Agrin promotes synaptic differentiation by
counteracting an inhibitory effect of neurotransmitter

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Synaptic organizing molecules and neurotransmission regulate synaptic development. Here, we use the skeletal neuromuscular junction to assess the interdependence of effects evoked by an essential synaptic organizing protein, agrin, and the neuromuscular transmitter, acetylcholine (ACh). Mice lacking agrin fail to maintain neuromuscular junctions, whereas neuromuscular synapses differentiate extensively in the absence of ACh. We now demonstrate that agrin’s action in vivo depends critically on cholinergic neurotransmission. Using double-mutant mice, we show that synapses do form in the absence of agrin provided that ACh is also absent. We provide evidence that ACh destabilizes nascent postsynaptic sites, and that one major physiological role of agrin is to counteract this “antisynaptogenic” influence. Similar interactions between neurotransmitters and synaptic organizing molecules may operate at synapses in the central nervous system.

Two classes of intercellular signals important for synaptogenesis are proteins that organize pre- and postsynaptic differentiation and neurotransmitters that elicit electrical responses. These signals presumably interact to shape the synapse, but they are generally studied separately. At the skeletal neuromuscular junction (NMJ), the neurotransmitter is acetylcholine (ACh), and a main synaptic organizing molecule is the glycoprotein agrin. The aim of the present study was to assess the interdependence between ACh and agrin during synaptic differentiation.

Agrin is synthesized and released by motoneurons at the NMJ, where it is believed to promote aggregation of ACh receptors (AChRs) and associated proteins to form a postsynaptic apparatus beneath the nerve terminal (1), (2). Agrin-deficient (agrn−/−) mice are born paralyzed because of profound synaptic deficits (3, 4); myotubes bear dramatically fewer and smaller AChR clusters than in controls, and most of the remaining clusters are unapposed by axons (3, 4). These findings supported the “agrin hypothesis,” which states that agrin is the master nerve-derived initiator of postsynaptic differentiation (1). Subsequent studies, however, showed that immature postsynaptic sites form in mice lacking agrin but then abort differentiation and disassemble (5, 6), reopening the question of agrin’s function. Moreover, AChR clusters persist longer in muscles lacking motor innervation entirely than in muscles innervated by agrn−/− axons (5, 6), suggesting that the nerve provides a second factor that acts to disperse postsynaptic sites.

ACh is a reasonable candidate for the dispersal factor because it, or the postsynaptic depolarization it elicits, regulates AChR subunit gene expression (2, 7) and stimulates AChR endocytosis (8). The existence of complex “postsynaptic” structures on myotubes cultured aneurally demonstrates that ACh is not required for postsynaptic differentiation (9). Moreover, NMJs differentiate extensively in mice that lack ACh due to targeted mutation of the gene that encodes its sole synthetic enzyme, choline acetyltransferase (chat−/− mice; refs. 10 and 11). Indeed, NMJs appear to develop precociously in the absence of neurotransmitter; they are larger and more complex in chat−/− embryos than in controls (10), suggesting that ACh could actually antagonize postsynaptic differentiation.

Here, using agrn−/− and chat−/− mice that we previously generated and characterized, we reassess agrin’s role and ask whether ACh is the nerve-derived factor that antagonizes synaptic differentiation. Using double-mutant mice, we show that synapses do form in the absence of agrin provided that ACh is also absent. We then provide evidence from cultured muscle cells that ACh destabilizes nascent postsynaptic sites, and that one critical physiological role of agrin is to counteract this “antisynaptogenic” influence.

Methods

Mutant Mice. chat−/− and agrn−/− mice were generated in our laboratory and have been described in detail (4, 10).

Histology. Embryos were decapitated and fixed by immersion in 4% paraformaldehyde in PBS. Tail samples were kept for genotyping. Tissues were postfixed for 12–24 h before staining. Images were acquired on a Bio-Rad MRC1024 or a FV500 Olympus (Melville, NY) confocal microscope and are represented as maximum intensity projections that were contrast-adjusted in PHOTOSHOP (Adobe Systems, San Jose, CA). Numbers of AChR clusters and muscle fibers were determined from images taken with a ×20 objective; AChR cluster size was measured from images taken with a ×60 objective.

Muscles were stained as described (10) by using the following reagents: anti-neurofilament (Chemicon), anti-synaptophysin (Zymed), Alexa594-labeled bungarotoxin (Btx) (2.5 μg/ml; Invitrogen), Alexa660-phalloidin (Invitrogen), anti-laminin-β2 (1117; gift of R. Timpl; Max Planck Institute of Biochemistry, Martinsried, Germany), anti-rapsyn (made in our laboratory), anti-muscle-specific kinase (MuSK) (gift of M. Ruegg; Biozentrum, University of Basel), anti-acetylcholinesterase (gift of T. Rosenberry; Mayo Clinic College of Medicine, Jacksonville, FL), and anti-SV2 (Developmental Studies Hybridoma Bank, Iowa City, IA). Secondary antibodies were from Jackson ImmunoResearch and Cappel (MP Biomedical, Irvine, CA). Muscle fiber number (Fig. 1d) was determined from rotation of confocal stacks through the entire thickness of the muscle. The number of AChR clusters was divided by the number of muscle fibers to obtain the number of AChR clusters per fiber. MuSK- and rapsyn-to-AChR density ratios were measured by determining the intensity of fluorescence over background in immunostained cryosections for either antigen and dividing by AChR density.

Tissue Culture. C2C12 myoblasts (American Type Culture Collection) were cultured as described (9). Reagents were from,

Abbreviations: ACh, acetylcholine; AChR, ACh receptor; NMJ, neuromuscular junction; CCh, carbachol; Btx, bungarotoxin; En, embryonic day n; MuSK, muscle-specific kinase; dko, double knockout.

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Sigma and were used as follows except where indicated: carbamylcholine (CCh; 100 μM for 12 h); α-Btx (2 μg/ml to block all AChRs, or 0.1 μg/ml for 30 min to block ~50% of AChRs before CCh treatment); veratridine (250–500 μM); scorpion toxin from Androctonus australis (100 μg/ml, ICN); QX-314 (500 μM), tetrodotoxin (5 μM), curare (1 mM), high potassium (50 mM, substituted for sodium to preserve osmolarity), and cycloheximide (20 μg/ml for 4 h before the addition of CCh and for another 6 h in the presence of CCh; ref. 12). Chinese hamster ovary cells expressing membrane-tethered Z+ or Z– rat agrin (13) or membrane-anchored cyan fluorescent protein (as controls) were added 24–36 h before CCh treatment. After fixation, cultures were stained with rat anti-AChR (mAb35; Sigma) and mouse anti-rat agrin (mAb131; Stressgen Biotechnologies, San Diego) and evaluated blindly.

Methods for preparing nerve–muscle cocultures were modified from published protocols (14, 15) as detailed in Supporting Text, which is published as supporting information on the PNAS web site. Motor neurons from one to two agrn–/– or agrn+/+ spinal cords were purified (14), added to 5- to 7-day-old chick primary myotubes, and cocultured for 2 days with or without Btx (3 μg/ml). Nerve–muscle cocultures were stained with anti-AChR, anti-neurofilament (NF-200; Sigma), and anti-SV2 antibodies (Developmental Studies Hybridoma Bank, Iowa City, IA). Fields (≥100, ×40) were scanned automatically by using a computer-controlled stage and scored blindly for each condition. Results are presented as mean ± SEM. Protocols for time-lapse imaging are detailed in Supporting Text.

Results
Neuromuscular Synapses Differentiate in Mice Lacking both Agrin and ACh. As a first step, we mated agrn null mutants to mice lacking choline acetyltransferase (ChAT) (chat–/–), the sole synthetic enzyme for ACh. NMJs form and mature through embryogenesis in chat–/– mice, although they exhibit some structural abnormalities (10). Remarkably, deleting choline acetyltransferase largely rescued the agrin defect in postsynaptic maturation; numerous AChR clusters were present in chat–/–, agrn–/– double knockout (dko) muscles >2 days after such sites had disassembled in agrn–/– animals (Fig. 1a). Similar results were obtained in each of several muscles examined, including intercostals, diaphragm, and triangularis sterni (Fig. 1a–c and Fig. 7a and b, which is published as supporting information on the PNAS web site). Most dko muscle fibers bore a single cluster, which was similar in size to those in controls (Fig. 1d and Fig. 7c). Rescue was imperfect in that some dko fibers lacked an AChR cluster, whereas others had multiple clusters (Fig. 2g and data not shown). Moreover, the central endplate band was wider in dko and chat–/– muscles than in controls. Nonetheless, the difference between agrn–/– and dko muscles was striking, supporting the notion that ACh is an AChR declustering factor in vivo.

Because AChR aggregates form and persist on uninnervated myotubes in vivo (5, 6) and in vitro (2), we considered the possibility that axons lacking both agrin and ACh failed to innervate muscles. However, dko axons formed nerve trunks that entered muscles, branched, and terminated on myotubes; exuberant growth already documented in chat–/– and agrn–/– mutants (3, 10) was also present in dko mice (Figs. 1b and c and 7). Interestingly, motor axons directly apposed 90% of dko AChR clusters (Figs. 1d and e and 7c) and formed varicose nerve terminals rich in synaptophysin and SV2, markers of synaptic vesicles, at such sites (Fig. 2a and f). Moreover, AChRs in dko embryos colocalized with multiple proteins normally concentrated at the postsynaptic apparatus (2), including rapsyn, MusK, laminin β2, and acetylcholinesterase (Fig. 2b–f). The densities of AChRs, MusK, and rapsyn in the postsynaptic membrane did not differ significantly between dkos and controls (Fig. 2h and i). Thus, surprisingly, NMJs differentiate extensively in the absence of agrin when ACh is also absent.

To determine when agrin–neurotransmitter interactions first become evident and how long they continue, we compared control, chat–/–, agrn–/–, and dko mice at different ages. At embryonic day (E)13, as synapses began to form, AChR aggregates were present in all genotypes, indicating that postsynaptic differentiation can initiate in the absence of both agrin and ACh (Fig. 3a). Subsequently (E15–17), AChR aggregates dispersed in agrn–/– embryos but persisted in dko littermates (Figs. 1c and d and 3b and c). Thus, without agrin, postsynaptic development begins but is curtailed by neurotransmission. Differences between agrn–/– and dko muscles were apparent until birth (the
Cholinergic Stimulation Disperses AChR Clusters. These results suggested the hypothesis that neurotransmitter acts to “decluster” AChRs, and that agrin locally protects against this effect. To test this idea, we first asked whether application of neurotransmitter leads to loss of AChR clusters that form spontaneously in a myogenic cell line (C2; 16). We used the cholinergic agonist CCh instead of ACh because it is nonhydrolyzable. Consistent with the results of Bloch (17), incubation of myotubes with CCh dispersed spontaneously formed AChR aggregates, as assessed by comparison of treated and untreated cultures (Fig. 4a). The ability of CCh to decluster AChRs depended in its ability to bind to them, in that the effect was blocked by the specific nicotinic antagonists α-Btx and curare (Table 1). The ED50 for CCh was ≤10 μM, cluster loss was apparent in <2 h, and ~80% of clusters were eventually lost (Fig. 4 b and c).

We used time-lapse imaging to further characterize the effects of CCh on AChR clusters. This method provided direct evidence that application of CCh enhanced the dispersal of AChR clusters that had formed in its absence (Fig. 5 a and b). CCh also decreased the formation of new AChR clusters (data not shown); this may reflect a separate action or may be secondary to CCh-dependent dispersal of undetected transient or small clusters that represent the first stage in growth of a large cluster. To determine the fate of AChRs removed from clusters, we used a double-label protocol in which surface-associated AChRs were labeled with red Btx at time 0, then the disposition of these AChRs was compared with that of surface AChRs labeled in its absence (Fig. 5 c–f). As shown in Fig. 5 c–e, declustering resulted, at least in part, from internalization of AChRs, presumably by endocytosis (8).

Although activation of AChRs can down-regulate AChR subunit gene expression (2, 7), the effect we observed was posttranslational, because CCh reduced cluster number in the presence of the protein synthesis inhibitor cycloheximide, and cycloheximide alone had little acute (10 h) effect on cluster number (Table 1). Moreover, CCh acted on AChRs already on the cell surface: when ~50% of AChRs were prelabeled with Btx, CCh activation of the remaining AChRs dispersed prelabeled receptors (Table 1, “low Btx”). This result also indicates that neurotransmitter can disaggregate inactive AChRs in the vicinity of active AChRs.

We next asked whether declustering required only ligand-induced conformational changes in AChR, or whether the resulting cation influx was also required (Table 1 and ref. 17). In support of the latter possibility, the open channel blocker, QX-314, prevented CCh-dependent declustering, whereas agents that increase sodium influx independent of the AChR (veratridine and A. australis [scorpion] toxin) caused declustering. Action potential propagation and consequent contractile activity were inessential, because CCh-dependent declustering occurred in the presence of the action potential blocker, tetro-
dependence of CCh-induced AChR cluster loss quantified per neural (ZAChR clusters associated with Chinese hamster ovary (CHO) cells expressing loss, we cocultured myotubes with heterologous (Chinese ham
whether agrin counteracts neurotransmitter-induced cluster this hypothesis difficult to test.
Ca2+
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Agrin Protects Against Cholinergic Dispersal of AChR Clusters. To ask whether agrin counteracts neurotransmitter-induced cluster loss, we cocultured myotubes with heterologous (Chinese ham-

dotoxin. We speculate that cation influx through the AChR leads directly (Ca2+) or indirectly (Na+) to increased intracellular Ca2+ levels that in turn stimulate disaggregation. However, the complex Ca2+ requirements for clustering per se (18, 19) make this hypothesis difficult to test.

Agrin Protects Against Cholinergic Dispersal of AChR Clusters. To ask whether agrin counteracts neurotransmitter-induced cluster loss, we cocultured myotubes with heterologous (Chinese ham-

ster ovary) cells engineered to stably express a membrane-anchored form of agrin (20). We used the isoform (Z+) that is most potent at clustering AChRs (see below) for these experiments. AChRs clustered at ~70% of contact sites between agrin-expressing cells and myotubes (Fig. 4 e and f). These clusters were larger than spontaneous clusters but nearly equivalen in AChR density (Fig. 8 a and b, which is published as
an unsuspected antagonistic relationship between these two aggn
of its accepted role as a clustering factor. declustering factor'' for AChRs either in addition to or instead
induced destabilization indicates that agrin serves as an “anti-
agrin-associated clusters are resistant to neurotransmitter-
these AChRs efficiently (Fig. 4
contact with a laminin-coated substrate (9). CCh disaggregated
they had formed. To test this possibility, we used a culture system
rather than agrin in particular, protected AChR clusters once
induced dispersal.
AChR aggregates formed at

AChR Clustering in Vitro Is Agrin-Independent If Neurotransmission Is
Blocked. As a critical test of the idea that agrin and ACh act
antagonistically at the synapse, we assessed their interaction in
a nerve–muscle coculture system (Fig. 6a). Motor neurons
purified from individual aggrn
embryos or aggrn
littermates
(14) were cultured with chick myotubes. After 2 days, cultures
were stained with antibodies to AChRs and neurites (Fig. 6b).
Consistent with previous studies using function-blocking anti-
bodies to agrin (22), the incidence of AChR clusters was
>15-fold higher at sites contacted by control neurites than
aggrn
neurites (Fig. 6 b, c, and e). If ACh declusters AChRs,
while agrin acts primarily as an antideclustering agent, blockade
of neurotransmission with Btx should restore the ability of
aggrn
neurites to elaborate AChR aggregates. In accordance
with this prediction, the incidence of neurite-associated AChR
aggregates in Btx-treated aggrn
cultures was similar to that in
aggrn
cultures (Fig. 6 d and e). Neither deleting agrin nor
adding Btx detectably affected neuronal survival or neurite
outgrowth (Fig. 6 f and g). Thus, agrin-deficient neurites can
form or stabilize AChR clusters provided neurotransmission is
blocked.

Discussion
This study began with the unexpected finding that NMJs differ-
entiate extensively in mice that lack both ACh and agrin. Based
on this finding, we undertook a set of studies in vitro that revealed
an unsuspected antagonistic relationship between these two
critical nerve-derived signals. Taken together, our results suggest
reinterpretations of previous findings that agrin is required for
postsynaptic differentiation and that neurotransmission is
dispensable (3–6, 10, 11): (i) Although agrin is necessary for

Fig. 6. AChR clusters form in apposition to agrin-deficient neurites if postsynaptic
activity is blocked. (a) Schematic of the experiment (motor neuron, green;
AChRs, red; myotube, gray). (b) Axons (green) from aggrn
motor neurons can
elaborate AChR aggregates (red) where they contact myotubes. Borders of
myotubes are outlined with dashed lines. (c) AGRN
axons are less commonly
associated with AChR aggregates. (d) When neurotransmission is blocked with
Btx, agrin
motor neurons regain the ability to elaborate clusters. (e) Summary
of results; each point derived from ≈100 random fields in a single coculture (*, P <
0.05, ANOVA). AChR aggregates were counted if they exceeded 20 μm² in size,
and a single neurite was considered to associate with no more than one aggregate
per myotube. (f and g) Neuron density and neurite outgrowth (per ×40 field)
were unaffected by genotype or the presence of Btx. (h) Schematic model showing
antagonistic effects of ACh and agrin on postsynaptic differentiation. ACh not only
activates AChRs but also leads to loss of AChR aggregates, at least in part by endocytosis. Agrin can cluster AChRs but acts in vivo, at least in part, by
inhibiting ACh-dependent declustering. (Scale bar in d, 20 μm; applies also to b and c.)
postsynaptic differentiation \textit{in vivo}, this requirement reflects the fact that neurotransmission occurs at developing NMJs rather than an inability of AChR clusters to form or persist in the absence of agrin. This interpretation is fully consistent with the finding that elaborate, aneurysmal postsynaptic specialization form on cultured myotubes (9). (ii) ACh plays a dual role at the NMJ, both activating and declustering AChRs. (iii) Although agrin can cluster AChRs, one of its primary roles during synapse formation is to counteract the antisyaptogenic effect of ACh. Thus, we suggest that agrin and ACh act in parallel to shape the postsynaptic apparatus, and that NMJ development depends critically on the interactions between these factors. A model summarizing the dual roles of ACh and agrin is shown in Fig. 6h.

The neurotransmitter-dependent dispersal of AChRs appears to require ACh-induced ion flux rather than merely a ligand-dependent conformation change in the AChR. While the present manuscript was being reviewed, a paper appeared suggesting that the kinase cdk5 may be an intermediate in this process (23). The spread of the ACh-initiated dispersal signal could result to some extent from extracellular diffusion of ACh but is more likely to result from intracellular spread of ions or other signaling intermediates. In this way, not only subneural AChR clusters but also nearby aneural clusters could be subject to the declustering effect of neurotransmitter. Time-lapse imaging revealed that at least some of the declustering occurs by endocytosis, but it is possible that some AChRs also disperse in the plane of the membrane.

In terms of agrin, we believe that in addition to its role in clustering AChRs, which has been demonstrated \textit{in vitro}, it also acts to antagonize the effect of ACh. Thus, it is an “antideclustering” agent, instead of or in addition to being a clustering factor. Our results \textit{in vivo} suggest that the antideclustering effects of agrin are physiologically crucial. Supporting this view, agrin’s antideclustering effects \textit{in vitro} require its z-exon (Fig. 4f and k), which has been shown to be required for postsynaptic differentiation \textit{in vivo} (2, 4, 5). The mechanisms downstream of MuSK by which agrin clusters and prevents declustering of AChRs remain to be determined; these two apparently distinct cellular effects may reflect a unitary molecular mechanism. For example, the ability of agrin to promote clustering in cultured myotubes might reflect an ability to stabilize “microclusters” that serve as nucleating centers for larger clusters.

The idea that neurotransmitter disperses postsynaptic receptors may seem counterintuitive but has a precedent in G protein-coupled receptor down-regulation, which involves ligand-induced receptor endocytosis (24). Moreover, when viewed as part of a balance with agrin, the antisyaptogenic effect of ACh may help explain several hitherto puzzling aspects of neuromuscular development, including the dispersal of clusters in \textit{agrn}−/− mutants (5, 6), the precocious growth of NMJs in \textit{chat}−/− mutants (10), the shrinkage of innervated clusters to conform to the size and shape of the nerve terminal (25), the perfectly coordinated growth of pre- and postsynaptic specializations as the synapse matures (26), and the loss of portions of the postsynaptic apparatus during synapse elimination (27). Developing central synapses face similar issues of apposition and coordinated growth (28–31). It is therefore possible that interactions between highly localized synaptic organizing molecules and neurotransmitters also help pattern central connectivity.

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