d.; not determined.

domain-containing proteins might be recruited to tyrosine-phosphorylated erbB2, erbB3 and erbB4 (Carraway and Cantley, 1994). Thus, NRG has the potential to stimulate different downstream signaling pathways at neuromuscular synapses.

Previously, we showed that C2 muscle cells respond to NRG and express erbB2 and erbB3 but not erbB4 mRNA. These results indicate that erbB3 is the NRG receptor in this cell line and that NRG signaling in C2 cells is mediated by a complex of erbB2 and erbB3. Moreover, these results indicate that erbB4 is not required for C2 cells to respond to NRG. The response of C2 cells to NRG, however, is small (~2.5-fold increase in AChR δ subunit expression), whereas expression of the AChR delta subunit gene in synaptic nuclei appears robust (Simon *et al.*, 1992; Tang *et al.*, 1994). Thus, it is possible that expression of erbB4 is required for maximal expression of certain AChR genes in synaptic nuclei.

Neuromuscular synapse formation begins at E15 of mouse development but synapses continue to mature during the first few weeks after birth (Hall and Sanes, 1993). This program involves expression of different genes at different stages of development. For example, the AChR γ subunit gene is expressed in synaptic nuclei in embryos and is inactivated postnatally, whereas the AChR ε subunit gene is expressed poorly in embryos and is induced markedly in synaptic nuclei following birth (Kues *et al.*, 1995). Similarly, sodium channel expression at synapses increases substantially following birth (Lupa *et al.*, 1993). The different temporal appearance of erbB3 and erbB4 at synapses during development raises the possibility that NRG could activate different signaling pathways at different stages of development and determine the temporal pattern of gene expression in synaptic nuclei.

NRG mRNA is detectable in motor neurons at E14 of mouse development (Corfas *et al.*, 1995). However, we did not detect NRG at synapses in newborn rats or mice. Indeed, we first detected NRG at synapses between the first and second weeks after birth. It is possible that the abundance of NRG mRNA increases substantially in motor

neurons postnatally and that the apparent difference in timing of NRG mRNA and protein expression is due to differences in the sensitivity of *in situ* hybridization and immunofluorescence assays. Alternatively, an increase in the rate of translation, rate of transport or an increase in stability of NRG protein during development might be responsible for a delayed appearance of NRG at synapses.

The mechanisms that localize erbB3 and erbB4 proteins to synapses are not known. We find comparable levels of erbB3 and erbB4 mRNAs in dissected synapse-enriched and synapse-free regions of skeletal muscle. Although these results do not support the idea that erbB genes are selectively transcribed in synaptic nuclei, the preponderance of non-synaptic nuclei in the synapse-enriched region (only ~3% of the nuclei in the dissected synapse-enriched region are truly synaptic nuclei) could obscure a potential enrichment of erbB mRNA near synaptic nuclei. Indeed, localization of rapsyn and N-CAM mRNAs at synaptic sites cannot be detected by comparing the level of mRNA expression in dissected synapse-enriched and synapse-free regions, but only by using *in situ* hybridization (Moscoso *et al.*, 1995). Thus, we cannot exclude the possibility that erbB mRNAs are concentrated near synaptic nuclei and that erbB mRNA localization has a role in clustering erbBs at synapses.

As we find erbB mRNA and protein in non-synaptic regions of skeletal muscle, it is possible that post-translational mechanisms are responsible for concentrating erbBs at synapses. It is possible that NRG, which is associated with the nerve terminal and with the synaptic basal lamina (Jo *et al.*, 1995), might capture its receptors and cause their localization to synapses. Alternatively, erbBs, like AChRs, could be redistributed from non-synaptic regions to synaptic sites by agrin. In this regard, it will be interesting to determine whether NRG and/or agrin can regulate the distribution of erbBs in skeletal muscle cells.

Materials and methods

Immunohistochemistry

Frozen sections of innervated and denervated skeletal muscle were incubated with primary antibodies (overnight at 4°C), washed in PBS, incubated with fluorescein-conjugated secondary antibodies (2 h at room temperature) and washed (Simon *et al.*, 1992). Sections were mounted in 80% glycerol, 100 mM sodium bicarbonate, pH 9 with 10 μg/ml *p*-phenylenediamine. Tetramethylrhodamine-conjugated α-bungarotoxin (TMR-α-BGT) was included in the secondary antibody incubation to mark synaptic sites (Burden, 1982; Woodruff *et al.*, 1987). The sections were viewed with filters selective for either rhodamine or fluorescein and with a Zeiss Planapo 63X objective.

Antibodies against erbB2 (C-18; Santa Cruz Biotechnology Inc.) were used at 1 μg/ml. We used two different antibodies against erbB3; C-17 (Santa Cruz Biotechnology Inc.) was used at 1 μg/ml and #3185 (Carraway *et al.*, 1994), generously provided by Dr Kermit Carraway, was diluted 1/500. Antibodies against erbB4 (#616, #618 and #622) were prepared against GST fusion proteins containing peptide sequences from mouse erbB4; antibody #616 is directed against a sequence that corresponds to residues 1185–1238 in human erbB4 (Plowman *et al.*, 1993); antibody #618 is directed against a sequence that corresponds to residues 1108–1136 (Plowman *et al.*, 1993); antibody #622 is directed against a sequence that corresponds to residues 1132–1182 (Plowman *et al.*, 1993).

Synaptic staining was eliminated by incubating antibodies against erbB2 (C-18) or erbB3 (C-17) with the appropriate peptide (5 μM; Santa Cruz Biotechnology Inc.). Absorption of antibody #616 with the appropriate GST fusion protein markedly diminished synaptic staining.

Analysis of erbB mRNA and protein expression

The abundance of erbB, AChR, actin and GAPDH mRNAs was measured by an RNase protection assay and quantitated with a PhosphorImager as described previously (Simon *et al.*, 1992; Jo *et al.*, 1995). ErbB2, erbB3 and erbB4 were immunoprecipitated as described previously (Jo *et al.*, 1995). ErbB2 was immunoprecipitated with antibody AB3 (Oncogene Sciences); erbB3 was immunoprecipitated with antibody C-17 and erbB4 was immunoprecipitated with antibody #616. Western blots of immunoprecipitated erbB2, erbB3 or erbB4 were probed with antibodies against erbB2 (C-18), erbB3 (C-17) or erbB4 (#616).

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