Neuregulin Inhibits Acetylcholine Receptor Aggregation in Myotubes*

The high local concentration of acetylcholine receptors (AChRs) at the vertebrate neuromuscular junction results from their aggregation by the agrin/MuSK signaling pathway and their synthetic up-regulation by the neuregulin/ErbB pathway. Here, we show a novel role for the neuregulin/ErbB pathway, the inhibition of AChR aggregation on the muscle surface. Treatment of C2C12 myotubes with the neuregulin epidermal growth factor domain decreased the number of both spontaneous and agrin-induced AChR clusters, in part by increasing the rate of cluster disassembly. Upon cluster disassembly, AChRs were internalized into caveolae (as identified by caveolin-3). Time-lapse microscopy revealed that individual AChR clusters fragmented into puncta, and application of neuregulin accelerated the rate at which AChR clusters decreased in area without affecting the density of AChRs remaining in individual clusters (as measured by the fluorescence intensity/unit area). We propose that this novel action of neuregulin regulates synaptic competition at the developing neuromuscular junction.

The mammalian neuromuscular junction (NMJ) is a cholinergic synapse composed of a muscle fiber, an axon terminal (located in a depression on the muscle surface known as the primary gutter), and a terminal Schwann cell. Within the postsynaptic membrane of the primary gutter, AChRs are concentrated at 10,000/μm². This density is 1000-fold higher than in extrajunctional regions of muscle. Two protein signaling pathways contribute to the enrichment of acetylcholine receptors (AChRs) at the NMJ. The first involves the release of agrin from the axon terminal, which binds to the receptor tyrosine kinase MuSK on the muscle surface, and ultimately results in clustering of AChRs at the NMJ (reviewed in Ref. 2). The second pathway involves neuregulin, which is released from the axon terminal and activates ErbB receptor tyrosine kinases on the muscle surface, resulting in the up-regulation of AChR mRNA synthesis by subsynaptic myonuclei (reviewed in Refs. 3 and 4). Alternative splicing results in at least 14 variants of the neuregulin-1 gene (hereafter referred to as neuregulin), which have been grouped into three major protein isoforms (types I–III). An extracellular epidermal growth factor (EGF)-like domain is common to all variants and is sufficient to activate ErbB receptors (5). The variants can be distinguished in part by the presence of either an amino-terminal Ig-like domain or a cysteine-rich domain. Different splice variants are expressed in many organs, including motor neurons and skeletal muscle (6). Most isoforms also contain a transmembrane domain and an intracellular carboxyl terminus. Upon cleavage of the transmembrane domain, neuregulin is incorporated into the extracellular matrix (7, 8).

During the early postnatal period in mammalian muscle, synaptic competition occurs between the multiple axons that initially innervate individual muscle fibers, with most muscle fibers become singly innervated by the second postnatal week (9). Synaptic competition likely involves a factor that destabilizes the nascent postsynaptic complex at soon to be eliminated synapses. Before the losing nerve terminal retracts from the muscle surface, the densities of AChR, utrophin, and rapsyn all decline immediately beneath the terminal (10–12). This destabilizing factor is either directly released from competing nerve terminals or generated in the muscle in response to activity at the competing terminal.

TrkB receptors are found postsynaptically at the NMJ. In myotube cultures, neurotrophin activation of TrkB receptors leads to inhibition of agrin-induced AChR clustering (13). However, inhibition of TrkB signaling both in myotube culture and in vivo has also been shown to disrupt AChR clustering (14); and therefore, the role of neurotrophin signaling in agrin-induced AChR clustering remains uncertain.

The receptor tyrosine kinases MuSK and TrkB are both able to regulate AChR aggregation. We therefore examined the ability of ErbB receptors to modulate AChR distribution in C2C12 myotubes. The mouse muscle cell line C2 and its subclone C2C12 have been used extensively to characterize AChR clustering on the surface of muscle cells (15–18). We demonstrate that the EGF domain of neuregulin-β1 is able to inhibit both spontaneous and agrin-induced AChR aggregation. This inhibition results in part from a decreased stability of individual AChR clusters.

EXPERIMENTAL PROCEDURES

Reagents—The mouse muscle cell line C2C12 was obtained from American Type Culture Collection (Manassas, VA). Assistant brand coverslips (12 and 22 mm, No. 1) were obtained from Carolina Biological Supply Co. (Burlington, NC), Alexa 594-α-bungarotoxin (α-Btx), Alexa 488-α-Btx, biotin-α-Btx, Alexa 594-streptavidin, Alexa 488-streptavidin, Alexa 594-conjugated goat anti-mouse, Alexa 594-conjugated goat anti-rabbit, and Alexa 647-conjugated goat anti-mouse immunoglobulins were obtained from Molecular Probes, Inc. (Eugene, OR). Rat tail collagen was obtained from BD Biosciences. All other tissue culture reagents were obtained from Invitrogen. Recombinant rat neural agrin (amino acids 1153–1948, including the three laminin G-like domains) and neuregulin (the EGF domain of human neuregulin-β1, amino acids

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The abbreviations used are: NMJ, neuromuscular junction; AChR, acetylcholine receptor; MuSK, muscle-specific kinase; EGF, epidermal growth factor; α-Btx, α-bungarotoxin; PBS, phosphate-buffered saline.
Assaying the Effect of Neuregulin on AChR Cluster Number—To assess the effect of neuregulin on the number of spontaneous AChR clusters, myotubes were treated for 12 h with 2 nM neuregulin, and the number of AChR clusters was then compared with that in untreated myotubes. To assess the effect of neuregulin on agrin-induced AChR clustering, myotubes were treated for 12 h with 100 pM agrin, and then the agrin was removed and replaced with either fresh differentiation medium or medium containing 2 nM neuregulin. The cells were incubated for 23 h. At the start of each experiment, the coverslips were coded to identify their specific treatment. The clusters in a visual field under each condition were then counted in a blind fashion using a microscope (Nikon Eclipse E800 equipped with a Micromax digital camera, and Metamorph software was used for computer control of the stage, shutter, and camera. Individual clusters were located by visualization through the eyepiece, and their locations were entered into the cluster area. The numbers of AChR clusters were then compared with that in untreated myotubes.

Cell Culture—Coverslips were coated with a 75 μg/ml solution of collagen in 2% acetic acid, which was evaporated to dryness. The coverslips were then rinsed twice with phosphate-buffered saline (PBS). C2C12 cells were plated on ice, and 22 nM Alexa 594-streptavidin or Alexa 488-streptavidin (22 nM) not used initially. The second Alexa-streptavidin was added. The myotubes were incubated for 7 h at 37 °C to allow for internalization of the AChRs. The cells were then incubated at 37 °C for 2 min, spun down, and replate at 50% confluence onto coverslips. Half of the medium was replaced daily. Myotubes were typically used 2–4 days following replating.

Light Microscopy—Epifluorescence images were obtained using a Nikon Eclipse E800 equipped with a Nikon ×60 Plan Apo objective (1.40 numerical aperture). Alexa 488 fluorescence was filtered through a Nikon 96176 fluorescein isothiocyanate (FITC) excitation and emission filters and a polychromatic beam splitter (8600 filter set, Chroma Technology Corp., Brattleboro, VT). The XYZ stage was from Prior Scientific (Rockland, MA), and the filter wheel and shutter were controlled using a Lambda 10-2 controller (Sutter Instrument Co., Novato, CA). Images were acquired with a Micromax digital camera, and Metamorph software was used for computer control of the stage, shutter, and camera. Individual clusters were located by visualization through the eyepiece, and their locations were entered into the stage control of the software. Generally four to eight clusters were imaged per experiment. Images were acquired for 600 ms using a neutral density filter that reduced the excitation intensity by 75%. Images were typically acquired every 30 min.

Quantitation of AChR Cluster Disassembly—Image analysis and quantitation were performed using Metamorph and Microsoft Excel. For each time series, an AChR cluster was defined as those pixels whose brightness exceeded an intensity threshold that was set to include the pixels that extended to the apparent perimeter of the cluster. Each image in a time series had the same threshold. The area of the cluster (total pixels above the threshold) and the total fluorescence intensity of the cluster (sum of the intensities of the pixels above the threshold) were measured at each time point. The fluorescence intensity/unit area was determined by dividing the cluster total fluorescence by the cluster area.

RESULTS

AChR Responses to Agrin—We examined the distribution of AChRs in C2C12 myotubes in the absence and presence of agrin by confocal microscopy to confirm that agrin induced aggregation of AChRs in our system. In the absence of agrin, there were infrequent AChR clusters (which were defined as aggregation of AChRs with a length of at least 4 μm). Treatment for 12 h with 100 pM agrin led to the robust formation of AChR clusters (Fig. 1, A2 and B2). When measured in terms of AChR clusters/field, agrin increased the number of clusters by 5–10-fold, with a half-maximal response at an agrin concentration of ~40 pM (Fig. 1C), in agreement with previously reported values (20). For myotubes treated with a saturating level of agrin, the maximal number of AChR clusters/field ranged between 25 and 50 from experiment to experiment.

As an additional confirmation that our C2C12 myotubes were responding to agrin in a similar fashion to that observed in other laboratories, we examined the distribution of utrophin at agrin-induced AChR clusters. In addition to co-localizing with the AChR in the primary gutter at the NMJ, utrophin has been shown to co-localize with AChR at agrin-induced clusters (21, 22). We confirmed that utrophin was enriched at agrin-induced AChR clusters (Fig. 1A1).

Due to the role of caveolae in organizing receptor tyrosine kinase signaling pathways (for a review, see Ref. 23) and the importance of tyrosine kinase activity in AChR clustering (24, 25), we examined the distribution of caveolin-3 compared with agrin-induced AChR clusters (Fig. 1B). After a 12-h treatment with agrin, there were numerous AChR clusters, but caveolin-3 was not enriched with them. Although there was caveolin-3 staining near AChR clusters, the pattern of caveolin-3 immunofluorescence did not correlate with the distribution of AChRs.

Neuregulin Inhibition of AChR Clustering—We examined the distribution of AChRs on the surface of C2C12 myotubes after treatment with neuregulin in the absence and presence of agrin. In the absence of agrin, myotubes treated for 12 h with 2 nM neuregulin displayed ~75% fewer AChR clusters com-
Neuregulin Inhibition of AChR Aggregation

AChRs were stained as described above. The EC$_{50}$ for AChR cluster formation was $\sim$100 µM (Fig. 2B). This concentration dependence was similar to that seen for the equilibrium binding of the neuregulin EGF domain to ErbB2 receptors ($K_d$ $\sim$ 105 pM) in breast tumor cells (26) and for the induction of expression of AChR subunit reporter genes (EC$_{50}$ $\sim$200--300 pM) in mouse primary myotubes (27) and in C2C12 myotubes (28).

To determine whether neuregulin also affects the agrin-induced formation of AChR clusters, myotubes were incubated for 12 h with 80 pM agrin with or without 2 nM neuregulin. Under these conditions, there were 60% fewer clusters in the neuregulin-treated myotubes (Fig. 2C). In addition, myotubes were incubated for 12 h with 100 pM agrin; the agrin was then removed; and the cells were incubated for 23 h in medium with or without 2 nM neuregulin. In these cultures, the myotubes treated with neuregulin contained 85% fewer AChR clusters (Fig. 2D).

**Visualization of Surface and Internalized AChRs**—To qualitatively characterize AChR redistribution during cluster formation, maintenance, and disassembly, we examined the subcellular distribution of AChRs during these processes. We used a protocol to differentiate between AChRs at the surface and those that had been recently internalized. This protocol involved labeling AChRs on the surface of live myotubes with biotin-α-Btx. The myotubes were then incubated for 7 h, during which a portion of the AChRs underwent internalization. During these 7 h, the myotubes were either left untreated or treated with agrin or neuregulin. At the end of the 7-h incubation, the biotin-α-Btx-labeled AChRs remaining on the surface were labeled with a fluorescent streptavidin for 30 min at 4°C; the cells were permeabilized; and the internalized biotin-α-Btx-labeled AChRs were detected with a different fluorescent streptavidin.

**AChR Internalization during Cluster Formation**—We initially examined AChR redistribution in response to agrin treatment. AChRs were labeled with biotin-α-Btx, and then the myotubes were treated with 100 pM agrin for 7 h. Following the sequential streptavidin staining protocol, 90–95% of AChR clusters were stained only by the initial application of fluorescent streptavidin (Fig. 3A). The failure of the second application of streptavidin to further label these clusters served as a control demonstrating saturated binding of the surface biotin-α-Btx by the first application of streptavidin. Since the initial application of streptavidin completely bound all surface-accessible biotin-α-Btx, any additional staining by the second application of streptavidin corresponded to internalized biotin-α-Btx, which was not accessible prior to permeabilization of the membrane. At $\sim$5–10% of AChR clusters, such internalized biotin-α-Btx-labeled AChRs were stained by the second streptavidin (Fig. 3B). This internal staining of AChRs was punctate in nature and distinct from that of the surface AChRs (as visualized by the initial streptavidin application).

**AChR Internalization during Cluster Maintenance and Disassembly**—Since agrin is known to reduce the turnover rate of AChRs (29), it was surprising to observe endocytosed AChRs associated with AChR clusters following agrin treatment. To determine whether AChRs become internalized from AChR clusters after cluster formation, myotubes were treated for 12 h with 100 pM agrin, labeled for 30 min with biotin-α-Btx, and then incubated for an additional 7 h in the continued presence of 100 pM agrin. During the second incubation phase, new AChR cluster formation was greatly reduced since the total number of AChR clusters does not continue to increase with longer exposure to agrin (30). If agrin-induced clusters are stable in the continued presence of agrin, then there should be a decrease in internalization near AChR clusters. However,
Myotubes were incubated with 100 nM agrin for 12 h and then treated with 100 nM fresh agrin during the 7-h internalization phase. Myotube cultures were incubated with 120 nM AChRs during agrin-induced AChR cluster formation, maintained with 100 nM fresh agrin during the 7-h internalization phase. The myotubes were incubated with 22 nM Alexa 594-streptavidin to label biotin-H9251—Btx at 37 °C for 30 min to label surface AChRs. After the removal of the excess biotin-α-Btx, the myotubes were incubated for 7 h at 37 °C to allow AChR internalization and then incubated for 30 min at 4 °C with 22 nM Alexa 488-streptavidin to label biotin-α-Btx remaining at the surface. The excess Alexa 488-streptavidin was then washed away. After fixation and permeabilization, the myotubes were incubated with 22 nM Alexa 594-streptavidin to label internalized biotin-α-Btx. A and B, to examine AChR distribution during cluster formation, previously untreated myotubes were incubated with 100 ps agrin during the 7-h biotin-α-Btx internalization phase. C, to examine AChR distribution during cluster maintenance, myotubes were incubated with 100 ps agrin for 12 h and then treated with 100 ps fresh agrin during the 7-h internalization phase. D, to examine AChR distribution during cluster disassembly, myotubes were treated for 12 h with 100 ps agrin. The agrin was removed and replaced with medium during the 7-h biotin-α-Btx incubation period. The distribution of Alexa 488-streptavidin (A1, B1, C1, and D1) and Alexa 594-streptavidin (A2, B2, C2, and D2) was determined with fluorescein isothiocyanate and rhodamine optics, respectively. Overlays of the two images for each myotube with surface AChRs (cyan) and internalized AChRs (magenta) are shown (A3, B3, C3, and D3). Scale bar = 10 μm.

FIG. 3. Biotin-α-Btx visualization of surface and internalized AChRs during agrin-induced AChR cluster formation, maintenance and disassembly. Myotube cultures were incubated with 120 nM biotin-α-Btx at 37 °C for 30 min to label surface AChRs. After the removal of the excess biotin-α-Btx, the myotubes were incubated in medium for 7 h at 37 °C to allow AChR internalization and then incubated for 30 min at 4 °C with 22 nM Alexa 488-streptavidin to label biotin-α-Btx remaining at the surface. The excess Alexa 488-streptavidin was then washed away; and after fixation and permeabilization, the myotubes were incubated with 22 nM Alexa 594-streptavidin to label internalized biotin-α-Btx. At this time, the myotubes were stained with antibody against caveolin-3 (A3) or AP-2 (B3), followed by Alexa 647-conjugated goat anti-mouse immunoglobulins. The distribution of Alexa 488-streptavidin (A1 and B1) and Alexa 594-streptavidin (A2 and B2) as well as Alexa 647-conjugated anti-mouse immunoglobulins for caveolin-3 (A3) and AP-2 (B3) was visualized using a Zeiss LSM 510 confocal microscope. Internalized AChRs, which were found both in close proximity to surface AChRs (arrows) and at a distance from surface clusters (arrowheads), were associated with caveolin-3 (A), but not with AP-2 (B). Scale bar = 10 μm.

FIG. 4. Distribution of surface and internalized AChRs compared with caveolin-3 and AP-2. Myotube cultures were incubated with 100 ps agrin for 12 h and then treated with 120 nM biotin-α-Btx at 37 °C for 30 min to label surface AChRs. After the removal of the excess biotin-α-Btx, the myotubes were incubated in medium for 7 h at 37 °C to allow AChR internalization and then incubated for 30 min at 4 °C with 22 nM Alexa 488-streptavidin to label biotin-α-Btx remaining at the surface. The excess Alexa 488-streptavidin was then washed away; and after fixation and permeabilization, the myotubes were incubated with 22 nM Alexa 594-streptavidin to label internalized biotin-α-Btx. A and B, to examine AChR distribution during cluster formation, previously untreated myotubes were incubated with 100 ps agrin during the 7-h biotin-α-Btx internalization phase. C, to examine AChR distribution during cluster maintenance, myotubes were incubated with 100 ps agrin for 12 h and then treated with 100 ps fresh agrin during the 7-h internalization phase. D, to examine AChR distribution during cluster disassembly, myotubes were treated for 12 h with 100 ps agrin. The agrin was removed and replaced with medium during the 7-h biotin-α-Btx incubation period. The distribution of Alexa 488-streptavidin (A1, B1, C1, and D1) and Alexa 594-streptavidin (A2, B2, C2, and D2) was determined with fluorescein isothiocyanate and rhodamine optics, respectively. Overlays of the two images for each myotube with surface AChRs (cyan) and internalized AChRs (magenta) are shown (A3, B3, C3, and D3). Scale bar = 10 μm.

under these experimental conditions, we still observed punctate internalized AChR staining at ~5–10% of AChR clusters. The punctate aggregates of internalized AChRs were most often seen associated with surface AChR clusters (Fig. 3C). These results suggest that even in the continued presence of agrin, individual AChR clusters are dynamic structures, with some of their AChRs undergoing internalization. However, there were spontaneous AChR clusters on the myotube surface in the absence of agrin, with agrin increasing the number of clusters by 5–10-fold (Figs. 1C and 2). Our experiments did not determine whether the punctate aggregates of internalized AChRs seen in the presence of agrin are associated with a small fraction of the agrin-induced clusters or preferentially with the small number of spontaneously formed AChR clusters that may also persist after treatment with agrin.

We also examined the internalization of AChRs during AChR cluster disassembly following the removal of agrin. The internalization protocol was repeated using myotubes that were treated for 12 h with agrin, labeled with biotin-α-Btx, and then incubated for 7 h in medium lacking agrin. As a population, agrin-induced AChR clusters are known to disassemble upon agrin removal with a half-life of between 6 and 12 h (31). In a similar fashion to the previous experiments, punctate aggregated internalized AChRs were found near 5–10% of surface clusters (Fig. 3D). We cannot be sure that the clusters we observed with internalized AChR staining (such as in Fig. 3D) were actually undergoing disassembly. However, under the conditions of this assay, the majority of clusters were undergoing disassembly.

Endocytosis of AChRs into Caveolae—To characterize the cellular compartment containing the internalized AChRs, we compared the distribution of surface and internalized AChRs with that of caveolae (as identified by caveolin-3) and clathrin-coated pits (as identified by the clathrin-associated adaptor protein AP-2). Internalized AChRs were co-localized with caveolin-3 (Fig. 4A1–3), whereas there was no co-localization between internalized AChRs and AP-2 (Fig. 4B1–3).

Effect of Neuregulin on AChR Internalization: Live Cell Imaging of Cultured Myotubes—The results of the population studies revealed that neuregulin clearly reduced the number of spontaneous and agrin-induced AChR clusters. When we examined AChR internalization during AChR cluster formation and disassembly in the presence of neuregulin, internalized AChRs were seen distributed in a punctate pattern and associated with surface AChR clusters (data not shown). However, the studies provided no insights into the mechanism by which
this occurred, and the assay was not well suited for a quantitative comparison of the differences in AChR internalization under different experimental conditions. To better characterize the effects of neuregulin on clustered AChRs, we examined AChR cluster disassembly in living myotubes in the presence and absence of neuregulin.

**Characterization of AChR Cluster Disassembly**—We visualized agrin-induced AChR clusters for 8 h following the removal of agrin. Myotubes were grown on coverslips and treated for 12 h with 100 μM agrin. The AChRs were then labeled with Alexa 594-α-Btx; the agrin-containing medium was replaced with fresh medium; and each coverslip was attached to a heated chamber and placed on the stage of an inverted microscope. Fig. 5 shows the 0-, 2-, 5-, and 8-h time points for a typical AChR cluster imaged once every hour for 8 h.

The AChR clusters displayed a range of morphologies upon removal of agrin from the medium. During the 8-h time period, 64% of the clusters (23 of 36) fragmented into small puncta, as shown in Fig. 5A. In this cluster, small puncta become evident by 2 h after the removal of agrin. The AChR clusters that underwent fragmentation did so as early as 1 h or as late as 7 h after the removal of agrin, with a median time of 5 h. The puncta were similar in shape to those seen in the internalization experiments. Whereas the latter AChR puncta were endocytosed AChRs, the puncta in the live cell imaging experiments originated from AChRs that were on the myotube surface at the beginning of the imaging period and may or may not have subsequently been endocytosed. The remaining 36% (13 of 36) of the clusters became smaller in area, but did not fragment. Qualitatively, the clusters appeared to decrease in area and total fluorescence intensity. The same protocol was used to characterize the effects of neuregulin on cluster disassembly, with 5 nM neuregulin added to the medium immediately prior to the start of imaging. A total of 28 clusters were imaged from four separate experiments. The clusters had a greater tendency to fragment into puncta (82%, 23 of 28) in the presence of neuregulin.

**Quantitation of AChR Cluster Disassembly**—Cluster area, total fluorescence intensity, and fluorescence intensity/unit area were measured as a function of time as described under “Experimental Procedures.” An example of the threshold procedure is shown in Fig. 5 (A and A'), corresponding to a cluster at 0, 2, 5, and 8 h. The black regions in Fig. 5A' represent the pixels in the panels in Fig. 5A above the fluorescence threshold.

For myotubes not exposed to neuregulin, the average AChR cluster area decreased by 57% during the 8-h time period after the removal of agrin (Fig. 5B, open circles). Treatment with neuregulin accelerated the decrease in the area of AChR clusters. When myotubes were treated with 5 nM neuregulin, there was an 80% decrease in AChR cluster area during the 8-h time period (p < 0.002) (Fig. 5B, closed diamonds).

When myotubes were not exposed to neuregulin, the total fluorescence decreased by 74% during the 8-h time period after the removal of agrin (Fig. 5C, open circles). When myotubes disassembled in the presence of 5 nM neuregulin, the total fluorescence decreased at a faster rate. At the end of 8 h, the total fluorescence had decreased by 88% (p < 0.02) (Fig. 5C, closed diamonds). Whereas neuregulin treatment led to a decrease in both the cluster area and total number of AChRs at individual clusters, there was no effect on the density of AChRs remaining in the cluster (as measured by the fluorescence/unit area). Over 8 h, the fluorescence intensity/unit area decreased by 40% and 39% in myotubes not exposed to neuregulin (Fig. 5D, open circles) and in neuregulin-treated myotubes (closed diamonds), respectively.

**DISCUSSION**

In this study, we used immunofluorescence microscopy to characterize the effects of neuregulin, the ErbB receptor ligand, on the stability of spontaneous and agrin-induced AChR clusters. We used a recombinant neuregulin containing the EGF domain, which has been shown to activate ErbB receptors and to induce expression of AChR subunit genes in myotube cultures (5, 27, 28). We demonstrated that this neuregulin
Neuregulin Inhibition of AChR Clustering—Neuregulin has been studied extensively for its ability to promote the synthesis of AChRs at the NMJ (reviewed in Ref. 4). However, an additional inhibitory effect of neuregulin on AChR clustering is not inconsistent with the role of neuregulin in the formation and maintenance of the NMJ; and indeed, there are several possible developmental roles that this inhibitory effect of neuregulin may play. As measured by 125I-labeled a-Btx binding, neuregulin and the neuregulin EGF domain have been shown to increase by 2–3-fold the rate of insertion of AChRs onto the surface of cultured myotubes in the absence of agrin (32, 33). Our experiments show that neuregulin reduced the number of spontaneous or agrin-induced AChR clusters and increased the disassembly of AChR clusters. In non- agrin-treated myotubes, spontaneous AChR clusters were infrequent, and neuregulin might selectively increase their endocytosis while increasing total surface AChR levels due to an overall increase in receptor synthesis and insertion.

Several observations led to the hypothesis that there exist factors from either the nerve or muscle that destabilize clusters of AChR. First, spontaneous AChR clusters are very scarce extra- junctionally in innervated muscle, but become more common following denervation (34). Second, during synapse elimination at the developing NMJ, in the absence of synaptic takeover, the postsynaptic apparatus appears to be lost underneath the axon terminal prior to its retraction (10–12). AChRs remain stabilized when the vacated synaptic site is reoccupied by the remaining axon, whereas a rapid loss of AChRs occurs when synaptic sites are not reoccupied (35). The relative stability of the postsynaptic complex in denervated muscle is evidence for the existence of a nerve-mediated destabilizing factor (36, 37). However, if a denervated myofiber is re-innervated and if the original NMJ is only partially reoccupied by the axon terminal, then the unoccupied portions of the postsynaptic apparatus become disassembled (38, 39). The factor is able to destabilize AChRs in neighboring inactive regions of muscle, whereas if it is generated in active regions of muscle, it does not destabilize the AChRs there. Presumably, stabilizing factors, which act more locally, overcome the destabilizing ones.

In mice that lack neuregulin expression in neurons innervating the diaphragm, AChR transcription and clustering along the central band are normal (40). ErbB receptor signaling appears to be maintained by muscle-derived neuregulin; and therefore, changes in AChR clustering would not necessarily be expected.

Mice have been generated lacking ErbB2 expression in both muscle and nerve (41). Although AChR clusters exist in the central band and contain rapsyn, MuSK, and dystroglycan, they display altered morphology. An ultrastructural examination of synapses showed impaired formation of junctional folds, consistent with a role of neuregulin in synaptic structural organization (42). However, the absence of Schwann cells at the NMJs in these mice complicates this interpretation since the lack of Schwann cells may more readily explain the impaired junctional fold formation.

Neurotrophins acting through the TrkB receptors can inhibit agrin-induced AChR clustering on cultured myotubes (Ref. 13; but see Ref. 14). Neuregulin and the ErbB receptors are also well positioned to have similar effects. The ErbB and TrkB receptors both activate the mitogen-activated protein kinase and phospholipase C pathways, although the specific modulation of these pathways by the two receptors is likely different. In addition, ErbB receptors activate phosphatase pathways in muscle. In C2C12 myotubes, neuregulin increases the association of ErbB receptors with the phosphatase SHP2 (43); and in cultured frog myotubes, phosphatase activity regulates AChR cluster stability (44). Modulation of Rho GTPase activity represents another mechanism by which ErbB signaling could regulate AChR clustering. In C2 myotubes, inhibition of Rho activity blocks the formation of agrin-induced AChR clusters and instead leads to an increase in the formation of punctate microclusters” (15).

AChR Internalization at AChR Clusters—The biotin-a-Btx internalization assay demonstrated that endocytosis of AChRs occurred near AChR clusters during both agrin-induced cluster formation and cluster disassembly after agrin removal. Upon removal of agrin, live cell imaging revealed that the AChR clusters disassembled, and the punctate structures in the area of the AChR clusters may correspond in part to recently endocytosed AChRs. However, even after agrin removal, internalized AChRs were seen at only a small fraction of AChR clusters (<10%), and further studies are required to determine whether those internalized AChRs are associated with agrin-induced clusters or, alternatively, with the small number of spontaneous clusters that may persist after agrin treatment.

AChRs became localized to caveolae upon internalization. Caveolae can serve as organizers of protein endocytosis and as integrators of receptor tyrosine kinase signaling (23). Further experiments will be required to determine whether the ErbB receptor tyrosine kinases signal through caveolae to direct internalization of AChRs.

Dynamics of Individual Cluster Disassembly—We visualized the reorganization of agrin-induced AChR clusters upon removal of agrin. Eight hours after the removal of agrin, 25% of the original AChRs remained at the cluster. This is in general agreement with the results of Kim and Nelson (45), who determined that 38% of the original AChRs remain at clusters after 8 h in primary rat myotubes. Clusters generally disassemble via fragmentation of the cluster into smaller puncta. In contrast, time-lapse imaging of AChR clusters in cultured frog muscle cells (44) revealed that when clusters disassemble, the overall morphology of the cluster remains intact, and the clusters lose AChRs uniformly, as expected if individual AChRs diffuse away from the cluster. The conflicting results are most likely attributable to underlying differences in synapse formation in frog versus mouse.

In our live cell imaging experiments, neuregulin increased the rate of AChR cluster disassembly, but had no effect on the density of AChRs remaining in the cluster (as measured by the fluorescence/unit area). This would occur if neuregulin acts by disrupting a higher order organization within individual clusters. In this scenario, clusters are composed of small structural units (seen as puncta) that are held together within a cluster by a cytoskeletal network. A small number of AChR and rapsyn molecules contribute to each puncta. The clusters would become smaller in area while maintaining their AChR surface density if neuregulin disrupts the interaction between the puncta and the cluster superstructure so that puncta are lost from the cluster while leaving the AChR-rapsyn interactions intact and the fluorescence intensity/puncta unchanged. A fragmenting phenomenon similar to ours has been observed in vivo at NMJs in mice labeled with fluorescent a-Btx (46). Puncta corresponding to internalized AChRs were observed in the perijunctional region within 8 h after addition of curare to block synaptic transmission. In this study, we have shown that activation of ErbB receptors by the EGF domain of neuregulin reduced the number of
spontaneous and agrin-induced AChR clusters in cultured myotubes. We propose that, in addition to regulating subsynaptic AChR transcription, neuregulin also mediates synaptic competition at the developing NMJ. In the future, it will be important to establish which specific neuregulin isoforms act in vivo to modulate agrin-induced AChR clustering.

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