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Recycling of acetylcholine receptors at ectopic postsynaptic clusters induced by exogenous agrin in living rats

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ABSTRACT

During the development of the neuromuscular junction, motor axons induce the clustering of acetylcholine receptors (AChRs) and increase their metabolic stability in the muscle membrane. Here, we asked whether the synaptic organizer agrin might regulate the metabolic stability and density of AChRs by promoting the recycling of internalized AChRs, which would otherwise be destined for degradation, into synaptic sites. We show that at nerve-free AChR clusters induced by agrin in extrasynaptic membrane, internalized AChRs are driven back into the ectopic synaptic clusters where they intermingle with pre-existing and new receptors. The extent of AChR recycling depended on the strength of the agrin stimulus, but not on the development of junctional folds, another hallmark of mature postsynaptic membranes. In chronically denervated muscles, in which both AChR stability and recycling are significantly decreased by muscle inactivity, agrin maintained the amount of recycled AChRs at agrin-induced clusters at a level similar to that at denervated original endplates. In contrast, AChRs did not recycle at agrin-induced clusters in C2C12 or primary myotubes. Thus, in muscles *in vivo*, but not in cultured myotubes, neural agrin promotes the recycling of AChRs and thereby increases their metabolic stability.

Introduction

The presence of a high density of neurotransmitter receptors (AChRs) in the postsynaptic muscle membrane is the hallmark of the neuromuscular junction. The molecular machinery that initiates and maintains receptor clusters is organized by agrin, a protein secreted from motor nerve terminals, which binds to the transmembrane protein LRP4 (low-density lipoprotein receptor-related protein 4, a member of the LDLR family) and stimulates MuSK (muscle skeletal receptor tyrosine-protein kinase) activity (DeChiara et al., 1996; Kim et al., 2008; Lin et al., 2001; Sanes and Lichtman, 2001; Zhang et al., 2008). The latter, through several pathways, acts both as a scaffold and a signaling molecule to form and stabilize the AChR clusters at the NMJ (Wu et al., 2010).

Recently, evidence has accumulated that the density of synaptic AChRs at the mature NMJ is maintained both through the insertion of newly synthetized AChRs and by the reinsertion of synaptic AChRs that had been internalized and then redirected back to the synaptic muscle membrane, a process called AChR recycling. Upon denervation, however, only a small fraction of the internalized AChRs are able to recycle back to the synaptic muscle membrane, and most internalized AChRs are degraded, a process that is reversed by muscle stimulation (Bruneau and Akaaboune, 2006). This shift to the degradation pathway by denervation and its reversal by muscle activity might explain the decrease in half-life of synaptic AChRs (from about 10–14 to 3–5 days as estimated through AChR pulse labeling) observed upon chronic denervation (Shyng and Salpeter, 1989). This change in receptor stability can also be reversed by muscle stimulation, apparently through Ca^{++} influx associated with action potential activity (Caroni et al., 1993). These data combined suggest that muscle activity, while suppressing extrasynaptic AChR expression (Lomo and Rosenthal, 1972), directs the recyling of internalized AChRs into the postsynaptic membrane (Bruneau and Akaaboune, 2006; Rotzler et al., 1991), thus increasing their metabolic half-life.

Neural agrin alone is sufficient to induce nerve-free, ectopic specializations of sub-synaptic nuclei and formation of the post-synaptic apparatus in non-synaptic regions of muscle fibers (Bezakova et al., 2001b; Cohen et al., 1997; Meier et al., 1997). These agrin-induced ectopic postsynaptic-like membranes are functional and contain most of postsynaptic proteins that are present at innervated NMJs (Jones et al., 1997). Neural agrin appears also to be involved in setting-up the machinery that controls the metabolic stability of synaptic AChRs, because high neural concentrations of recombinant agrin can enhance the life-time of AChRs at ectopic

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agrin-induced clusters in both innervated and denervated muscles to levels comparable to those at innervated NMJs (Bezakova et al., 2001a; Bezakova and Lomo (2001); Bezakova et al., 2001b). Thus, we hypothesized that the effect of high agrin to stabilize AChRs could be mediated by increasing *the amount of recycled AChRs.*

In the current work we sought to test whether recombinant agrin is sufficient to induce the recycling of AChR at nerve-free, ectopic postsynaptic membranes. Analysis of receptor pools at such ectopic clusters showed that agrin is sufficient to induce the recycling in a dose-dependent manner, but that it is insufficient to trigger recycling at receptor clusters in cultured myotubes.

Experimental procedures

Intramuscular injection of plasmid containing agrin

Adult female Wistar rats (~250 g body weight) or C57/BL6 mice (30 g) were anaesthetized by i.p. injection of ketamine and xylazine (0.4 ml/100 g body weight). Soleus muscles were exposed and different concentration of plasmids containing full length chicken agrin (NtAcagrin748) and nls-GFP (nuclear marker) were injected into individual muscle fibers as described previously (Jones et al., 1997). Mouse sternomastoid muscles were studied using identical methods.

NMJ labeling and confocal microscopy

Three to four weeks after injection, the soleus muscle was exposed; AChR were labeled with a saturating dose of α-bungarotoxin coupled to biotin (BTX-biotin; 5 μg/ml, 60 min) and then washed out with Ringer's. Muscles were then bathed with a saturating dose of streptavidin-Alexa 594 and the incisions were sutured and the animals returned back to their cage. On subsequent days (typically 4–5 days later), the animals were re-anesthetized and muscles were exposed and bathed with a second saturating dose of streptavidin-Alexa 488 and BTX-Alexa 430 to label the recycled and newly synthesized AChRs respectively. Control experiments for ruling out dissociation of streptavidin from biotin on the surface of the muscle cells were described in our previous work (Bruneau et al., 2005). Muscles were removed and fixed with 4% paraformaldehyde, mounted on coverslips and scanned with a confocal microscope (Leica; model SPE) using a 100×, 1.46 numerical aperture (NA) oil-immersion objective (Leica; HCX Apochromat). The *z*-stacks were then collapsed and the contrast of images were adjusted with Adobe Photoshop CS2.

In another set of experiments, soleus muscles were exposed and bathed with a low dose of BTX-biotin (1 μg/ml, 30 min, so synapses remain fully functional), followed by a saturating dose of streptavidin Alexa 594 or unlabeled streptavidin (10 μg/ml, 3 h). Four to five days later, the animals were anaesthetized and muscles were relabeled with streptavidin Alexa 488 to label specifically the recycled AChR pool. The analysis of fluorescence intensity of both recycled and pre-existing AChR pools was performed by using Image J (Version 1.45). Only receptor clusters that did not exhibit fluorescence saturation at ectopic clusters and original NMJs were analyzed. The magic wand function was used to select clusters of fluorescently labeled receptors and a region of the image with no signal, which was used to calculate a background level that was subtracted from fluorescence intensity of measured receptor clusters.

Denervation

Soleus muscles of rat were denervated by removing a 5 mm piece of sciatic nerve at the level of the thigh to prevent muscles from reinnervation and then injected with agrin-cDNA. Three to four weeks later the muscles were exposed and labeled as described above.

Immunocytochemistry

Soleus muscles injected with agrin cDNA (three to four weeks after injection) were labeled with BTX-biotin followed by streptavidin Alexa 594 (to label pre-existing AChRs) and four to five days later recycled AChRs were labeled with streptavidin Alexa 660. The muscles were then removed, permeabilized with 1% Triton X-100 for 15 min, incubated for 10 min with 100 mM glycine, blocked for 30 min with 1% BSA in PBS, incubated overnight at 4 °C with rabbit anti-agrin antibody, washed 3 times in 1 h with in PBS containing 1% BSA, and incubated for 1 h with Alexa488-conjugated anti-rabbit antibody. The bundles were then examined with a confocal laser–scanning microscope (Leica; model SPE).

Primary and C2C12 muscle cells

Primary and C2C12 myotubes (American Type Cell Culture) were cultured on laminin-coated dishes focally impregnated with agrin (DMEM supplemented with 20% fetal bovine serum at 37 °C), as described previously (Jones et al., 1996). Cells were differentiated 2 days later by replacing the media with DMEM supplemented with 5% horse serum. 4–6 Days after differentiation, cells were bathed with a saturating dose of BTX-biotin followed by a saturating dose of fluorescently tagged streptavidin as describe above. 6 h after initial labeling myotubes were bathed with (red) streptavidin-Alexa594 to label recycled AChR and with BTX-Alexa430 to label newly synthesized AChRs and receptor clusters were imaged.

Electron microscopy

Plasmid injected muscles were removed and prefixed for 1 h with 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.4, at room temperature. Muscles were stained for acetylcholine esterase (AChE) activity according to Koelle and Friedenwald (1949), except that acetyl thiocholine concentration was reduced to keep AChE-dependent histochemical reaction product minimal. Muscles were then washed in PBS as soon as reaction product was observed, and they were postfixed in glutaraldehyde/cacodylate buffer overnight. Pieces of tissue were cut as described in Meier et al. (1998) and then were dehydrated and embedded in Epon. Ultrathin sections were prepared and stained with uranylacetate and lead citrate, and viewed in a Hitachi 7100 electron microscope.

Results

Recycled receptors at agrin-induced ectopic clusters in vivo

To examine whether internalized AChRs were able to recycle to agrin-induced ectopic AChR clusters, we co-injected fibers of rat soleus muscle with expression plasmids for nls-GFP (allowing later identification of injected fibers) and for full length chicken agrin (NtAcagrin748) (Denzer et al., 1995) at a concentration of 1 mg/ml, i.e., a high concentration inducing clusters of relatively high AChR density. Three to four weeks later, the muscles were bathed *in situ* with a saturating dose of bungarotoxin-biotin (BTX-biotin) to label surface receptors, followed by a saturating dose of streptavidin-Alexa594 (red: see Fig. 1) to label all biotin sites as described by Bruneau et al. (2005). Four days later, the soleus muscle was exposed and sequentially labeled with streptavidin-Alexa488 (green) to specifically label the recycled receptor pool (receptors that had lost their initial streptavidin-Alexa594 tag while retaining BTX-biotin during the process of internalization and reinsertion in the postsynaptic membrane receptors) and BTX-Alexa430 (blue) to