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Report

Acetylcholine Receptor Clustering Is Required for the Accumulation and Maintenance of Scaffolding Proteins

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Summary

The maintenance of a high density of postsynaptic receptors is essential for proper synaptic function. At the neuromuscular junction, acetylcholine receptor (AChR) aggregation is induced by nerve-clustering factors and mediated by scaffolding proteins [1]. Although the mechanisms underlying AChR clustering have been extensively studied [2–5], the role that the receptors themselves play in the clustering process and how they are organized with scaffolding proteins is not well understood. Here, we report that the exposure of AChRs labeled with Alexa 594 conjugates to relatively low-powered laser light caused an effect similar to chromophore-assisted light inactivation (CALI) [6, 7], which resulted in the unexpected dissipation of the illuminated AChRs from clusters on cultured myotubes. This technique enabled us to demonstrate that AChR removal from illuminated regions induced the removal of scaffolding proteins and prevented the accumulation of new AChRs and associated scaffolding proteins. Further, the dissipation of clustered AChRs and scaffold was spatially restricted to the illuminated region and had no effect on neighboring nonilluminated AChRs. These results provide direct evidence that AChRs are essential for the local maintenance and accumulation of intracellular scaffolding proteins and suggest that the scaffold is organized into distinct modular units at AChR clusters.

Results

Characterization of Photo Dissipation of AChRs from Individual Clusters

Evidence for laser-induced AChR dissipation was obtained from the following experiments: AChRs on primary mouse myotubes (Figure S1 available online) or on C2C12 myotubes cultured on plastic or laminin-coated plates were saturated with bungarotoxin-Alexa 594 (BTX-594), and entire clusters or small regions of individual clusters were bleached either with an Argon (488 nm) laser mounted to an upright epifluorescent microscope or with a DPSS (561 nm) scanning laser on a confocal microscope. When the cells were imaged 6 hr later (or 20 hr later, see Figure S2), we were surprised to find that there was neither recovery of fluorescence at bleached clusters from laterally migrating BTX-594-labeled receptors nor any new receptor labeling above background (after the addition of a distinctly colored fluorescent BTX conjugate), even if not all of the fluorescence was removed (Figure 1A, arrowheads) (see also Figure S3). At the same time, the accumulation of receptors occurred normally at neighboring unbleached clusters (Figure 1A). Further, when only small regions within BTX-594-labeled receptor clusters were illuminated with a focused laser, only background AChR staining was observed at the bleached region after the addition of the same or distinctly colored fluorescent BTX (see arrowheads, Figure 1B), whereas the accumulation of newly synthesized receptors at neighboring unbleached clusters and unbleached regions within the illuminated cluster were completely unaffected (Figures 1A and 1B). The same effect was observed when AChRs were labeled with bungarotoxin conjugated to biotin (BTX-biotin) and streptavidin-Alexa-594 (strept-594) (Figure 1C), whereas AChRs labeled with anti-AChR antibody (mAb35) and secondary antibody conjugated to Alexa 594 were not disrupted by laser illumination (Figure 1D). This indicates that the distance between Alexa 594 and the AChR may be crucial to Alexa-594-mediated AChR dissociation. Finally, dissipation of BTX-594-labeled receptors also occurred on clusters that had been treated with c-terminal agrin (data not shown), indicating that the dissipation of AChRs can occur even in the presence of a neural clustering factor.

To test whether the removal of AChRs was specific to the Alexa 594 fluorophore, cultured C2C12 myotubes were labeled with BTX-488 or BTX-555 and receptor clusters were imaged and then laser illuminated for long enough to bleach all the fluorescence from individual clusters (~3 s per cluster). Six hours after original bleaching, we found that the recovery of original fluorescence from the lateral migration of surface receptors and the accumulation of new receptors that were subsequently labeled with a distinct BTX conjugate, were both readily apparent around the circumference of the bleached clusters, as previously reported [8] (Figures 1E and 1F) (it should be noted that continued exposure of BTX-488- or BTX-555-labeled clusters to high-intensity laser light for >60 s sometimes resulted in partial disruption of the cluster). Similarly, receptor accumulation at bleached sites was normal if receptors were labeled with BTX-tetramethylrhodamine or BTX-647 (data not shown).

The dissipation of BTX-594-labeled AChRs from clusters was not due to laser damage of the muscle membrane (Figure S4); however, when an AChR antibody that does not compete for AChR binding with bungarotoxin was added immediately after AChR-BTX-594 illumination, the fluorescent intensity at bleached clusters decreased to ~60% to that of neighboring unbleached clusters, implying that AChR dissipation might be mediated by a change in receptor conformation (Figure S5).

AChRs Are Required for the Maintenance and Accumulation of Postsynaptic Scaffolding Proteins at Individual Clusters

To investigate whether receptors are necessary for the maintenance of the postsynaptic scaffold, we examined the localization of rapsyn, β-dystroglycan, utrophin, and actin after laser illumination of BTX-594-labeled receptor clusters. We focused on these proteins because they have been shown to serve as a link between the AChR and the intracellular cytoskeleton

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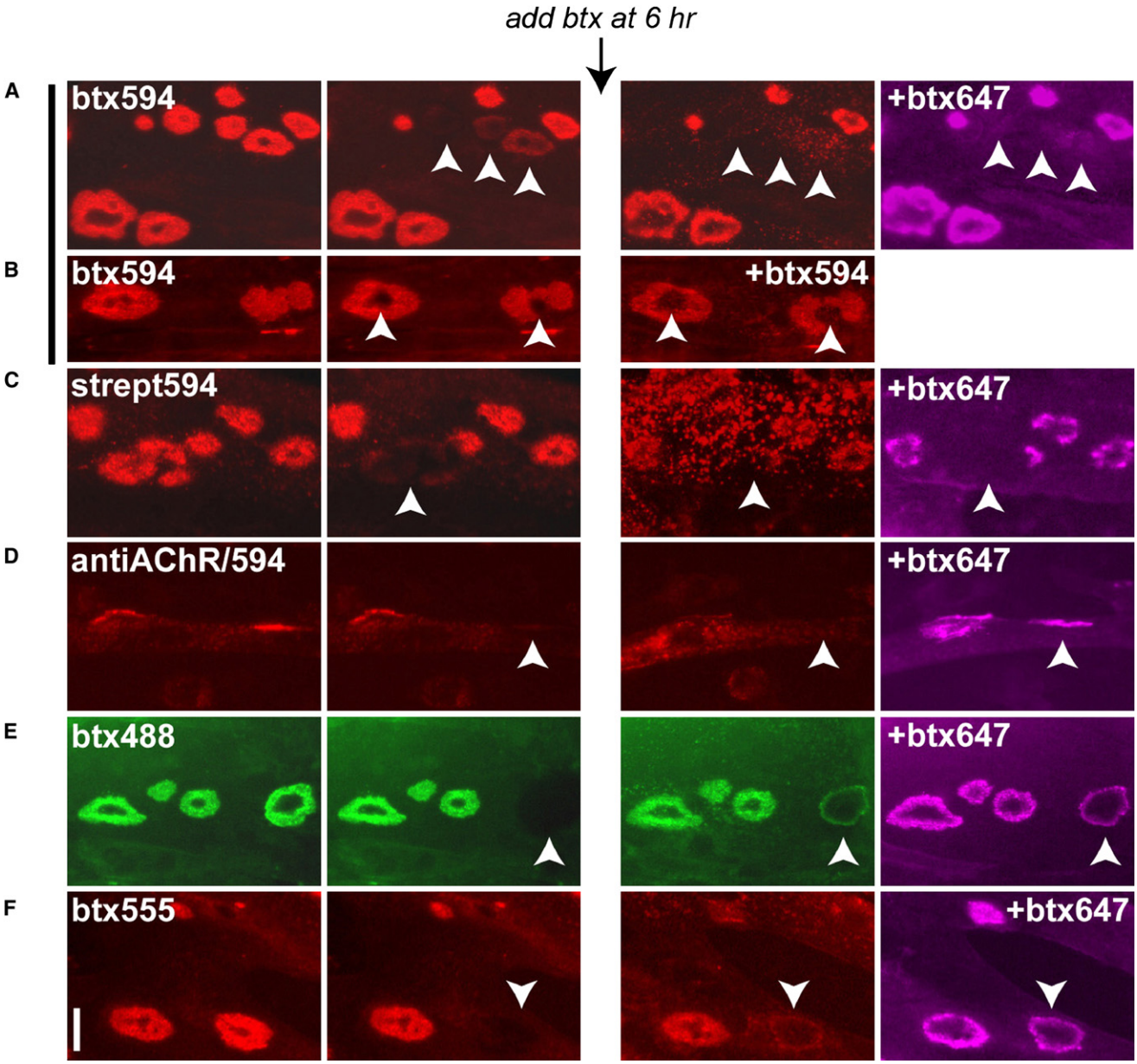

Figure 1. Alexa 594-Mediated Dissipation of Illuminated AChR Clusters on Cultured Myotubes

C2C12 myotubes were incubated in fluorescent BTX-488, BTX-555, or BTX-594 or bungarotoxin-biotin/streptavidin-Alexa 594, and individual labeled clusters on the myotube surface were imaged and then illuminated with an Argon laser (488 nm). Six hours later, the cells were incubated in BTX-647 or BTX-594 and then imaged again to identify newly synthesized receptors inserted over this time.

(A) Example of a myotube labeled with BTX-594 in which 3 clusters were illuminated for various times to cause different amounts of bleaching (arrowheads). Six hours later, after the addition of BTX-647, no originally labeled or newly inserted receptors had accumulated at any of the illuminated cluster sites, whereas high-density accumulation at unbleached control clusters was normal.

(B) Small regions within AChR clusters on myotubes labeled with BTX-594 were illuminated and then relabeled 6 hr later with new BTX-594. Note that the accumulation of newly synthesized AChRs was only prevented at the illuminated spots, indicating that the disruption of AChR clustering after laser illumination is a local effect.

(C) The accumulation of newly synthesized receptors also was prevented when AChR clusters labeled with bungarotoxin-biotin/streptavidin-Alexa 594 were illuminated. Note that the direct internalization and accumulation of streptavidin-Alexa 594 in intracellular puncta after labeling dramatically increases intracellular fluorescence.

(D) When AChRs on living myotubes were labeled with anti-AChR antibody and secondary antibody conjugated to Alexa 594, bleached, and then labeled 6 hr later with BTX-647, we found that AChRs were inserted normally.

(E and F) Examples of myotubes that were labeled with BTX-488 or BTX-555 and then illuminated with the same Argon laser used above. Six hours after laser exposure, the illuminated clusters had recovered significant amounts of fluorescence around the periphery of the cluster (as previously reported [8, 22]), both from the lateral migration of originally labeled receptors and the insertion of new receptors, indicating that laser illumination of BTX-488- or BTX-555-labeled AChRs did not noticeably alter AChR accumulation. Scale bar = 20 μm.

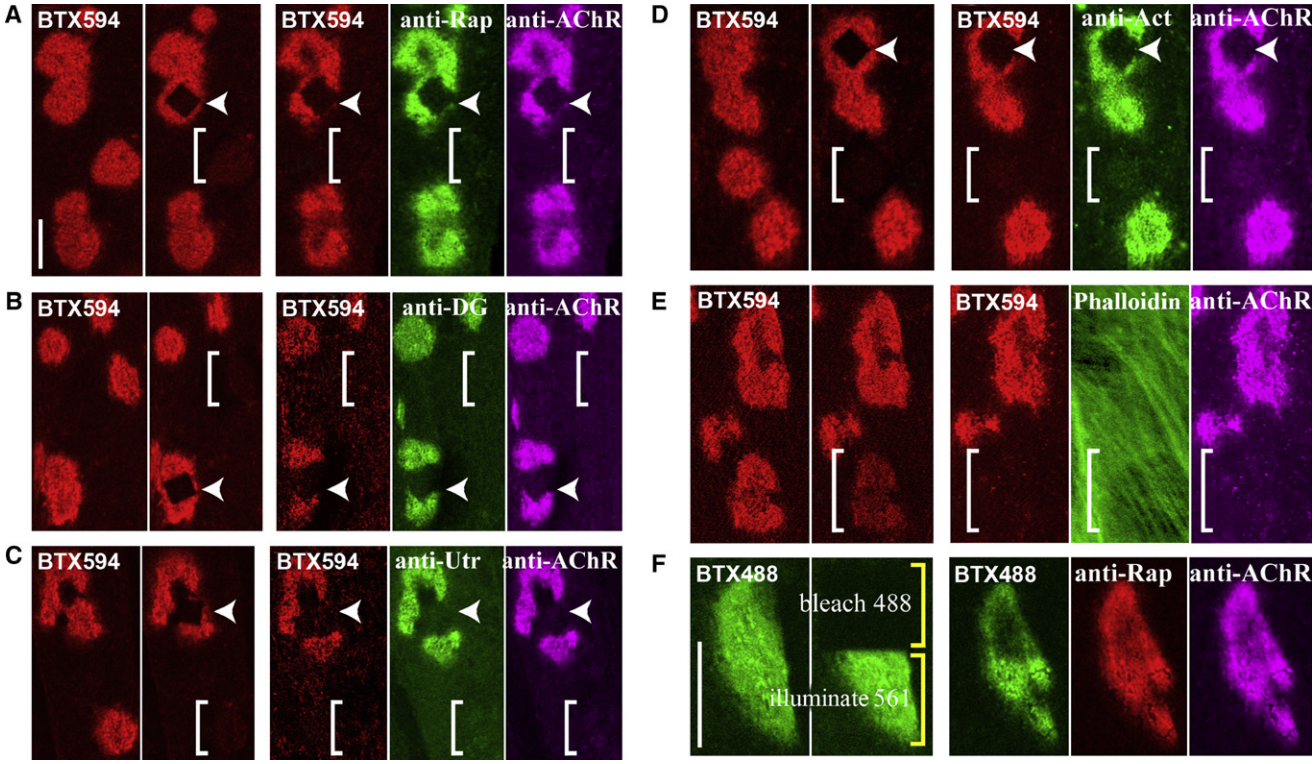

Figure 2. Laser Illumination of BTX-594-Labeled Clusters Results in the Complete Dissipation of the AChR Scaffold

Myotubes were labeled with BTX-Alexa 594 and then individual clusters (open bracket) and regions of individual clusters (arrowheads) were illuminated with a scanning confocal microscope using DPSS (561 nm) laser emission. Six hours later the myotubes were fixed and labeled with anti-AChR antibody and one of the following: anti-rapsyn (Rap), anti-β-dystroglycan (DG), anti-utrophin (Utr), anti-actin (Act), or Phalloidin-FITC.

(A–D) Because antibodies label both existing proteins and the proteins inserted during the 6 hr incubation, complete lack of staining from Rap, DG, Utr, and Act at whole illuminated clusters or illuminated regions within clusters indicates that the elimination of AChRs results in the local dissipation of the intracellular scaffold and also prevents the insertion of new scaffolding proteins.

(E) The presence of normal phalloidin staining after 6 hr at illuminated sites in which AChR clusters have been induced to dissipate indicates that the underlying cytoskeleton is unaffected by this laser illumination. Note that for (A)–(E), different levels of illumination/bleaching were able to induce complete dissipation.

(F) Clusters labeled with BTX-488 were also bleached with either the Argon (488 nm) or DPSS (561 nm) laser of the scanning confocal microscope and then fixed and labeled as above. Note that complete bleaching of the Alexa 488 with the Argon laser or exposure of the cluster to the DPSS laser (for the same amount of time used to dissociate clusters in [A]–[E]) did not result in receptor dissipation. Each confocal image was taken on a single plane on the muscle surface. Scale bars = 20 μm.

[1, 9–12]. To do this, receptors on cultured myotubes were labeled with BTX-594 and then entire clusters or small regions within individual clusters were illuminated with the DPSS (561 nm) laser of a scanning confocal microscope. Six hours later, cells were fixed and immunostained with anti-AChR (mAb35) antibody, and one of the following: anti-rapsyn, anti-β-dystroglycan, anti-utrophin, anti-actin (JLA20) (which we found to bind specifically to scaffold-specific actin), or phalloidin-FITC (which has been shown previously to bind to cytoskeletal actin [13]) (Figure S6). We found that the removal of the BTX-594-labeled AChRs from the entire illuminated cluster or from a small illuminated region within a cluster was accompanied by the disappearance of rapsyn, β-dystroglycan, utrophin, and scaffold-specific actin from the same bleached sites (Figures 2A–2D). The underlying cytoskeleton (labeled with phalloidin-FITC), however, was unaffected (Figure 2E). Illumination of BTX-488-labeled AChRs at individual clusters either with the Argon laser, or exposure of these clusters to the confocal DPSS laser for the same amounts of time that were used for BTX-594-mediated dissipation, had no effect on the migration of AChRs into bleached regions or the accumulation of either AChRs or rapsyn at the cluster after 6 hr (Figure 2F). These data indicate that AChRs are required for the maintenance and accumulation of rapsyn, β-dystroglycan, utrophin, and actin molecules at clusters in the muscle membrane.

Time-Lapse Imaging of Intracellular Scaffolding Protein Dissipation

To examine the time course of removal of labeled AChRs and AChR scaffold proteins from illuminated clusters, we monitored AChR and associated scaffolding proteins at various time points after laser illumination. As an initial step, we examined the removal of rapsyn from illuminated clusters by transfecting myotubes with rapsyn-GFP and then labeling the AChRs on these cells with BTX-594. The GFP or the Alexa 594 from individual clusters was then excited by using the Argon or DPSS lasers of a confocal microscope, respectively. In this way we were able to simultaneously follow rapsyn-GFP removal over time from clusters that were illuminated with the DPSS (561 nm) laser (which induced BTX-594-labeled AChRs to dissipate but did not bleach the rapsyn-GFP) and determine the insertion of rapsyn-GFP into clusters that were illuminated briefly with the Argon (488 nm) laser (which bleached the GFP

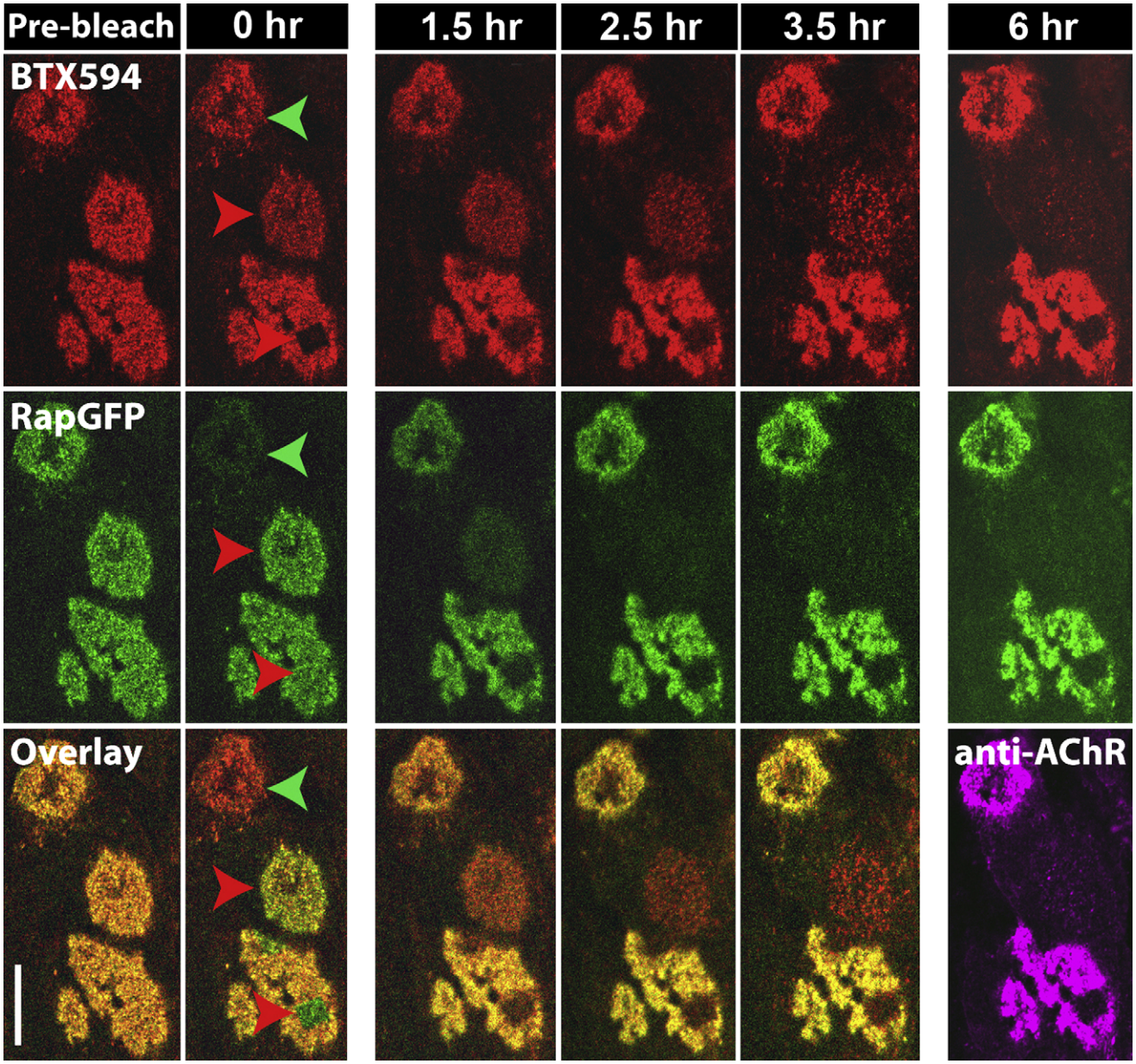

Figure 3. Time-Lapse Imaging of Rapsyn-GFP after Laser-Induced AChR Dissipation

Myotubes were transfected with rapsyn-GFP and labeled with BXT-594. Individual clusters were imaged and then illuminated either with a brief exposure to the Argon (488 nm) laser to bleach the GFP fluorescence (green arrowheads) or more prolonged exposure to the DPSS (561 nm) laser to excite Alexa 594 fluorescence (red arrowheads). The same clusters were then imaged from 0 to 3.5 hr later after fresh applications of new BTX-594 between imaging time points. Cells were then fixed and labeled with anti-AChR antibody after 6 hr. At clusters in which only rapsyn-GFP was bleached (green arrowhead), rapsyn-GFP fluorescence rapidly recovered, and fluorescence had nearly reached original levels when cells were fixed 6 hr later. At clusters in which the Alexa 594 fluorescence was illuminated and AChRs were therefore induced to dissociate (red arrowheads), rapsyn-GFP fluorescence rapidly decreased over time as rapsyn dissipated from the bleached cluster, and rapsyn-GFP was completely absent from the cluster site after cells were fixed at 6 hr. The absence of anti-AChR staining at 6 hr indicates that complete cluster dissipation had occurred. Each confocal image was taken on a single plane of the muscle surface. Scale bar = 20 μm.

but left labeled AChRs relatively unbleached) on the same myotube. At clusters where only AChR-Alexa 594 was excited, time-lapse imaging revealed that rapsyn-GFP fluorescence decreased rapidly after only 1.5 hr and was nearly undetectable after 3.5 hr. In contrast to visible internalized Alexa 594 puncta, no internal GFP signal was seen at these clusters. At the same time, insertion of rapsyn-GFP into clusters in which the GFP (and not the Alexa 594) was bleached with the Argon laser was normal and similar to previous reports [14] (Figure 3). These data indicate that net rapsyn dissipation (the combination of rapsyn removal and the prevention of new rapsyn insertion) occurs very rapidly from clusters after photo dissipation of clustered AChRs.

To confirm the above results and extend them to the other scaffolding proteins, BTX-594-labeled receptor clusters were illuminated, fixed 1 hr later, and then immunostained with anti-rapsyn, anti-utrophin, anti-β-dystroglycan or anti-actin (JLA20) antibodies. We found that rapsyn, utrophin,

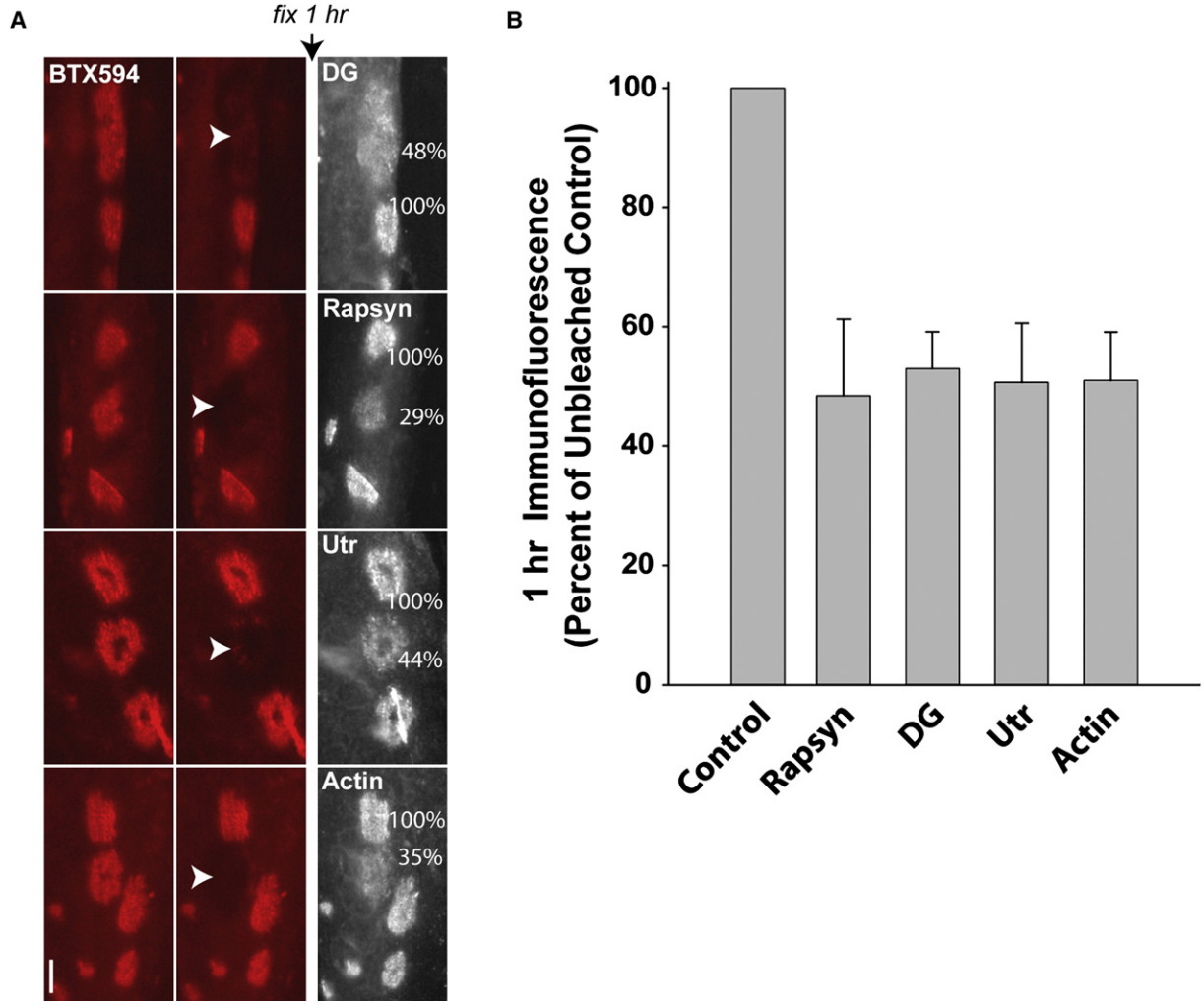

Figure 4. Rapsyn, β-Dystroglycan, Utrophin, and Actin Are All Removed at Similar Rates from Illuminated Clusters

Myotube cultures were labeled with BTX-594 and imaged, and then individual clusters were illuminated with an Argon (488 nm) laser. One hour later the cells were fixed; immunostained with antibodies for rapsyn, β-dystroglycan, utrophin and actin; and imaged again. The average immunofluorescence from each bleached cluster was then compared to the average immunofluorescence at a neighboring unbleached control cluster.

(A) Representative images from individual myotubes show that after 1 hr, approximately half of the population of each scaffolding protein at bleached clusters had been removed.

(B) Graph represents the quantification of immunofluorescence from all antibodies 1 hr after illumination. Note that the immunofluorescence from each protein decreased similarly. All data represented as mean ± SD. Scale bar = 20 μm.

β-dystroglycan, and actin immunofluorescence at bleached clusters had all decreased after only 1 hr to nearly half the amount at neighboring unbleached control clusters (48 ± 13%, n = 7; 53 ± 6%, n = 6; 51 ± 10%, n = 19; and 51 ± 8%, n = 13, respectively) (Figures 4A and 4B). When cells were fixed immediately after bleaching, negligible changes in antibody binding for scaffolding components were seen (Figure S5 and data not shown). When cells were fixed 2 hr after illumination, bright intracellular puncta made quantification difficult, and at time points after 2 hr the immunofluorescence from all antibodies was too dim to quantify; immunofluorescence was completely absent at illuminated clusters after 6 hr.

Discussion

This work demonstrates that low-power laser illumination of the Alexa 594 fluorophore conjugated to bungarotoxin not only causes the removal of the AChRs and simultaneous or subsequent loss of scaffold proteins from illuminated regions but also prevents the accumulation of new synaptic proteins at that region. This phenomenon is spatially restricted to the illuminated region, leaving unbleached regions of the same cluster unaffected.

The effect observed here is similar to chromaphore-assisted light inactivation (CALI) in which illumination of a specific protein/fluorophore pair causes the inactivation of the labeled protein. CALI has been shown to result from the generation of free radicals that alter proteins only within a very close proximity (less than 100 Å) [15, 16]. Consistent with this, we found that the Alexa 594-mediated dissipation occurred only if the fluorophore was relatively close to the AChR (separated by BTX or BTX-biotin-streptavidin), but not if the fluorophore was conjugated to a secondary antibody. Further, even if the laser light was intense enough to temporarily damage the muscle membrane, it still did not alter AChR clustering in regions immediately adjacent to the illuminated region.

Although it is not clear how illumination of the Alexa 594 fluorophore is able to induce the dissipation of clustered receptors, it is possible that the absorption of photons by the fluorophore could cause structural modification of the AChR (as suggested by Figure S5). Although it is possible that the illuminated and structurally modified AChRs remain stuck in the membrane, our data are more consistent with a model in which subsequent untethering of affected AChRs from the cluster site could then lead to an AChR density below a critical level necessary to signal the accumulation of AChRs, which would lead to the dispersal of all clustered AChRs at the illuminated region and the prevention of newly synthesized AChR accumulation. Whatever the mechanism by which laser illumination causes Alexa-594-labeled AChR dissipation, we used this as a tool to directly investigate the role of the AChR in the maintenance and integrity of the intracellular scaffold. When receptors were induced to disperse, all other proteins of the scaffold examined also were removed from the cluster and newly synthesized proteins were not accumulated at the illuminated region. Consistent with this observation, rapsyn fails to cluster in AChR-deficient muscles in zebrafish [17, 18] and in C2C12 myotubes lacking AChRs [19], and mice lacking the adult epsilon AChR subunit fail to form mature synapses and show marked decreases in scaffolding proteins [20].

One concern with all laser illumination studies is that the laser may be causing nonspecific cell damage. The following data indicate that this is likely not the case in the present study. First, although laser illumination caused AChR dissipation, it did not result in muscle membrane damage (Figure S3). Second, at clusters that were induced to dissipate, the intracellular actin cytoskeleton was not altered after laser illumination (Figure 3E). Third, AChRs that were within microns of the laser illumination (even extensive illumination that temporarily damaged the muscle membrane) were not affected. Fourth, exposing rapsyn-GFP to high laser power for prolonged time did not disrupt rapsyn insertion. Fifth, illumination of BTX-488-labeled AChRs with the Argon or DPSS scanning confocal laser with the same parameters that caused dissipation of BTX-594-labeled AChRs did not alter AChR clustering or rapsyn localization (Figure 3F); Finally, 6 hr after laser exposure the background fluorescence from new fluorescent BTX at illuminated regions was similar to staining from diffuse AChRs elsewhere on the muscle surface (Figure 1 and Figures S1 and S2), and even nonlabeled AChRs within illuminated regions were dissipated after laser illumination (Figure S3), indicating that receptors were able to move freely into and out of the laser-illuminated membrane. These observations suggest that laser illumination caused the selective dissociation of clustered receptors, which in turn resulted in the local disassembly of the intracellular scaffold without disrupting either the intracellular cytoskeleton or the membrane integrity or fluidity.

Here, we show directly that AChRs are not only necessary for the clustering of rapsyn in mammalian myotubes but also that a threshold density of AChRs likely provides a critical signal that enables proteins of the dystrophin-glycoprotein complex and intracellular scaffold to remain aggregated and that allows the accumulation of newly synthesized proteins into the existing cluster. The fact that Alexa-594-mediated dissipation occurs only within small illuminated sections of individual clusters further implies that the high density of AChRs provides a local clustering signal. Together, these data indicate that the postsynaptic scaffold may be organized not as a matrix of cross-linked proteins but as discrete units of intracellular scaffolding proteins associated with individual receptors (Figure S7). Consistent with this model, local removal of clustered AChRs and scaffold proteins has been observed during synaptic remodeling during development or after nerve damage [1, 21]. It will be interesting to see if AChR dissipation is responsible for precipitating these types of synaptic remodeling events.

Supplemental Data

Experimental Procedures and seven figures are available at http://www.current-biology.com/cgi/content/full/18/2/109/DC1/.

Acknowledgments

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