Induction of multiple signaling loops by MuSK during neuromuscular synapse formation

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At the neuromuscular junction, two motor neuron-derived signals have been implicated in the regulation of synaptogenesis. Neuregulin-1 is thought to induce transcription of acetylcholine receptor (AChR) genes in subsynaptic muscle nuclei by activating ErbB receptors. Neural agrin aggregates AChRs by activating the receptor tyrosine kinase MuSK. Here, we show that these two signals act sequentially. Agrin, by activating MuSK, induces the synthesis and aggregation of both MuSK and ErbB receptors. ErbB acts downstream of MuSK in synapse formation. In this way, MuSK activation leads to the establishment of a neuregulin-1-dependent signaling complex that maintains MuSK, ErbB, and AChR expression at the synapse of electrically active muscle fibers.

The formation and maintenance of the neuromuscular junction requires the expression of MuSK in the muscle fibers and the secretion of agrin from motor nerve terminals (1–3). Their interaction in the muscle membrane can mediate the aggregation of acetylcholine receptors (AChRs) and other subsynaptic components at the synaptic site. In fetal muscles, both MuSK and AChRs are expressed constitutively (4), and synaptic-like local AChR synthesis and clustering occur in the absence of the nerve (5) and agrin but require MuSK (6, 7). Later in development, however, expression of MuSK and AChRs is down-regulated by electrical impulse activity except in subsynaptic nuclei (8, 9), where they are maintained by neural signals such as agrin and neuregulin-1 (NRG-1) (10). An isoform of NRG-1 derived from motor neurones is thought to maintain synaptic AChR expression by activating ErbB receptor tyrosine kinases (10) clustered in the subsynaptic membrane (11). In contrast, the neural signal maintaining MuSK expression is not known. Likewise, it is not known whether synaptic ErbB accumulations involve the synapse-specific synthesis of ErbBs and, if so, how this regulation is mediated.

In addition to aggregating AChRs, agrin can cause the transcription of their genes in the absence of a nerve terminal (12). One way to reconcile this observation with the results described above would be to suppose that MuSK can stimulate the transcription of *AChR* genes via a pathway other than NRG/ErbB. Alternatively, agrin/MuSK could organize a secondary NRG/ErbB signaling pathway with NRG derived from muscle. Indeed, agrin-induced ectopic AChR clusters contain NRG-1, ErbB2, and ErbB3 (13–15), but it is not known whether they form a functional pathway *in vivo*.

In the present paper, we demonstrate that nerve and agrin-activated MuSK induce the synapse-specific expression of *musk*, *erbB2*, and *erbB3* genes and can regulate the expression of *AChR* genes via the induction of a secondary NRG/ErbB signaling loop. In cultured myotubes, the same pathway can regulate the expression of MuSK (16) and ErbB2 mRNA, suggesting that it regulates the synthesis of MuSK and ErbB2 at the synapse. We propose that, in this way, agrin/MuSK signaling sets up multiple loops feeding back to maintain an elevated synapse-specific expression of their own components as well as of AChRs in electrically active muscle fibers.

Materials and Methods

Experiments were carried out on male Wistar rats (120–160 g) or on *erbB2 fl/fl* mice anesthetized with Nembutal. *ErbB2fl/fl* mice carried an allele of the *erbB2* gene where the first coding exon was flanked by two loxP sites (M.L., unpublished work) and was excised selectively in muscle fibers by the expression of Cre recombinase on injection of a Cre expression vector, pCA-13-Cre (U.M., unpublished work), leading to a null allele of the *erbB2* gene (17).

Expression plasmids pcagrin7A4B8 (18), pneuneuTMuSK, or pMuSKneuTMuSK (13) were injected intracellularly into single muscle fibers of innervated rat soleus muscle fibers, as described (12). The latter are constitutively active MuSK receptors composed of extracellular and transmembrane domains of neuT, an oncogenic variant of rat ErbB2, and of intracellular MuSK kinase domain or intra- and extracellular MuSK domains linked by transmembrane domain of neuT, respectively. Alternatively, 70 μl of 2 μM cAgrin7A4B8 was injected into the proximal endplate-free region of the soleus muscle.

Mouse soleus muscle fibers were injected with pHER4, pHER4KM (19), and/or pCA-13-Cre and/or pnlsGFP (GFP, green fluorescent protein) (13) (each at 100 ng/μl). In addition, rat NtAagrinX12Y4Z19 placed under the control of a doxycycline-dependent expression system was injected as described (20). In some animals, the soleus nerve was crushed with fine forceps near its entry into the muscle. Muscles were examined 3–5 weeks later. Cre-mediated recombination in *erbB2fl/fl* mice was tested by PCR amplification, by using primers annealing upstream and downstream of loxP sites.

***In Situ* Hybridization.** Synaptic or ectopic AChR clusters were localized by staining with Texas red-α-bungarotoxin (Molecular Probes) in 14-μm-thick frozen sections and hybridized with ^{35}S-labeled cRNA according to ref. 21 with few modifications. Briefly, paraformaldehyde (4%)-fixed sections were treated with proteinase K (20 μg/ml in 50 mM Tris·HCl/5 mM EDTA) for 8.5 min at room temperature, incubated for 10 minutes in 2.5% acetic anhydride in 0.1 M triethanolamine (pH 7.5), dehydrated through graded ethanol, and prehybridized for 3 h in hybridization buffer at 50°C. They were then hybridized overnight at 50°C with cRNA probes containing ^{35}S-labeled CTP and UTP (Amersham Pharmacia) not crossreacting with products derived from injected plasmids (see below), by using riboprobe concentrations between 40,000 and 180,000 cpm μl^{-1}. After washing with sodium-Tris-EDTA (NTE) buffer, sections were treated with RNase A (20 μg/ml) for 45 min at 37°C and subsequently washed with increasing stringency [NTE and 2 × sodium-sodium citrate (SSC) buffer at 37°C/0.1 × SSC at 50°C]. After ethanol dehy-

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Abbreviations: AChR, acetylcholine receptor; NRG-1, neuregulin-1; GFP, green fluorescent protein.

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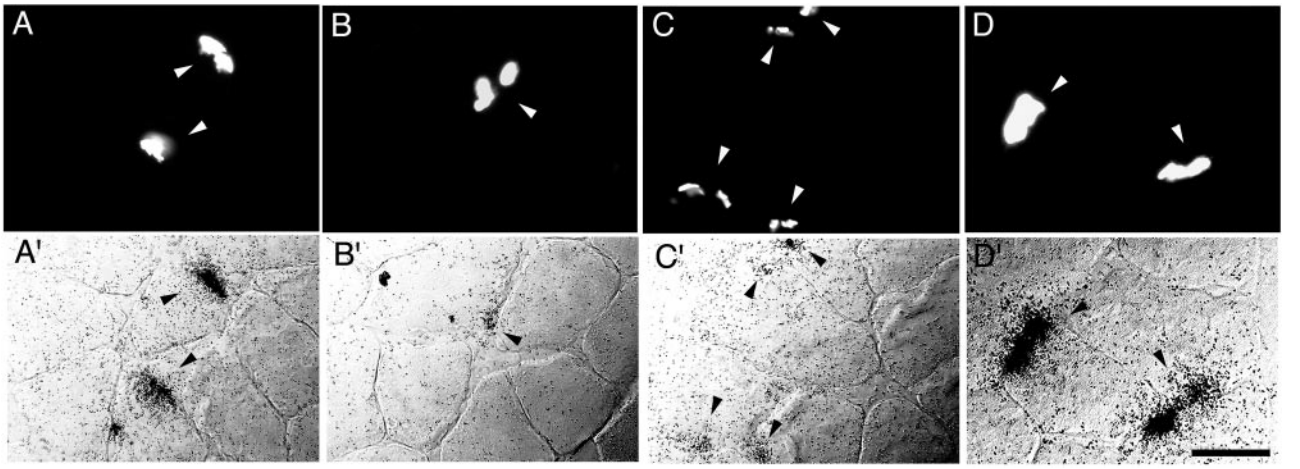

Fig. 1. Subsynaptic localization of mRNA expression for MuSK (*A*, *A'*), ErbB2 (*B*, *B'*), ErbB3 (*C*, *C'*) and AChR ε-subunit (*D*, *D'*) at the rat soleus neuromuscular junction. Synapses are marked by staining AChR clusters. (Bar = 30 μm.) Frozen cross sections (14 μm) of rat soleus muscle were stained with Texas red-α-bungarotoxin (Molecular Probes) and processed for *in situ* hybridization by using ^{35}S-labeled cRNA probes derived from cDNA clones encoding the extracellular domain of MuSK, intracellular domains of neu/ErbB2 and ErbB3, respectively, full length AChR ε-subunit, and a protocol modified from ref. 21.

dration, they were dipped in Kodak NTB2 emulsion and exposed for 5–7 weeks at 4°C. For quantification of the hybridization signals, nuclei were stained with hematoxylin. AChR clusters lacking muscle nuclei were excluded from the analysis. In the remaining fiber profiles, the numbers of silver grains located above the hematoxylin stain denoting the nuclei colocalized with an AChR cluster were counted at 630× and compared with those above other nuclei in the same and in neighboring fiber profiles and in background.

Hybridization Probes. The cRNA probes used were derived from full length cDNA encoding rat AChR ε-subunit and ErbB2 or from cloned cDNA fragments encoding the extracellular domain of rat MuSK (Met-1 to Ser-435) and the intracellular domains of ErbB2 (Leu-1103 to Val-1260) and ErbB3 (Met-1197 to Tyr-1325), respectively.

Quantitative Analysis of mRNA by Reverse Transcription–PCR. Recombinant heregulin β1 (NeoMarkers, Fremont CA) was added for 48 h at 1 nM concentration to Sol8 myotubes after 3 days of differentiation. Total RNA was extracted by using the Qiagen RNeasy RNA extraction kit (Qiagen AG, Basel). First-strand cDNA synthesis and TaqMan quantitative PCR were performed according to ref. 13, where primers and fluorescent probes for AChR ε-subunit and β actin are described. Primers for ErbB2 cDNA were 5′-ggcagtggattggctctgat-3′ (forward) and 5′-ggtcccaaggtacagtgtgtacaa-3′ (reverse) and flanked the TaqMan oligonucleotide probe 5′-(FAM) caccgcaacgcccatctctgc (TAMRA)-3′. Primers for mouse muscle creatine kinase (MCK) cDNA were 5′-cgagactggcccgatgc-3′ (forward) and 5′-tcacccacacaaggaagcttt-3′ (reverse) and flanked the TaqMan oligonucleotide probe 5′-(FAM) cgtggcatctggcacaacgacaac (TAMRA)-3′. For mouse GADPH cDNA, primers and TaqMan oligonucleotide probes were 5′-ggcatggccttccgtgt (forward), 5′-ggtttctccaggcggca-3′ (reverse), and 5′-(FAM)cctacccccaatgtgtc-cgtcgtg (TAMRA)-3′, respectively. The levels of mRNAs encoding AChR ε-subunit, ErbB2, MCK, and GAPDH were normalized relative to β-actin mRNA as described (13).

Results

Synaptic and MuSK-Induced Accumulations of AChR, MuSK, and ErbB mRNAs. We first examined whether ErbB2 and ErbB3 mRNAs, like that of MuSK (4), accumulate specifically at neuromuscular junctions, indicative of local synthesis induced by the motor neuron. *In situ* hybridization (ISH) of cross sections of adult rat soleus muscle fibers, by using ^{35}S-labeled cRNA probes followed by autoradiography, revealed an accumulation of ErbB2, ErbB3, and MuSK mRNAs associated with synapses (Fig. 1), consistent with nerve-induced transcription of their respective genes. Because neural agrin is a major organizer of synaptic differentiation, we next asked whether agrin can induce the expression of its own receptor MuSK. Nonsynaptic regions of rat soleus muscle fibers were injected with an expression vector for a neural isoform of chicken agrin, pcagrin7A4B8 (18), leading to the local secretion of agrin from the injected fibers and the formation of postsynaptic-like membranes within days (12). The injected fibers remained innervated and thus electrically active during the entire experiment via their original synapses to keep nonsynaptic expression of MuSK and AChRs down-regulated. Indeed, ISH revealed accumulations of MuSK mRNA at the ectopic AChR clusters (Fig. 2*A*), indicating the local induction of *musk* gene by agrin in electrically active fibers, as it is observed at normal synapses. We next asked whether this involves activation of MuSK. Therefore, we overexpressed by expression plasmid injection a constitutively active MuSK receptor, neuneuTMuSK (13), instead of agrin in mature muscle fibers. Expression of endogenous MuSK was analyzed with an ISH probe not cross-reacting with the MuSK mRNA expressed from the injected plasmid. Constitutively active MuSK induced the local expression of both endogenous MuSK (Fig. 2*B'*) and of AChR ε-subunit mRNA (Fig. 2*E'*) and has previously been shown to induce the accumulation of ErbB2 and ErbB3 proteins at ectopic AChR clusters (13). We now observed that this accumulation was also accompanied by local synthesis of ErbB2 and ErbB3 mRNAs (Fig. 2 *C'* and *D'*). Quantification by counting autoradiographic silver grains (Fig. 2 *E* and *F*) showed that mRNAs were restricted to nuclei associated with ectopic AChR clusters. The specific increase in grain numbers per "synaptic" nucleus was pronounced relative to that in "nonsynaptic" nuclei where grain number was indistinguishable from background. In summary, activated MuSK induces the local synthesis and accumulation of AChR ε-subunit, MuSK, ErbB2, and ErbB3 mRNAs and protein and, as shown previously (13), of NRG-1.

ErbB2 mRNA Expression Is Stimulated by NRG-1 in Cultured Myotubes. Like AChR mRNA, MuSK mRNA is up-regulated in cultured myotubes exposed to NRG-1, the ligand of ErbBs (16), consistent with a common MuSK-dependent pathway regulating the

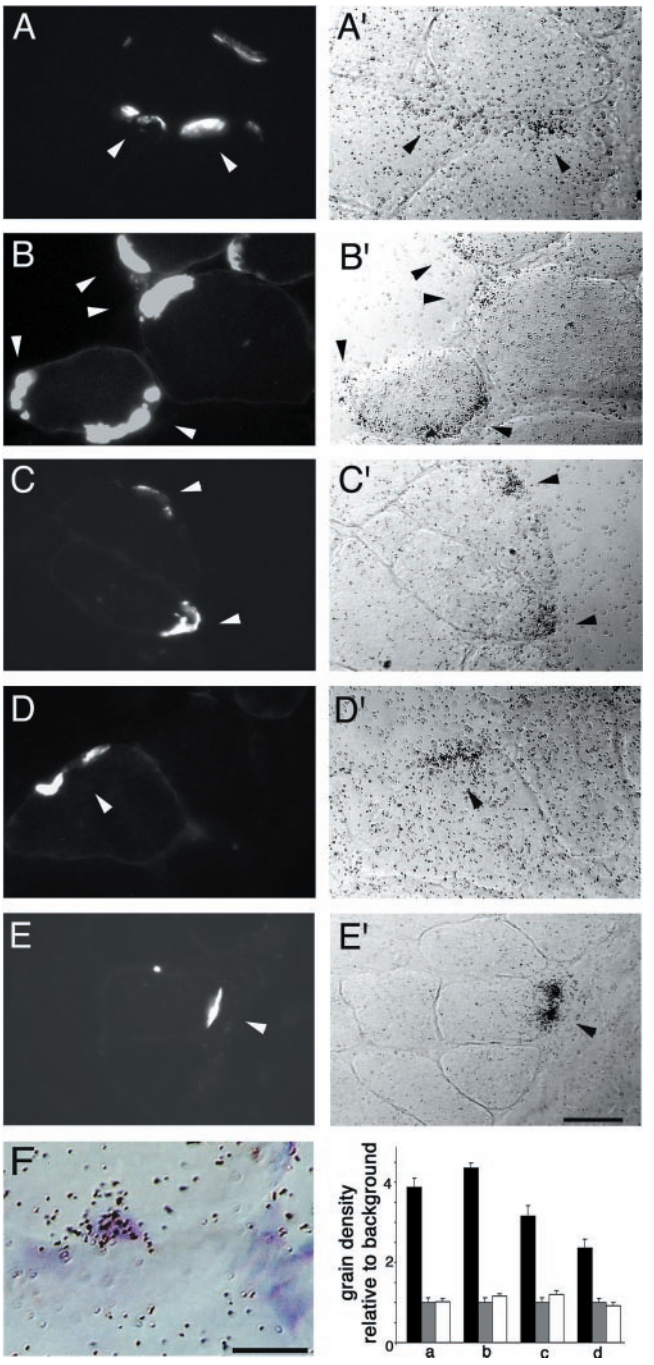

Fig. 2. Colocalization of ectopic AChR clusters induced by neural agrin and neuT/MuSK chimeras, respectively, with mRNAs for MuSK, ErbB2, ErbB3, and AChR ε-subunit; (Bar = 30 μm.) AChR clusters were induced by cagrin7A4B8 (*A*), neuneuTMuSK (*B*, *D*, and *E*), and MuSKneuTMuSK (*C*), respectively. cRNA probes used were specific for extracellular domain of MuSK mRNA (*A*′, *B*′), ErbB3 mRNA (*D*′), and full length ErbB2 and AChR ε-subunit mRNAs (*C*′, *E*′), respectively. (*F*) Example of nuclear accumulation of grains (ErbB2 mRNA) (Bar = 10 μm) and histogram of grain numbers ($\pm$SE) per nucleus at AChR clusters (black columns) relative to that at adjacent nuclei (gray columns); white columns represent grain numbers per nuclear profile area of background. Note that grain numbers at nuclei not associated with AChR clusters and background are similar, indicating absence of specific expression at these nuclei. a–d refer to experiments illustrated in *A*–*D*. Grain numbers per nucleus associated with AChR clusters are elevated significantly compared with grain numbers at adjacent muscle nuclei (Student's *t* test; $P < 0.001$ to 0.05, $n = 11$ to 39 in the experiments described).

activation of the *AChR* and *musk* genes. To test whether the induction of ErbB2 expression by activated MuSK could also be regulated via NRG-1/ErbB, we examined whether ErbB2 mRNA was increased by NRG-1 in Sol8 muscle cells. Extraction of mRNA followed by reverse transcription and real-time PCR of cDNAs showed that ErbB2 mRNA was increased significantly [1.93 $\pm$ 0.38-fold, standard error (SE), $n = 3$] in myotubes treated with 1 nM NRG-1. In the same cultures, NRG-1 induced an increase in AChR ε-subunit mRNA by 17.3 $\pm$ 7.8-fold, whereas muscle creatinine kinase and GAPDH mRNAs were unchanged (1.06 $\pm$ 0.08 and 1.02 $\pm$ 0.09, respectively; SE, $n = 3$), indicating that stimulation of *erbB2* gene by NRG-1 was specific. Thus, the data suggest that the agrin/MuSK pathway leads to the aggregation of NRG-1 and ErbB receptors in the subsynaptic membrane, organizing a secondary NRG-1/ErbB signaling pathway that in turn stimulates the expression of *musk*, *erbB2*, and *AChR* genes.

Agrin-Induced AChR Expression Is Blocked by Interfering with NRG/ErbB Signaling. The mechanism proposed above depends on whether NRG-1 and ErbB aggregated ectopically by agrin/MuSK interact to induce expression of *AChR*, *musk*, and *erbB* genes. For AChR expression, we have tested this *in vivo* by examining whether agrin-induced AChR clustering in innervated soleus muscle fibers was blocked by interfering with NRG/ErbB signaling. NRGs signal by stimulating heterodimerization of ErbB3 with ErbB2, ErbB4, or homodimerization of ErbB4 (22, 23). Because ErbB2 is the preferred heterodimer partner of the two other members (19, 24), we sought to block NRG/ErbB signaling by deleting the *erbB2* gene. The early lethality of generalized *erbB2* deletion (17) was circumvented by its selective deletion in individual muscle fibers. For this purpose, we injected a Cre expression vector, pCA-13-Cre, into soleus muscle fibers of *erbB2fl/fl* mice. Cre-induced excision of loxP-flanked DNA sequences in muscle fibers was ascertained in two ways: (*i*) in fibers of mice carrying a *lacZ* transgene that is only expressed on Cre-mediated recombination (25), Cre induced β-gal activity (Fig. 3*B*), and (*ii*) in DNA from *erbB2fl/fl* fibers injected with pCA-13-Cre, a fragment of predicted size of the mutated *erbB2* gene could be resolved by PCR (Fig. 3*C*). However, quantitative data on the extent of Cre-mediated excision in fibers injected with pCA-13-Cre could not be obtained, because isolated Cre-injected fibers yielded copy numbers of the gene too low for analysis. Next, innervated fibers of *erbB2fl/fl* mice were injected with pCA-13-Cre and a combination of expression vectors placing the expression of rat neural agrin under the control of doxycycline (Dox) (Fig. 3*A*; ref. 27). In this way, agrin expression could be delayed after the injection by delaying Dox administration by 14 days to allow muscle fibers to recover from injury caused by the injection (20). pnls-GFP (13) was also coinjected to mark injected fibers. Three weeks after Dox administration, GFP-positive fibers were examined for the presence of agrin-induced AChR clusters. Of the *erbB2fl/fl* fibers injected with pCA-13-Cre, about 20% showed AChR clusters. In contrast, 45% of the fibers injected with pCMV (CMV, cytomegalovirus) control vector revealed clusters of AChRs (Fig. 3 *D*–*F*). This reduction in agrin-induced AChR cluster formation in *erbB2*$-/-$ fibers reflects inhibition of *AChR* gene transcription, because in innervated fibers, basal levels of extrasynaptic AChRs are insufficient for agrin to allow the formation of AChR clusters by posttranslational mechanisms alone. Specifically, whereas exposure of 4-day-predenervated soleus muscles *in vivo* to neural agrin (1 μM for 2 h; ref. 28) induces ectopic AChR clusters within 1 day only, AChR clusters do not appear in innervated fibers until 3 days after agrin exposure, when AChR ε-subunit mRNA expression in agrin-injected muscle was significantly up-regulated (5.0 $\pm$ 0.42-fold, $n = 3$). Considering that a minority only of muscle nuclei

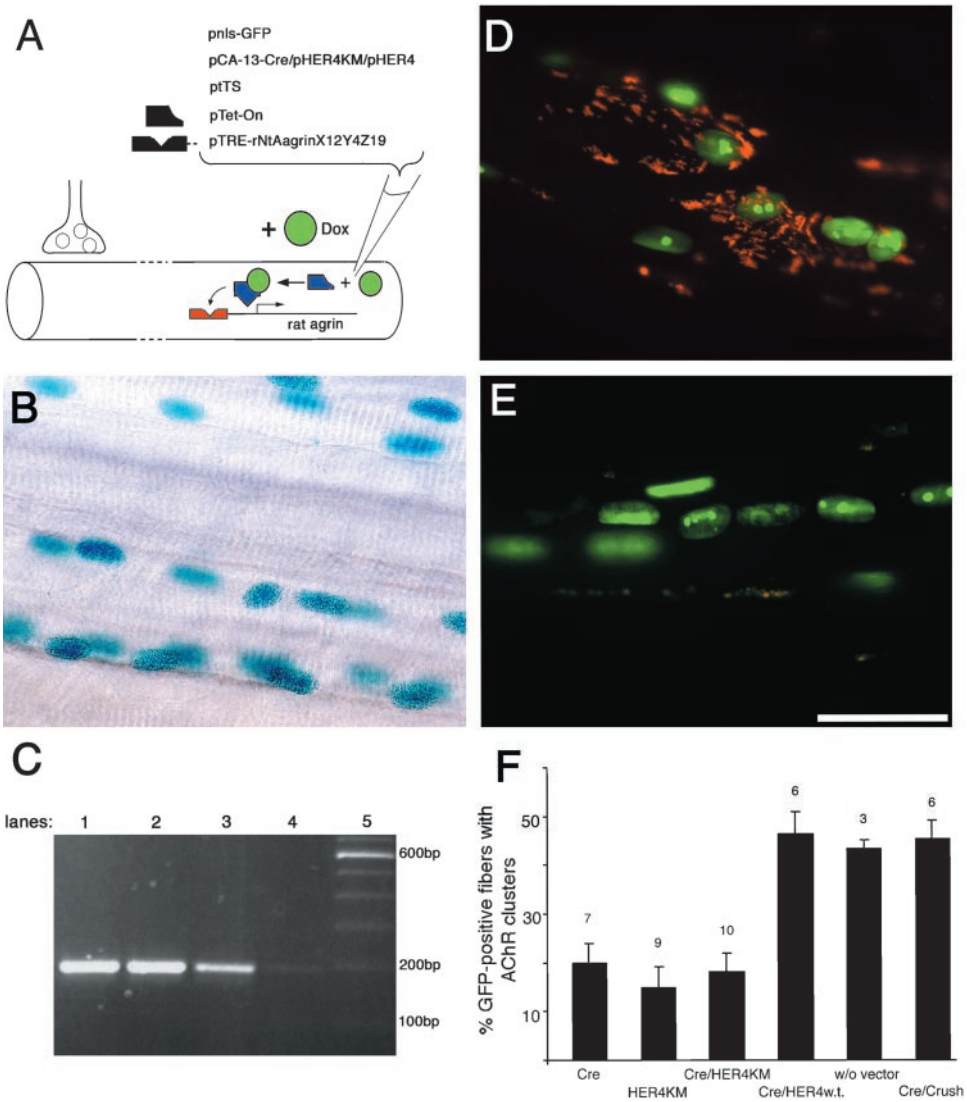

Fig. 3. Blockade of NRG-1/ErbB signaling in muscle fibers inhibits ectopic AChR synthesis but not clustering by rat neural agrin. (*A*) Schematic illustration of plasmid injection protocol. *erbB2fl/fl* muscle fibers were injected with expression vectors for nls-GFP and for rat agrin (NtAragrinX12Y4Z19, red) placed under the control of doxycycline (green) administration as described in ref. 20, as well as for HER4, HER4KM, and/or Cre recombinase (each at 100 ng/μl). Two weeks later, doxycycline (2 mg/ml) was added to the drinking water, and 5 weeks later, GFP-positive fibers were examined for ectopic AChR clusters at 630×. (*B*) Muscle fibers carrying a *lacZ* transgene that is expressed only on Cre-mediated recombination (25) express β-galactosidase on injection of Cre-expression vector. (*C*) Injection of Cre-expression vector into *erbB2fl/fl* fibers causes excision of floxed DNA fragment containing exon 1 of *erbB2*. The Cre-mediated recombination allows amplification by PCR of a DNA fragment of predicted size of 170 bp (lanes 1 and 2). Lane 3, PCR product amplified from recombined DNA from brain of *erbB2fl/fl* mice expressing Cre driven by the brain-specific nestin promoter (26). Lane 4, DNA from Cre-free *erbB2fl/fl* muscle is not amplified because of large size of fragment (approximately 2 kb) flanked by loxP. Size markers in lane 5. (*D* and *E*) Examples of *erbB2fl/fl* fibers injected according to *A*, in the absence (*D*) or presence (*E*) of pCA-13-Cre. Cre inhibits formation of ectopic postsynaptic membranes by agrin. (*F*) Mean percentages (±SE) of GFP-positive fibers exhibiting AChR clusters from muscles treated as indicated. The numbers of muscles analyzed and respective treatments are indicated above and below the columns, respectively. Only muscles containing ≥10 GFP-positive fibers are included. Note that inhibitions produced by deletion of *erbB2* and by overexpression of HER4KM are similar in magnitude but are not additive, and that inhibition by *erbB2* deletion is rescued by overexpression of HER4 wild type or crushing the nerve. Inhibition of AChR clustering by deletion of *erbB2* was increased from 2- to 4-fold when the amount of pTRE-rNtAagrinX12Y4Z19 injected was reduced.

colocalized with AChR clusters are activated (12), the up-regulation in AChR mRNA expressed per activated nucleus was much higher. Moreover, inhibition of agrin-induced *AChR* gene transcription in innervated soleus muscle by overexpression of an inactive mutant of the transcription factor GABP inhibits agrin-induced ectopic AChR cluster formation (29) and interfering with NRG/ErbB signaling in cultured myotubes inhibits agrin-induced *AChR* gene transcription (30). These observations combined strongly argue that the decrease in agrin-induced AChR cluster formation observed in *erbB2−/−* fibers originated not from inhibition of AChR aggregation but rather from the inhibition of the agrin-induced *AChR* gene transcription. Accordingly, when *erbB2−/−* muscles were transiently denervated by nerve crush at the time of plasmid injection, leading to extrasynaptic AChR synthesis independently of NRG/ErbB signaling, the induction of AChR clusters by agrin was not affected by the injection of pCA-13-Cre (Fig. 3*F*).

The residual ectopic AChR clusters in *erbB2−/−* fibers could be caused by several factors. For example, NRG-1 signaling may be mediated by ErbB4 receptors that become also localized at ectopic agrin-induced AChR clusters (not shown). Indeed, deletion of *erbB2* does not abolish nerve-induced *AChR* gene

transcription in fetal muscle (31), perhaps because of signaling via high levels of ErbB4 at fetal neuromuscular junctions (11). To test this possibility, we inhibited ErbB4 by overexpression of HER4KM, a kinase-inactive mutant of HER4, the human orthologue of ErbB4 (19). Coinjection of pHER4KM, independently of whether *erbB2* was deleted, produced an inhibition of agrin-induced AChR clustering similar to deletion of *erbB2* alone. Conversely, expressing high levels of human ErbB4 by coinjection of pHER4 rescued the inhibition on deletion of *erbB2*. These data are summarized in Fig. 3*F*; they suggest that the residual formation of ectopic AChR clusters in muscle fibers lacking ErbB2 was not solely because of signaling via ErbB4, but that overexpression of ErbB4 can compensate for *erbB2* deletion. Another possibility is that leakiness of the Dox-dependent expression system (27), leading to agrin expression before inactivation of *erbB2*, also contributed to residual AChR clustering. This was suggested by a decrease in the number of fibers with AChR clusters in *erbB2*−/− muscle but not in control fibers, when the amount of the injected Dox-inducible agrin expression vector was reduced (data not shown). Finally, as we were unable to quantitate the extent of ErbB2 loss in fibers injected with Cre, the residual ACLR clusters in these fibers may be due to incomplete loss of ErbB2. In summary, agrin/MuSK can induce AChR expression *in vivo* via NRG/ErbB signaling, but ErbB-independent signaling of agrin/MuSK to the nuclei cannot be excluded at this time.

Discussion

Previous attempts to resolve synapse-specific expression of ErbB3 mRNA have failed (32), probably because of insufficient resolution of the methods used. The present experiments show, to our knowledge for the first time, that not only *AChR* genes but also signaling components regulating their activity, i.e., *erbB2* and *erbB3*, are expressed specifically at the neuromuscular junction and at ectopic postsynaptic-like membranes induced by constitutively active MuSK.

We further show that agrin/MuSK induce the expression of *musk* gene, implying that synapse-specific MuSK expression is regulated in a positive feed-back loop activated by MuSK. This loop may involve the activation of ErbBs because, (*i*) MuSK mRNA expression in cultured myotubes is stimulated by NRG-1 (16), and (*ii*) genomic DNA contains in the vicinity of the first exon of the *musk* gene an N-box, which is thought to mediate synapse-specific and NRG-1-dependent activation of AChR subunit genes (33–35). Indeed, activation of MuSK promoter fragments in cultured myotubes by agrin depend on the presence of this N-box (E. Lacazette and H.R.B., unpublished work).

Our experiments further show that in cultured myotubes, *erbB2* gene expression is stimulated by NRG-1, as are *AChR* and *musk* genes (10, 16), consistent with the notion that synapse-specific expression of AChRs, MuSK, and ErbBs may be regulated via a common pathway involving ErbB activation. Finally, interfering with NRG/ErbB signaling by deletion of *erbB2* or by overexpression of an inactive mutant of ErbB4, HER4KM, inhibits ectopic agrin-induced AChR cluster formation in innervated muscle fibers. This inhibition reflects inhibition of *AChR* gene transcription rather than of AChR aggregation via post-translational mechanisms alone. We propose, therefore, that agrin regulates *AChR* gene expression via the MuSK-induced assembly of an NRG/ErbB receptor pathway, i.e., that NRG and ErbBs accumulated by agrin interact to induce the transcription of *AChR* and perhaps of *musk* and *erbB2* genes. A model for agrin-regulated expression of subsynaptic gene expression genes is presented in Fig. 4.

It has been shown recently that fetal muscle fibers can express AChR clusters colocalized with sites of elevated *AChR* gene expression before they are innervated (5–7). The formation of these early synaptic sites requires MuSK but is independent of the presence of the nerve or of agrin, suggesting that initial stages of synaptogenesis may be determined by muscle-intrinsic mechanisms (5–7). Their formation may be supported by the high levels of MuSK, ErbBs, NRG, and AChRs constitutively expressed in fetal muscle. Later in development, however, the establishment of stable neuromuscular synapses requires the stable interaction of nerve-derived agrin with MuSK, whereas AChR clusters remaining nerve-free are dispersed (6, 7), perhaps by down-regulation of MuSK and AChRs, by spontaneous or nerve-induced electrical muscle activity. Thus, the most important aspect of synapse stabilization involves the local expression of the signaling components regulating synaptic MuSK and AChR synthesis and aggregation in a manner resistant to electrical muscle activity, i.e., MuSK, ErbBs, and NRG-1. The model proposed in Fig. 4 offers a mechanism for how this stabilization may be regulated. The primary role of agrin/MuSK would be to cluster preexisting ErbBs and NRG to a MuSK-based scaffold (36), as is observed at ectopic AChR clusters induced by agrin or by constitutively active MuSK, rather than to act directly on subsynaptic muscle nuclei. As a result, ErbB receptors are activated to induce locally the expression of *musk*, *erbB*, and *AChR* subunit genes in a manner resistant to electrical muscle activity. The feedback loops are closed by the ability of the MuSK-induced subsynaptic apparatus also to cluster locally synthetized MuSK (13). The involvement of other pathways cannot be excluded, however.

According to Fig. 4, synaptic expression of ErbBs depends on MuSK activation, and synaptic MuSK expression depends on ErbB activation. Therefore, MuSK, NRG, and ErbBs must be expressed constitutively at a basal level for the feedback loops (Fig. 4) to be initiated by agrin. Indeed, MuSK (4, 20), ErbBs (11), and an NRG-1 isoform thought to be involved in synaptic AChR expression (30) can be resolved in nonsynaptic regions of both fetal and adult muscle. Unlike in fetal muscle, where protosynapses form in the absence of the nerve, the basal extrasynaptic expression of MuSK, NRG, and ErbBs in the adult is not sufficient to induce ectopic postsynaptic membranes in the absence of agrin. However, when agrin is present, the coupling

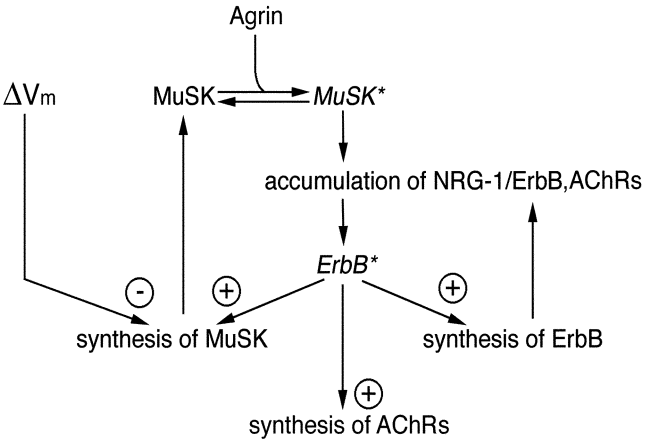

Fig. 4. Model for agrin-induced stabilization of postsynaptic membrane after onset of electrical activity in the muscle fiber. * denotes activated forms of respective kinases. MuSK is locally stabilized by agrin in its active conformation, thus aggregating components of the subsynaptic apparatus including preexisting NRG-1 and ErbBs to a MuSK-dependent scaffold. Thereby, ErbBs are activated to induce the local expression of *AChR*, *musk*, and *erbB* genes and perhaps *nrg-1*. Activation of ErbBs by up-regulation of their synthesis alone is suggested by the increase in AChR ε-subunit expression in cultured myotubes on overexpression of HER2, the human orthologue of ErbB2 (30). In nonsynaptic fiber regions, MuSK expression is down-regulated by electrical muscle activity (ΔVm).

of stable MuSK activation to MuSK synthesis appears to be sufficiently strong to enable ectopic agrin to induce an ectopic postsynaptic-like membrane in spite of the low levels of MuSK initially present in electrically active muscle (4).

The establishment of a functional secondary NRG/ErbB signaling loop by agrin/MuSK to induce synaptic gene expression is demonstrated by the inhibition of ectopic agrin-induced AChR cluster formation on injection of pCre and/or pHER4KM into *erb2fl/fl* fibers. Although the present finding that these inhibitions are incomplete and do not add may reflect the involvement of pathways other than NRG/ErbB, other explanations are also possible. For example, Cre may not be expressed at sufficient levels to block NRG/ErbB in all fibers, as is the case for the expression of GFP, which can be observed in about 60% only of fibers injected with its expression plasmid (unpublished observation). Nevertheless, the present data show that synaptic expression of *AChR* genes in muscle *in vivo* can occur by activating ErbBs but independently of nerve-derived NRG-1, consistent with elevated *AChR* gene expression levels in presumptive innervation regions at early aneural stages of normal synaptic development (5–7).

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