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**Abstract**

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**Disciplines**

Biological Psychology | Communication | Molecular and Cellular Neuroscience | Neurology | Neurosciences | Social and Behavioral Sciences

# The Dynamics of the Rapsyn Scaffolding Protein at Individual Acetylcholine Receptor Clusters*

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**Rapsyn, a cytoplasmic receptor-associated protein, is required for the clustering of acetylcholine receptors (AChRs). Although AChR dynamics have been extensively studied, little is known about the dynamics of rapsyn. Here, we used a rapsyn-green fluorescent protein (GFP) fusion protein and quantitative fluorescent imaging to study the dynamics of rapsyn in transfected C2C12 myotubes. First, we found that rapsyn-GFP expression at clusters did not alter AChR aggregation, function, or turnover. Quantification of rapsyn immunofluorescence indicated that the expression of rapsyn-GFP proteins at clusters does not increase the overall rapsyn density compared with untransfected myotube clusters. Using time lapse imaging and fluorescence recovery after photobleaching, we demonstrated that the recovery of rapsyn-GFP fluorescence at clusters was very fast, with a halftime of about ~1.5 h (~3 times faster than AChRs). Inhibition of protein kinase C significantly altered receptor insertion, but it had no effect on rapsyn insertion. When cells were treated with the broad spectrum kinase inhibitor staurosporine, receptor insertion was decreased even further. However, inhibition of protein kinase A had no effect on insertion of either rapsyn or receptors. Finally, when cells were treated with neural agrin, rapsyn and AChRs were both directed away from preexisting clusters and accumulated together in new small clusters. These results demonstrate the remarkable dynamism of rapsyn, which may underlie the stability and maintenance of the postsynaptic scaffold and suggest that the insertion of different postsynaptic proteins may be operating independently.**

The efficacy of synaptic transmission depends upon the high density and number of postsynaptic neurotransmitter receptors at sites of neurotransmitter release (1, 2). This density can be maintained in several ways. For example, phosphorylation/dephosphorylation events mediated by different kinases are able to alter receptor synthesis and dynamics (2–5), and a number of postsynaptic scaffolding proteins have been found to be essential either for the clustering or the maintenance of postsynaptic receptors at cluster sites at both central and peripheral synapses (2, 6–11).

The formation of the postsynaptic receptor density is best studied at the neuromuscular junction. Among the molecules involved in the clustering of AChRs[2] is rapsyn. Rapsyn (43 kDa) was initially purified from the torpedo electric organ and is thought to be associated with AChRs in a 1:1 stoichiometry at the muscle surface (12, 13). Rapsyn is responsible for mediating the effect of agrin, a neuronal clustering factor that activates the muscle-specific kinase and initiates AChR clustering in a tyrosine kinase-dependent manner (14–18). In mice lacking rapsyn, receptor clustering does not occur, and neuromuscular junctions fail to form (19). Rapsyn mutations also cause AChR deficiency in patients with congenital myasthenia syndrome (20–22), highlighting the importance of rapsyn for the formation and organization of the postsynaptic receptor density. Although the availability of highly specific receptor ligands has enabled the study of AChR dynamics on the living muscle *in vitro* and *in vivo* (23, 24), the study of scaffolding protein dynamics at postsynaptic density has been hindered by their inaccessibility to extracellular ligands.

In this study, we investigated the dynamics of rapsyn at individual clusters on cultured myotubes. Our interest specifically in rapsyn stems from its close association with AChRs and its role in receptor clustering at the postsynaptic membrane. By monitoring the fluorescence of rapsyn-GFP fusion proteins clustered on the surface of transfected C2C12 myotubes, we were able to determine that rapsyn is markedly more dynamic than AChRs and is not affected by pharmacological manipulation that alters AChR dynamics, although both rapsyn and AChRs are similarly modulated by the nerve clustering factor, agrin.

## EXPERIMENTAL PROCEDURES

*GFP Fusion Construct*—The rapsyn-GFP fusion construct was kindly provided by Dr. Jonathan Cohen (Harvard Medical School).

*Cell Culture*—To generate laminin-coated dishes, 5 $\mu$g/ml polyornithine (Sigma) was added to 35-mm culture dishes and allowed to evaporate overnight. The following day, 10 $\mu$g/ml of EHS laminin (Invitrogen) in L-15 medium supplemented with 0.2% sodium bicarbonate was added to the dishes, and they were placed in an incubator overnight until ready for plating.

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[2] The abbreviations used are: AChR, acetylcholine receptor; GFP, green fluorescent protein; PKA, protein kinase A; PKC, protein kinase C; BTX, bungarotoxin; BTX-488, bungarotoxin-Alexa 488.

C2C12 myoblasts (ATCC, Manassas, VA) were plated and then grown in 20% fetal bovine serum with penicillin and streptomycin in Dulbecco's modified Eagle's medium. Cells were transfected at 70–100% confluence with 1 $\mu$g of rapsyn-GFP using FUGENE6 transfection reagent (Roche Applied Science). Cells were then differentiated by replacing the fetal bovine serum solution with 5% horse serum medium with penicillin and streptomycin in Dulbecco's modified Eagle's medium. Medium was changed every 2 days, and cells were imaged 3–4 days after differentiation.

*Immunofluorescence Microscopy*—Transfected myotubes were fixed with 2% paraformaldehyde and then treated for 20 min with 1% Triton X-100 in phosphate-buffered solution. Cells were then incubated with mouse anti-rapsyn primary (monoclonal antibody 1234; Sigma) and goat anti-mouse Alexa 594 secondary antibodies (Invitrogen). After extensive washing, cells were imaged. Background fluorescence was approximated by selecting a boundary region around the cluster and subtracting it from the original image, and the mean of the total fluorescence intensity (which corresponds to density) was measured. Image analysis was performed by using a procedure written for Matlab (The Mathworks, Natick, MA) (25). The acquisition and quantification of images were done with the experimenter blind to rapsyn-GFP expression at each cluster.

*Electrophysiology*—A rapsyn-GFP-transfected myotube was located, and a whole cell patch clamp recording was obtained under voltage clamp. The AChR agonist carbachol (100 $\mu$M) was then ejected into the medium with a puffer pipette placed near the patched cell, and the resulting inward current was measured. As a control for the specificity of carbachol, the AChR antagonist curare (10 $\mu$M) was applied by perfusion into the cell medium to block these inward currents. Measurements were made as above on untransfected myotubes for comparison.

*Receptor Loss*—AChR loss was determined by labeling AChRs of untransfected myotubes or myotubes expressing rapsyn-GFP with bungarotoxin-Alexa 594 (5 $\mu$g/ml, 1 h). Initial images of rapsyn-GFP and/or clustered receptors were taken, and 4 h later, the same clusters were found and reimaged. The loss of fluorescence was then assayed using quantitative fluorescence imaging (24, 26). Clusters expressing different levels of GFP were then grouped into three categories according to mean GFP expression: low (0–25th percentile of mean fluorescence), medium (26–49th percentile), and high (50–100th percentile).

*Fluorescent Recovery after Photobleaching*—To determine the insertion of rapsyn into receptor clusters over time, the green fluorescence at individual rapsyn-GFP-expressing clusters was removed by illuminating the cluster with an argon laser passed through a neutral density filter that blocked 50% of the light. Laser illumination of myotube clusters has been shown previously not to affect receptor dynamics or muscle cell integrity (8, 23). The recovery of fluorescence was assayed, and the amount of fluorescence recovered at each cluster was measured.

To determine the insertion of AChRs into clusters over time, receptors on untransfected myotubes were saturated with bungarotoxin-Alexa 488 (BTX-488) (5 $\mu$g/ml, 1 h) (Molecular Probes, Inc., Eugene, OR), and the green fluorescence was com-pletely removed with an argon laser. Since newly synthesized receptors inserted into the cluster over time were unlabeled, BTX-488 was then added before each time point to label these receptors, excess BTX-488 was washed away, and the clusters were again imaged (as described by Bruneau *et al.* (23)). In this way, the total insertion of receptors (both previously labeled receptors that had laterally migrated into the cluster and the newly labeled receptors that had been inserted into the cluster) could be determined and compared with the insertion and lateral migration of rapsyn-GFP. For the purposes of this paper, "insertion" will refer to the accumulation of proteins at clusters both through direct incorporation from intracellular locations and from the lateral migration of surface-associated proteins.

*Agrin Treatment*—To determine the effect of agrin on rapsyn-GFP expression at clusters, myotubes grown on laminin-coated dishes transfected with rapsyn-GFP were incubated with BTX-594 to label all AChRs. After initial images were taken, 100 ng/ml C-terminal agrin (R & D Systems, Minneapolis, MN) was added to the culture dish. At 4 and 8 h, images were taken to determine the location of rapsyn-GFP and AChRs.

*Kinase Inhibition*—To determine the effect of various kinase inhibitors on rapsyn-GFP insertion over time, the GFP signal from clusters on myotubes transfected with rapsyn-GFP was carefully removed with an argon laser, and one of the following drugs was added to the medium: 100 nM H-89 (to block PKA), 20 $\mu$M H-89 (to inhibit both PKA and PKC), 10 nM calphostin C (to inhibit PKC), or 20 nM staurosporine (to block a broad spectrum of kinases, including PKA and PKC) (drug specificity obtained from the manufacturer, Sigma). Images were taken immediately before and after photobleaching, and 4 h later, the same clusters were reimaged, and the fluorescent intensity at bleached and nonbleached clusters was determined. Rapsyn-GFP recovery was determined by comparing the GFP signal at bleached clusters after 4 h with their fluorescence immediately prior to bleaching. Since the expression of rapsyn-GFP was variable over time and between different myotubes in these experiments, rapsyn-GFP insertion at each cluster was normalized to the changes in expression (if any) at control, unbleached clusters on the same myotube. Overall changes in expression in each culture dish were comparable across all treatment groups.

We also performed a series of experiments to determine the loss and insertion of receptors at individual clusters in the presence of the same pharmacological agents. To measure the insertion rate of receptors, untransfected myotubes were saturated with BTX-488 (5 $\mu$g/ml, 1 h), and the fluorescence was carefully removed from individual clusters with an argon laser. Initial images were taken, and one of the above kinase inhibitors was added to the cultures. Four hours later, after incubating with new BTX-488 to label new receptors inserted since initial imaging, the same clusters were found and imaged. The insertion of AChRs was determined by comparing cluster fluorescence at 4 h with the original fluorescence prior to bleaching.

To measure the loss rate of receptors, untransfected myotubes were saturated with BTX-488 (5 $\mu$g/ml, 1 h), and individual clusters were immediately imaged and incubated with one of the above mentioned inhibitors. The same clusters were then reimaged 4 h later, and their fluorescence intensity was measured.

## Rapsyn Dynamics

### RESULTS

*Rapsyn-GFP Does Not Affect Acetylcholine Receptor Clustering, Function, or Removal Rate*—First, in order to verify that rapsyn-GFP was able to localize to laminin-associated AChR clusters on our cultured myotubes, we transiently transfected C2C12 myoblasts with a rapsyn-GFP fusion protein (27). Three to four days after differentiation into myotubes, cultured cells were bathed with bungarotoxin conjugated to Alexa 594 (BTX-594) to label AChRs, and receptor clusters were imaged. Rapsyn-GFP clusters were found to always perfectly co-localize with AChRs (Fig. 1A).

We also wanted to determine if the insertion and accumulation of rapsyn-GFP at laminin-induced receptor clusters affected AChR function. To examine this question, we compared inward current across the muscle membrane in response to the application of the AChR agonist, carbachol, on myotubes expressing rapsyn-GFP and nontransfected myotubes. Under direct visual control, we obtained a whole cell patch on a myotube, clamped voltage, and stimulated with carbachol. We found that the inward currents were similar in both transfected and untransfected cells and were both completely blocked by the addition of the AChR antagonist curare (Fig. 1B). These results indicate that the surface expression of rapsyn-GFP does not disrupt receptor function.

Next, we asked whether the overall density of rapsyn molecules at clusters (endogenous rapsyn plus exogenous rapsyn-GFP) was increased by rapsyn-GFP expression in transfected myotubes. To examine this, myotube cultures that were transfected with rapsyn-GFP were fixed and permeabilized and then immunostained with an anti-rapsyn antibody. We used an anti-rapsyn antibody that has been shown previously to recognize both rapsyn and rapsyn-GFP fusion proteins (28). Since transfection efficiency of myotubes in each culture dish was variable, we were able to use the same culture dish to determine the relative number of rapsyn molecules at nontransfected clusters and at rapsyn-GFP-expressing clusters over a wide range of expression levels (Fig. 1C). Quantitative analysis of the mean fluorescence intensity of anti-rapsyn immunostaining revealed that the average density of total rapsyn at rapsyn-GFP-expressing clusters was $12.5 \pm 3.2$ (S.D.) (arbitrary units), $n = 198$. This is nearly identical to the density at clusters containing only endogenous rapsyn ($11.8 \pm 3.2$ (S.D., $n = 123$) (Fig. 1, C–E). Even over a nearly 10-fold range of rapsyn-GFP expression at clusters, the difference in total rapsyn density was negligible (Fig. 1E). It is possible that the number of rapsyn-GFP molecules at transfected clusters is negligible compared with the total number of endogenous rapsyn molecules. In this case, the presence of exogenous rapsyn-GFP would not detectably alter overall rapsyn density. Alternatively, it is possible that exogenous rapsyn-GFP replaces the endogenous rapsyn, allowing overall rapsyn density at clusters to remain constant (see "Discussion").

Finally, we wanted to determine whether rapsyn-GFP expression levels at clusters had any effect on AChR stability. To do this, transfected myotubes were saturated with BTX-594, and receptor loss was monitored at clusters expressing low, medium, and high levels of rapsyn-GFP on two separate myo-
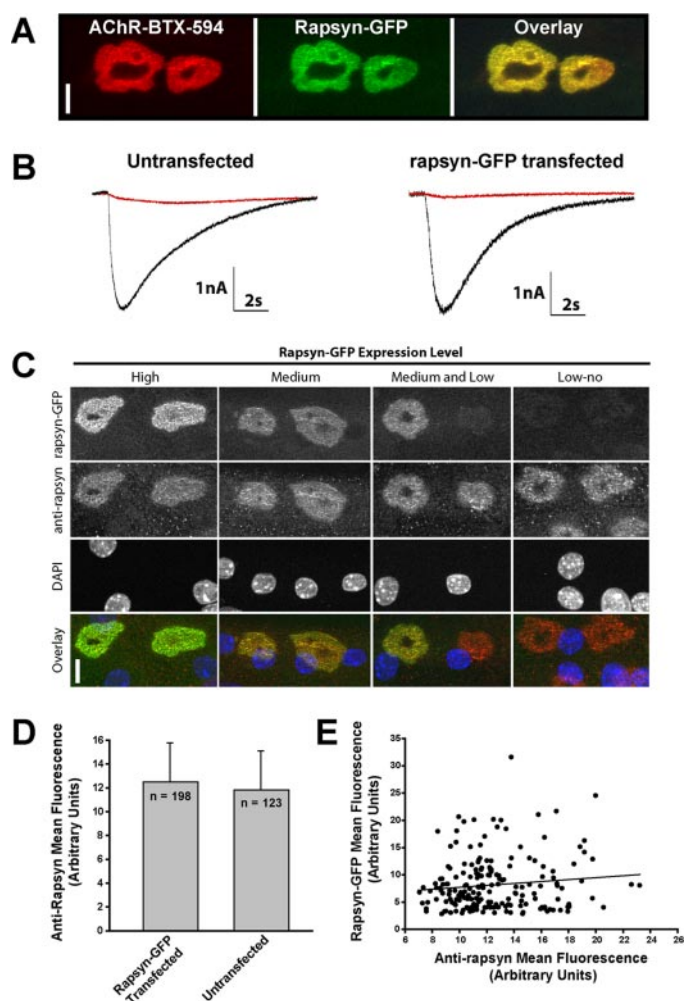


FIGURE 1. **Rapsyn-GFP cluster expression does not alter AChR clustering, stability, or activity.** *A*, example of a myotube transfected with rapsyn-GFP and labeled with bungarotoxin-Alexa 594. Shown are representative clusters in which rapsyn-GFP perfectly co-localizes with acetylcholine receptors. *Scale bar*, 20 $\mu$m. *B*, untransfected myotubes and myotubes expressing rapsyn-GFP responded similarly to the application of the AChR agonist, carbachol, and this response was blocked after the addition of the AChR antagonist, curare, indicating that AChR function is not disrupted by rapsyn-GFP. *C*, example of myotubes expressing different levels of rapsyn-GFP at clusters that were labeled with anti-rapsyn antibodies and 4',6-diamidino-2-phenylindole (*DAPI*) to stain nuclei. Images illustrate that overall rapsyn density is the same in rapsyn-GFP-expressing clusters and in clusters that do not express rapsyn-GFP. *Scale bar*, 20 $\mu$m. *D*, *histogram* showing the mean fluorescence intensity of anti-rapsyn staining from many clusters of nontransfected myotubes and myotubes transfected with rapsyn-GFP. Note that the mean fluorescence is comparable in both transfected and untransfected myotube clusters. Histograms represent mean fluorescent intensity ± S.D. *E*, quantification of rapsyn immunofluorescence and rapsyn-GFP for many clusters on transfected myotubes. As shown by the *scatter plot*, rapsyn-GFP fluorescent expression at clusters did not correlate with total rapsyn density.

tube cultures. After 4 h, we found that $65 \pm 15\%$ (S.D., $n = 29$) of fluorescently labeled AChRs remained at clusters expressing low rapsyn-GFP fluorescence, $60 \pm 13\%$ (S.D, $n = 42$) remained at clusters expressing medium rapsyn-GFP, and $62 \pm 15\%$ (S.D., $n = 44$) remained at clusters expressing high rapsyn-GFP. These results are similar to fluorescence remaining after 4 h at AChR clusters expressing no rapsyn-GFP ($63 \pm 17\%$ (S.D., $n = 77$)) (Fig. 2, A–C). This indicates that rapsyn-GFP expression has no effect on AChR stability in our culture system.
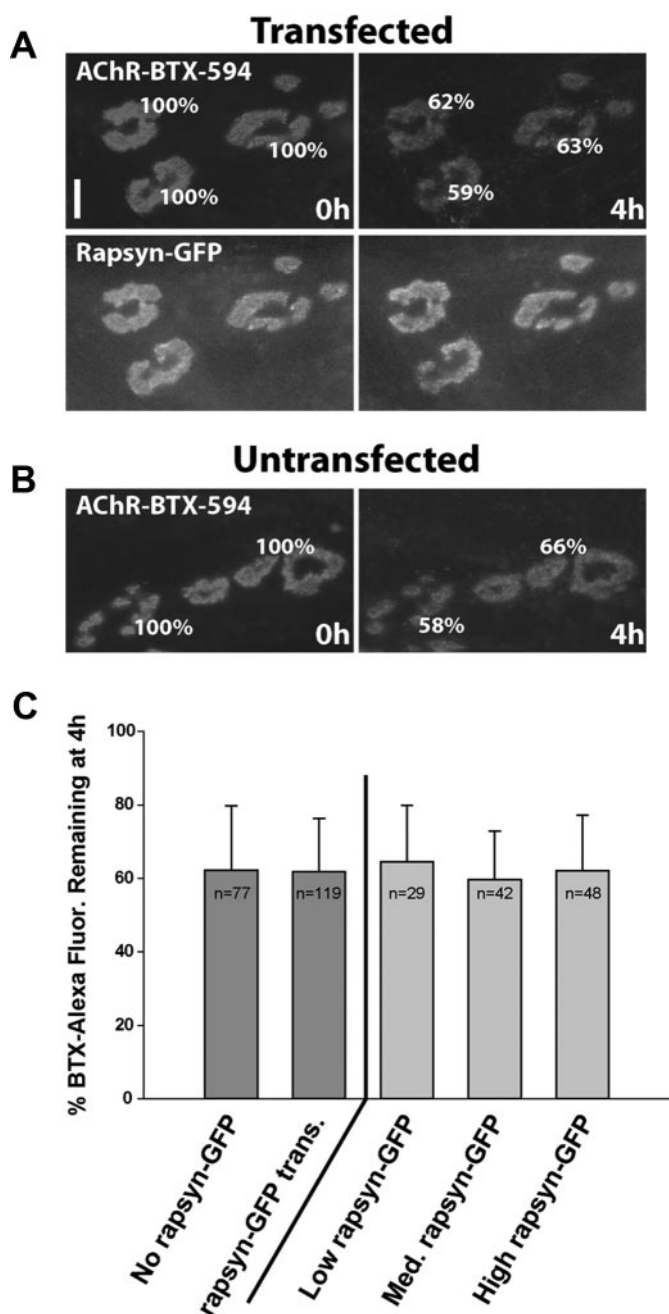
## A

### Transfected

AChR-BTX-594

100% 62%

100% 63%

59%

100% 0h 4h

Rapsyn-GFP

## B

### Untransfected

AChR-BTX-594

100% 66%

100% 58% 4h
0h

## C



FIGURE 2. **The cluster expression of rapsyn-GFP has no effect on AChR removal.** Nontransfected myotubes and myotubes transfected with rapsyn-GFP were labeled with BTX-Alexa 594, and the loss of AChR-BTX-594 was monitored over 4 h. *A*, example of clustered AChRs on rapsyn-GFP transfected myotubes that were labeled with BTX-594, imaged, and imaged again 4 h later to determine the receptor loss rate. *B*, clustered AChRs on untransfected myotubes were labeled as above and also followed over 4 h. *C*, histogram summarizes data obtained from many clusters on transfected and untransfected myotubes. Note that the change in fluorescently labeled AChR levels monitored over 4 h on untransfected myotubes and at myotube clusters expressing a wide range of rapsyn-GFP were not significantly different ($p > 0.3$). All values represent mean $\pm$ S.D.

*Recovery Rate of Rapsyn-GFP at Individual Myotube Clusters*—Having found that rapsyn-GFP has no effect on the clustering, function, or turnover of AChRs, we wanted to determine the lifetime of rapsyn at individual clusters. To do this, transfected myotubes expressing rapsyn-GFP were imaged, and then the GFP at individual clusters was removed with an argon laser.

Confirmation of the removal of fluorescence was done by acquiring a second image (Fig. 3*A*). We then monitored the recovery of fluorescence at bleached clusters over time from a number of cultures (data from 3–5 culture dishes at each time point). We found that rapsyn clusters recovered fluorescence very rapidly, gaining $61 \pm 11\%$ (S.D., $n = 40$) of original fluorescence after only 2 h, $71 \pm 19\%$ (S.D., $n = 56$) after 4 h, and $75 \pm 18\%$ (S.D., $n = 58$) after 6 h (Fig. 3, *A* and *B*). The recovery at each data point was nearly identical for rapsyn-GFP clusters from myotubes not grown on laminin (2 h: $63 \pm 12\%$ (S.D., $n = 35$); 4 h: $73 \pm 17\%$ (S.D., $n = 15$); 6 h: $78 \pm 21\%$ (S.D., $n = 33$)) (Fig. 3, *A* and *B*). This recovery was not due to reversible bleaching of GFP, because no fluorescence was observed after bleaching rapsyn-GFP clusters on fixed myotubes. In addition, we found that the recovery of rapsyn-GFP over 4 h was not affected by rapsyn-GFP expression levels (Fig. 3*C*).

Given this rapid rate of rapsyn insertion and past reports suggesting that rapsyn is co-transported with AChRs to the cell surface (29, 30), we wanted to determine the rate of AChR insertion into individual clusters using the same fluorescence recovery after photobleaching method. To do this, myotubes were bathed with a single saturating dose of BTX-488, and then all fluorescence was carefully removed from individual clusters with an argon laser. After 2, 4, or 6 h, a second dose of BTX-488 was added to label all AChRs that had been inserted into clusters over this time. We found that $15 \pm 4\%$ (S.D., $n = 30$) of original fluorescence was returned to the clusters after 2 h, $25 \pm 7\%$ (S.D., $n = 54$) after 4 h, and $28 \pm 9\%$ (S.D., $n = 39$) after 6 h (data obtained from 3–5 cultures per data point) (Fig. 3, *A* and *B*). The number of AChR molecules inserted over time was therefore significantly lower than the number of rapsyn-GFP molecules inserted over the same time period (see "Discussion").

Given the large amount of rapsyn-GFP recovery during the first 2 h, we wanted to monitor more closely the insertion of rapsyn-GFP immediately after photobleaching. To do this, rapsyn-GFP clusters on transfected myotubes were bleached, and time lapse imaging was used to monitor the fluorescent recovery every 15 min at room temperature. We found that rapsyn-GFP recovery was $11 \pm 4$, $17 \pm 5$, $23 \pm 9$, and $27 \pm 11\%$ at 15, 30, 45, and 60 min (S.D., $n = 15$ clusters/3 culture dishes) (Fig. 3, *D* and *E*). The rapid recovery of fluorescence observed in clusters is not due to reversible bleaching of the fluorophore, since rapsyn-GFP clusters on fixed myotubes that are bleached show no spontaneous fluorescence recovery, even over days (data not shown). We also found that the labeling of AChRs with BTX-647 had no effect on rapsyn insertion dynamics, since rapsyn insertion measured by fluorescence recovery after photobleaching was identical at rapsyn-GFP-expressing clusters that were not labeled with any BTX and at rapsyn-GFP-expressing clusters that were saturated with BTX-647 prior to photobleaching (data not shown).

*The Clustering of AChRs and Rapsyn Are Altered Similarly by Agrin Treatment*—In order to determine if rapsyn and AChRs are regulated similarly despite the difference in dynamics, we attempted to manipulate AChR stability and insertion using the neuronal clustering factor, agrin. Our previous results showed that when agrin was added to myotubes grown on a laminin
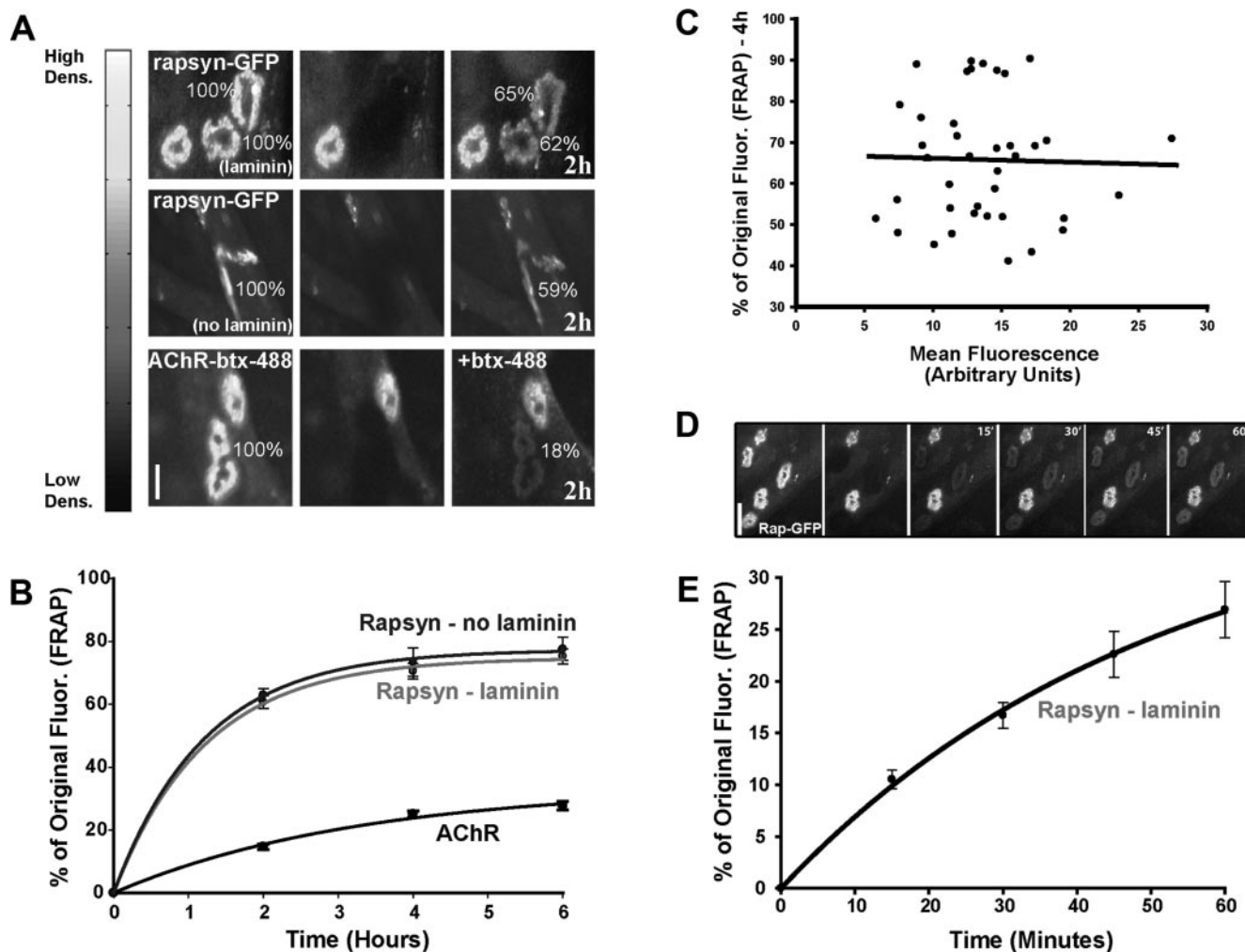
FIGURE 3. **Rapsyn is inserted rapidly into individual clusters.** *A*, the insertion of rapsyn-GFP into individual clusters on myotubes grown either on laminin-coated dishes or uncoated dishes was measured by determining the fluorescent recovery after photobleaching using quantitative fluorescent imaging. The insertion of rapsyn-GFP into bleached laminin-associated clusters (*top panels*) and nonlaminin clusters (*middle panels*) is rapid and nearly identical over 2 h in both cases. In contrast, acetylcholine receptor clusters that were labeled with bungarotoxin-Alexa 488, bleached, and then incubated in new bungarotoxin-488 (*bottom panels*) show far fewer inserted AChRs over the same time period. *B*, graph summarizes the data from the recovery of rapsyn and AChRs at 2, 4, and 6 h time points. *C*, the rate of rapsyn-GFP insertion is independent of the level of rapsyn-GFP expression. *D*, sample images from a time lapse experiment in which the fluorescent recovery at a number of clusters was determined every 15 min for 60 min. *E*, results from three experiments performed as in *D* ($n = 15$ clusters). Note that the recovery of fluorescence is >25% after 1 h, although cells were maintained at room temperature for the duration of the experiment. All graph points for *B* and *E* represent mean ± S.E., and all data were fit well by single exponential curves. *Scale bars*, 20 $\mu$m.

substrate, most new receptors were not directed into preexisting laminin-induced clusters but instead formed numerous small aggregates on the entire muscle surface (23). Since rapsyn is required for the clustering of AChRs, and its interaction with the receptor is highly regulated and increased by agrin (31), we wanted to know whether newly inserted rapsyn molecules are also directed away from preexisting laminin-associated clusters and into new, agrin-induced clusters when agrin is added to the cultures. To investigate this issue, transfected myotubes expressing rapsyn-GFP were labeled with BTX-594, imaged, treated with agrin, and then imaged again 4 and 8 h later. Over the time course of our study, we found that rapsyn clustering was altered similarly to AChRs, being directed away from the same regions on laminin-associated clusters and inserted into the same agrin-induced clusters (Fig. 4).

*Effect of Kinase Inhibition on Rapsyn-GFP and AChR Dynamics*—Having found that AChRs and rapsyn respond similarly to agrin treatment, we wanted to extend our anal-

ysis to determine if alterations in kinase activity could affect rapsyn and AChRs in a similar manner. Previous studies have shown that agrin-induced clustering of AChRs can be inhibited by the broad spectrum kinase inhibitor, staurosporine (32–34). To further investigate the role of kinase activity on receptor and rapsyn dynamics at single clusters, we performed a series of experiments using various kinase inhibitors. As a first step, we used the broad spectrum kinase inhibitor, staurosporine. To study the effect of staurosporine on receptor insertion, cultured myotubes were labeled with BTX-488, and clusters were imaged and photobleached. Cells were then incubated with 20 nM staurosporine, and 4 h later, the cells were bathed with new BTX-488 to label all new receptors. We found that the number of AChRs accumulated at bleached clusters was significantly decreased to $18 \pm 5\%$ (S.D., $n = 13$, $p < 0.0001$) of the original receptor density, compared with $28 \pm 6\%$ (S.D., $n = 20$) for control clusters (Fig. 5, *A* and *B*).
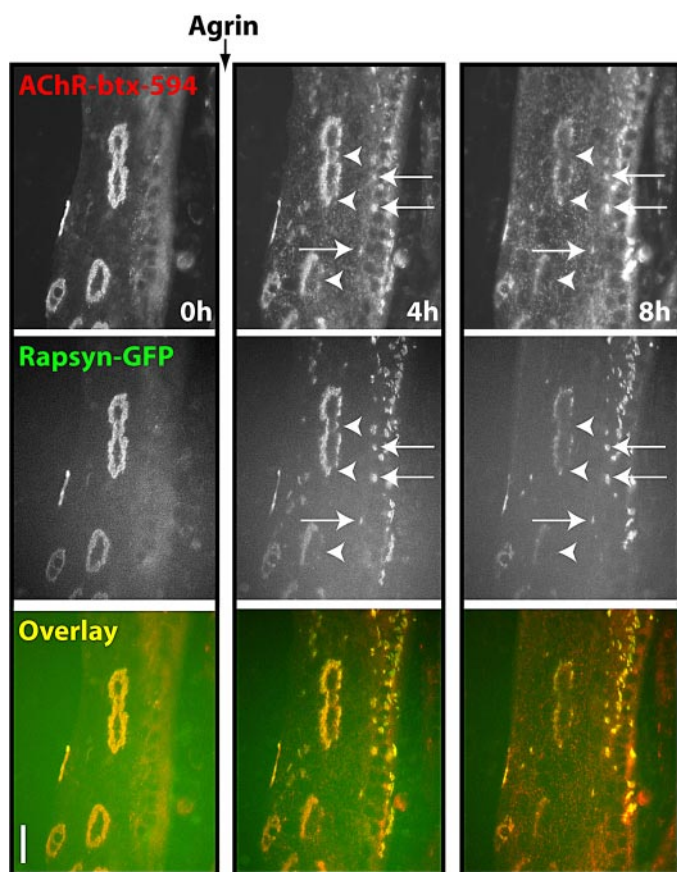
FIGURE 4. **Agrin redirects both AChRs and rapsyn away from preexisting laminin clusters and into new agrin-induced clusters.** Myotubes expressing rapsyn-GFP were incubated with BTX-594 to label all AChRs. Agrin was then added to the culture medium, and rapsyn and receptors were imaged 4 and 8 h later. Note that both AChRs and rapsyn were directed away from some locations on the preexisting laminin-associated clusters (*arrowheads*) and were also aggregated together into new, agrin-induced clusters (*arrows*). *Scale bar*, 20 $\mu$m.

Since staurosporine inhibits PKC, PKA, and other kinases, we wanted to more specifically determine the effect of PKA and PKC on receptor insertion. To do this, myotubes were labeled with BTX-488 as above, and the recovery of fluorescence in bleached clusters was monitored in the presence of 100 nM H-89 (to specifically block PKA), 20 nM H-89 (to block PKA and PKC), or 10 nM calphostin C (to specifically block PKC). We found that 100 nM H-89 had no effect on receptor insertion ($27 \pm 8\%$ (S.D., $n = 20$)) compared with controls. However, in the presence of 20 $\mu$M H-89, the accumulation of new receptors at bleached clusters decreased to $23 \pm 4\%$ (S.D., $n = 21$, $p < 0.005$). Similar results were obtained when PKC was more specifically inhibited by 10 nM calphostin C ($21 \pm 6\%$ (S.D., $n = 20$, $p < 0.005$)). At higher concentration of calphostin C ($>50$ nM), most cells were dead after 4 h. These experiments suggest that PKC activity is somehow involved in the insertion of receptors (Fig. 5B).

Next we asked whether AChR loss was also affected by kinase inhibition. To answer this question, cells were labeled with BTX-488, and individual receptor clusters were imaged and then incubated with one of the same drugs used in the above experiments before being imaged again at 4 h. At clusters on untreated control myotubes, $72 \pm 8\%$ (S.D., $n = 15$) of origi-

nal fluorescence remained after 4 h. When cells were treated with 100 nM H-89, 20 $\mu$M H-89, or 10 nM calphostin C, there was no difference in receptor loss compared with control ($68 \pm 9\%$ (S.D., $n = 23$), $71 \pm 10\%$ (S.D., $n = 10$), $73 \pm 12\%$ (S.D., $n = 20$)) (Fig. 5D). However, incubation in 20 nM staurosporine resulted in an increase in receptor removal from clusters, with only $52 \pm 10\%$ (S.D., $n = 21$, $p < 0.0001$) of original fluorescence remaining at clusters after 4 h (Fig. 5, C and D). These results suggest that staurosporine, in addition to inhibiting AChR insertion, increases receptor removal from clusters in a non-PKC/PKA-dependent manner.

Finally, we investigated whether rapsyn-GFP insertion is similarly affected by kinase inhibition. To do this, myotubes expressing rapsyn-GFP at clusters were imaged, and the fluorescence was removed from individual clusters using an argon laser. Bleached clusters were then imaged; staurosporine (20 nM), H-89 (100 nM or 20 $\mu$M), or calphostin C (10 nM) was added to the cultures, and the rate of rapsyn-GFP insertion into the bleached clusters was determined at 4 h. We found that bleached clusters regained $67 \pm 15\%$ (S.D., $n = 37$), $64 \pm 15\%$ (S.D., $n = 10$), $67 \pm 15\%$ (S.D., $n = 16$), and $76 \pm 11\%$ (S.D., $n = 19$) of their original fluorescence, respectively (Fig. 5, E and F). The insertion at bleached clusters was normalized to the changes in expression (if any) at control, unbleached clusters on the same myotube. It should be noted that higher concentrations (200 nM) of staurosporine did result in a significant decrease in rapsyn-GFP insertion over 2 h (clusters were too dim to be imaged after 4 h) that was similar to the decrease in AChR insertion measured over the same time period. However, it seems likely that this was due to decreased cell viability, as has been observed previously (34).

## DISCUSSION

In this study, we investigated the dynamics of rapsyn in cultured myotubes and found that rapsyn is remarkably dynamic compared with AChRs. Further, although the localization of both rapsyn and receptor were similarly affected by agrin treatment, their dynamics were differentially regulated by kinase inhibition.

One unexpected finding from this work is the rapid recovery of rapsyn-GFP fluorescence at individual clusters after photobleaching. For a number of reasons, we believe that the fused GFP is not interfering with rapsyn function and that the recovery in fluorescence observed in our studies therefore reflects the turnover rate of rapsyn. First, it has been shown previously that the rapsyn-GFP construct used in the present studies is indistinguishable from wild-type rapsyn in its ability to associate with and cluster AChRs in heterologous cells (27). Second, the present work demonstrates that rapsyn-GFP expression at clusters does not alter receptor function, clustering or turnover in muscle cells. Third, the rapid recovery of fluorescence after photobleaching was independent of rapsyn-GFP expression at myotube clusters (Fig. 3). Finally, the accumulation of rapsyn-GFP at clusters was regulated by agrin in a similar manner as AChRs. Consistent with these observations, it has been shown that fluorescent rapsyn also recovers rapidly after photobleaching in QT-6 cells (28). The remarkable dynamism of rapsyn at surface clusters is further supported by biochemical studies
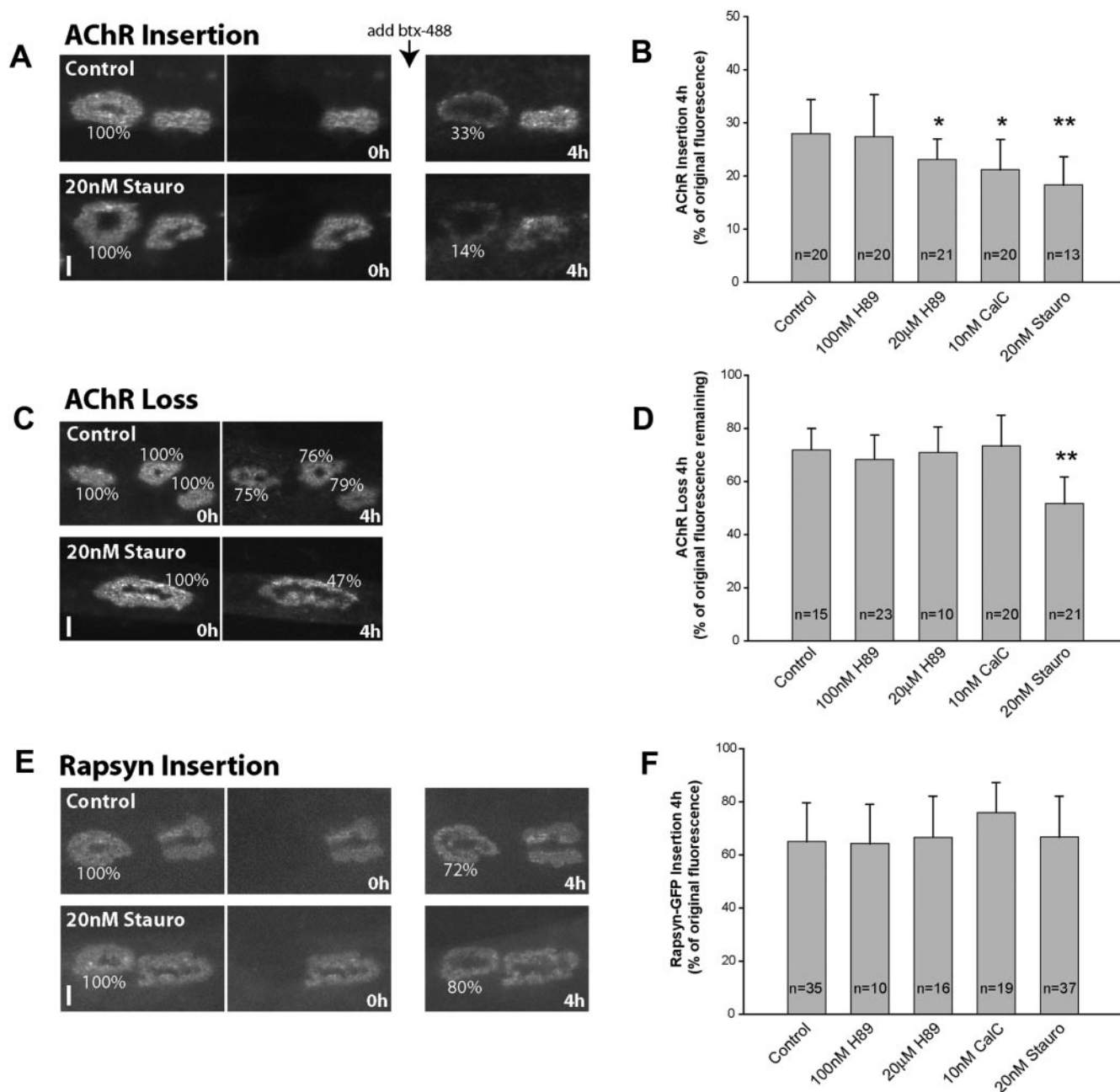
**A   AChR Insertion**    add btx-488

Control    100%    0h    33%    4h

20nM Stauro    100%    0h    14%    4h

**B**

**C   AChR Loss**

Control    100%    100%    0h    76%    79%    4h
100%    100%    75%    79%

20nM Stauro    100%    0h    47%    4h

**D**

**E   Rapsyn Insertion**

Control    100%    0h    72%    4h

20nM Stauro    100%    0h    80%    4h

**F**

FIGURE 5. **Kinase inhibitors alter AChR dynamics but not the dynamics of rapsyn-GFP.** Laminin-associated clusters on rapsyn-GFP transfected myotubes were bleached, and on nontransfected myotubes, AChRs were labeled with BTX-488 before bleaching individual clusters. Cells were then incubated in either 100 nM H-89, 20 $\mu$M H-89, 10 nM calphostin C, or 20 nM staurosporine. Myotubes were imaged, and the fluorescence at each cluster was compared with its original fluorescence. *A*, example of clusters that were bleached on either control or 20 nM staurosporine-treated myotubes. *B*, *graph* summarizes data obtained from many clusters in untreated control cultures and in cultures incubated with one of the above drugs. Note that PKA inhibition alone had no effect on AChR insertion, whereas the blockade of PKC (with higher concentrations of H-89 and with calphostin C) resulted in a significant decrease in AChR insertion. Inhibition of a broader spectrum of kinases along with PKC by staurosporine caused a slightly greater inhibition of AChR insertion. *C*, representative clusters showing loss of receptors from control myotubes or myotubes treated with 20 nM staurosporine. *D*, AChR loss from individual clusters in the presence of the same kinase inhibitors shows that only incubation with staurosporine caused a decrease in overall receptor density remaining at clusters after 4 h, indicating that the blockade of kinases other than PKA and PKC with staurosporine treatment resulted in the accelerated removal of AChRs from clusters. *E*, representative images of control and staurosporine-treated myotubes that are expressing rapsyn-GFP. *F*, clusters expressing rapsyn-GFP were bleached, and the insertion of rapsyn was measured by determining the recovery in fluorescence over time in the presence of the same drugs as used above. Note that the insertion of rapsyn-GFP was not altered by any of the drug treatments. Insertion into clusters was normalized to the changes in fluorescence at neighboring, unbleached clusters on the same myotube. All *bar graphs* represent mean ± S.D. *, $p < 0.005$; **, $p < 0.0001$ (Student's *t* test). *Scale bars*, 10 $\mu$m.

that show that the metabolic turnover of rapsyn is short compared with AChRs, with a metabolic half-life of ~3 h (35). Since our studies were done on aneural receptor clusters, it is possible that the turnover of rapsyn might be decreased under the influence of the nerve. Further studies investigating the dynamics of

rapsyn at the neuromuscular junction will be necessary to determine if rapsyn is indeed stabilized by innervation. Although we cannot definitively exclude the possibility that GFP may diminish the affinity of rapsyn for the AChR or other scaffold proteins, these results argue strongly that rapsyn-GFP

behaves as endogenous rapsyn and that rapsyn therefore turns over very rapidly in muscle cells.

Similar to our findings that the acetylcholine receptor-associated protein turns over more rapidly than AChRs, recent studies on the turnover of receptor-associated proteins at glutamatergic synapses have yielded analogous results. For example, PSD-95, a dynamic protein affiliated with the AMPA and NMDA receptors (36–41), when tagged with GFP and measured with fluorescence recovery after photobleaching, was shown to turn over very rapidly relative to the glutamate receptors with which it associates (42, 43). This result opens the possibility that the rapid turnover of rapsyn observed in muscle cells in the present study may be similar to the behavior of receptor-associated proteins in the central nervous system.

Previously it has been shown that AChRs and rapsyn are transported together in the same intracellular vesicles from the Golgi complex to the membrane surface both in COS cells co-transfected with AChRs and rapsyn and at the mature torpedo electric organ (a structure analogous to the mammalian neuromuscular junction) (29, 30). Although the ratio of rapsyn to receptor during intracellular trafficking remains undetermined, estimates from our results suggest that multiple rapsyn molecules may accompany each AChR to the membrane surface in aneural myotubes.

In addition to highlighting the difference in turnover between receptors and rapsyn, the present study also shows that the expression of exogenous rapsyn at clusters does not increase receptor stability. The effect of exogenous rapsyn on AChR dynamics has previously been studied in heterologous cells. In these studies, the effect of rapsyn on AChR stability was determined by comparing the degradation of radioactive bungarotoxin in heterologous cells devoid of endogenous rapsyn with cells transfected with rapsyn cDNA (15, 29, 30). They found that the metabolic half-life of receptors was increased upon co-expression with rapsyn. A recent study also indicates that the overexpression rapsyn through electroporation of rapsyn-GFP into muscle cells *in vivo* can stabilize receptors (28). This *in vivo* result is further supported by findings that mutant rapsyn results in a decrease in receptor stability in some forms of myasthenia gravis (21). By contrast, our results indicate that rapsyn-GFP expression at clusters did not alter AChR stability. This result can be explained by two possibilities. First, it is conceivable that that exogenous rapsyn-GFP is able to replace endogenous rapsyn at clusters without increasing rapsyn density, as demonstrated by quantitative immunofluorescence (Fig. 1, *C–E*). It is also possible that despite the strong GFP fluorescence signal, the number of exogenous rapsyn-GFP molecules inserted at clusters was negligible compared with the density of endogenous rapsyn. It is important to mention that the anti-rapsyn antibody used for assaying the total rapsyn content has been shown to recognize both endogenous rapsyn and exogenous rapsyn-GFP (28).

Despite the difference in turnover between rapsyn and AChRs, our work indicates that both rapsyn and AChRs are subject to similar modulation following treatment with neural agrin. Previously, work from our laboratory has shown that when AChRs at laminin-associated clusters are treated with agrin, most new receptors are not directed into preexisting laminin-induced clusters but instead form numerous small aggregates on the entire muscle surface (23). The present work extends this observation and demonstrates that when agrin is added to myotubes, rapsyn-GFP is directed into the new aggregates to co-localize with AChRs, suggesting that rapsyn and AChRs are responding to agrin similarly and simultaneously. It is possible that the redirection of AChRs into small aggregates is driven by the rapsyn, since rapsyn is the bridge between biological activity following agrin treatment and receptor clustering. This idea is supported by experiments showing that AChRs fail to associate with cytoskeletal proteins in agrin-treated myotubes devoid of rapsyn (17, 18). In fact, AChRs fail to cluster at all *in vitro* or *in vivo* when rapsyn is not present (19, 27, 44). On the other hand, there is also reason to believe that rapsyn could instead follow AChRs to clusters. For example, in the absence of receptors, rapsyn fails to cluster (45, 46).[3]

Although rapsyn and AChR are intimately associated and similarly directed by agrin, the mechanisms regulating their delivery and removal from individual clusters are at least partially distinct. Although PKA inhibition with 100 nM H-89 had no effect on the insertion of rapsyn or AChRs or on the loss of AChRs from individual clusters, inhibition of PKC with higher concentrations of H-89 (20 μM) or 10 nM calphostin C significantly decreased AChR insertion without affecting AChR loss or rapsyn insertion. The broad spectrum kinase inhibitor staurosporine was able to further decrease AChR insertion and resulted also in an increase in AChR removal without altering rapsyn insertion. Previous work has shown that the *in vivo* inhibition of PKC by calphostin C injection retards the development of neuromuscular synapses (47). Although our cells are aneural, they were plated on laminin, which has been shown to mimic, at least in some respects, the development of the neuromuscular junction (48). Therefore, it is possible that the mechanism by which PKC inhibition decreases AChR insertion in our system could account for the alterations in postsynaptic growth observed when PKC is blocked *in vivo* by the same pharmacological agent. The fact that staurosporine increases the removal of AChRs in addition to decreasing their insertion implies that staurosporine may act to increase AChR dispersal through a non-PKA/PKC-dependent mechanism. In support of this conclusion, it has been shown previously that non-PKC-dependent tyrosine phosphorylation contributes to receptor clustering. For example, Src class kinases promote the phosphorylation of the β subunit and increase the association of AChRs with the intracellular cytoskeleton (3, 15, 16, 49), and agrin-induced receptor clusters are removed rapidly in the absence of Src class kinases (15).

Our results show that rapsyn-GFP insertion was not affected by any of the kinase inhibitors used. This may indicate that the synthesis and delivery of rapsyn to individual clusters is independent of the phosphorylation either of rapsyn or of proteins that are necessary for rapsyn exocytosis. Previous reports have shown that receptors and rapsyn are co-transported in the same vesicle and inserted into the plasma membrane. It is possible that the difference in the amount of receptor and rapsyn

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[3] E. Bruneau and M. Akaaboune, unpublished results.

insertion when cultured cells were treated with PKC inhibitor could therefore be due to the selective impairment of receptor transcription.

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