Agrin Regulates Rapsyn Interaction with Surface Acetylcholine Receptors, and This Underlies Cytoskeletal Anchoring and Clustering*

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Martijn Moransard‡, Lucia S. Borges§, Raffaella Willmann‡, P. Angelo Marangi‡, Hans Rudolf Brenner‡, Michael J. Ferns‡, and Christian Fuhrer‡

From the ‡Department of Neurochemistry, Brain Research Institute, University of Zürich, CH-8057 Zürich, Switzerland, the §Department of Neurology & Neurosurgery, McGill University, Montreal, Quebec H3A 2T5, Canada, and the ¶Department of Physiology, University of Basel, CH-4051 Basel, Switzerland

The acetylcholine receptor (AChR)-associated protein rapsyn is essential for neuromuscular synapse formation and clustering of AChRs, but its mode of action remains unclear. We have investigated whether agrin, a key nerve-derived synaptogenic factor, influences rapsyn-AChR interactions and how this affects clustering and cytoskeletal linkage of AChRs. By precipitating AChRs and probing for associated rapsyn, we found that in denervated diaphragm rapsyn associates with synaptic as well as with extrasynaptic AChRs showing that rapsyn interacts with unclustered AChRs in vivo. Interestingly, synaptic AChRs are associated with more rapsyn suggesting that clustering of AChRs may require increased interaction with rapsyn. In similar experiments in cultured myotubes, rapsyn interacted with intracellular AChRs and with unclustered AChRs at the cell surface, although surface interactions are much more prominent. Remarkably, agrin induces recruitment of additional rapsyn to surface AChRs and clustering of AChRs independently of the secretory pathway. This agrin-induced increase in rapsyn-AChR interaction strongly correlates with clustering, because staurosporine and herbimycin blocked both the increase and clustering. Conversely, laminin and calcium induced both increased rapsyn-AChR interaction and AChR clustering. Finally, time course experiments revealed that the agrin-induced increase occurs with AChRs that become cytoskeletonally linked, and that this precedes receptor clustering. Thus, we propose that neural agrin controls postsynaptic aggregation of the AChR by enhancing rapsyn interaction with surface AChRs and inducing cytoskeletal anchoring and that this is an important precursor step for AChR clustering.

Clustering of neurotransmitter receptors in the postsynaptic membrane is a fundamental aspect of synapses and is thought to originate from receptor interactions with scaffolding proteins that mediate binding to the cytoskeleton. At the neuromuscular junction (NMJ),¹ the 43-kDa scaffolding protein rapsyn plays a pivotal role in clustering of acetylcholine receptors (AChRs). This is best illustrated by rapsyn –/– mice, which lack differentiated NMJs and fail to cluster both AChRs and cytoskeleton-interacting components such as utrophin and dystroglycan (1). Furthermore, mutations in rapsyn can lead to congenital myasthenic syndrome in humans and gradual loss of synaptic AChRs (2).

Rapsyn’s mode of action remains unclear, although rapsyn is sufficient to drive clustering of AChRs upon expression in heterologous cells. In these cells rapsyn forms aggregates in the absence of AChRs and, upon coexpression, colocalizes with AChRs, MuSK, and β-dystroglycan in clusters (3–6). These observations have led to the concept that rapsyn recruits AChRs into cytoskeleton-bound clusters, and that rapsyn interacts with AChRs only in clusters (reviewed in Refs. 7 and 8).

In muscle cells, however, several observations indicate that AChR aggregation is a precisely regulated process. First, AChR clustering in muscle is regulated by the motor nerve in a process that requires neural agrin (9). Agrin acts via the receptor tyrosine kinase MuSK, and initiates a signaling mechanism that leads to rapsyn-dependent AChR clustering (13, 14). Some spontaneous AChR clusters are still observed in aneural muscle in vivo (10, 12) and in cultured myotubes in vitro, however (11). Rapsyn colocalizes with these spontaneous clusters in myotubes (11) and, consistent with this, can also be detected in complexes with the AChR (15). Secondly, unlike in heterologous cells, rapsyn does not cluster in the absence of AChRs in muscle but needs some form of association with the AChR in order to form aggregates (16, 17). Thirdly, in myotubes, the relative expression levels of rapsyn and AChRs are critical parameters for clustering. Transfected myotubes that slightly overexpress rapsyn form more (although smaller) AChR clusters, whereas strong overexpression of rapsyn abolishes clustering (18, 19). Although these studies establish the importance of the general rapsyn to AChR expression ratio, they do not address the ratio and regulation of rapsyn-AChR interaction and the possible importance of this in receptor clustering.

Regulation of AChR-rapsyn interactions could involve secretory mechanisms, because in Torpedo electric organ, rapsyn and AChRs are co-transported in post-Golgi vesicles suggesting that the secretory pathway may deliver rapsyn with AChRs to the plasma membrane (20). It remains unknown, however, whether rapsyn actually interacts with these intracellular AChRs and whether the post-Golgi vesicles deliver rapsyn and

¹The abbreviations used are: NMJ, neuromuscular junction; AChR, acetylcholine receptor; α-BT, α-bungarotoxin, TGN, trans-Golgi network; NGS, normal goat serum; mAb, monoclonal antibody.
AChRs directly into the postsynaptic membrane or rather into extrasynaptic membrane areas.

Thus, it is currently unclear where rapasin interacts with AChRs within developing mammalian muscle, and whether rapasn binds only to clustered receptors or also to unclustered surface AChRs. It is further unclear how rapasin-AChR interactions are modulated by agrin in the process of clustering and how such interactions may contribute to the formation of the postsynaptic density.

We have therefore investigated the interaction of rapasin with unclustered AChRs at the muscle surface and its regulation by agrin. We find that rapasin associates with extrasynaptic, unclustered surface AChRs, that synaptic AChRs are associated with more rapasin, and that these interactions are much more prominent than intracellular interactions. Interestingly, we find that agrin increases the amount of rapasin associated with surface AChRs in myotubes in vitro. The agrin-induced increase in the rapasin-AChR interaction requires tyrosine kinase activity, occurs independently of the secretory pathway, parallels the rapid cytoskeletal linkage of AChRs, and correlates highly with the subsequent clustering of the AChR. Thus, our data suggest that the enhanced interaction of rapasin with surface AChRs is a crucial precursor step in the clustering process triggered by agrin.

EXPERIMENTAL PROCEDURES

Cell Cultures—C2 (C2C12), S26, S27, and Sol8 cell lines were propagated and fused as previously described (21, 22). Constructs encoding the C-terminal half of neural and muscle agrin isoforms (C-Ag12,4,8 and C-Ag12,0,0,0, respectively) were expressed in COS cells as described earlier (23). To achieve the 20 °C block, myotubes were shifted to Dulbecco's modified Eagle's medium containing 20 mM Hepes, pH 7.5, and incubated at 20 °C either in a cooled water bath or in a cooling incubator. To inhibit tyrosine kinases, C2 myotubes were preincubated for 5 h with 15 μM herbimycin A, followed by agrin treatment in the presence of inhibitors (23).

Rapsyn Antibodies—Two novel antibodies, Rap1 and Rap2, were produced by Research Genetics (Huntsville, AL) in rabbits against the C-terminal half of neural and muscle agrin isoforms (C-Ag12,4,8, and C-Ag12,0,0, respectively) were expressed in COS cells as described earlier (23). To achieve the 20 °C block, myotubes were shifted to Dulbecco's modified Eagle's medium containing 20 mM Hepes, pH 7.5, and incubated at 20 °C either in a cooled water bath or in a cooling incubator. To inhibit tyrosine kinases, C2 myotubes were preincubated for 5 h with 15 μM herbimycin A, followed by agrin treatment in the presence of inhibitors (23).

Preparation of Diaphragms—Diaphragms were removed from decapitated adult Wistar rats of at least 150 g body weight. For denervation, rats (6 in total) were anesthetized with isoflurane and the left phrenic nerve was severed. The left hemidiaphragm was cut 5–6 days later, diaphragms were removed. From both denervated and control animals, synaptic areas of the left hemidiaphragm were microscopically dissected from extrasynaptic areas, by observing the location of the nerve and by using AChEesterase staining of parallel control samples. From each preparation a control piece (4–5 mm wide) was analyzed by serial diaphragm sections (Fig. 2 and data not shown). The reactivities of Rap1 and Rap2 in immunoblots allowed detection with higher sensitivity than antibodies 5943P and mAb1254, well characterized rapsyn antibodies that we obtained from Dr. J. S. Sanes (Washington University, St. Louis, MO) and Dr. S. C. Froehner (University of Washington, Seattle, respectively).

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Rapsyn Antibodies—Two novel antibodies, Rap1 and Rap2, were produced by Research Genetics (Huntsville, AL) in rabbits against peptides corresponding to amino acids 133–153 (Rap1) and to the C-terminus of rapsyn (Rap2). These antibodies were affinity-purified with immobilized amino acids 133–153 and 139–153, respectively and used in immunoblotting and immunofluorescence microscopy.

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were reprobed with AChR/H9252 recognize a single band corresponding to rapsyn. Serotypes (or affinity-purified antibodies (Fig. 1a)) or biotin-labeled antibodies (Fig. 1b) or biotin-labeled antibodies were analyzed by immunoblotting using Rap1 and Rap2 crude sera. Both antibodies, but not their preimmune sera, precipitated AChRs (Fig. 1a; data for Rap2 not shown). Blots were reprobed with AChR β-subunit-specific antisera (lower panels). Rap1 specifically detects AChR-bound rapsyn. lysates from C2 myotubes were analyzed by immunoblotting using Rap1 and Rap2 crude sera (ser) or affinity-purified antibodies (pur). Purified Rap1 and Rap2 recognize a single band corresponding to rapsyn.

Rap2 antisera. Both antibodies, but not their preimmune sera, identified a single band corresponding to rapsyn that was co-precipitated with AChRs (Fig. 1a; data for Rap2 not shown). Precipitation of the AChR was confirmed by reprobing the blots with antibodies against the AChR β-subunit (Fig. 1a, bottom panel). In addition, Western blotting with Rap1 antisera, after precipitating AChRs with an antibody against the AChR α-subunit (mAbAb35), identified a similar prominent band corresponding to rapsyn (Fig. 4e). Immunoblot analysis of C2 lysates without AChR precipitation showed that affinity-purified Rap1 and Rap2, but not the crude sera, identify a single band at the molecular weight of rapsyn (Fig. 1b).

Thus, Rap1 and Rap2 antibodies allow specific and efficient detection, by immunoblotting, of AChR-associated rapsyn and total cellular rapsyn in cultured myotubes. The sensitivity of this detection appears much higher than in our previous studies using other rapsyn antibodies (15), enabling us now to detect subtle changes in the rapsyn-AChR interaction.

**Synaptic AChRs Interact with More Rapsyn than Extrasynaptic AChRs in Vivo**—Rapsyn interacts with the receptor in cultured myotubes not treated with agrin (Fig. 1 and Ref. 15), but it is unclear whether this association occurs only with AChR in spontaneous clusters or also with unclustered AChR (15). To investigate this question, we first studied rapsyn-AChR interaction in extrasynaptic versus synaptic areas of diaphragms of adult rats. Extrasynaptic and synaptic areas were microscopically dissected and divided into two parts. The smaller control parts were cryostat sectioned and serial transverse or longitudinal sections throughout the piece of tissue were double labeled for AChRs and rapsyn using the affinity-purified Rap2 anti-rapsyn antiserum. This showed that synaptic preparations are rich in co-localizing clusters of AChRs and rapsyn (Fig. 2a). Extrasynaptic preparations, in contrast, were completely devoid of clustered AChRs and rapsyn along the whole length of these preparations, stretching from the medial to the lateral edges of the diaphragm muscle (Fig. 2a).

The major parts of the synaptic and extrasynaptic preparations were lysed and AChRs precipitated using biotin-α-BT. Immunoblotting with Rap1 revealed that rapsyn interacts with synaptic AChRs (Fig. 2c). The low amounts of extrasynaptic AChR and rapsyn (see Fig. 2b) prohibited assessment of a possible interaction of the two proteins in these regions. Therefore, we denervated the left hemi-diaphragm, leaving the right hemi-diaphragm intact. Western blot analysis 5 days after denervation showed that rapsyn and AChRs were up-regulated in lysates made from extrasynaptic areas of the denervated left hemi-diaphragm but not in lysates from extrasynaptic innervated diaphragm (Fig. 2b). The extent of up-regulation was higher for AChRs than for rapsyn, in agreement with the different degree of up-regulation of their respective mRNAs after denervation (24). This up-regulation, however, was not sufficient to allow immunohistochemical detection of AChRs and rapsyn in extrasynaptic areas of denervated hemi-diaphragm (data not shown). Importantly, clusters of AChRs and rapsyn were never observed in these denervated extrasynaptic preparations, either. In fact, immunostainings for rapsyn and AChRs of denervated extrasynaptic areas yielded results indistinguishable from those of innervated extrasynaptic areas as shown in Fig. 2a.

Precipitation of total AChRs from these denervated diaphragms showed that rapsyn was associated with AChRs in both extrasynaptic and synaptic areas (Fig. 2d). Interestingly, significantly more rapsyn (73.9 ± 2.8% more) was co-precipitated with AChRs from synaptic than from extrasynaptic areas, although the amounts of isolated AChR were equal (Fig. 2e). These data demonstrate that rapsyn interacts with unclustered AChRs in extrasynaptic areas of the diaphragm muscle in vivo. However, synaptic AChR-rapsyn interactions are more pronounced, because AChRs isolated from muscle areas that contain synaptically clustered AChRs are bound to more rapsyn.

**Rapsyn Interacts with Unclustered Surface AChRs in Cultured Myotubes**—The observed interaction of rapsyn with extrasynaptic AChRs in vivo may be due to interactions of rapsyn with intracellular AChRs and/or unclustered receptors at the cell surface. To investigate this question, we first tested whether rapsyn interacts with unclustered AChRs at the cell surface using myotube cultures of non-agrin-treated C2 cells, as well as two proteoglycan mutants derived from C2, S27, and S26. In contrast to C2, S27 and S26 cells completely lack spontaneous clusters of rapsyn and AChRs, and these proteins are therefore distributed in a diffuse, unclustered manner in these two cell lines (Fig. 3a and Ref. 11). We isolated total AChRs from myotube extracts using α-BT, and then immunoblotted for bound rapsyn. We found that comparable amounts of rapsyn were co-precipitated with receptors in non-agrin-treated C2, S26, and S27 cells (Fig. 3, b and c). These results demonstrate that rapsyn interacts with unclustered AChRs in cultured myotubes.
Second, we tested whether rapsyn interacts with intracellular and/or surface AChRs. For this purpose, we separately precipitated either surface or intracellular AChRs (22), and analyzed bound rapsyn by Rap1-immunoblotting. In order to precipitate surface AChRs, intact myotube cultures were incubated with biotinylated α-BT. After washing and lysis of the cells, surface AChRs were isolated by adding streptavidin-agarose. Intracellular AChRs, in contrast, were isolated with biotinylated α-BT and streptavidin-agarose after surface AChRs were first blocked with free α-BT. In each case, precipitated AChRs were detected by immunoblotting for the AChR β-subunit, and AChR-bound rapsyn was detected using Rap1 antibodies. Probing for the AChR revealed that the majority of the receptor is located at the plasma membrane with only a relatively small fraction of all AChRs located intracellularly. Furthermore, the surface and intracellular AChRs pools were additive to yield total AChRs (data not shown).

We next found that, although rapsyn co-precipitated with both surface and intracellular AChRs, it was preferentially associated with the surface receptor pool. Thus in C2, the size of the intracellular AChR pool is ~40% of the surface receptor pool, but the amount of rapsyn bound to intracellular AChRs is only ~10% of the amount of rapsyn bound to surface AChRs (Fig. 3, d and e). S27 yielded similar results (Fig. 3, d and e). Rapsyn therefore interacts with intracellular receptors, but the average amount of rapsyn bound per AChR is much lower intracellularly than at the surface.

Taken together, these findings indicate that rapsyn predominately associates with surface AChRs. In addition, the S27 data indicate that rapsyn interacts efficiently with unclustered AChRs. Since the level of rapsyn-AChR interaction is very similar for S27 and C2, this suggests that in non-agrin-treated C2, the majority of rapsyn-AChR-complexes are unclustered.

**Neural Agrin Increases the Interaction of Rapsyn with the AChR, and This Precedes and Correlates with Clustering**—We show that, in diaphragm, synaptic AChRs interact with more rapsyn than unclustered extrasynaptic AChRs (Fig. 2). To investigate whether this difference is caused by neurally derived agrin, which is concentrated at NMJs (25), we treated C2 myotubes with recombinant neural agrin and determined the amount of rapsyn co-precipitated with total AChRs by Western blotting with the Rap1 antiserum. Neural agrin indeed caused an increase in the amount of rapsyn bound to AChRs (Fig. 4a).

A similar increase in the rapsyn-AChR interaction was seen
Fig. 3. Intracellular and unclustered AChRs at the surface of myotubes interact with rapsyn. a, double labeling with rhodamine-α-BT and rapsyn antibodies (mAb1234) shows that spontaneous clusters of AChRs and rapsyn are absent in S27 but present in C2 myotubes. Photographic exposure times for S27 were 3-fold longer than for C2. Scale bar, 20 μm. b and c, α-BT-Sepharose precipitation of total AChRs, followed by rapsyn (5943p antibodies) and AChR immunoblotting, shows that rapsyn is equally associated with AChRs in C2, S26, and S27 myotubes. As controls, 10 μM free α-BT was added (+T), and a fraction of the starting lysate was analyzed (L). Quantitation represents mean ± S.E. of six experiments; values for S26 and S27 were combined. d, surface and intracellular AChRs were isolated from C2 using biotin-α-BT, followed by rapsyn and AChR β immunoblotting; one-fifth of the surface precipitation was loaded to allow comparison. e, for quantitation of blots as in d, surface AChRs and of rapsyn bound to surface AChRs were set to 100%, and intracellular values were calculated accordingly. Data represent mean ± S.D. of four experiments. * differ significantly from intracellular AChRs (p < 0.05 by two-tailed Student’s paired t test), indicating that rapsyn associates to a higher degree with surface than with intracellular AChRs.

Agrin Regulates Rapsyn-AChR Interactions

when α-BT-precipitated total AChRs were probed with another rapsyn antibody (5943p) to detect AChR-bound rapsyn (Fig. 4b). Furthermore, isolation of total AChRs by immunoprecipitation with an antibody against the AChR α-subunit (mAb35), followed by Rap1 immunoblotting, revealed a comparable agrin-induced increase in the rapsyn-AChR interaction (Fig. 4c). Finally, this increase is specific for neural agrin since treatment with muscle agrin isoforms had no effect (Fig. 4d).

We next quantitated this increased interaction in response to neural agrin and determined its dose-dependence and time course. Treatment of C2 myotubes with a low concentration (100 pm) of neural agrin for 1 h was sufficient to cause a maximal increase, ~70%, in the amount of rapsyn bound to total AChRs (Fig. 5a). In time course experiments, this increase occurred rapidly, reached its maximum within 40 min of neural agrin treatment and stayed elevated thereafter (Fig. 5b). Taken together, these data show that neural agrin rapidly increases the amount of rapsyn associated with AChRs, and that this precedes clustering of the AChR which is first detectable after ~4 h of agrin treatment (26).

To investigate the significance of the increase in rapsyn-AChR interaction for clustering of the AChR, we treated C2 myotube cultures with herbimycin and staurosporine, two tyrosine kinase inhibitors that prevent agrin-induced AChR clustering (26). Assessment of the amount of rapsyn co-precipitated with total AChRs revealed that both inhibitors attenuated the agrin-induced increase in rapsyn-AChR association without affecting the pre-existing interaction (Fig. 6a). In addition, we used S27 myotubes, which are not able to form AChR clusters in response to even high concentrations of agrin (26). In these cells, agrin did not affect rapsyn-AChR association, not even at 50 nM (Fig. 6b and data not shown).

If the increase in rapsyn-AChR interaction is required for clustering, one can expect that induction of clustering by alternative agents and pathways will induce a similar increase in the interaction. Therefore, we treated C2 myotubes with Ca2+ or laminin-1. These treatments induce AChR aggregation in myotubes although through different mechanisms because Ca2+ causes phosphorylation of MuSK while laminin does not (27–29). Lammin, nevertheless, requires rapsyn and the activity of downstream tyrosine kinases to induce AChR clustering in myotubes (30). In our experiments, both Ca2+ and laminin elicited a clear increase in the amount of rapsyn co-precipitated with total AChR, and this increase was comparable to the increase triggered by agrin (Fig. 6c).

These results demonstrate a strong correlation between increased rapsyn-AChR interaction and clustering of these proteins, suggesting that this is a common required precursor step in formation of AChR clusters in muscle.

Agrin-induced Increase in Rapsyn-AChR Interaction Occurs at the Cell Surface and Is Independent of the Secretory Pathway—Since rapsyn interacts predominantly with AChRs at the surface, we next examined whether the agrin-induced increase in interaction occurs with surface AChRs. Agrin treatment of C2 myotubes followed by selective precipitation of surface AChRs revealed that agrin indeed increases the interaction of rapsyn with surface AChRs (Fig. 7b). The extent of this increase was identical to the increase in association of rapsyn with total AChRs (see Fig. 5). Furthermore, precipitating intracellular AChRs after agrin treatment did not reveal an increase in rapsyn-intracellular AChR interaction showing that agrin specifically increases the association of rapsyn with
surface AChRs (data not shown). It has been proposed that rapsyn and AChRs are co-trans-
ported through the secretory pathway to the postsynaptic apparatus in Torpedo electrocytes (20, 31). This, together with our observed intracellular rapsyn-AChR complexes (Fig. 3), raised the question of whether the secretory pathway may contribute to the agrin-induced increase in rapsyn-surface AChR interaction and to AChR clustering. To assess the role of the secretory pathway in these processes, we first established the effect of 20 °C incubation on AChR surface delivery. This temperature selectively blocks formation of post-Golgi transport vesicles, results in accumulation of newly made plasma membrane proteins in the trans-Golgi network (TGN) and therefore prevents surface delivery of AChRs (32). C2 myotubes were incubated with free α-BT to block pre-existing surface AChRs, followed by biotin-α-BT to identify newly inserted surface receptors (Fig. 7a). At 37 °C new AChRs were readily detected while at 20 °C insertion of receptors was inhibited (Fig. 7a, lane 1), even when this temperature was only applied during the biotin-α-BT incubation. These data show that a 1-h incubation at 20 °C is sufficient to block transport of the AChR from the TGN to the surface in our myotube cultures. This temperature is thus a useful tool to specifically block the secretory pathway at the level of the TGN.

We found that application of agrin at 20 °C still increased the interaction of rapsyn with AChRs in C2 myotubes. a and b, total AChRs were precipitated from myotube cultures treated with neural agrin (0.25 nM) for 1 h or from control cultures. AChR-bound rapsyn was visualized by immunoblotting with Rap1 (a) or 5943p (b) followed by reprobing with AChR β-subunit-specific antisera. c, alternatively, total AChRs were immunoprecipitated with antibodies against the AChR α-subunit (mAb35) followed by immunoblotting with Rap1 and reprobing with AChR δ/γ-subunit-specific antisera (mAb 88b). As controls, the mAb35 antibody was omitted from the precipitation (No Ab) or the myotube lysate was omitted (LB). The amount of rapsyn per AChR in neural agrin-treated cultures is higher than in control cultures. d, muscle agrin (0.25 nM) was applied overnight, followed by the same procedure as in a. Muscle agrin does not increase the rapsyn-AChR interaction. Data represent mean ± S.D. of at least three experiments.

Fig. 4. Neural agrin increases the interaction of rapsyn with AChRs in C2 myotubes. a and b, total AChRs were precipitated from myotube cultures treated with neural agrin (0.25 nM) for 1 h or from control cultures. AChR-bound rapsyn was visualized by immunoblotting with Rap1 (a) or 5943p (b) followed by reprobing with AChR β-subunit-specific antisera. c, alternatively, total AChRs were immunoprecipitated with antibodies against the AChR α-subunit (mAb35) followed by immunoblotting with Rap1 and reprobing with AChR δ/γ-subunit-specific antisera (mAb 88b). As controls, the mAb35 antibody was omitted from the precipitation (No Ab) or the myotube lysate was omitted (LB). The amount of rapsyn per AChR in neural agrin-treated cultures is higher than in control cultures. d, muscle agrin (0.25 nM) was applied overnight, followed by the same procedure as in a. Muscle agrin does not increase the rapsyn-AChR interaction. Data represent mean ± S.D. of at least three experiments.

Fig. 5. Dose and time-dependence of the agrin-induced increase in rapsyn-AChR interaction. a, myotubes were treated with increasing concentrations of agrin (neural isoform) for 1 h. Precipitation of total AChRs was followed by rapsyn and AChR immunoblotting. The amount of rapsyn bound to total AChRs was quantitated (lower panel). 100 psf of agrin is sufficient to elicit a ~70% increase in the rapsyn-receptor interaction. b, neural agrin (0.25 nM) was applied for increasing times, and AChR-bound rapsyn was analyzed as in a. Neural agrin causes maximal increase in rapsyn-receptor interaction within 40 min. This increase does not decline thereafter. Data represent mean ± S.D. of at least three experiments. Significant differences from controls: *, p < 0.05; **, p < 0.01, by two-tailed Student’s paired t test.
since only exocytosis but not endocytosis is blocked at 20 °C.

These data demonstrate that the secretory pathway is not required for agrin-induced clustering of pre-existing surface AChRs, and that the events critical for clustering thus occur at the cell surface. Together, the results show that agrin causes an increase in the interaction of rapsyn with surface AChRs independently of the secretory pathway, and this increase in itself appears sufficient for clustering in the absence of secretion.

**Rapsyn Interaction Correlates with Linkage of the AChR to the Cytoskeleton**—In the clustering process triggered by agrin, AChRs become linked to the cytoskeleton (33). We analyzed whether the increased rapsyn-AChR interaction following agrin treatment might mediate this anchoring. In cultured myotubes, cytoskeletal linkage of AChRs can be assayed by a differential extraction procedure in which AChRs are first extracted in low detergent buffer, followed by re-extraction of insoluble receptors in a higher detergent buffer. Surface AChRs are then precipitated separately from these extractions using biotin-α-BT (22). In cultured SO8 myotubes, 1-h agrin treatment induced a decrease in the amount of surface AChR that was extracted in low detergent, with more receptor being shifted to the less extractable, cytoskeletal-associated fraction (Fig. 8, a and b). Strikingly, rapsyn was predominantly associ-

![Fig. 6. Increased rapsyn-AChR interaction correlates with clustering.](image)

**Fig. 6.** Increased rapsyn-AChR interaction correlates with clustering. Total AChRs were precipitated from C2 myotubes, and receptor-bound rapsyn detected by Rap1-immunoblotting. a, herbinycin (Herb) and staurosporine (Stau) inhibit the agrin-induced increase in rapsyn-AChR association in C2 cells (0.25 nM agrin for 1 h). b, in S27 myotubes, 0.25 nM agrin does not affect rapsyn-AChR interaction. c, increased rapsyn-receptor associations are observed after treatment of C2 with Ca2⁺ (5 mM overnight) or laminin (100 mM overnight, Lm), conditions that induce AChR clustering (not shown). Data are mean ± S.D. from at least three experiments. Significant differences from controls: *, p < 0.05; **, p < 0.01; by two-tailed Student’s paired t test.

Figure 7 shows agrin increases the interaction of rapsyn with surface AChRs and causes AChR clustering independently of the secretory pathway. a, C2 myotubes were incubated for 1 h at 20 °C or 37 °C with free α-BT (Tx), followed by 1 h with biotin-α-BT (biotin Tx) at the indicated temperature. Surface AChRs were isolated with streptavidin-Sepharose and visualized by AChR β immunoblotting. At 20 °C, surface transport of newly synthesized AChRs is blocked within 1 h. b, C2 myotubes were incubated with 0.25 nM agrin at 37 °C or 20 °C for the indicated times; agrin was added 1 h after the shift to 20 °C. Surface AChRs were isolated, subjected to rapsyn and AChR-immunoblotting, and rapsyn bound to AChRs quantified, using untreated cells (black bar) as controls. Agrin increases this interaction at the surface, even when surface delivery of AChRs is blocked by shifting the myotubes to 20 °C. c, C2 myotubes were treated with 0.25 nM agrin for 20 h at 20 °C or 37 °C, and AChRs were visualized with rhodamine-α-BT. d, the average number of AChR clusters per myotube was determined by counting clusters (as shown in c) in 20 visual fields per treatment. Agrin increases the number of AChR clusters significantly even at 20 °C, although these clusters have a thinner and less bright appearance. Data represent mean ± S.D. from at least three experiments. Significant difference from control: *, p < 0.05; **, p < 0.01; ***, p < 0.001; by two-tailed Student’s paired t test.

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**Fig. 7.** Agrin increases the interaction of rapsyn with surface AChRs and causes AChR clustering independently of the secretory pathway. a, C2 myotubes were incubated for 1 h at 20 °C or 37 °C with free α-BT (Tx), followed by 1 h with biotin-α-BT (biotin Tx) at the indicated temperature. Surface AChRs were isolated with streptavidin-Sepharose and visualized by AChR β immunoblotting. At 20 °C, surface transport of newly synthesized AChRs is blocked within 1 h. b, C2 myotubes were incubated with 0.25 nM agrin at 37 °C or 20 °C for the indicated times; agrin was added 1 h after the shift to 20 °C. Surface AChRs were isolated, subjected to rapsyn and AChR-immunoblotting, and rapsyn bound to AChRs quantified, using untreated cells (black bar) as controls. Agrin increases this interaction at the surface, even when surface delivery of AChRs is blocked by shifting the myotubes to 20 °C. c, C2 myotubes were treated with 0.25 nM agrin for 20 h at 20 °C or 37 °C, and AChRs were visualized with rhodamine-α-BT. d, the average number of AChR clusters per myotube was determined by counting clusters (as shown in c) in 20 visual fields per treatment. Agrin increases the number of AChR clusters significantly even at 20 °C, although these clusters have a thinner and less bright appearance. Data represent mean ± S.D. from at least three experiments. Significant difference from control: *, p < 0.05; **, p < 0.01; ***, p < 0.001; by two-tailed Student’s paired t test.

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L. S. Borges and M. J. Ferns, unpublished data.
induced decrease in extractability of the AChR therefore parallels the kinetics of the increase in rapsyn-AChR interaction (see Fig. 5b).

These data demonstrate that rapsyn-associated AChRs are preferentially linked to the cytoskeleton and that the agrin-induced increase in rapsyn binding to all isolated AChRs (7%), was set to 100%, and the percentages of newly recruited rapsyn molecules were calculated for the more extractable (0.05% detergent) and less extractable (1% detergent) AChRs. Thus, agrin-induced rapsyn-AChR complexes are preferentially linked to the cytoskeleton. Data are mean ± S.D. of five experiments. *p < 0.02 by two-tailed Student’s t test.

d. In C2 cells, the total agrin-induced increase in rapsyn binding to all isolated AChRs (72%), was set to 100%, and the percentages of newly recruited rapsyn molecules were calculated for the more extractable (0.05% detergent) and less extractable (1% detergent) AChRs. Thus, agrin-induced rapsyn-AChR complexes are preferentially linked to the cytoskeleton. Data are mean ± S.D. of five experiments. *p < 0.02 by two-tailed Student’s t test.

DISCUSSION

In this study, we have investigated how rapsyn-AChR interactions relate to clustering. We show that, in vivo, extrasynaptic (unclustered) AChRs are associated with rapsyn and that synaptic AChRs are associated with more rapsyn. We therefore investigated how rapsyn-AChR interactions are regulated in myotube cultures, and find that neural agrin rapidly induces an increase in the interaction of rapsyn with surface AChRs. This increase requires tyrosine kinase activity, parallels enhanced cytoskeletal linkage of the AChR, correlates strongly with subsequent receptor clustering, and (along with clustering) occurs independently of the secretory pathway. These observations suggest that agrin-induced increases in rapsyn-AChR interactions trigger cytoskeletal anchoring and clustering of AChRs.

Rapsyn and the AChR Form Pre-assembled Complexes—A long-standing question in synapse formation concerns the sequence of events in which postsynaptic components are assembled. At the NMJ much evidence suggests that MuSK forms a primary synaptic scaffold to which rapsyn is recruited followed by AChRs and other proteins, implying that rapsyn interacts with AChRs only in clusters at the synapse (7, 8). However, we recently reported that, in cultured C2 myotubes that were not treated with agrin, AChRs already interact with several postsynaptic proteins including rapsyn (15). Such interactions may have originated from spontaneously clustered AChRs or from diffusely distributed receptors. We investigated this issue and show here that rapsyn interacts with unclustered AChRs at the surface of S27 myotubes, a mutant derivative of C2 that completely lacks clusters. The comparable extent of the rapsyn-receptor interaction between C2, S26, and S27 cells strongly suggests that the majority of surface AChR-rapsyn complexes in non-agrin-treated C2 are unclustered. We also find that rapsyn interacts with unclustered AChRs in diaphragm in vivo. Based upon the predominant association of rapsyn with surface AChRs in C2 and S27 myotubes, it seems likely that the extrasynaptic rapsyn-AChR complexes in diaphragm reside at the plasma membrane rather than intracellularly. Taken together, these data show for the first time that rapsyn interacts with AChRs outside of clusters in pre-assembled, diffusely distributed complexes, both in cultured myotubes and in diaphragm muscle.

These unclustered, pre-assembled surface complexes appear to be important for clustering for the following reasons: Firstly, rapsyn does not aggregate in the absence of AChRs in ectopically injected myofibers in vivo (17). Secondly, we recently estimated that in cultured C2 myotubes ~50% of total cellular rapsyn are constitutively bound to AChRs while the residual 50% represent free rapsyn (17), indicating that pre-assembled rapsyn-AChR complexes are very prominent. The pool of free...
Agrin Increases the Interaction of Rapsyn with Surface AChRs, and This Correlates with AChR Clustering and Anchoring—We show that in denervated muscle fibers in vitro, in which agrin remains concentrated at NMJs (25), AChRs isolated from synaptic areas have more (~70%) rapsyn associated than extrasynaptic AChRs. The higher amount of rapsyn bound to synaptic AChRs most likely stems from the action of motorneuron-derived agrin, as we find that neural agrin elicits the same increase in rapsyn-AChR interaction in cultured muscles. The agrin-induced increase in interaction occurred only on surface AChR and was not blocked at 20 °C, indicating that it does not require the secretory pathway. At 20 °C, AChRs were clustered in response to agrin, albeit in less densely packed aggregates, showing that clustering per se does not depend on continuous surface delivery of rapsyn and AChRs. The functional relevance of co-transport of rapsyn and AChR through the secretory pathway (20, 31) thus remains to be established. While it may maintain a high density of pre-assembled surface rapsyn-AChR complexes, it is as such not required for agrin-induced clustering. Rather, our present data show that the critical events for AChR clustering occur independently of secretion at the plasma membrane, where agrin regulates the extent of interaction between a free pool of rapsyn (17) and surface AChRs (Fig. 9).

The agrin-induced increase in rapsyn-AChR interaction could potentially reflect de novo formation of more rapsyn-AChR complexes, higher affinity binding between rapsyn and the AChR, or more rapsyn molecules bound per individual AChR. The formation of additional 1:1 rapsyn-AChR complexes (or the stabilization of such complexes) seems possible, as the AChR and rapsyn have been shown to be present in approximately equimolar amounts in Torpedo electric organ and in cultured muscle cells (34). Ultrastructural studies also support a 1:1 stoichiometry and a direct interaction between rapsyn and AChRs (35, 36). On the other hand, the precise ratio of rapsyn to AChR in clusters remains to be determined and our increased rapsyn-AChR interaction may reflect binding of more rapsyn molecules to each AChR. If this is the case, the additional rapsyn would necessarily bind at a different site on the AChR, creating a distinct form of rapsyn-AChR complex with a 2:1 stoichiometry (see Fig. 9).

This notion is supported by our observation that the agrin-induced increase in rapsyn-AChR interaction correlated strongly with AChR clustering in several different experimental conditions. First, in S27 cells, agrin, even at high concentrations, failed to induce increased interaction as well as clustering. Second and third, herbimycin and staurosporine blocked both the agrin-induced increase in the rapsyn-AChR interaction and AChR clustering in C2 cells. Herbimycin inhibits agrin-induced phosphorylation of MuSK, while staurospo-
rine blocks a kinase downstream of MuSK, possibly a member of the Src-family (21, 23). Thus, our results suggest that such a kinase may regulate rapsyn-AChR interactions downstream of MuSK activation. Fourth, the agrin-induced increase in rapsyn interaction occurs rapidly (within 40 min in C2), and parallels early signaling events that are important for the subsequent clustering of the AChR, such as tyrosine phosphorylation of the AChR β subunit (22). This temporal correlation raises the possibility that rapsyn binding to the AChR is regulated by β subunit phosphorylation, and that they are linked precursor steps in the clustering pathway, a hypothesis we are currently investigating. Fifth, laminin-1, which is thought to act independently of MuSK in AChR clustering (27, 28), also leads to increased rapsyn-AChR interaction. Sixth, extracellular Ca²⁺, which acts via MuSK to induce AChR β-subunit phosphorylation and receptor clustering (29), induces a similar increase in rapsyn-AChR binding. Finally, agrin-induced rapsyn-AChR interactions and clustering still occur at 20 °C, when secretion and new AChR insertion into the plasma membrane are blocked. This shows that the events critical for clustering occur at the cell surface and suggests that increased interaction of rapsyn with surface AChRs may be sufficient to drive clustering in the absence of secretion.

Rapsyn has long been proposed to mediate a cytoskeletal link of the AChR. For example, extraction of rapsyn from clusters on rat myotubes or from Torpedo electrocytes increases AChR mobility in the membrane (38, 39). Furthermore, rapsyn mediates interaction of AChRs with the dystrophin/utrophin glycoprotein complex, which binds to F-actin (15). We now demonstrate that rapsyn is preferentially associated with AChRs that are bound to the cytoskeleton, and that the agrin-induced increase in interaction of rapsyn with AChRs correlates strongly with increased cytoskeletal linkage of the AChR. This increased cytoskeletal linkage occurs in parallel with AChR-associated signaling events (such as tyrosine phosphorylation of AChRs, of AChR-bound MuSK, and Src-type kinases) (21), and precedes detectable clustering of the AChR. The emerging picture is thus that the AChR acts as a scaffold onto which anchoring proteins like rapsyn and several signaling proteins are rapidly recruited by agrin. Within the resulting AChR-protein complexes, rapsyn appears as the most abundant postsynaptic protein based on its high expression level (34) and its much higher extent of binding to the AChR relative to other postsynaptic components (15). Together, these observations strongly imply that it is the increased rapsyn-AChR interaction that enhances the cytoskeletal anchoring of the AChR in the postsynaptic membrane, and so promotes AChR clustering.

In summary, the data presented here, together with our previous studies (17), suggest a model for AChR clustering in which pre-assembled, unclustered AChR-rapsyn complexes occur at the plasma membrane and are required for subsequent clustering (Fig. 9, step 1). Neural agrin, released by the nerve terminal, then signals through MuSK and further increases the interaction of rapsyn and the AChR in the postsynaptic membrane, either by increasing the number of AChR-rapsyn complexes or by increasing the amount of rapsyn interacting with an individual AChR. This occurs through a kinase-dependent step, independently of the secretory pathway, and by recruitment of rapsyn from a free pool. We propose that the increased rapsyn-AChR association then leads to enhanced cytoskeletal linkage, and to the progressive postsynaptic clustering of the AChR (Fig. 9, step 2).

Pre-assembly of postsynaptic complexes may also play a role in formation of central synapses. At excitatory synapses in hippocampal neurons, clusters of PSD-95 first appear in dendritic shafts or dynamic spine precursors before moving, through cytoskeletal rearrangement, to their final position in the mature spine head (40). At inhibitory synapses, much like AChRs and rapsyn at the NMJ, gephrin and γ-aminobutyric acid type A (GABA_A) receptors are mutually important for clustering, most likely by forming interdependent components of a protein complex (41). Our data substantiate the possibility that postsynaptic specializations of synapses are in general constructed from pre-assembled complexes of their components. Such pre-assembly may ensure that postsynaptic membranes can be formed and modified rapidly according to physiological needs.

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