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Formation of Functional Synaptic Connections between Cultured Cortical Neurons from Agrin-Deficient Mice

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ABSTRACT: Numerous studies suggest that the extracellular matrix protein agrin directs the formation of the postsynaptic apparatus at the neuromuscular junction (NMJ). Strong support for this hypothesis comes from the observation that the high density of acetylcholine receptors (AChR) normally present at the neuromuscular junction fails to form in muscle of embryonic agrin mutant mice. Agrin is expressed by many populations of neurons in the central nervous system (CNS), suggesting that this molecule may also play a role in neuron–neuron synapse formation. To test this hypothesis, we examined synapse formation between cultured cortical neurons isolated from agrin-deficient mouse embryos. Our data show that glutamate receptors accumulate at synaptic sites on agrin-deficient neurons. Moreover, electrophysiological analysis demonstrates

that functional glutamatergic and gamma-aminobutyric acid (GABA)ergic synapses form between mutant neurons. The frequency and amplitude of miniature postsynaptic glutamatergic and GABAergic currents are similar in mutant and age-matched wild-type neurons during the first 3 weeks in culture. These results demonstrate that neuron-specific agrin is not required for formation and early development of functional synaptic contacts between CNS neurons, and suggest that mechanisms of interneuronal synaptogenesis are distinct from those regulating synapse formation at the neuromuscular junction. © 1999 John Wiley & Sons, Inc. *J Neurobiol* 39: 547–557, 1999

Keywords: agrin; neuron–neuron synapse formation; cortical neuron

The accumulation of neurotransmitter receptors in the postsynaptic membrane is a critical step in the formation of chemical synapses in both the peripheral nervous system (PNS) and central nervous system (CNS). The molecular mechanisms underlying this process are currently best understood at the neuromuscular junction (NMJ), where short-range signals emanating from the nerve trigger the accumulation of

acetylcholine receptors (AChR) and other synaptic components to the region of the muscle fiber membrane immediately subjacent to the nerve terminal. Agrin, an extracellular matrix protein, originally identified by its ability to induce clustering of AChR on cultured myotubes, plays a key role in this process (reviewed in Hall and Sanes, 1993; Sanes, 1997).

Agrin is synthesized by motor neurons (Rupp et al., 1991; Tsim et al., 1992) and is present at high concentrations at the NMJ, where its appearance coincides with the earliest forming AChR clusters during development (Reist et al., 1987; Fallon and Gelfman, 1989). Molecular cloning studies have revealed

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that a single agrin gene gives rise to a family of protein isoforms through alternative pre-mRNA splicing at several sites. One site, located in the C-terminus of the protein and designated the z site, is especially important in that splicing at this site is cell specific and regulates agrin's biological activity. Isoforms that contain either one or both exons 32 and 33 (agrin_{z8}, -_{z11}, or -_{z19}) are neuron specific and have high AChR clustering activity. Agrin lacking an insert at the z site (agrin_{z0}) is expressed by nonneuronal cells and has little or no AChR clustering activity (Ferns et al., 1992, 1993; Ruegg et al., 1992). Gautam et al. (1996) used gene targeting to develop an agrin mutant mouse in which exons 32 and 33 were deleted. Mice homozygous for this mutation, which lack the neuron-specific active agrin isoforms, exhibit a dramatic reduction in the number and size of AChR clusters in developing muscle, providing strong evidence to support the hypothesis that agrin is essential for NMJ formation.

Agrin expression is not limited to motor neurons in the peripheral nervous system (PNS). Virtually all neuronal populations in the brain express agrin, including isoforms with high AChR aggregating activity (O'Connor et al., 1994; Stone and Nikolics, 1995). In the developing rodent brain, agrin expression peaks during periods of synapse formation (Hoch et al., 1993; Li et al., 1997) and persists in regions of the mature brain that maintain a high level of synaptic plasticity throughout life (O'Connor et al., 1994). Immunohistochemical studies have further shown that agrin is present at synaptic sites between some neurons (Kröger et al., 1996; Mann and Kröger, 1996). Given the structural and biochemical similarity between the NMJ and synapses mediating fast synaptic transmission between neurons, and the temporal/spatial pattern of agrin expression in brain, it is tempting to speculate that agrin might also play a specific role in neuron–neuron synapse formation.

We hypothesized that if the role of agrin at inter-neuronal synapses is similar to its action at the NMJ, agrin-deficient mice should exhibit deficits in the clustering of neurotransmitter receptors in the postsynaptic membranes of neurons and related alterations in synaptic transmission in the CNS. This prediction is difficult to test *in vivo* as mice homozygous for the mutant agrin gene exhibit a perinatal lethal phenotype (Gautam et al., 1996). An alternative approach, however, was suggested by a previous study from our laboratory demonstrating that neurons dissociated from the cortices of newborn mice exhibit a pattern of agrin expression during the first 3 weeks in culture that is similar to that observed during the corresponding period of postnatal development *in vivo* (Li et al.,

1997). In the present study, we used immunohistochemical and electrophysiological techniques to compare synapse formation between cortical neurons from agrin-deficient and wild-type embryos developing in dissociated cell culture. The results of this study show clusters of glutamate receptors at synaptic sites and functionally active synapses between neurons lacking “active” agrin that were indistinguishable from those in wild-type neurons during the first 3 weeks in culture. These data suggest that neuron–neuron synapse formation does not show the same agrin-dependence as the NMJ.

MATERIALS AND METHODS

Preparation of Cultures

Somatosensory cortical neurons were grown in defined medium on glass coverslips placed on top of a confluent layer of nonneuronal cells growing in 35-mm tissue culture dishes as described (Li et al., 1997). Briefly, small pieces (≈ 1 mm²) of somatosensory cortex were harvested from individual 18-day-gestation (E18) mouse embryos and incubated 30 min at 37°C in a balanced salt solution (BSS) containing 10 U/mL papain (Worthington Biomedical Co.) and 50 mM DL-2-amino-5-phosphonovaleric acid (APV; RBI). Tissues were washed several times in BSS containing APV, trypsin inhibitor, and bovine serum albumin as described (Li et al., 1997), then twice in neurobasal medium with B27 supplements (NBM + B27; Life Technologies), followed by trituration through glass micropipettes and plating onto poly-D-lysine-coated glass coverslips. Neurons were maintained at 37°C in humidified 5.0% CO₂ atmosphere overnight, at which time the coverslips were transferred to 35-mm dishes containing confluent nonneuronal feeder cells in NBM + B27. Coverslips were transferred to new dishes of feeder cells every 3–4 days thereafter. Non-neuronal feeder cells were prepared from cortices obtained from P0–3 mice (ICR; Harlan Sprague-Dawley), plated onto 35-mm poly-D-lysine-coated culture dishes, and maintained in minimal essential medium supplemented with 10% fetal bovine serum (FBS-MEM) (Li et al., 1997). Prior to use as feeder layers, FBS-MEM was replaced with NBM+B27 and the cells were allowed to condition the medium for 24 h before addition of neuronal coverslips.

Immunocytochemistry

Neuronal cultures were rinsed with phosphate-buffered saline, pH 7.2 (PBS), fixed in 4% paraformaldehyde-PBS for 1 h on ice, washed in PBS, and then permeabilized in PBS containing 0.1% Triton X-100 and 4% bovine serum albumin (BSA) for 30 min on ice. Primary antibody incubations were carried out in 4% BSA-PBS containing 0.05% sodium azide overnight at 4°C. Secondary antibody incubations were performed in 4% BSA-PBS for 2 h at room tempera-

ture. Cultures were washed three times in BSA-PBS (10–15 min/wash) after each incubation step. SV2 antibody was obtained from the Developmental Studies Hybridoma Bank as hybridoma supernatant and used at 1:20 dilution. SVP-38, a monoclonal antibody against synaptophysin (Sigma) was used at 1:100 dilution. A rabbit antiserum, AB 1504 (Chemicon), against the glutamate receptor subunit GluR1 was used at 1:40 dilution. Fluorescein isothiocyanate (FITC)-conjugated anti-mouse and Texas-red-conjugated anti-rabbit antibodies (Vector Laboratories) were used at 1:200 dilution. Images were acquired using a Spot (Diagnostic Instruments) cooled CCD mounted on a Nikon Optiphot-2 microscope and prepared for presentation in Adobe PhotoShop.

Electrophysiology

Electrophysiological recordings were obtained using the whole-cell recording technique as previously described (Li et al., 1997). Internal solutions to record spontaneous postsynaptic currents (sPSCs) contained (in mM): 120 KCl, 0.1 CaCl₂, 2 MgCl₂, 20 NaCl, 1.1 EGTA, and 10 Hepes, pH 7.2. The external solution contained (in mM): 140 NaCl, 1 CaCl₂, 3 KCl, 5 Hepes, pH 7.2. To record GABAergic miniature postsynaptic currents (mPSCs) in isolation 5 μ M 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX), 20 μ M APV, and 1 μ M tetrodotoxin (TTX) were added to the bath. To record glutamatergic mPSCs in isolation, K-gluconate replaced KCl in the pipette solution and 2 μ M bicuculine methchloride (BMC) and 1 μ M TTX were added to the bathing solution. Data were collected and analyzed using a List EPC-7 patch-clamp amplifier, a Dell 386/486 computer, and either pCLAMP (Axon Instruments, v.5.5.1) or SCAN/SES (University of Strathclyde) software. Biophysical properties of mEPSCs were determined from records acquired at 20 kHz and filtered at 2.5 kHz using a trigger amplitude threshold of 10 pA. Mean amplitudes were determined by averaging the values obtained from 50 or more single events for each neuron. All recordings were performed at room temperature.

Polymerase Chain Reactions (PCR)

The genotype of the cultures used in this study was established by PCR analysis of genomic DNA. DNA was isolated using the TriReagent (Molecular Research Center), according to the manufacturer's directions, from small pieces of liver tissue harvested at the time of culturing. Approximately 1 μ g of DNA was amplified for 45 cycles (94°C, 1 min; 62°C, 1 min; 72°C, 3.05 min) using primers E32 and E33 embedded in exons 32 and 33 of agrin, respectively, and N1 and N2 in the neomycin resistance gene. PCR products were analyzed by electrophoresis on a 6% nondenaturing polyacrylamide gel.

Levels of agrin mRNA expression were determined by competitive PCR as previously described (Smith et al., 1997). For each determination, cDNA from 500 ng of total RNA was coamplified with 0.01–3.0 pg of the competing

template pRA_{Bam}, which contains a unique *Bam*HI restriction site, using the F25/B12 primer pair as described (Smith et al., 1997). PCR products were labeled by addition of approximately 5×10^5 cpm of ³²P-labeled F25 into each reaction. Two microliters of the amplified mixture was subjected to overdigestion with *Bam*HI followed by electrophoresis through 6% nondenaturing polyacrylamide gel. Product yields from the native and competing templates were determined by analysis on a PhosphorImager (Molecular Dynamics) and levels of mRNA expression calculated by linear estimation based on the amount of competing template added to each reaction.

The relative abundance of alternatively spliced agrin mRNAs was determined by PCR using aliquots of cDNA synthesized from 100 ng of total RNA. To investigate the pattern of alternative splicing at the y site, samples were subjected to a single round of amplification using oligonucleotide primers F111/B112 which flank the y site (Smith et al., 1997). Alternative splicing at the z site was examined using two rounds of amplification using nested primers. During the first amplification, the forward primer F110, whose 3'-end consisted of the 12 nucleotide sequence encoding four-amino-acid insert at the y site, was used in combination with the reverse primer B6 located 3' of the z site to specifically amplify agrin_{y4} mRNAs. Aliquots of the first-round PCR were subsequently diluted 1000-fold and reamplified using the second primer pair F24/B2 flanking the z site (Smith et al., 1997). PCR products were labeled by inclusion of approximately 5×10^5 cpm of ³²P-labeled F24 in each amplification reaction, separated by electrophoresis on 8% nondenaturing polyacrylamide gels and analyzed using a phosphorimager.

Polymerase chain reaction primers used in this study were: N1, 5'-CTATTCGGCTATGACTGGGCACAAC-3'; N2, 5'-CATTGCATCAGCCATGATGGATAC-3'; E32, 5'-CGAGCTGACCAATGAGATCCCAGC-3'; E33, 5'-AGGGCCCGGAATCCAGAGTTTCC-3'; F111, 5'-TGGTCAGGGTATTCTTGGA-3'; B112, 5'-AGGTTGAGCATGGTGTGCGG-3'; F110, 5'-GAATCTCCGAATCCCGCAAG-3'; B6, 5'-TGGTGTTGACCTTCA-CAGTGGAG-3'; F24, 5'-AGTCAGTGGGGACCTAGAAACAC-3'; B2, 5'-AAGCTCAGTTCAAAGTGGTTGCTC-3'.

RESULTS

Agrin-Deficient Cortical Neurons Develop Normally in Cell Culture

Homozygous agrin-deficient mice appear to develop normally until the last fetal day (E18) but die *in utero* or are stillborn (Gautam et al., 1996). To examine the role of agrin in neuronal synapse formation between CNS neurons, we prepared dissociated cell cultures from somatosensory cortices of individual E17/18 embryos resulting from mating of heterozygous agrin-deficient parents. The genotype of each culture was subsequently

established by PCR analysis of liver genomic DNA isolated from each embryo. Within a few hours of plating numerous phase-bright cells could be seen adhering to the coverslips (Fig. 1). Many of these cells appeared to be neurons that survived the dissociation procedure with processes still attached. As development in culture proceeds, the neurons extended neurites that contact the cell bodies and processes of other neurons. Neurons appeared healthy for at least 3 weeks in culture with no obvious morphological differences at any stage of development between cultures prepared from cortices of homozygous wild-type control or agrin-deficient embryos.

Agrin Gene Expression in Agrin-Deficient Neurons

Mutant mice homozygous for the targeted disruption of exons 32 and 33 of the agrin gene lack agrin

isoforms containing inserts at the z site and are severe hypomorphs for other forms of agrin (Gautam et al., 1996). To verify the effects of the deficiency on agrin expression in cultured neurons, we used reverse transcription (RT)-PCR to analyze the pattern of alternative splicing and level of agrin mRNA in RNA isolated from agrin-deficient and control neurons. Consistent with our earlier study, RT-PCR analysis using primers flanking the alternatively spliced y site indicates that virtually all of the agrin mRNA isolated from control cultures included exon 28 [Fig. 2(A)] and was thus derived from neurons (Ruegg et al., 1992; Hoch et al., 1993; Stone and Nikolics, 1995). Similar results were obtained from RNA isolated from agrin-deficient neurons. In contrast, analysis of the z site in transcripts containing the y4 insert revealed that whereas both control and agrin-deficient neurons express agrin_{z0}, the _{-z19} transcript which is relatively abundant in control neurons is below detection in the

Figure 1 Differentiation of agrin-deficient cortical neurons in cell culture. Neurons were dissociated from somatosensory cortices of E17/18 embryos, plated on poly-D-lysine-coated coverslips, and maintained in NBM+B27 medium over a feeder layer of nonneuronal cells. At 2 h after dissociation, neurons could be recognized by their large phase-bright cell bodies, many of which still had processes attached. At 7 and 21 days in culture, cells had extended numerous long, overlapping processes. No obvious morphological differences were apparent between cultures prepared from homozygous wild-type (+/+) or agrin-deficient (-/-) embryos at any stage of development in culture.

Figure 2 Expression of “active” agrin was abolished and total agrin mRNA levels were reduced in agrin-deficient cortical neurons. (A) PCR analysis of alternative splicing indicated that >90% of all agrin mRNA in wild-type (+/+), heterozygous (+/-), and agrin-deficient (-/-) cultures includes the four-amino-acid exon 28 at the y site found only in neurons. Wild-type, heterozygous, and agrin-deficient cortical neurons expressed agrin_{y4z0} mRNA lacking alternatively spliced exons at the z site, whereas transcripts containing exons 32 and/or 33 (agrins_{y4z8}, -_{y4z11}, and -_{y4z19}) were absent from the agrin-deficient neurons. The faint band between the agrins_{y4z11} and -_{y4z19} markers is an artifact present in some amplification reactions. (B) Competitive PCR analysis indicated that at 8 days in culture, total agrin mRNA levels in agrin-deficient cortical neurons were approximately 20% of those in wild-type neurons.

agrin-deficient cells. Quantitative measurements using competitive RT-PCR showed that although not eliminated entirely, the level of agrin mRNA expression was significantly reduced by the mutation to about 20% of control values [Fig. 2(B)].

Pre- and Postsynaptic Markers Are Colocalized in Agrin-Deficient Neurons

Neuromuscular development is severely disrupted in agrin-deficient mice with alterations in presynaptic as well as postsynaptic differentiation (Gautam et al., 1996). As a first step toward examining the effect of

this mutation on synaptogenesis between CNS neurons, we used immunohistochemistry to identify synaptic sites and their colocalization with high-density clusters of neurotransmitter receptors on cultured neurons. Staining with antibodies against the synaptic vesicle components synaptophysin (Fig. 3) or SV2 (data not shown) revealed numerous boutonlike structures associated with neuronal processes and cell bodies in both control and agrin-deficient cultures, presumed to represent differentiating nerve terminals. Double labeling with antibodies against the GluR-1 subunit indicated that the majority of these putative synaptic sites colocalized with high-density clusters of glutamate receptors (Fig. 3). No difference in the morphology, size, or density of nerve terminals or their associated clusters of glutamate receptors was apparent between neurons cultured from control or agrin-deficient cortices.

Functional Synaptic Contacts Form between Agrin-Deficient Neurons

Although the data described above suggest that morphological correlates of pre- and postsynaptic differentiation are normal in agrin-deficient neurons, they leave open the possibility that mutation of the agrin gene might result in altered synaptic function. To address this question, whole-cell recordings were performed to examine synaptic transmission between cultured cortical neurons. At a holding potential of -75 mV, with a pipette solution containing high chloride, two kinetically distinct classes of sPSCs were observed in agrin-deficient and control neurons [Fig. 4(A)]. These sPSCs resulted from both action-potential-dependent and action-potential-independent release of neurotransmitter. An action-potential-independent component, or miniature postsynaptic currents (mPSCs), could be recorded from both genotypes as well [Fig. 4(B,C)]. The rapidly decaying mPSCs resulted from activation of glutamate receptors based on their reversible blockade by bath application of APV and CNQX [Fig. 4(B)]. The more slowly decaying mPSCs were GABAergic as they were blocked by BMC [Fig. 4(C)] and reversed at the chloride equilibrium potential (data not shown). Thus, agrin isoforms containing inserts at the z site are not necessary for formation of spontaneously active glutamatergic or GABAergic synapses on cortical neurons.

To determine whether disruption of the agrin gene resulted in quantitative differences in glutamatergic or GABAergic synaptic transmission, we measured the fraction of neurons receiving functional synaptic contacts, as well as the frequency and amplitude of syn-

Figure 3 Glutamate receptors were clustered at synaptic sites in agrin-deficient cortical neurons. Cultures were double stained for the synaptic vesicle marker synaptophysin (Syp; fluorescein channel) and glutamate receptor subunit (GluR1; Texas red channel). The upper panels show low-power views of homozygous wild-type (Ag^{+/+}) and mutant (Ag^{-/-}) cultured neurons in which the fluorescein and Texas red channels were combined. Regions of overlap between the synaptophysin-rich nerve terminals and high-density clusters of glutamate receptors appear yellow. Lower panels show higher-power views of the regions indicated by the boxes, illustrating the colocalization of the punctate labeling for synaptophysin and glutamate receptors separately.

aptic currents as a function of age in culture. Since the goal of our study was to examine the possible role of agrin in neuronal synaptogenesis, we focused our analysis on mPSCs to eliminate the contribution of potential genotype-specific differences in neuronal excitability that might influence the frequency or amplitude of action-potential-dependent synaptic events. To avoid experimenter bias, all recordings were performed blind with respect to the genotype of the culture, which was revealed only after analysis of the electrophysiological data was complete.

By 4 days in culture, glutamatergic mPSCs were recorded in approximately 30% of wild-type neurons examined [Fig. 5(A)]. The number of functionally innervated neurons increased rapidly with time such that in cultures 8 days and older, essentially all wild-type neurons examined received glutamatergic input. A similar developmental profile of glutamatergic innervation was observed in neurons cultured from agrin-deficient embryos [Fig. 5(A)]. Functional GABAergic mPSCs were also detected as early as 4 days in culture for wild-type neurons. Approximately

half the neurons received GABAergic contacts by 8 days, and by 12 days GABAergic mPSCs were detected in all neurons examined [Fig. 5(B)]. Again, the time course and incidence of GABAergic input in agrin-deficient cultures were indistinguishable from those seen in wild-type cultures.

Together with the increase in the fraction of neurons receiving glutamatergic and GABAergic mPSCs, there was also a dramatic increase in the mPSC frequency recorded from each neuron over the first 3 weeks in culture. However, no difference in mPSC frequency for either neurotransmitter was apparent between control or agrin-deficient neurons [Fig. 5(C,D)]. Analysis of mPSC amplitudes showed no significant change during this period, nor did it reveal a difference between the wild-type and mutant cells [Fig. 5(E,F)]. Consistent with our immunohistochemical findings, these data suggest that the density of receptors at glutamatergic and GABAergic synapses on agrin-deficient neurons is similar to wild type, although we cannot rule out the possibility that a change in neurotransmitter sensitivity might be com-

Figure 4 Agrin-deficient cortical neurons formed functional synaptic contacts. (A) Typical whole-cell records obtained from control (+/-) or agrin-deficient (-/-) cortical neurons maintained for 12–14 days in culture. Neurons were held at -70 mV with KCl inside the recording pipette. Both rapidly and slowly decaying spontaneous PSCs were apparent in records obtained from control or agrin-deficient neurons. (B) Pharmacological analysis in the presence of TTX showed that mPSCs with rapid decay kinetics were reversibly blocked by application of APV and CNQX, indicating they were mediated by activation of glutamate receptors. (C) In contrast, mPSCs with slow decay kinetics were GABAergic and reversibly blocked by BMC.

Figure 5 Development of functional synapses in agrin-deficient neurons. The number of cortical neurons receiving functional synaptic contacts is expressed as a percentage of cells in which one or more glutamatergic (A) or GABAergic (B) PSCs could be detected during a 30-s recording period. Experiments were performed in the absence of TTX to include PSCs triggered by spontaneous activity in the culture as well as mPSCs. Although glutamatergic synapses appeared earlier in development than GABAergic synapses, no difference was apparent between the rates of synapse formation between wild-type (open bars) and agrin-deficient (filled bars) neurons. To estimate the level of synaptic input received by cultured neurons growing in culture, the mean frequency of mPSCs was determined. The frequency of both glutamatergic (C) and GABAergic (D) mPSCs increased steadily as a function of time in culture and was similar in wild-type (squares) and agrin-deficient (circles) neurons. In contrast, the mean mPSC amplitude (E,F) did not change significantly over time and was similar for both wild-type and agrin-deficient neurons. Each chart summarizes the data obtained from three to four independent platings of neurons from agrin-deficient embryos and their wild-type littermates. Mean mPSC amplitudes were determined using the SCAN program and are based on a minimum of 50 events ≥ 10 pA recorded from each of at least 10 neurons.

compensated for by a change in quantal size. Taken together, these results suggest that neither the rate of formation nor early differentiation of glutamatergic and GABAergic synaptic contacts is influenced by the altered agrin expression in the mutant neurons. Future studies examining evoked synaptic currents will be necessary to determine whether agrin plays a role in other aspects of synaptic transmission such as long-term potentiation or depression.

DISCUSSION

The pattern of agrin expression in brain is consistent with a role for agrin in the formation of neuron–neuron synapses. To test this possibility, we analyzed morphological and functional correlates of synapse formation occurring between cortical neurons isolated from agrin-deficient mouse embryos developing in dissociated cell culture. The results of this study indicate that neuron–neuron synapse formation does not show the same agrin dependence as neuromuscular synaptogenesis. In particular, the accumulation of excitatory neurotransmitter receptors occurs in the absence of the active agrin isoforms at neuron–neuron synapses but not at the NMJ. We further show that functional synapses, using either excitatory or inhibitory neurotransmitters, form between the agrin-deficient neurons. The effect of the agrin deficiency on neuromuscular synaptic transmission remains to be tested.

Neurons used in the present study were maintained in a defined medium conditioned by a feeder layer of cells dissociated from cortices of normal mice, raising the possibility that agrin produced by the feeder layer might compensate for the lack of agrin expression in the agrin-deficient neurons. Two observations make this unlikely. RT-PCR analysis indicates that agrin transcripts containing inserts at either the y or z site are below detection in RNA isolated from the feeder layer cultures (Li, unpublished observations). Moreover, functional synaptic contacts still formed between agrin-deficient neurons maintained above agrin-deficient feeder layers (data not shown). Thus, even under conditions in which all possible sources of agrin containing inserts at the z site were eliminated, neuronal synapse formation and function was apparently normal.

Although neuronal synapse formation is not dependent on the presence of z+ agrin, we cannot rule out the possibility that the residual level of agrin_{z0} expression is sufficient. It is interesting to note that large differences in AChR aggregating activity between agrin_{z+} and agrin_{z-} isoforms are evident only for

soluble agrin and that such differences become less marked when full-length agrin expressed on the cell surface is assayed (Ferns et al., 1992, 1993). Consistent with this observation are the results of recent studies showing that neuroblastoma-cell-induced AChR aggregation on cultured myotubes is mediated by agrin_{y0,z0}, and suggest further that cell-specific postranslational modification may also play an important role in modulating agrin function (Pun and Tsim, 1997). Residual expression of agrin_{z0} in agrin-deficient mice, however, is not able to support neuromuscular differentiation (Gautam et al., 1996). Thus, if the similarly low levels of agrin_{z0} expression observed in agrin-deficient cultured cortical neurons are sufficient to direct neuronal synapse formation, the underlying mechanisms of agrin action in the brain would seem to differ from those at the NMJ. Reports that the muscle-specific kinase MuSK, which serves as a key component of the functional receptor for agrin in muscle (DeChiara et al., 1996; Glass et al., 1996), is barely detectable in the brain (Valenzuela et al., 1995) support this conclusion.

Agrin is an adhesive substrate that may act as a stop and differentiation signal for some neurons (Campagna et al., 1995; Chang et al., 1997). The observation that patterns of intramuscular nerve branching and nerve terminal differentiation are abnormal in agrin-deficient mice (Gautam et al., 1996) also argues for a role for agrin in presynaptic differentiation. If agrin plays a similar role in the CNS, then several measures in the present study might reasonably have been expected to be affected. Staining with antibodies against various synaptic vesicle antigens suggest that size and density of nerve terminals on cortical neurons were similar in cultures made from agrin-deficient and littermate control embryos. Moreover, the time course of synapse formation and frequency of mPSCs, which might signal changes in number, surface area, or maturity of presynaptic contacts, were indistinguishable between the two genotypes. All agrin isoforms appear equally competent in inhibiting neurite outgrowth and stimulating nerve terminal differentiation (Campagna et al., 1995; Chang et al., 1997), and it is conceivable that residual agrin_{z0} expression might be sufficient to support any presynaptic functions agrin might have. That similarly reduced levels of agrin expression produce an altered presynaptic phenotype in motor neurons *in vivo* (Gautam et al., 1996), however, suggests that this function may not be shared in the CNS.

The results of this study show that formation and early differentiation of glutamatergic and GABAergic synapses do not show the same agrin dependence as the NMJ. Although additional experiments will be

required to eliminate a possible role for agrin_{z0}, these data suggest that alternate strategies for identifying agrin's role in brain need to be explored. Our understanding of agrin function at the NMJ has been greatly enhanced by studies of the signal transduction pathway that mediates its effects (reviewed in Sanes et al., 1998). In light of the results of the present study, and given agrin's widespread pattern of expression, it is difficult to predict which cells in brain might be the target cells for this molecule. A first step toward characterizing a CNS receptor for agrin will be to identify cells in brain that respond to agrin.

NOTE ADDED IN PROOF

Consistent with the results presented here, a recent immunohistochemical analysis (Serpinskaya et al. 1999. *Dev Biol* 205:65–78) concluded that z+ agrin isoforms are not required for differentiation of glutamatergic and GABAergic synapses on cultured hippocampal neurons.

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