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The effect of agrin and laminin on acetylcholine receptor dynamics in vitro

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Abstract

Using optical imaging assays, we investigated the dynamics of acetylcholine receptors (AChRs) at laminin-associated clusters on cultured myotubes in the absence or presence of the nerve-derived clustering factor, agrin. Using fluorescence recovery after photobleaching (FRAP) on fluorescent bungarotoxin-labeled receptors, we found that ~9% of original fluorescence was recovered after 8 h as surface AChRs were recruited into clusters. By quantifying the loss of labeled receptors and the recovery of fluorescence after photobleaching, we estimated that the half-life of clustered receptors was ~4.5 h. Despite the rapid removal of receptors, the accumulation of new receptors at clusters was robust enough to maintain receptor density over time. We also found that the AChR half-life was not affected by agrin despite its role in inducing the aggregation of AChRs. Interestingly, when agrin was added to myotubes grown on laminin-coated substrates, most new receptors were not directed into preexisting laminin-induced clusters but instead formed numerous small aggregates on the entire muscle surface. Time-lapse imaging revealed that the agrin-induced clusters could be seen as early as 1 h, and agrin treatment resulted in the complete dissipation of laminin-associated clusters by 24 h. These results reveal that while laminin and agrin are involved in the clustering of receptors they are not critical to the regulation of receptor metabolic stability at these clusters, and further argue that agrin is able to rapidly and fully negate the laminin substrate clustering effect while inducing the rapid formation of new clusters.

Keywords: C2C12; Myotube; Laminin; Agrin; Receptor turnover; Receptor insertion; Receptor removal; Receptor half-life

Introduction

Through the course of neuromuscular development the most prominent emerging feature of the post-synaptic membrane surface is the high density of acetylcholine receptors (AChRs) at the nerve endplate. In rodents, early in embryonic development AChRs are distributed evenly over the muscle surface (Bevan and Steinbach, 1977). By around E13, nerve-independent clustering of receptors occurs in the general region of eventual innervation (Lin et al., 2001). Upon innervation a few days later, the receptor clusters that lie outside of muscle innervation sites disappear and new clustering of receptors is rapidly induced in direct and tight apposition to each nerve endplate (Lin et al., 2001). The receptor clusters continue to morphologically differentiate post-natally, resulting in a dramatic increase in AChR density to >10,000/μm^2 at the mature neuromuscular junction (NMJ) that drops to <10/μm^2 within a few microns of the NMJ boundary (Fambrough, 1979; Fertuck and Salpeter, 1974).

The metabolic stability of AChRs has been extensively studied both in vivo and in vitro. Using conjugates of bungarotoxin (a snake venom that binds specifically and quasi-irreversibly to AChRs), it has been shown that AChR turnover in cultured aneural myotubes is rapid, with a half-life of 7–24 h depending on experimental conditions (Devreotes and Fambrough, 1975; Trinidad and Cohen, 2004; Wang et al., 1999). At innervated and functional adult neuromuscular junctions, however, receptor stability dramatically increases ($t_{1/2}$ ~10–14 days) (Akaaboune et al., 1999; Salpeter and Loring, 1985). What remains unclear is the dynamics of receptors during the early stages of embryonic development and whether these dynamics are modulated by neuronal factors.

A crucial factor that affects AChR clustering during development in vivo is agrin, a nerve-derived heparin sulphate

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proteoglycan that activates both a muscle-specific kinase (MuSK), and rapsyn, an intracellular protein that is found in 1:1 stoichiometry with AChRs (Burden et al., 1983; Sanes and Lichtman, 2001; Sealock et al., 1984; Valenzuela et al., 1995). Indeed, mice deficient in either MuSK or rapsyn fail to form any AChR clusters either during development or on dissociated myotubes (DeChiara et al., 1996; Gautam et al., 1995). Agrin knockout mice, however, do form receptor clusters in the endplate zone by E14.5, but these clusters disappear after failing to stabilize or reform under nerve endplates after innervation (Lin et al., 2001). Based on these observations it was suggested that agrin may act to stabilize AChR clusters rather than initiate their formation.

Since it would be difficult to study receptor dynamics in utero directly, we have used a culture system that produces post-synaptic receptor clusters that are remarkably similar in morphology and maturation to those seen during post-synaptic apparatus development in vivo (Kummer et al., 2004). This culture system was developed by growing myotubes on another factor known to influence receptor clustering in vitro: the nerve-independent extracellular matrix protein laminin (Vogel et al., 1983). In this work we sought to study the effect of agrin on AChR dynamics directly using fluorescence imaging assays, and used the same assay to analyze the effect of laminin per se on the metabolic stability of clustered AChRs. This approach has led to the observation that agrin and laminin do not alter AChR metabolic stability, and it unexpectedly revealed that agrin is able to induce the accumulation of new receptors into new cluster sites while preventing the maintenance of receptor density at preexisting laminin-associated clusters.

Materials and methods

Cell culture

C2C12 myotubes obtained from American Type Cell Culture were grown in DMEM supplemented with 20% fetal bovine serum at 37°C, split into 35-mm culture dishes and differentiated 2 days later at cell confluence by replacing the media with DMEM supplemented with 5% horse serum. Media was then changed every 2 days and cells were imaged 4–6 days after differentiation. For laminin-coated cultures, culture dishes were coated with 5 μg/ml polyornathine (Sigma-Aldrich) in distilled water, air dried and then incubated overnight at 37°C with EHS laminin (10 μg/ml) (Invitrogen) in L-15 media supplemented with 0.5% sodium bicarbonate. Dishes were then aspirated of excess laminin media immediately before plating cells. Laminin-coated plates caused rapid differentiation and growth of muscle cells. Consequently, imaging of AChRs on myotubes grown on laminin substrate was done at time points slightly earlier than those for non-laminin substrate myotubes (3–5 days).

Fluorescence microscopy

Images were taken with a digital CCD camera (Retiga EXi, Burnaby, BS, Canada) using a water immersion objective (20X UAPO 0.7 NA Olympus BW51, Optical Analysis Corporation, NH). Images were captured at 25% light intensity through a neutral density filter (to limit photobleaching effects) on an Olympus51 microscope using IPLab, and images were then quantified using Matlab and converted into figures in Photoshop.

To study the rate of AChR removal from receptor clusters, cells were incubated with α-bungarotoxin conjugated to an Alexa fluorophore (BTX-Alexa 594) (5 μg/ml, 1 h) and washed gently three times in differentiation media; a second dose of BTX-Alexa 488 was added to ensure that all receptors were saturated and then cultures were washed gently three times and imaged at time 0. Residual BTX left in the culture media for the duration of the experiment after time 0 was therefore primarily BTX-Alexa 488 and not BTX-Alexa 594. This ensured that any new receptors that were inserted into the membrane would bind residual BTX-488 and therefore would not contribute to BTX-Alexa 594 fluorescence detected at later time points. From 2 to 8 h later, the same clusters were re-imaged and their fluorescence intensity was assayed using a quantitative fluorescence imaging technique, as described by Turney et al. (1996), with minor modifications. Control myotubes were saturated with BTX-594, imaged and then immediately incubated in BTX-488 until imaging at 8 h. Loss of fluorescence at the clusters on these myotubes was indistinguishable from the loss of fluorescence at tubes subjected to the BTX-488 labeling only immediately before imaging at 8 h. This implies both that the unbinding and re-binding of BTX conjugates does not occur at a measurable rate over 8 h, and that blocking new functional receptors does not affect receptor removal. This last point was supported by the fact that the addition of the sodium channel blocker tetrodotoxin also did not alter the removal of receptors from clusters over the same time period.

To study the rate of AChR insertion into receptor clusters, cells were saturated with BTX-Alexa 594 as described above, washed gently three times and then immediately imaged. Four and/or 8 h later, the same clusters were re-imaged and their fluorescence intensity was assayed, and then a second dose of the same BTX-Alexa 594 was added to saturate the new AChRs inserted at each time point, and fluorescence intensity was again determined. Clusters that had "rolled" over to the edge of the tube as it grew and therefore existed in multiple focal planes, or clusters on myotubes that were dying or shrinking at the later time point were discarded. This did not present a problem on laminin-coated dishes as the clusters in these preparations were large and easy to differentiate from each other, and they always formed at the laminin–muscle cell boundary and therefore were consistently in a single focal plane.

To study the lateral migration of AChRs, cells were saturated with BTX-Alexa 488 and washed gently three times. Immediately after imaging, the fluorescence of single clusters was carefully removed with a 10-mW argon laser emitting at 488 nm and passed through neutral density filters that blocked 87.5% of the laser emission. Photobleaching typically took less than 5 s/cluster. At later time points, the bleached and non-bleached neighboring "sister" clusters were found and re-imaged. The FRAP experiments were done in the presence of either unlabeled BTX or BTX-Alexa 594 to prevent re-binding of photo-unbound BTX-Alexa 488 (Akaaboune et al., 2002).

Agrin treatment

Either 100 ng/ml or 500 ng/ml of C-terminal agrin (R&D Systems, Minneapolis, MN) was added to culture dishes either 16 h prior to initial imaging or immediately before the initial images were taken. After all washing steps, additional agrin was added to maintain the concentration throughout the imaging session.

Laminin treatment

Cell cultures were treated with a soluble laminin (10 μg/ml) immediately before initial imaging and through the imaging period. After all washing steps, additional laminin was added to maintain the concentration throughout the imaging session. Cells were labeled and imaged as describe above.

Results

Substrate laminin does not affect the rate of receptor removal

To estimate the receptor removal rate at individual spontaneous AChR clusters over time, C2C12 myotube cultures were labeled with BTX-Alexa 594 (5 μg/ml, 1 h) and then incubated in BTX-Alexa 488 (5 μg/ml, 10 min) to ensure that saturation was achieved. The fluorescence intensities of clusters were then assayed immediately after saturation

and then 4 and/or 8 h later. 4 h after initial labeling, fluorescent intensity had decreased to 56 ± 19% SD (n = 94 cluster/5 cultures) of original fluorescence, and had decreased further to 38 ± 20% SD (n = 39 clusters/4 cultures) of original fluorescence at 8 h (Figs. 1A and C). We next wanted to determine if receptor removal is altered in clusters found on the surface of myotubes that are grown on a laminin substrate. These clusters show some striking similarities to mature neuromuscular junctions in terms of size, topology and the general morphological development from "plaque" to "open" to "pretzel-like" shapes, as described by Kummer et al., 2004. Further, these clusters form between muscle and dish and so lie in a single focal plane and do not tend to change location as muscles grow or even contract, making these clusters relatively easy to image when labeled with fluorescent molecules. When the fluorescence intensity of labeled receptor clusters was assayed at 4 h, we found that 61 ± 12% SD (n = 92 clusters/4 cultures) of original fluorescence remained, and at 8 h 44 ± 14% SD (n = 71 clusters/4 cultures) of original fluorescence remained (Figs. 1B and C). This loss was not due to the

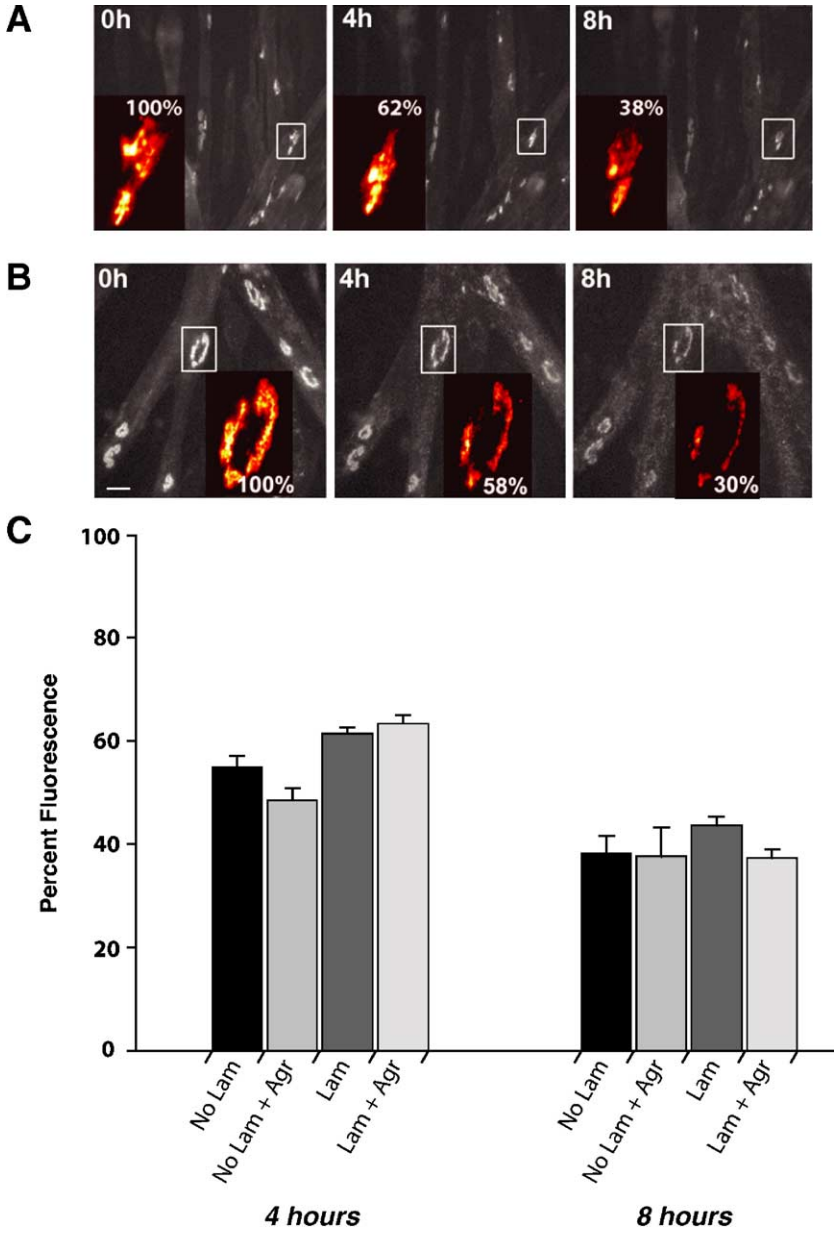

Fig. 1. Receptor removal at single acetylcholine receptor clusters in vitro is unaffected by laminin or agrin. (A) Example of a number of myotubes displaying small, spontaneous AChR clusters. Receptors were labeled once with BTX-Alexa 594 and the loss in fluorescence was assayed at 4 and 8 h. (B) Example of a single fused myotube grown on a laminin substrate displaying multiple laminin-induced AChR clusters and assayed for fluorescent loss as in panel A. Note that these clusters are larger and more topologically complex than the non-laminin clusters in panel A. Scale bar = 20 μm. (C) A histogram summarizing the fluorescence at 4 and 8 h of clusters on myotubes grown in the presence or absence of laminin substrate and in the presence or absence of agrin. Receptors at clusters on the surface of myotubes grown on a laminin substrate show approximately the same stability as receptors at clusters on myotubes lacking substrate. Agrin treatment 16 h prior to or immediately after initial imaging had little effect on the removal rate of clustered receptors on myotubes grown in the presence or absence of a laminin substrate. All plots represent mean ± SEM.

photobleaching of fluorescent bungarotoxin bound to receptors, as the same control cluster imaged several times experienced little if any loss of fluorescence (<1% fluorescence loss at each sequential image). The loss of fluorescence was also not dependent upon spontaneous unbinding of BTX from AChRs, since BTX-labeled receptor complexes that remained bound to the laminin as "ghost" clusters (after the muscle they were originally clustered on had died) did not bind to new BTX-Alexa of a different color added days later. This is in agreement with studies that have shown that the BTX-AChR bond is essentially irreversible (Devreotes and Fambrough, 1975, Sanes and Lichtman, 2001). Therefore, these results suggest that while substrate laminin dramatically alters receptor cluster size and morphology, it does not increase AChR half-life at these clusters.

Since the effect of c-terminal agrin on increasing the number of spontaneous clusters is well documented (Ferns et al., 1993), we wanted to investigate whether the clustering activity of agrin is also involved in altering the rate of receptor removal. To examine this possibility, cultured myotubes were grown either on a laminin substrate or non-coated culture dishes and incubated with c-terminal agrin (100 ng/ml) either the night before imaging ($t = -16$ h) or immediately prior to initial imaging ($t = 0$ h). In both cases, agrin concentration was maintained in the culture through the duration of the experiment and BTX-Alexa labeling was done as detailed above. When we measured receptor loss from spontaneous clusters at 4 h in the presence of agrin, we found that 48 ± 20% SD ($n = 39$ clusters/4 cultures) of the original fluorescence was retained, and that the fluorescence dropped to 38 ± 20% SD ($n = 12$ clusters/3 cultures) at 8 h, which was nearly identical to the rate of fluorescence lost from clusters on myotubes in the absence of agrin (Fig. 1C). In many cases it was difficult to accurately identify or quantify the original clusters at later time points either because clusters often "rolled" over the side of the muscle out of a single focal plane as the myotube grew, or because they fragmented over time. We therefore only used clusters in this study that could be clearly distinguished from neighboring clusters and accurately quantified at 4 and 8 h.

When myotube cultures expressing laminin-induced clusters were treated with c-terminal agrin (hereafter referred to as "agrin", unless otherwise noted) and fluorescence loss was examined at both 4 and 8 h, we found that the agrin had no effect on the rate of receptor removal: at 4 h the fluorescent intensity was 63 ± 13% SD ($n = 74$ clusters/4 cultures) of original fluorescence, and at 8 h fluorescent intensity was 37 ± 14% SD ($n = 74$ clusters/4 cultures) of original fluorescence (Fig. 1C). While agrin did cause clear and dramatic increases in receptor cluster number (consistent with previous studies), these results indicate that agrin does not affect AChR stability at spontaneous clusters or laminin-induced clusters on C2C12 myotubes.

Lateral migration of AChRs

Given that the rate of receptor removal from receptor clusters was nearly identical for myotubes grown in the presence or absence of laminin substrate, and since laminin-induced clusters are larger, easier to identify and lie in the same focal plane, we carried out most of the following experiments only on myotubes grown on laminin substrate. First we were interested to know whether the diffusible AChRs contribute to cluster density. If lateral migration of receptors accounts for an appreciable amount of fluorescence over time then measurements of fluorescence at later time points would not allow an accurate computation of receptor half-life. To determine the contribution of laterally migrating AChRs to cluster density, we saturated all preexisting AChRs on the myotube surface with BTX-Alexa 488 and then used an argon laser to selectively remove the fluorescence from all the receptors at single laminin-induced clusters. In this way, only the preexisting diffusible AChRs on the membrane remained fluorescently labeled. All photobleaching experiments were performed in the presence of unlabeled bungarotoxin or BTX-Alexa 594 to prevent the re-binding of any BTX-Alexa 488 that may have been induced to unbind by the laser (Akaaboune et al., 2002).

Since photobleaching was done on living myotubes, we performed a number of controls to confirm that the argon laser did not have deleterious effects in our experimental design in vitro. We found that myotube growth was normal, and nuclei under photobleached regions appeared healthy, even on myotubes that were completely photobleached over their entire surface. Bleached clusters themselves also appeared to be unaffected by the bleaching process, as the accumulation of new receptors measured with a distinctly colored BTX-Alexa at later time points revealed that the addition of new receptors at bleached clusters was equal to that at non-bleached neighboring "sister" clusters on the surface of the same myotube. Photobleaching therefore had no observable deleterious effect on the muscle cell or bleached cluster. Bleaching of sister clusters by residual laser light was also minimal.

From 2 to 8 h after photobleaching the recovery of fluorescence was monitored. We found that the fluorescence recovery was circumferential around the original cluster area, consistent with a diffusion-trap process, and that bleached clusters gradually gained back ~9% (9.4 ± 4% SD, $n = 49$) of their original fluorescence intensity after 8 h (Fig. 2A). Since previous work indicates that recycling does not occur in cultured myotubes (Gardner and Fambrough, 1979), we assume that these laterally migrating receptors diffuse solely through the membrane surface. After 2, 4 and 6 h, fluorescence recovery was 5.6% (±2 SD, $n = 39$), 9.4% (±4 SD, $n = 23$) and 9.7% (±5 SD, $n = 21$), respectively. These data were fit well by a single exponential curve (Fig. 2B) with $R^2 = 0.98$. When sister clusters were not laser illuminated, 44% of fluorescence remained after 8 h, consistent with our calculations of fluorescence loss at clusters on myotubes not exposed to the laser (Fig. 2A). Since the remaining fluorescence at a non-bleached sister cluster is a combination of both the preexisting labeled receptors and the recovery from laterally migrating receptors, we subtracted out the amount of fluorescence signal due to lateral migration to determine the amount of fluorescence due only to originally clustered receptors (44% − 9% = 35% of receptors remaining after 8 h). After collecting data for

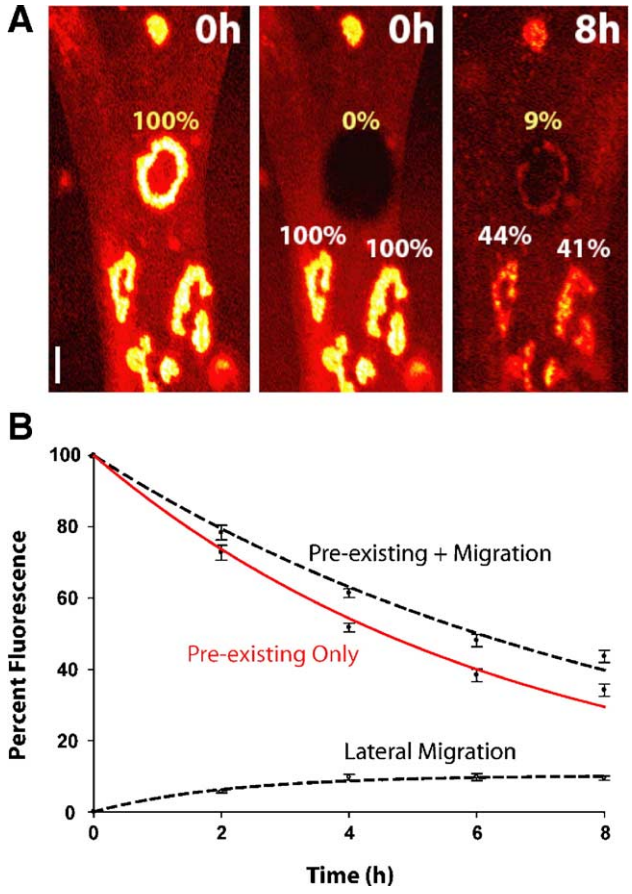

Fig. 2. Contribution of diffusible acetylcholine receptors to laminin-induced clusters. (A) Example of a single myotube grown on laminin substrate that was imaged prior to, immediately after and 8 h after photobleaching. At 8 h the fluorescence recovery at bleached clusters was ~9%, indicating that a significant number of diffusible preexisting labeled receptors had moved into the bleached cluster. "Sister" clusters on the myotube that were not exposed to the laser lost more than half of their fluorescence after 8 h, as expected (see Fig. 1). Scale bar = 20 μm. (B) Data from a number of experiments performed at 2, 4, 6 and 8 h time points. At each time point the average fluorescence due to lateral migration was subtracted from the total average fluorescence remaining to obtain the number of preexisting receptors remaining at a cluster. All data were fit by single exponential curves. All points represent mean ± SEM.

the loss of fluorescence at unbleached clusters at the 2-, 4-, 6- and 8-h time points (78 ± 13% SD, n = 42; 61 ± 11% SD, n = 92; 48 ± 8% SD, n = 20; 44 ± 14% SD, n = 71, respectively), this correction for lateral migration was done at each data point to give a new curve that allowed us to estimate receptor half-life (Fig. 2B). This curve was fit well by a single exponential with R^2 = 0.99. Interestingly, the only point that was slightly elevated compared to the exponential fit was the 8-h time point, which was also the time at which aggregations of intracellular fluorescent puncta became prevalent. Although every effort was made to only image clusters that did not have these puncta near them, it seems likely that this intracellular fluorescence might have contributed to the total cluster fluorescence, thus causing this slight elevation above the actual fluorescence from clustered receptors at this time point. The receptor half-life of 4.5 h derived from this corrected decay is far more rapid than the uncorrected half-life calculated from our experiments that measured fluorescence loss only ($t_{1/2}$ ~7 h), and approximately two times shorter than previous estimates that have ignored lateral migration and trapping of diffusible surface AChRs.

Accumulation of new receptors compensates for removal of old receptors to maintain receptor density at AChR clusters

While the rapid loss of receptors from laminin-induced AChR clusters is partially offset by the trapping of laterally migrating receptors, we wanted to determine whether the accumulation of new receptors at myotube clusters is able to provide enough additional receptors to match receptor removal and maintain receptor density over time. To determine the amount of new receptor accumulation at clusters, myotubes were labeled with BTX-Alexa 594 (5 μg/ml, 1 h), and clusters were imaged immediately. Four hours later, the same clusters were imaged to determine the amount of fluorescence lost, and new BTX-Alexa 594 was then added to the myotube culture to saturate all new receptors that had accumulated at clusters since the initial BTX-Alexa 594 saturation. We found that on myotubes grown on laminin substrate and on myotubes grown without substrate, accumulation of new receptors at existing clusters allowed receptor density to be maintained: laminin-associated clusters were at 101% of original fluorescence at 5 h ± 21% SD (n = 72 clusters/5 cultures) (Figs. 3A and B), and non-laminin clusters were at 95% of original fluorescence at 5 h (±20 SD, n = 22 clusters/3 cultures) (Fig. 3B). These results suggest that the accumulation of new receptors at clusters on myotubes grown on both laminin- and non-laminin substrates is rapid and robust enough to maintain receptor density at individual clusters even as muscle and cluster morphology often change over time. Similar results were obtained at 8 h, see Fig. 6.

Agrin causes new cluster formation and disrupts laminin substrate-associated clusters

Although the experiments above showed that laminin substrate and soluble agrin alone do not alter receptor half-life at individual AChR clusters, laminin and agrin are known to interact directly and indirectly to induce receptor clustering (Denzer et al., 1997; Montanaro et al., 1998; Sugiyama et al., 1997). We therefore wanted to determine if the clustering effect of laminin is affected by the addition of agrin. To do this, myotubes grown on a laminin substrate were saturated with BTX-Alexa 594 (5 μg/ml, 1.5) and exposed to a high dose of agrin (100–500 ng/ml). Either 16 h after or immediately following agrin treatment, initial images for each condition were taken to identify clusters saturated with BTX-Alexa 594 (Fig. 4, "preexisting", pseudo-color). For each condition, 8 h after initial imaging the same myotubes were re-imaged and then incubated with BTX-Alexa 488 to label all new receptors. On control myotubes grown on laminin substrate and not treated with agrin, no new clusters formed over the 8-h time period (Fig. 4A) while old receptors were selectively removed from the inside of developing cluster (Fig. 4A), as previously described by Kummer et al. (2004). Interestingly, on the myotubes incubated with agrin, immediately after initial

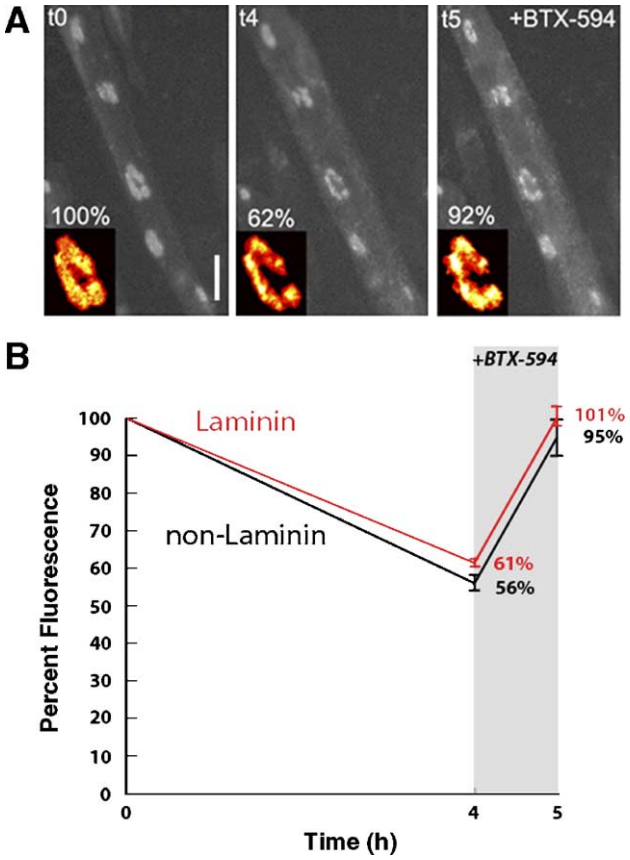

Fig. 3. Accumulation of new receptors at single acetylcholine receptor clusters in vitro. (A) Myotubes grown on a laminin substrate that were saturated with BTX-Alexa 594 immediately before the first image. A sample cluster is shown in the inset that was quantitatively imaged at time 0 and 4 h later. The loss of fluorescence was assayed, and then the cluster was imaged a third time after new BTX-Alexa 594 was added to label new AChRs. Note that even though the overall morphology of laminin-associated clusters can change markedly over 4 h, the receptor density is maintained by the accumulation of new receptors. Scale bar = 20 μm. (B) Summary of data from multiple experiments with both laminin and non-laminin substrates. Note that insertion allows both laminin-associated and non-laminin clusters to maintain receptor density over a 5-h time period. All points represent mean ± SEM.

imaging we found that while the fluorescence from preexisting laminin-induced clusters labeled with BTX-Alexa 594 (red) disappeared over time at a normal rate (37 ± 14% SD, n = 74 clusters/4 cultures fluorescence remaining at 8 h), numerous small clusters formed from prelabeled AChRs on the surface of the myotubes (Fig. 4B, arrowheads). This is consistent with previous studies that have found that agrin-induced receptor aggregation is independent of new protein synthesis (Wallace, 1988). However, new AChRs that were identified with BTX-Alexa 488 (green) were also found to be concentrated at these clusters (Fig. 4B, arrowheads). Cells preincubated in agrin the night before initial imaging displayed many of these small clusters but lacked discernible laminin-induced clusters when imaged the next day (Fig. 4C, time 0 “preexisting”). The agrin-induced clusters, however, were stable over 8 h and no new clusters appeared at 8 h that had not existed at time 0 (Fig. 4C). This implies that the c-terminus of agrin induces either a preferential targeting of new AChRs to agrin-induced clusters over laminin-induced clusters, or that receptors, once inserted randomly into the membrane, are trapped by agrin-induced clusters more effectively than laminin-induced clusters as they diffuse laterally in the membrane.

In order to gain a better temporal resolution of the agrin effect on the redirection of new receptors, we examined the agrin effect over a 3-h time period using time-lapse imaging. Myotubes were grown on a laminin substrate and incubated with agrin at the time of initial imaging, and then re-imaged 1 and 3 h later (Fig. 5A). After only 1 h, small clusters of preexisting receptors labeled with BTX-Alexa 488 could be detected, and these clusters became more defined by 3 h (Fig. 5B, first panels). While some of the clustering of preexisting receptors clearly occurred at places where AChR cluster “seeds” existed at 0 h (arrowheads), others appeared at regions that were not obviously seeded by receptors (arrows). When BTX-Alexa 594 was added to label new receptors, labeling was clearly visible at laminin-induced clusters but was not able to be resolved at the agrin-induced clusters at 1 h. However, when the same myotube was re-imaged again at 3 h after new BTX-Alexa 594 incubation, new receptors were clearly visible at the agrin-induced clusters (Fig. 5B, second panels). These results indicate that the effect of agrin is rapid, occurring within 1 h of incubation. This effect was also specific to agrin, as soluble laminin (10 μg/ml) added in the same manner as agrin failed to induce new cluster formation over the same time period (Fig. 5C).

Effect of agrin on the accumulation of new receptors at agrin-induced and laminin substrate-induced clusters

Having found that agrin does not alter the rate of receptor removal from membrane clusters (see Fig. 1), we wanted to determine whether agrin modulates the accumulation of new AChRs into clusters. To do this we continually labeled all receptors with the same fluorescent BTX conjugate and quantified the amount of fluorescence at new agrin-induced clusters and large distinct laminin substrate clusters over time. Myotubes were saturated with BTX-Alexa 594 and then imaged in the presence of agrin (100 ng/ml). The same clusters were re-imaged 4 and 8 h later after being re-saturated with new BTX-Alexa 594 (5 μg/ml, 1 h) to label any new receptors that had accumulated at clusters over each time period (Fig. 6A). In this way, the full complement of clustered receptors (both preexisting and new) was imaged at each time point. While total fluorescence at 4 and 8 h remained near 100% at laminin-associated myotube clusters in the absence of agrin, we found that the total receptor number at laminin-induced clusters treated with agrin decreased to 89% ±17 SD (n = 71 clusters/3 cultures) of original fluorescence at 4 h and 61% ±23 SD (n = 78 clusters/3 cultures) of original fluorescence at 8 h (Fig. 6B). In addition we found that less than 5% of original fluorescence was recovered after photobleaching at laminin-induced clusters in the presence of agrin compared to approximately 10% in the absence of agrin, indicating that the lateral migration of diffuse preexisting AChRs into preexisting clusters was altered by agrin treatment.

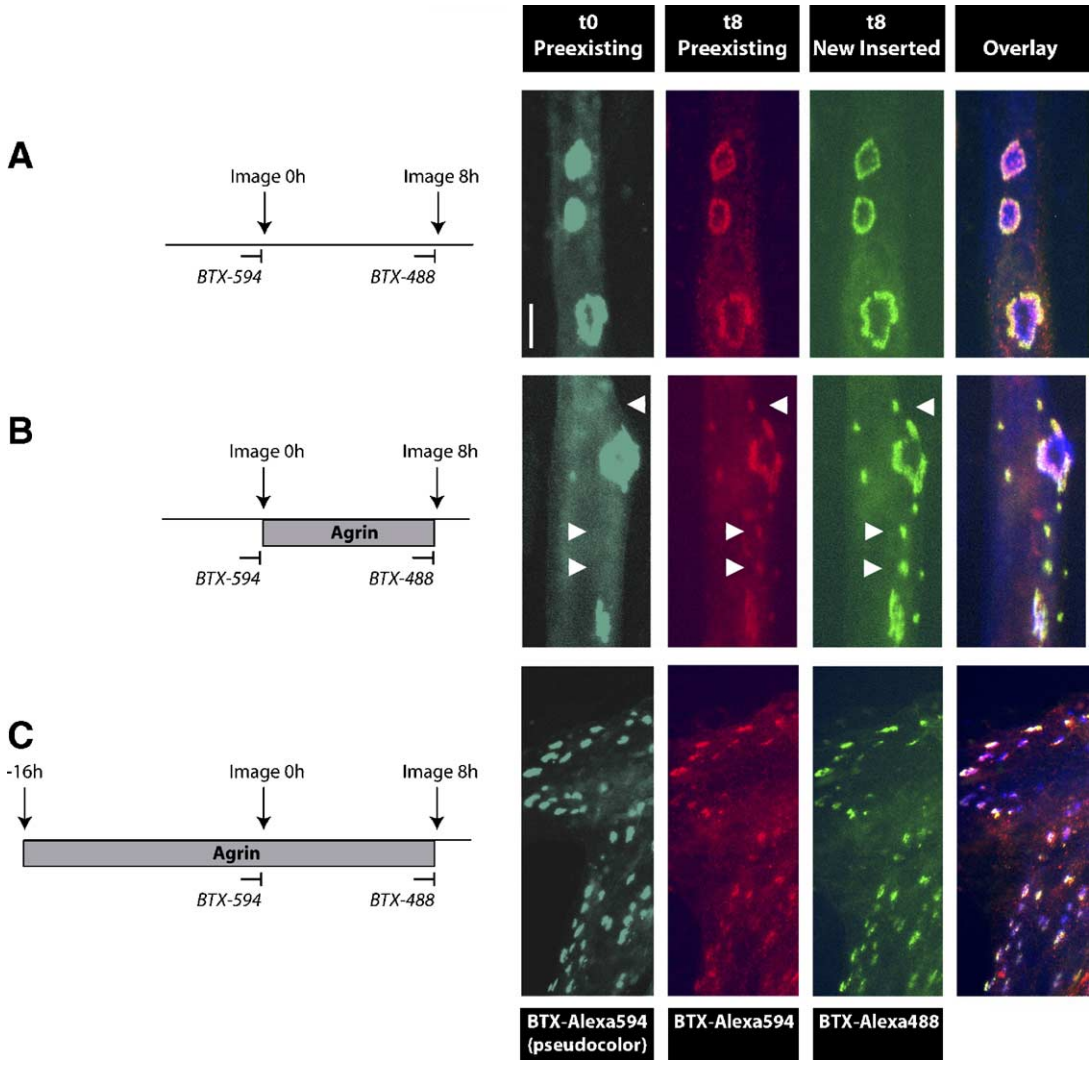

Fig. 4. Agrin decreases the accumulation of new receptors at laminin-associated clusters and induces clustering of preexisting and new receptors at novel cluster sites. (A–C) Labeling schemes and sample images for control myotubes and myotubes incubated with a high dose of agrin either at the time of initial imaging or 16 h prior to initial imaging, and then re-imaged 8 h later. (A) In non-agrin-treated cells, laminin-associated myotubes display large clusters (left panel), typical removal of preexisting receptors (second panel), and a normal pattern of receptor insertion (third panel). Overlay (right panel) reveals that preexisting receptors are preferentially removed from the center of the developing cluster and new receptors accumulate around the entire cluster. (B) When agrin is added to the culture immediately before the first image is taken, preexisting receptors can be seen to aggregate into new clusters after 8 h that did not exist at the time of initial imaging (second panel, arrowheads). New receptors are also preferentially added to these new agrin-induced clusters (third panel, arrowheads). (C) When incubated in agrin for 16 h prior to initial imaging (and then for the duration of the experiment), myotubes are devoid of laminin-induced clusters at time 0 (left panel), the small agrin clusters are maintained over the next 8 h (second panel) and new receptors accumulate only at these preexisting clusters (third panel). Scale bar = 20 μm.

We next wanted to estimate the number of new receptors that were integrated into clusters by lateral migration versus targeted insertion. To do this we first calculated the contribution in original fluorescence of new receptors by subtracting the preexisting receptors remaining at clusters after 8 h from the full complement of receptors (preexisting + new) found at clusters at this time. For example, at 8 h preexisting + new receptors at laminin-associated clusters account for 97% of original fluorescence, and preexisting receptors alone account for 44% of original fluorescence. Therefore, 53% of the original fluorescence is due to new receptors at 8 h. Assuming that <10% of original fluorescence is due to the lateral diffusion of new receptors, >43% must be due to direct insertion of new receptors. However, in agrin-challenged cultures, only 24% of original fluorescence is contributed by new receptors (61–37%) at 8 h. Assuming that <5% of original fluorescence is due to the lateral diffusion of new receptors, direct insertion of new receptors must account for at least 15% of original fluorescence at 8 h. Therefore, while laminin-associated clusters disappear over time and are absent 24 h after agrin treatment, this appears not to be the result of increased receptor removal or cluster fragmentation but rather because the accumulation of new receptors through both diffusion/trap and targeted insertion is decreased markedly within 8 h of agrin treatment, presumably dropping to undetectable levels within 24 h.

Finally, we observed that this agrin effect occurred even if the agrin was removed after imaging at 8 h. Indeed a single 30-min incubation with agrin was sufficient to produce an effect similar to that seen when agrin was used for the duration of the

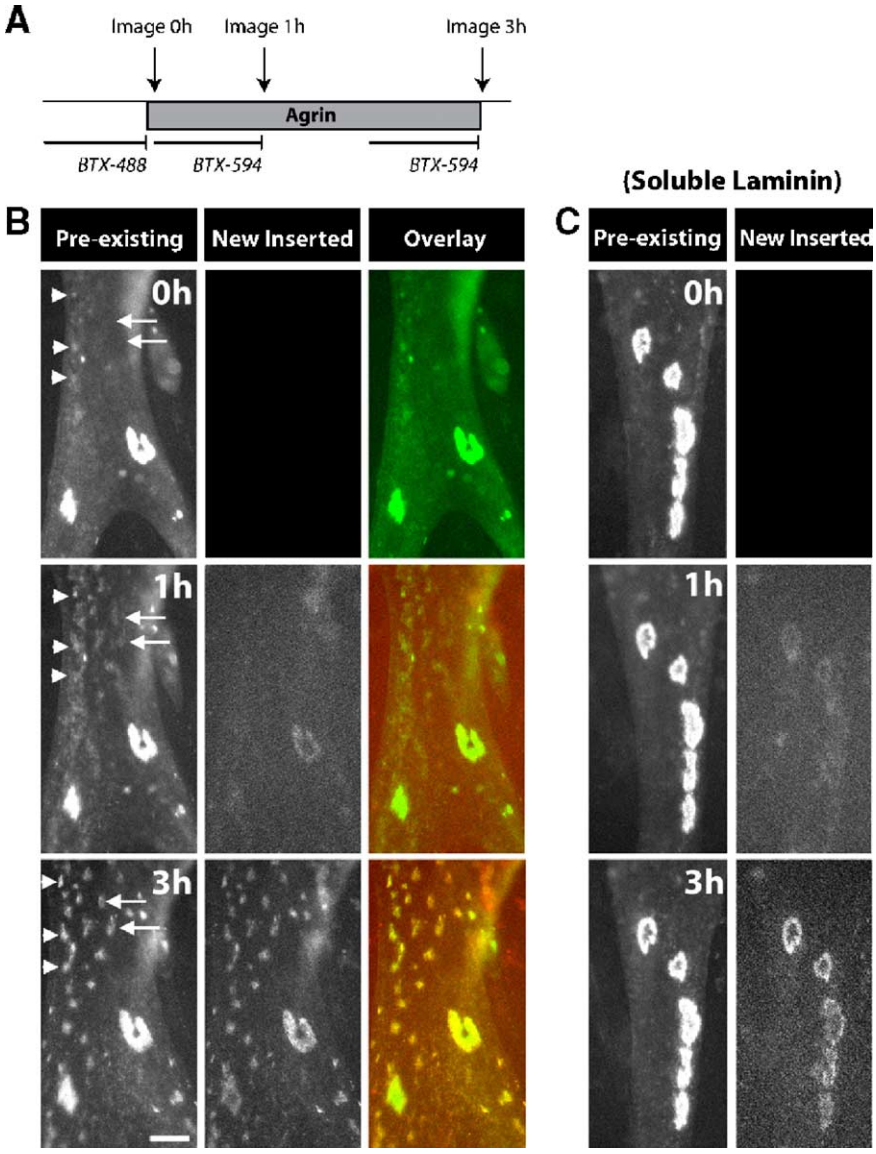

Fig. 5. Time-lapse imaging of agrin-induced cluster formation on the surface of myotubes grown on laminin substrate. (A) Scheme for the BTX-Alexa labeling and agrin treatment of myotubes. (B) Example of a single myotube treated with 100 ng of agrin and imaged 3 times. First panels (left) represent preexisting receptors labeled at time 0 and imaged at 0, 1 and 3 h later. Second panels (from the left) show the same myotube labeled with BTX-Alexa 594 to stain new receptors that have accumulated at the clusters between time 0 and each of the later time points. Third panels are overlays of the first two panels at each time point. Note that by 1 h small receptor aggregates can be detected on the muscle surface and that at 3 h these preexisting receptors have clustered and new receptors are clearly visible and co-localized at these agrin-induced clusters. Arrowheads indicate clusters that formed where cluster "seeds" existed at time 0; arrows indicate clusters that formed at regions on the surface where no receptor cluster seeds could be seen at time 0. (C) When soluble laminin was added instead of agrin to myotubes grown on laminin substrate no new clusters could be detected, indicating that the formation of new clusters is specific to agrin-treatment. Scale bar = 20 μm.

experiment, albeit at a reduced effect, suggesting that a single agrin treatment is sufficient to activate downstream machinery necessary for the redirection of new AChRs and the formation of new clusters.

Discussion

In this work, we used quantitative fluorescence imaging and FRAP techniques to study AChR dynamics at single spontaneous and laminin-induced clusters over time. While receptors on myotubes grown on a laminin substrate aggregate into large, differentiated clusters, we demonstrated that receptor half-life at these clusters was hardly affected, having a $t_{1/2}$ of about 4.5 h. In addition we found that the total number of receptors at individual laminin-induced and non-laminin clusters remained nearly constant over time. Furthermore, we found that c-terminal agrin is not involved in regulating the metabolic stability of receptors, although its critical role in clustering receptors is undisputed. Finally, agrin treatment of laminin-induced clusters resulted in the formation of numerous small agrin clusters on the myotube surface while the accumulation of new receptors at preexisting laminin clusters was significantly decreased.

The metabolic stability of surface AChRs on developing cultured myotubes has been studied previously and these receptors were found to have a $t_{1/2}$ of 7–24 h, depending on cell type and experimental design. Most of these previous

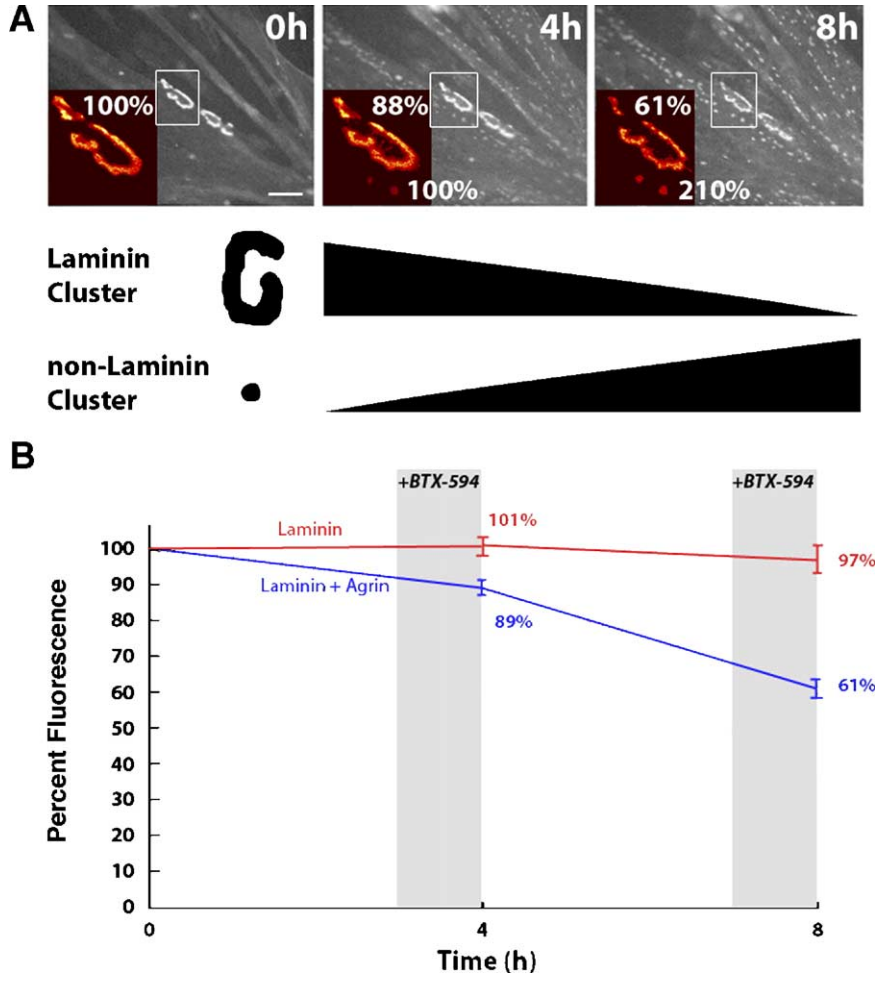

Fig. 6. Agrin treatment decreases the accumulation of new receptors at preexisting laminin-induced clusters and increases insertion of new receptors into agrin-induced clusters, as revealed by quantitative fluorescence imaging. (A) A sample image shows the decrease in total receptor number at laminin-associated clusters and the subsequent appearance and increase in total receptor number at agrin-clusters beginning at 4 h. Scale bar = 20 μm. (B) AChR clusters on myotubes grown on a laminin substrate were saturated with the same fluorescent bungarotoxin three times (before each of the three imaging time points at 0 h, 4 h and 8 h) to ensure that all receptors (both preexisting and new) were quantitatively imaged. This showed that receptor density is maintained at control clusters formed on myotubes grown on laminin substrate over 8 h (red trace). Agrin treatment immediately before initial imaging resulted in a marked decrease in total receptor number at laminin-induced clusters over 8 h (blue trace); since agrin does not increase receptor removal (see Fig. 1), this must be due to a decrease in receptor insertion. At the same time, agrin resulted in a marked increase in new receptor aggregation at agrin-induced clusters that had appeared by 4 h. All points represent mean ± SEM.

studies used radio-labeled bungarotoxin (I^{125}-BTX), estimating the time course of AChR removal by comparing the amount of radioactivity released into the media to the total amount of the radioactivity at the time of initial labeling. However, this method does not distinguish between diffuse and clustered receptors and the estimate of receptor half-life is derived by pooling together all receptors from the entire population of cells in a culture (Miller, 1984; O'Malley et al., 1993). In addition, this technique does not distinguish between the surface AChRs and the receptors that are removed from the membrane but not yet degraded (Devreotes and Fambrough, 1975), potentially inflating the calculations of surface receptor half-life. More recently a few attempts have been made to study the removal of receptors from individual clusters in cultured myotubes using fluorescence assays. These studies have found that the $t_{1/2}$ of removal of clustered receptors ranges from 7 to 20 h (Kim and Nelson, 2000; Trinidad and Cohen, 2004). These studies did not, however, take into account the pool of diffuse receptors that are able to laterally migrate into the receptor clusters over time. In our work, we also used fluorescent bungarotoxin to assay receptor removal, but we used FRAP to determine the amount of lateral migration into individual clusters over time. Before taking into account the contribution of laterally diffusing AChRs to cluster density, we found that receptor half-life was ~7 h, and after correcting for lateral migration we estimated the half-life of AChRs at clusters to be ~4.5 h. This rate is more rapid than previous estimates that have ignored the migration of diffuse AChRs.

Our work also demonstrated that the accumulation of new receptors at preexisting clusters was quite rapid, matching receptor removal to maintain receptor density over time. It further implied that a significant number of these new receptors are targeted directly to the clusters: Since results from our FRAP experiments clearly show that the diffusion of preexisting receptors accounts for <10% of original fluorescence

accumulated at clusters over 8 h, one would assume that new receptors would laterally migrate at a similar rate, also contributing <10% of original fluorescence after 8 h. If this assumption is correct, then a significant number of new AChRs, which account for >50% of original fluorescence after 8 h, must be inserted directly into the preexisting clusters. This result is consistent with previous studies that showed that a significant number of new inserted receptors are targeted directly to clusters (Bursztajn et al., 1985).

One interesting finding demonstrated by this work is that c-terminal agrin is not involved in the maintenance of receptors at either spontaneous or elaborated laminin-induced clusters on myotubes, even when a large dose of agrin (100–500 ng/ml) was added to the cultures. Previously it has been shown that the initiation of receptor clustering occurs in agrin deficient mice, but that these clusters disappear later on which suggests that agrin might have a role in maintaining receptor clusters rather than initiating them (Lin et al., 2001). In transfected denervated muscle too, agrin has been shown to stabilize receptors at ectopic clusters (Bezakova et al., 2001). This could be due to the fact that this study used full-length agrin rather than the c-terminal agrin that was used in the present study. While the c-terminal region of agrin is known to be responsible for the receptor aggregation affect seen in vitro, it is possible that other regions on agrin might alter receptor stability at those clusters.

While some studies hint at the action of agrin on receptor stability in vivo, most of the studies that have investigated the effect of c-terminal agrin on clustering in vitro have quantified clustering by the number of clusters per myotube (Cornish et al., 1999; Gesemann et al., 1995; Nitkin et al., 1987), using an increase in the number of clusters as an indirect indicator of increased AChR "stability" rather than examining agrin's effect on receptor turnover at the clusters themselves. Here, by showing explicitly that c-terminal agrin has no effect on AChR metabolic stability at individual clusters, we provide direct evidence that the clustering region of agrin is not involved in regulating AChR half-life in myotube clusters, suggesting that c-terminal agrin does not act as a stabilizer of metabolic stability in this culture system, but only as a cluster inducer.

Since agrin and laminin are clustering factors known to effect the aggregation of AChRs during development in living animals, an obvious question arises from the current work: does the laminin/agrin interaction observed here potentially shed light on any developmental phenomenon in vivo? While speculative, there are some interesting parallels between our observations and the development of the nerve-endplate region during embryonic development. The initial clustering of AChRs on muscle fibers at the future endplate region is induced by nerve-independent factors. One such factor that is able to induce cluster formation is laminin, which when added in solution to muscle cell cultures does not induce large, complex clusters, but when allowed to form as a substrate on laminin-coated dishes does result in such formation (Kummer et al., 2004; Sugiyama et al., 1997). As laminin exists as a part of the extracellular matrix that forms a layer in between muscle fibers in vivo, it seems plausible that the substrate laminin represents a more likely mimic of endogenous laminin deposition than soluble laminin applied to dissociated muscle cells. As such, laminin becomes a candidate for the factor responsible for the early, nerve-independent clustering observed at ~E14 in mice, as has been suggested (Sugiyama et al., 1997). By E18.5, nerve endplates have contacted the muscle cells and released high local concentrations of agrin. Subsequent to this innervation are the disappearance of extrasynaptic clusters and then the formation of clusters directly under the nerve endplate. While our observations of decreased AChR accumulation in laminin-induced clusters after agrin treatment is consistent with this developmental progression, it must be stressed that the agrin used in our study is c-terminal agrin, which contains the receptor aggregation activity of agrin but lacks the laminin-binding site, so the applicability of the laminin and agrin clustering interactions to in vivo remains highly speculative. Still, it will be interesting to see if these factors do indeed interact during this intriguing developmental transition.

In sum, while agrin appears to induce the formation of multiple new clusters, even while it causes the degradation of existing laminin clusters, it does not contribute to receptor stability at these clusters. Agrin does have a rapid and dramatic effect on clusters that form on myotubes grown on laminin substrate, however, significantly decreasing accumulation into these clusters and eventuating in their dissolution after 24 h, while resulting in the simultaneous induction of numerous small clusters at novel clustering sites. These results therefore support the "induction" but not the "stabilizing" hypothesis of c-terminal agrin action on clustered acetylcholine receptors.

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