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Synapse Formation by Hippocampal Neurons from Agrin-Deficient Mice

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Agrin, a proteoglycan secreted by motoneurons, is a critical organizer of synaptic differentiation at skeletal neuromuscular junctions. Agrin is widely expressed in the nervous system so other functions seem likely, but none have been demonstrated. To test roles for agrin in interneuronal synapse formation, we studied hippocampi from mutant mice that completely lack the z^+ splice form of agrin essential for neuromuscular differentiation and also exhibit severely (~90%) reduced levels of all agrin isoforms (M. Gautam *et al.*, 1996, *Cell* 85, 525–535). The brains of neonatal homozygous agrin mutants were often smaller than those of heterozygous and wild-type littermates, but were morphologically and histologically indistinguishable. In particular, antibodies to pre- and postsynaptic components of glutamatergic synapses were similarly coaggregated at synaptic sites in both mutants and controls. Because mutants die at birth due to neuromuscular defects, we cultured neurons to assess later stages of synaptic maturation. In primary cultures, the agrin-deficient neurons formed MAP2-positive dendrites and tau-1-positive axons. Synaptic vesicle proteins, AMPA- and NMDA-type glutamate receptors, $GABA_A$ receptors, and the putative synapse-organizing proteins PSD-95, GKAP, and gephyrin formed numerous clusters at synaptic sites. Quantitatively, the number of SV2-labeled contacts per neuron at day 5 and the number of PSD-95 clusters per dendrite length at day 18 in culture showed no significant differences between genotypes. Furthermore, exogenous z^+ agrin was unable to induce ectopic accumulation of components of central glutamatergic or GABAergic synapses as it does for neuromuscular cholinergic synapses. These results indicate that the z^+ forms of agrin are dispensable for glutamatergic and GABAergic synaptic differentiation in the central nervous system.

INTRODUCTION

Many of the cellular events that underlie synapse formation between neurons of the vertebrate central nervous system (CNS) parallel those at the well-studied neuromuscular junction (NMJ) (reviewed by Hall and Sanes, 1993; Sanes and Lichtman, 1999). Presynaptically, growth cones differentiate into specialized boutons in which arrays of transmitter-filled synaptic vesicles are clustered at a membrane-associated fusion apparatus. Postsynaptically, neurotransmitter receptors and a variety of cytoskeletal proteins aggregate in postsynaptic densities that lie opposite transmitter release sites. The densities are localized to specialized domains called junctional folds at the NMJ and dendritic spines at excitatory CNS synapses. At the NMJ, the acetylcholine receptor (AChR) is enriched ~1000-fold in the postsynaptic membrane relative to extrasynaptic muscle membrane (Fertuck and Salpeter, 1976). Although the degree of enrichment is not as high for neurotransmitter receptors at postsynaptic sites on CNS neurons, it is clear that inhibitory glycine and $GABA_A$ receptors (Triller *et al.*, 1985; Craig *et al.*, 1994; Nusser *et al.*, 1995) as well as excitatory AMPA/kainate, NMDA, and metabotropic glutamate receptors (Petralia and Wenthold, 1992; Petralia *et al.*, 1994; Rao and Craig, 1997; Baude *et al.*, 1993; Nomura *et al.*, 1994) are enriched at postsynaptic sites on CNS neurons. The molecules that directly mediate receptor clustering on the postsynaptic side are thought to be different for each of these neurotransmitter receptors: rapsyn for skeletal muscle AChR (Sanes, 1997), gephyrin for glycine and possibly $GABA_A$ receptors (Kirsch *et al.*, 1993; Craig *et al.*, 1996; Feng *et al.*, 1998), and PDZ domain proteins of the PSD-95/SAP90 and GRIP families for NMDA- and AMPA-type glutamate receptors (reviewed by Kornau *et al.*, 1997).

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In contrast, the *trans*-synaptic molecular signals that induce postsynaptic differentiation are known only for the vertebrate NMJ. ARIA/neuregulin and electrical activity regulate AChR transcription (Duclert and Changeux, 1995). The signal that induces the aggregation of AChRs and many other aspects of postsynaptic differentiation is agrin (McMahan, 1990; Ruegg and Bixby, 1998), which acts through MuSK and rapsyn (Glass and Yancopoulos, 1997; Sanes, 1997). Agrin is released from motor nerve terminals, induces AChR aggregation when added to cultured muscle cells, and is required *in vivo* for NMJ differentiation. Agrin is a proteoglycan with a 200-kDa protein backbone, but only the C-terminal region containing an EGF and a laminin-like domain is required for AChR-aggregating activity, and this activity is regulated by alternative splicing. Agrin isoforms with an insert at the z splice site (of 8, 11, or 19 aa; referred to as z^+ agrin) are up to 1000-fold more active in inducing AChR clustering than z^- agrin isoforms (Ruegg *et al.*, 1992; Ferns *et al.*, 1993). While many nonneuronal cells, including muscle and glial cells, produce z^- agrin, only neurons produce z^+ agrin (McMahan *et al.*, 1992; Smith and O'Dowd, 1994; Escher *et al.*, 1996; Hager *et al.*, 1997). Moreover, mutant mice lacking only the z^+ forms of agrin have neuromuscular defects as severe as those of mice that lack all forms of agrin (Burgess *et al.*, 1998).

Interestingly, expression of z^+ agrin mRNA is not restricted to motoneurons or even to cholinergic pathways but is widespread by neurons throughout the peripheral and central nervous systems (O'Connor *et al.*, 1994). This observation suggests that agrin may play roles in the development of neuron–neuron synapses. In support of this idea, z^- and z^+ forms of agrin mRNA are expressed in all brain regions (O'Connor *et al.*, 1994). In developing somatosensory cortex, peak levels of z0, z11, and z19 agrin occur during the time of maximal synaptogenesis (Li *et al.*, 1997b), and in the hippocampus the z0, z8, and z19 forms are highest during development and can be reinduced in the adult following acute depolarizing stimulus (Cohen *et al.*, 1997). Moreover, agrin is concentrated at synapses in the retina (Kröger *et al.*, 1996; Mann and Kröger, 1996) and z^+ agrin specifically is abundant in the plexiform layers of the developing retina at the time of synaptogenesis (Kröger, 1997). In addition, synaptic localization of z^+ agrin has been suggested by protein binding to hippocampal cultures. By this method, z^+ agrin binds to synaptic sites, whereas z^- agrin binds nonsynaptically (Wells and Fallon, 1997), suggesting a possible function specifically for z^+ agrin in CNS synapse formation. Finally, agrin has been proposed to act as a stop signal for motor axons and to promote their differentiation into nerve terminals (Campagna *et al.*, 1995, 1997; Chang *et al.*, 1997), and might serve similar functions at central synapses.

Here, we have sought roles for agrin in development of the CNS by analyzing agrin-deficient "knockout" mice. These mice express no z^+ agrin and also have levels of z^- agrin that are reduced ~90% relative to wild type. Agrin-mutant mice have gross defects in both pre- and postsynaptic differentiation at their NMJs, which lead to their death during the last fetal day (E18) or immediately after birth (Gautam *et al.*, 1996). We therefore analyzed hippocampal neurons from these agrin-mutant mice, both *in vivo* at E18 and in primary culture, to permit assessment of roles agrin might play in postnatal development. We also asked whether exogenous recombinant agrin affected synaptic differentiation in the cultures.

MATERIALS AND METHODS

Mice. Mice in which the alternatively spliced z exons of the agrin gene (exons 32 and 33) were replaced with the neomycin resistance gene were generated by Gautam *et al.* (1996) and maintained as heterozygotes on a C57/129 hybrid background. Embryos were genotyped by PCR using the following primer sets: 1a, CATTGCATCAGCCATGATGGATAC, 1b, CTATTCGGCTATGACTGGGCACAAC, 2a, AGGGCCCGGGAATCCAGAGTTTCC, and 2b, CGAGCTGACCAATGAGATCCCAGC. Primer set 1, from the deleted segment, gave a 617-bp band for the intact agrin gene and no band for the mutant allele. Primer set 2, for the neomycin insert, gave a 305-bp band for the mutant allele and no band for the wild-type allele. PCR was performed on crude proteinase K-digested tissue extracts using Advantage cDNA polymerase (Clontech) for 35 cycles of 94°C for 1 min, 62°C for 1 min, 72°C for 3.5 min, followed by a 10-min 72°C extension.

Antibodies. The following mouse monoclonal antibodies were used: SV2 (supernatant at 1:50, gift from K. Buckley, Harvard University; and from Developmental Studies Hybridoma Bank, University of Iowa), tau-1 against a dephosphorylated form of tau (1:400; Boehringer Mannheim), bd17 against the $GABA_A$ receptor β2/3 subunits (5 μg/ml; Boehringer Mannheim), 7a against gephyrin (2 μg/ml; Boehringer Mannheim), and 54.1 against NR1 (1:300–1:5,000 depending on lot; Pharmingen). Rabbit antibodies were used against synapsin (1:2000, No. G357, gift from A. Czernik, Rockefeller University), synaptophysin (1:20,000, gift from P. De Camilli, Yale University), microtubule-associated protein 2 (MAP2; antibody 266, 1:20,000, gift from S. Halpain, Scripps Institute), glutamic acid decarboxylase (GAD) (No. K2, 1:2,000; Chemicon), and GKAP (No. 9589, 1:300, gift from M. Sheng, Harvard University). Guinea pig antibodies were used against GluR1 (serum at 1:1,600, gift from R. L. Huganir, Johns Hopkins University) and PSD-95 (1:300, gift from M. Sheng, Harvard University).

Histology. For conventional histological analysis, brains were dissected from embryonic day 18 mice, fixed in 4% paraformaldehyde for 4–6 h, cryoprotected with 15 and 30% sucrose, frozen, and sectioned in the coronal plane at 20 μm in a cryostat. Sections were stained with 0.25% toluidine blue for 2 min. For immunofluorescence, brains were frozen without fixation and sectioned at 10 μm in a cryostat. Sections to be stained with antibodies to SV2, synaptophysin, GluR1, PSD-95, or $GABA_A$ receptors were fixed with 2% paraformaldehyde and then permeabilized with 0.2% Triton X-100 before application of antibody. Sections to be stained with antibodies to GKAP and gephyrin were fixed and permeabilized with methanol at −20° for 10 min. Primary and secondary antibodies were each applied for 1–2 h.

Cell culture. Cultures were prepared from hippocampi of individual mouse embryos at E16–E17 following methods for low-density rat hippocampal cultures (Goslin and Banker, 1998).

Briefly, hippocampi were dissociated by trypsinization and trituration and plated at 2400 cells/cm^2 onto poly-L-lysine-coated glass coverslips (~15–20 coverslips per embryo). After cells were allowed to attach in MEM with 10% serum, neuron-bearing coverslips were transferred into dishes of rat glial feeders and maintained in defined N2.1 media. Coverslips were suspended above the glial feeders with wax dots so that neurons were not in contact with the glia. For analysis of NMDA receptors, neurons were treated twice a week with the NMDA receptor antagonist 2-amino-5-phosphonovalerate (100 μM) to induce the synaptic distribution (see Rao and Craig, 1997).

Analysis of cultures. For analysis of NMDA receptors, neurons were simultaneously fixed and permeabilized in −20°C methanol for 10 min. For all other immunocytochemical studies, neurons were fixed for 15 min in warm 4% paraformaldehyde, 4% sucrose in PBS and permeabilized for 5 min with 0.25% Triton X-100. Nonspecific staining was blocked for 30 min in 10% BSA and neurons were immunolabeled in 3% BSA, usually by overnight incubation at room temperature with agitation for primary antibodies and a 45-min incubation for secondary antibodies. For most double-staining experiments both primary antibodies were incubated together. In the experiments involving GAD immunocytochemistry, cross-reactivity was troublesome, so cultures were incubated sequentially with anti-gephyrin and then anti-GAD. Secondary antibodies were combinations of Texas red- or fluorescein isothiocyanate-conjugated horse anti-mouse, goat anti-rabbit, or goat anti-guinea pig IgG (2.5–7.5 μg/ml; Vector). Coverslips were mounted in Tris–HCl, glycerol, polyvinyl alcohol with 2% 1,4-diazobicyclo[2,2,2]octane. Fluorescence and phase-contrast images were captured with a Photometrics Series 200 cooled CCD camera mounted on a Zeiss Axioskop with 40× 1.3 NA and 63× 1.4 NA lenses using Oncor imaging software. Images were prepared for presentation in Adobe PhotoShop.

For quantitation of presynaptic specializations (Fig. 4), 5-day cultures were labeled for MAP2 and SV2. Images of randomly selected neurons were acquired at 40×. The MAP2 image was used to define the somatodendritic domain, and all SV2-labeled puncta in contact with the somatodendritic domain were counted. Puncta were defined by an intensity thresholding function in Oncor Image to all select regions in a given image above a given intensity. For quantitation of PSD-95 clusters at 18 days in culture (Fig. 7), images of randomly chosen dendrites were acquired at 63×. Dendrite length was measured, and PSD-95 fluorescence images were subjected to the thresholding function to select puncta of about twofold or greater intensity above the dendrite shaft labeling. Measurements were analyzed using Microsoft Excel, StatView, and CricketGraph.

For analysis of the effects of exogenous C-agrin, COS cell conditioned medium containing C-Ag0,0 or C-Ag4,8 was generously donated by J. E. Sugiyama and Z. W. Hall. The C-Ag4,8 was active at 1:1000 in inducing AChR clusters on cultured muscle cells. For addition to hippocampal cultures, coverslips were flipped neuron side up, C-Ag-containing medium was added at final dilutions of 1:1000 to 1:50, the coverslips were flipped back with neurons facing the glia after 30–90 min, and neurons were fixed and analyzed 20–48 h later.

RESULTS

Brains of Agrin-Mutant Mice Appear Morphologically Normal

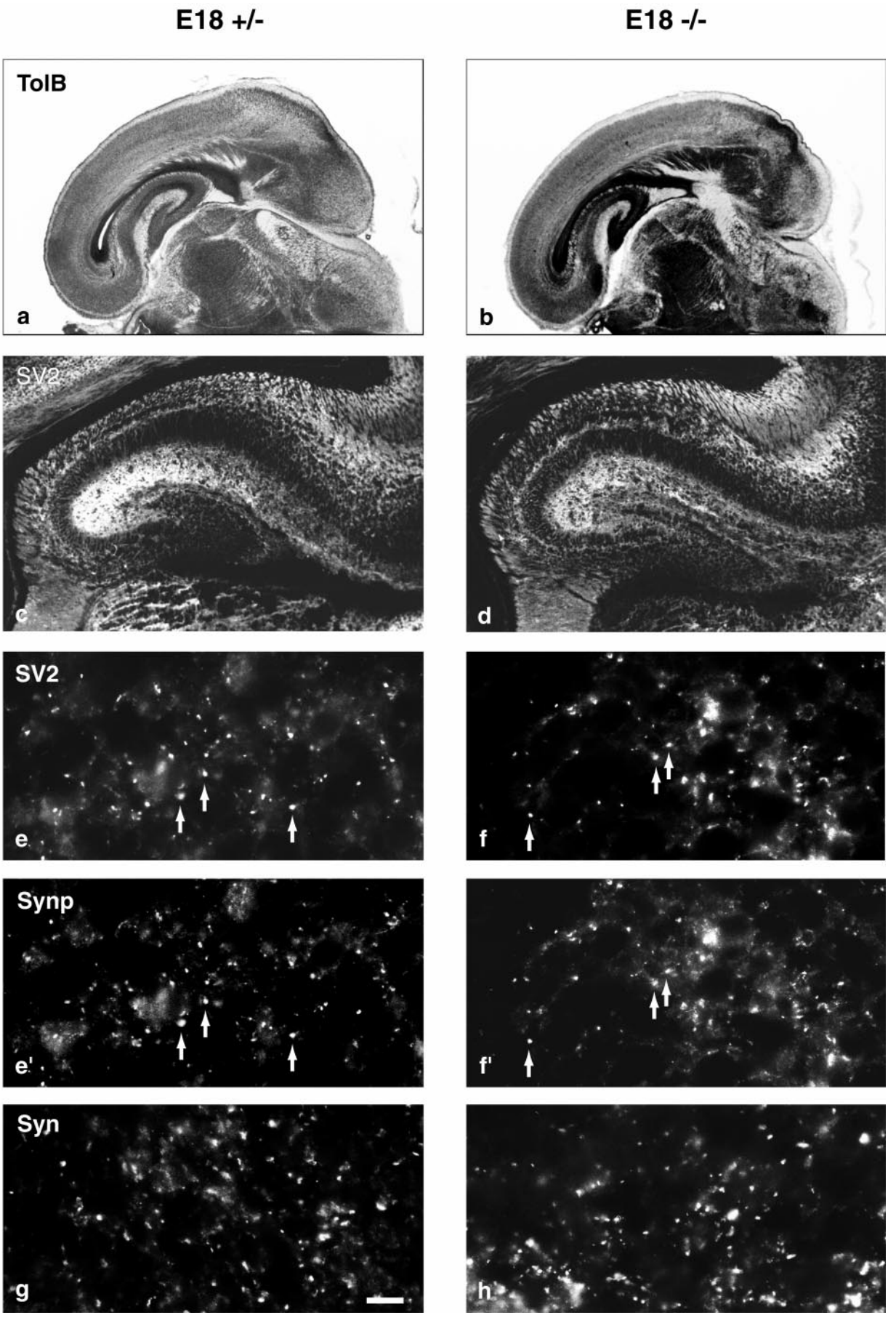
Brains of E18 agrin −/− mice appeared externally normal. In some cases, agrin −/− brains were slightly smaller than those of littermates, but this difference was variable and paralleled an overall decrease in weight of agrin −/− embryos relative to controls. To seek more subtle histological differences, brains were sectioned in the sagittal and coronal planes. Again, no consistent differences between mutants and controls were detectable (Figs. 1a and 1b and data not shown).

Synapses Differentiate in Hippocampi of Neonatal Agrin-Mutant Mice

To estimate the density and distribution of synapses in the brain, we stained sections with antibodies to three components of synaptic vesicles—SV2, synaptophysin, and synapsin. These antigens have been widely used as synaptic markers both *in vivo* and *in vitro*. In E18 brain, they all stained numerous 0.5- to 2-μm-diameter puncta within synapse-rich layers of the neuropil (Figs. 1c, 1e, 1e′, and 1g). Double labeling with contrasting fluorophores showed that the majority of SV2-rich puncta were also synaptophysin-rich, supporting their identity as synapses (Figs. 1e and 1e′). The number, size, and distribution of such synapses did not differ detectably between agrin −/− and control brains (Figs. 1c–1h).

Although both pre- and postsynaptic development are perturbed at agrin −/− neuromuscular junctions, the best characterized effects of z^+ agrin are on aggregation of AChRs and associated cytoplasmic proteins in the postsynaptic membrane. To ask whether z^+ agrin exerted similar effects at central synapses, we stained brains with a panel of antibodies to postsynaptic markers of the major synapse types, excitatory glutamatergic synapses and inhibitory GABAergic synapses. The GluR1 subunit of the AMPA-type glutamate receptor, the $GABA_A$ receptor β2/3 subunits, the synapse-organizing proteins PSD-95 and gephyrin, and the PSD-95-associated protein GKAP all were

FIG. 1. Brains of agrin-mutant mice appear normal and develop clusters of synaptic vesicle antigens by E18. Sections of whole brain (a and b) or hippocampus (c–h) from heterozygous littermate controls (a, c, e, e′, g) and agrin mutants (b, d, f, f′, h) were stained with toluidine blue (a, b) or immunolabeled for synaptic vesicle proteins SV2 (c–f), synaptophysin (e′, f′; double label with SV2), and synapsin I + II (g, h). The synaptic vesicle antigens were all concentrated in the neuropil regions in puncta typical of presynaptic specializations. No differences were seen in brain morphology or in the distribution of synaptic vesicle proteins between agrin-deficient neurons and controls. Scale bar represents 400 μm in a and b, 100 μm in c and d, and 10 μm in e–h.


E18 +/-
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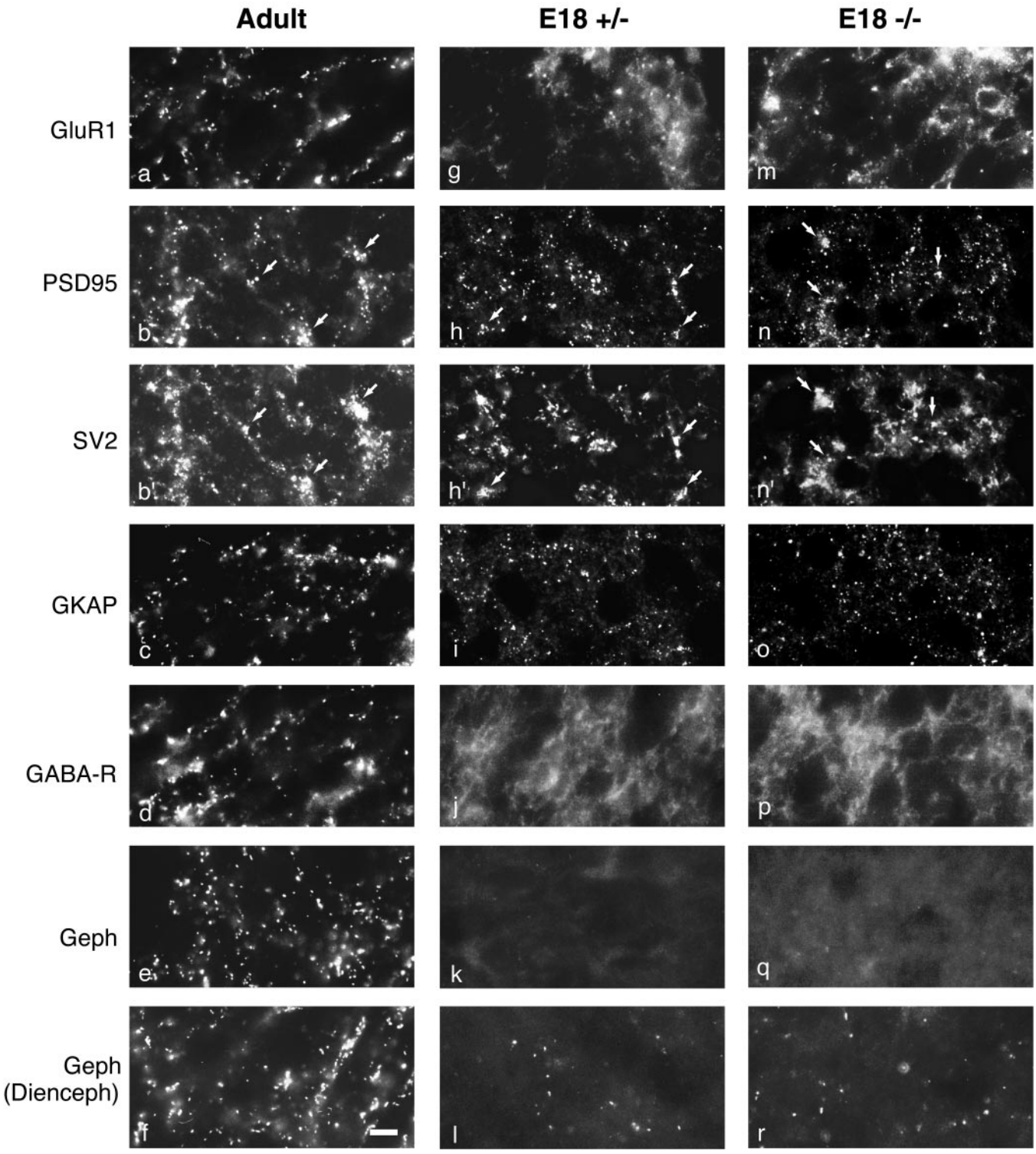
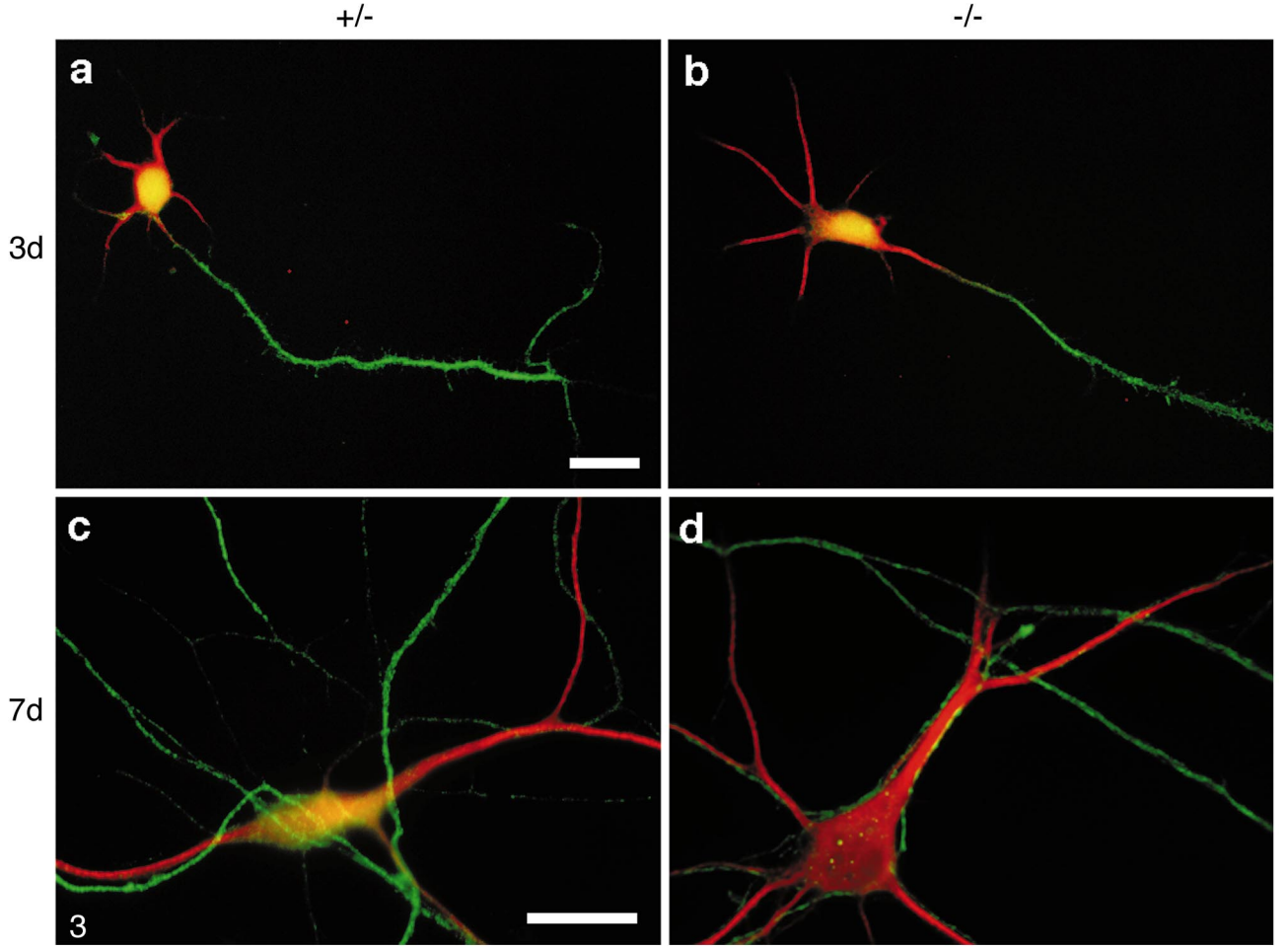


FIG. 2. Agrin-mutant mice cluster the glutamate postsynaptic proteins GluR1, PSD-95, and GKAP and the GABA/glycine postsynaptic protein gephyrin. Hippocampal sections from adult (a–e), E18 agrin +/− (g–k), and E18 agrin −/− (m–q) mice were immunolabeled for the GluR1 subunit of the AMPA-type glutamate receptor (a, g, m), postsynaptic density protein PSD-95 (b, h, n), SV2 (b′, h′, n′; double label with PSD-95), PSD-95 binding protein GKAP (c, i, o), $GABA_A$ receptor β2/3 subunits (d, j, p), and gephyrin (e, k, q). GluR1, PSD-95, and GKAP formed clusters by E18 in controls and in agrin mutants; many of these clusters were present at synaptic sites, as shown in the double labeling for SV2 and PSD-95 (arrows). $GABA_A$ receptors and gephyrin formed synapse-associated clusters in adult hippocampi but not at E18 in either agrin mutants or controls. Synapse-associated gephyrin-immunoreactive puncta were present in thalamus by E18 (f, l, r). For all of these postsynaptic markers, staining patterns were indistinguishable in mutants and controls. Scale bar, 10 μm.



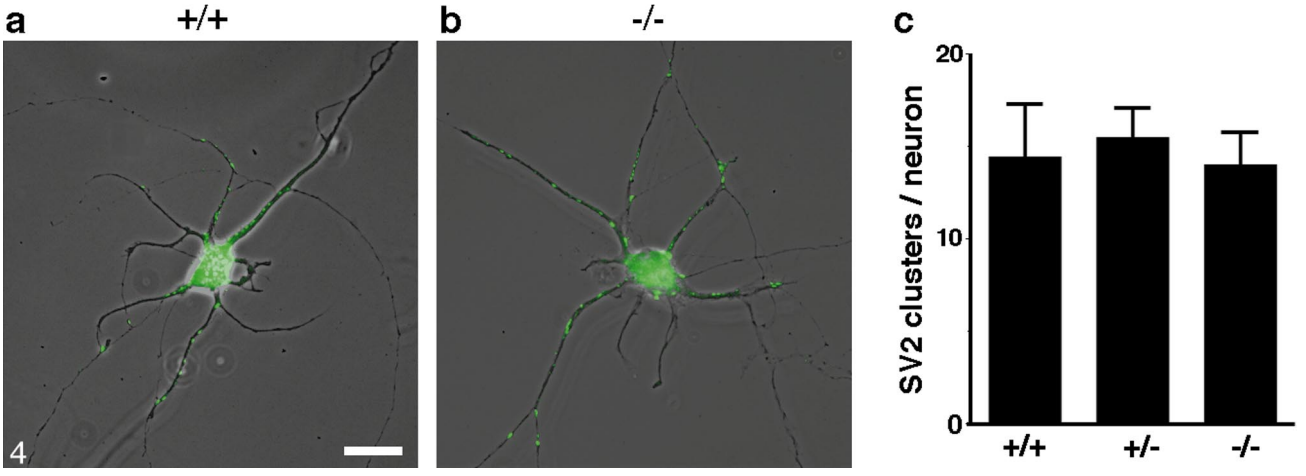


FIG. 3. Agrin-mutant neurons develop normal MAP2-positive dendrites and tau-1-positive axons. Hipppocampal neurons from control heterozygous (a and c) and homozygous (b and d) agrin-mutant mice were cultured for 3 days (a, b) or 7 days (c, d) and immunostained with antibodies against microtubule-associated proteins. MAP2 (red) was polarized to dendrites and dephospho-tau recognized by tau-1 (green) was polarized to axons with a normal developmental time course in the mutants. Scale bars, 20 μm.

FIG. 4. Agrin-mutant hippocampal neurons show no deficits in development of presynaptic specializations. Phase-contrast images are shown of hippocampal neurons cultured for 5 days from wild-type (a) and mutant (b) agrin-deficient mice superimposed with immunostaining for the synaptic vesicle protein SV2 (green). Synaptic vesicle proteins accumulated at synapses forming between axons and dendrites or cell bodies. (c) There was no significant difference between genotypes in the number of presynaptic specializations as defined by the number of SV2 clusters made onto each MAP2-positive somatodendritic region at 5 days in culture ($P > 0.5$ by t test between all groups; $n = 20$ cells from 1 animal for +/+, 38 cells from 2 animals for +/−, and 53 cells from 3 animals for −/−). Scale bar, 20 μm.

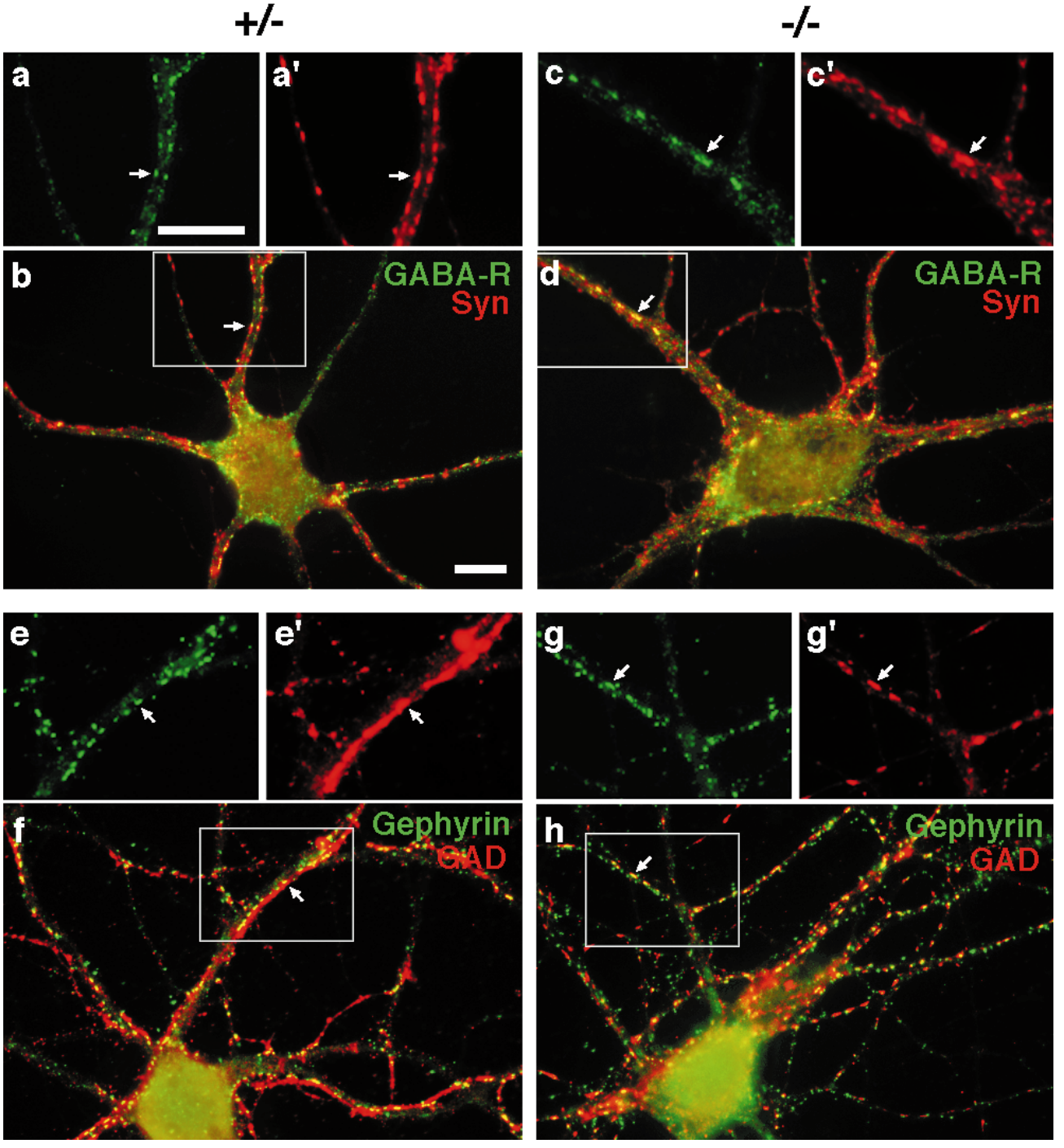


FIG. 5. Agrin mutants form normal GABAergic synapses including postsynaptic clusters of $GABA_A$ receptors and gephyrin. (a–d) Double-label immunostaining of 2-week hippocampal cultures for the $GABA_A$ receptor $\beta 2/3$ subunits (green) and the synaptic vesicle protein synaptophysin (red) revealed overlap between the presynaptic marker and postsynaptic GABA receptor clusters (arrows) in both heterozygotes and agrin mutants. Yellow in the double-color images indicates overlap. Single-channel images (a, c $GABA_AR$; a′, c′ synaptophysin) are shown for the boxed regions of the larger double-color images (b, d). (e–h) Double-label immunochemistry of 3-week cultured neurons for gephyrin (green) and glutamic acid decarboxylase (GAD, red) also showed a correlation between sites of GABAergic input and large clusters of gephyrin at postsynaptic sites (arrows). Gephyrin was also present in smaller clusters at nonsynaptic sites. No differences were observed in the distributions of gephyrin or $GABA_A$ receptors between agrin mutant and control neurons. Scale bars, 10 μm.

concentrated at synapses in adult brain (Figs. 2a–2f), as previously reported by others (Petralia and Wenthold, 1992; Baude *et al.*, 1995; Fritschy *et al.*, 1992; Nusser *et al.*, 1995; Cho *et al.*, 1992; Triller *et al.*, 1985; Cabot *et al.*, 1995; Sassoè-Pognetto *et al.*, 1995; Naisbitt *et al.*, 1997). GluR1, PSD-95, and GKAP were also concentrated in small puncta at E18 (Figs. 2g–2i), most of which were identifiable as synapse associated by double labeling with anti-SV2 or anti-synaptophysin (Figs. 2h and 2h′ and data not shown). In contrast, $GABA_A$ receptors were diffusely distributed on neurons at E18 (Fig. 2j). Little gephyrin was detectable in hippocampi at this age, but synapse-associated puncta were present in thalamus (Figs. 2k and 2l), presumably because synapses mature earlier in the diencephalon than in the telencephalon. For all of these postsynaptic markers, staining was indistinguishable in mutants and control (Figs. 2m–2r).

Hippocampal Neurons Cultured from Agrin-Mutant Mice Form Axons and Dendrites

The perinatal lethality of the agrin mutation restricted our ability to assess late steps in synapse formation *in vivo*. To circumvent this limitation, we turned to low density primary neuron cultures. E17 hippocampal neurons were grown on coverslips suspended above normal rat glial feeder layers, using methods previously developed to study neuronal polarity and synaptogenesis (Banker and Cowan, 1977; Goslin and Banker, 1998; Craig *et al.*, 1994; Rao *et al.*, 1998). The glial feeder layers do not express z^+ agrin but do produce z^- agrin isoforms which can be deposited on the neuron-bearing coverslips (Smith and O'Dowd, 1994; Escher *et al.*, 1996; Hager *et al.*, 1997; Wells and Fallon, 1997). The mutant neurons may also produce low levels of z^- agrin. Therefore, these experiments tested the function of the z^+ isoforms of agrin.

We analyzed cultures from 7 agrin homozygous mutants and 5 wild-type and 22 heterozygous littermates. We followed neuronal development both by morphology and by immunocytochemistry for molecular markers of axons (tau-1 antibody against dephospho-tau) and dendrites (MAP2). The agrin-deficient neurons were indistinguishable from controls at all stages tested, from 1 to 28 days in culture. Both control and agrin −/− neurons developed molecularly polarized axonal and dendritic compartments by 3 days in culture (Figs. 3a and 3b). By 1 week in culture, the dendrites had begun to take on their typical branched and tapered morphology, and the axons continued to elongate and formed an elaborate network, often coursing along the dendrites of other neurons in the culture (Figs. 3c and 3d).

Agrin-Mutant Neurons Develop Normal Presynaptic Specializations in Culture

Neurons lacking the z^+ agrin formed presynaptic specializations with a normal developmental time course. The presynaptic specializations were visualized as immunocytochemical puncta of synaptophysin or SV2 immunoreactivity where axons contacted dendrites or cell bodies (Figs. 4–7). Previous immunoelectron microscopic analysis has shown that these puncta correspond to morphological presynaptic specializations (Fletcher *et al.*, 1991). These presynaptic specializations were observed in both control and agrin-mutant neurons as early as 3 days in culture (not shown) and were very numerous by 3 weeks in cultures from all genotypes tested (Figs. 5–7).

We chose an early developmental time point, 5 days in culture, for a quantitative comparison of the number of presynaptic specializations made between neurons of each genotype. Figures 4a and 4b show typical phase-contrast images of agrin −/− and wild-type control neurons overlaid with the SV2 immunofluorescence images. SV2 formed puncta in axons at sites of contact with cell bodies and dendrites; in these neurons, the axons often grow alongside the dendrites (as seen in Figs. 3c and 3d but not always resolved by phase contrast), making many en passant contacts (as described by Bartlett and Banker, 1984). The 5-day neurons were double-labeled for SV2 and MAP2, neurons were chosen at random for analysis, and the number of SV2-labeled presynaptic specializations made onto each MAP2-labeled somatodendritic domain was counted. There were no differences between genotypes (Fig. 4c, $P > 0.5$, pairwise t tests between all groups).

Agrin-Mutant Neurons Form Differentiated GABAergic Synapses

Hippocampal cultures are composed primarily of glutamatergic pyramidal neurons plus about 7% GABAergic interneurons (Benson *et al.*, 1994); each cell type can synapse on itself and on the other cell type. We assessed GABAergic synapse formation between agrin −/− neurons with two double-immunolabeling protocols. In one, antibodies to the postsynaptic $GABA_A$ receptor $\beta 2/3$ subunit were combined with antibodies to the general presynaptic marker synaptophysin (Figs. 5a–5d). In the other, antibodies to the inhibitory synapse-organizing molecule gephyrin were combined with antibodies to the enzyme that synthesizes GABA, GAD (Figs. 5e–5h). Neurons lacking z^+ agrin were indistinguishable from controls with respect to all four of these synaptic antigens. $GABA_A$ receptors clustered normally at postsynaptic sites at 2–3 weeks in culture (Figs. 5a–5d). Gephyrin also formed large clusters on dendrites specifically opposite GAD-labeled boutons in the agrin mutants, in addition to the nonsynaptic smaller clusters found in axons and then dendrites during development (Figs. 5e–5h).

Agrin-Mutant Neurons Form Differentiated Glutamatergic Synapses

To assess the role of z^+ agrin in glutamatergic synapse formation, we stained cultures from agrin −/− embryos and

littermate controls with antibodies to four markers of glutamatergic postsynaptic sites: the GluR1 subunit of the AMPA-type glutamate receptor, the essential NR1 subunit of the NMDA type glutamate receptor, and the putative synapse-organizing proteins PSD-95 and GKAP (Figs. 6 and 7). In each case, cultures were double-labeled with one antibody to a glutamatergic postsynaptic marker plus an antibody to a synaptic vesicle protein. GluR1 was polarized to dendrites and clustered at postsynaptic sites on pyramidal cell spines (Figs. 6a–6d) and on GABAergic cell shafts of agrin −/− neurons (data not shown). Numerous spiny clusters of GluR1 were evident in mutants by 14 days in culture, indicating that excitatory synaptogenesis proceeded with little or no delay in mutants relative to controls. Consistent with previous findings on rat hippocampal cultures (Rao and Craig, 1997), NR1 was present in a mostly nonsynaptic pattern in 3-week spontaneously active cultures of control or agrin mutant mouse hippocampal neurons (data not shown) and chronic blockade of the NMDA receptor led to a more clustered, spiny, and synaptic distribution of NR1. There was no difference between agrin −/− and control neurons in the NR1 patterns; agrin mutants also formed numerous postsynaptic clusters of NR1 following chronic NMDA receptor blockade (Figs. 6e–6h).

Whereas the AMPA and NMDA receptors form clusters at subsets of excitatory postsynaptic sites in hippocampal cultures, the NMDA receptor binding protein PSD-95 and its associated protein GKAP cocluster with both types of receptor, form postsynaptic clusters earlier than either receptor type, and in general appear to be more ubiquitous markers of excitatory postsynaptic sites (Rao *et al.*, 1998). We therefore used PSD-95 as a marker to quantitate excitatory postsynaptic sites in hippocampal cultures of agrin −/− versus control embryos. PSD-95 formed numerous synaptic clusters in the mutants lacking z^+ agrin, as shown by double-labeling for PSD-95 and synaptophysin (Figs. 7a and 7b). Quantitation of dendrites from randomly selected neurons revealed no differences in the number of PSD-95 clusters per dendrite length between genotypes (Fig. 7c, $P > 0.5$, *t* test). The PSD-95 binding protein GKAP was also clustered at synaptic sites in the agrin mutant neurons in a pattern indistinguishable from controls (data not shown).

Exogenous Soluble C-Agrin Has No Effect on the Distribution of Synaptic Antigens in Hippocampal Cultures

An alternative strategy for uncovering potential roles of agrin in CNS synaptogenesis is to add agrin to hippocampal cultures and test for effects on glutamate or GABA synapse formation. Addition of soluble or cell-attached agrin to muscle cells in culture or *in vivo* induces ectopic AChR aggregates and other aspects of neuromuscular postsynaptic differentiation (reviewed in Ruegg and Bixby, 1998). Thus, we added soluble C-terminal agrin fragments C-Ag0,0 or C-Ag4,8 to wild-type hippocampal neurons in culture and immunolabeled 20–48 h later for glutamatergic and GABAergic presynaptic and postsynaptic antigens. The agrin was added in a range of concentrations from 1 to 20× the effective concentration for C-Ag4,8 on muscle. We performed these experiments from 9 days in culture when postsynaptic specializations are just beginning to form to 21 days in culture when postsynaptic specializations are reliably present. In the example shown in Fig. 8, C-Ag4,8 was added to neurons at 15 days and again at 16 days in culture (at 10× effective concentration for muscle), and the neurons were fixed and immunolabeled at 17 days in culture. In contrast to its effect on muscle cultures, the soluble agrin had no apparent effect on neuronal development in any of these experiments. Clusters of GluR1 and $GABA_A$ receptor $\beta 2/3$ subunits were seen in dendrites only at sites of contact with axons double-labeled for synaptic vesicle antigens (Fig. 8). Postsynaptic antigens were never observed at ectopic sites regardless of the presence of C-agrin. In addition, coculture of neurons with CHO cells expressing full-length agrin-4,8 on their surface had no apparent effect on the localization of synaptic markers (M. E. McGrath, J. T. Campanelli, and A.M.C., unpublished results). Thus exogenous z^+ agrin was unable to induce ectopic accumulation of components of central glutamatergic or GABAergic synapses as it does for neuromuscular cholinergic synapses.

DISCUSSION

Our main result is that z^+ agrin is not required for neuronal differentiation or for molecular differentiation of glutamatergic or GABAergic synapses in the hippocampus. In both mutants and controls hippocampi were morphologically and immunochemically differentiated at birth. Likewise, synaptophysin, SV2, GluR1, PSD-95, GKAP, and gephyrin formed synaptic clusters by E18 *in vivo* and by 1–2 weeks in culture. NR1 and $GABA_A$ receptor also formed synaptic clusters with further maturation in culture. No difference was found between genotypes in the timing or extent of axon and dendrite outgrowth or development of pre- or postsynaptic specializations. Quantitative analysis for SV2-labeled contacts at 5 days in culture and for PSD-95clusters at 3 weeks in culture revealed no differences between genotypes. In addition, exogenous z^+ agrin was unable to induce ectopic accumulation of glutamate orGABA receptors on hippocampal neurons as it does for AChRs on the muscle surface. Thus, although z^+ agrin is widely expressed in the nervous system, it is not essential or sufficient for differentiation of the major synapse types in brain.

Agrin: Not a Synaptic Differentiation Factor in the CNS?

Our results show conclusively that z^+ agrin is dispensable for the morphological and molecular specialization of glutamatergic and GABAergic synapses. It is possible that z^+ agrin does play some role in CNS synapse formation but is redundant or can be compensated during development of the agrin mutants or promotes some aspect of differentia-

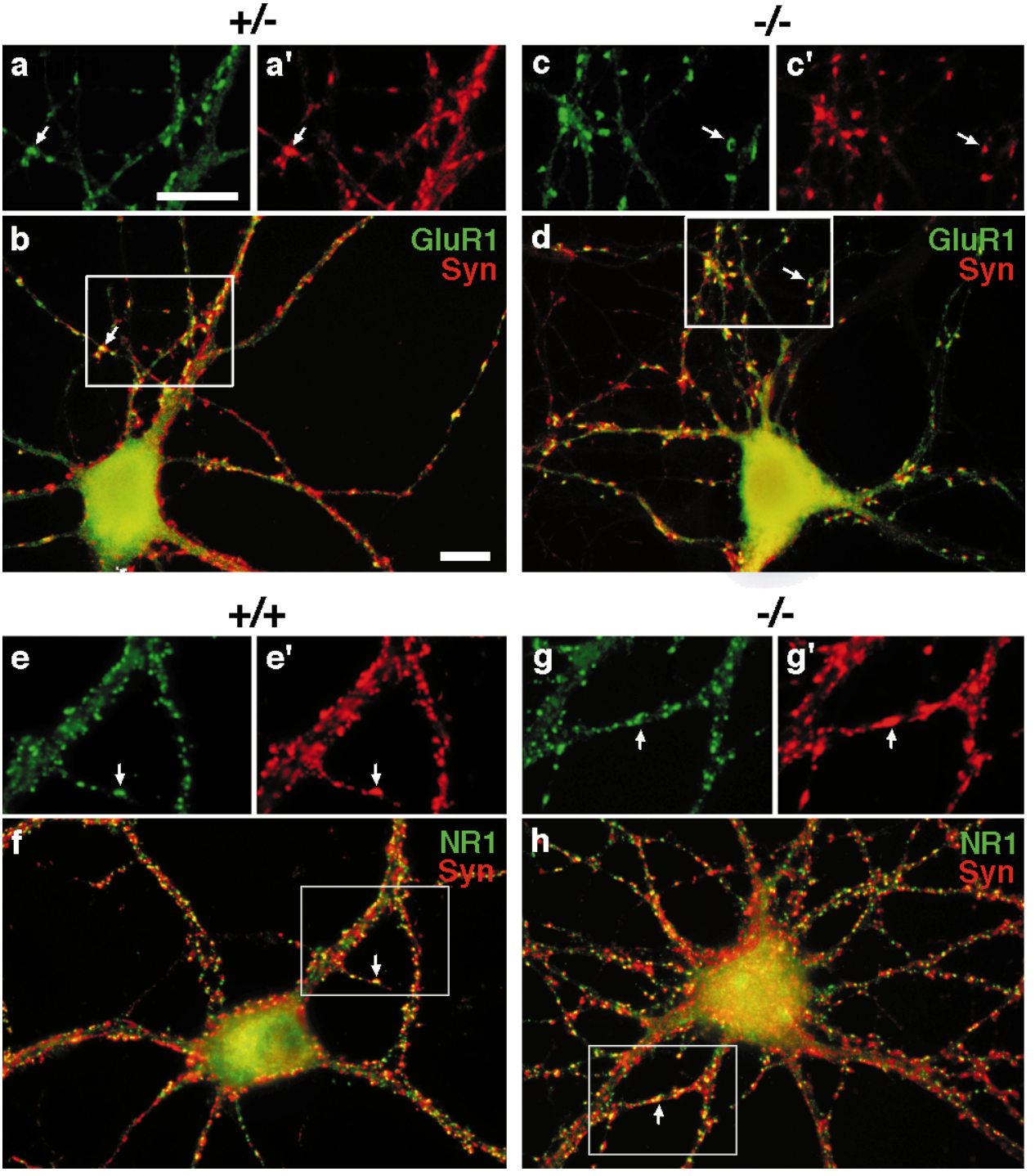


FIG. 6. AMPA- and NMDA-type glutamate receptors form numerous postsynaptic clusters in agrin-mutant neurons. (a–d) Double-label immunostaining of 2-week hippocampal cultures for the AMPA glutamate receptor subunit GluR1 (green) and the synaptic vesicle protein synaptophysin (Syn, red) revealed GluR1-enriched spines opposed to presynaptic terminals (arrows). (e–h) Immunocytochemistry of 3-week hippocampal cultures for the essential NMDA receptor subunit NR1 (green) and synaptophysin (red) revealed numerous NMDA receptor clusters at postsynaptic sites (arrows). No differences were observed between agrin mutants and controls in the distributions of either glutamate receptor. Scale bars, 10 μm.

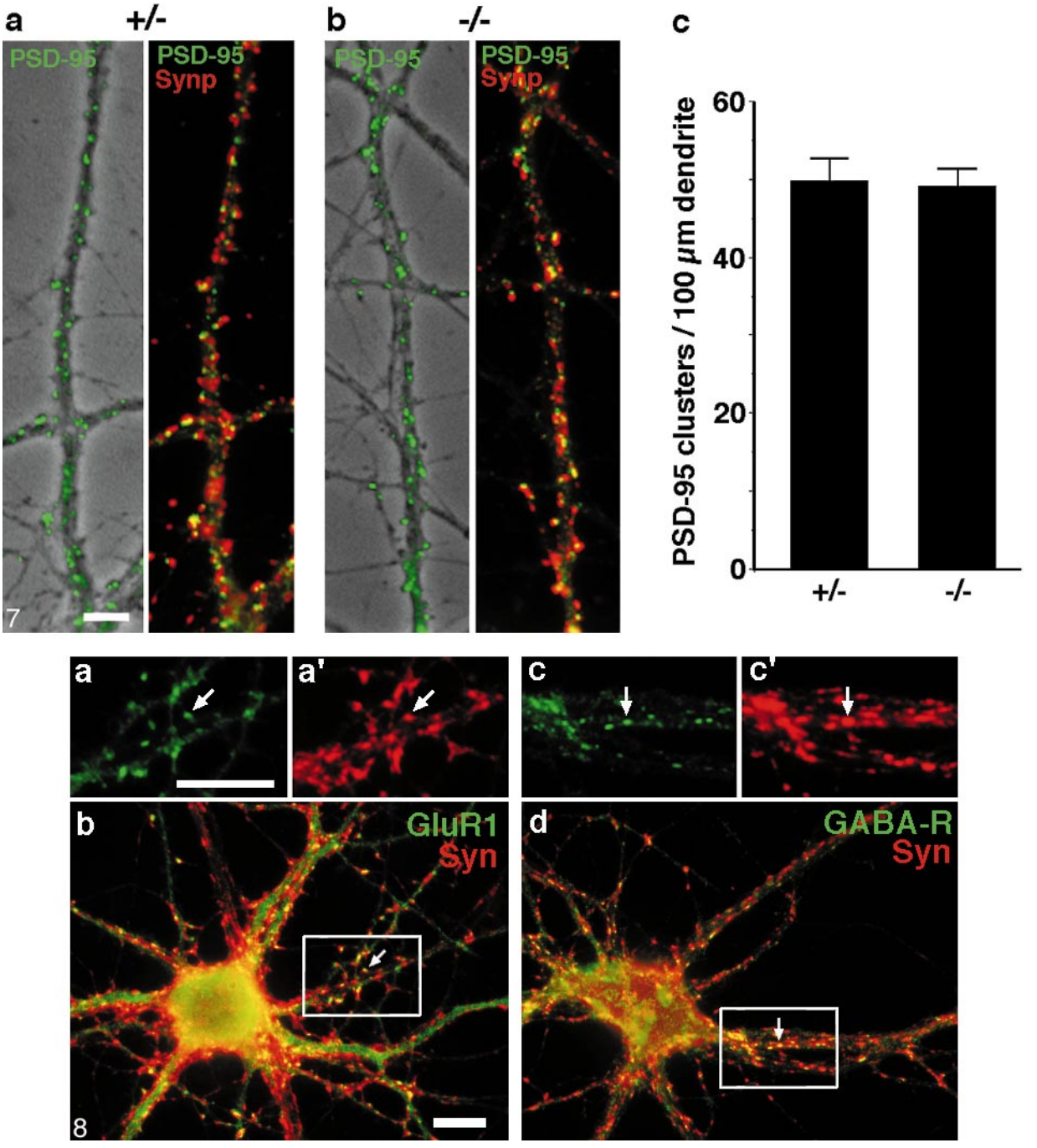


FIG. 7. Agrin-mutant neurons show no deficits in the development of glutamatergic postsynaptic specializations. (a and b) The PDZ domain protein PSD-95 (green) clustered on dendrites at sites of contact with axons as seen in overlay of the immunofluorescence image on the phase-contrast images. These clusters occurred at postsynaptic sites opposed to a subset of synaptophysin (Synp, red)-labeled terminals. Equivalent results were found for hippocampal neurons cultured for 18 days from heterozygous (a) and homozygous (b) agrin-deficient mice. (c) There was no significant difference between genotypes in the number of glutamatergic postsynaptic specializations as defined by the number of PSD-95 clusters per dendrite length ($P > 0.5$ by t test; $n = 23$ cells from 2 animals for $+/-$ and 21 cells from 2 animals for $-/-$). Scale bar, 5 μm.

FIG. 8. Exogenous soluble C-Ag4,8 has no effect on the distribution of glutamate or GABA receptors. Hippocampal cultures were exposed to two aliquots of C-Ag4,8, each at 10× the effective concentration for muscle, on days 15 and 16 and immunolabeled on day 17 for GluR1 (green) and synaptophysin (red, a and b) and for $GABA_A$ receptor $\beta 2/3$ subunits (green) and synaptophysin (red, c and d). Both receptor types exhibited only the normal pattern of clusters opposed to presynaptic specializations (arrows); ectopic clusters were not observed. Scale bars, 10 μm.

tion we have not assessed. Homologs of agrin have not yet been reported, and we found none in a search of public databases, so there is no reason to suspect redundancy in that sense. On the other hand, it is possible that z^- forms of agrin are important for synapse formation. Since the z^- forms are not specifically localized to synapses in the CNS (Kröger, 1997; Wells and Fallon, 1997) and are synthesized by glia as well as neurons (McMahan *et al.*, 1992; Smith and O'Dowd, 1994; Escher *et al.*, 1996; Hager *et al.*, 1997), it seems unlikely that they perform a specific *trans*-synaptic signaling function analogous to the role of z^+ agrin at the NMJ. z^- agrin might function in a permissive role in neuronal or synapse maturation. However, preliminary studies of a newly generated agrin-null mutant have also failed to detect defects in CNS development (R. Burgess and J.R.S., unpublished). The inability of soluble or cell-attached z^- or z^+ agrin to induce ectopic clusters of postsynaptic markers in wild-type hippocampal neurons indicates that agrin is not a sufficient signal to induce molecular aspects of glutamatergic or GABAergic synaptic differentiation. This is in clear contrast to the ability of exogenous z^+ agrin to induce molecular aspects of postsynaptic specializations on cultured muscle cells. If compensation masked an effect of z^+ agrin in the mutant, we might have expected to see an effect of exogenous agrin on wild-type neurons. These results, taken together, suggest that redundancy or compensation in the mutants is not masking an effect of agrin in CNS synapse formation.

We have assessed essentially every aspect of glutamate and GABA synapse formation for which molecular markers are available, including clustering of presynaptic vesicles, clustering and alignment of postsynaptic receptors and associated synapse-organizing or signal-transducing proteins, and activity-dependent synaptic localization of NMDA receptors. Independent evidence indicates that physiological maturation of glutamatergic synapses can also occur in the absence of z^+ agrin. In neocortical cultures from agrin mutant mice, Li *et al.* (1997a) found no defects in functional glutamatergic synaptic transmission and actually a slight increase in the frequency of glutamatergic miniature postsynaptic currents. Thus it seems unlikely that z^+ agrin is required for any aspect of glutamate or GABA synapse formation.

A final possibility is that agrin may function in the development of other synapse types not assessed here. One obvious candidate is the neuronal nicotinic cholinergic synapse, which is the central synapse most closely related to the skeletal NMJ. To date, however, only subtle defects have been observed in nicotinic cholinergic synapses of sympathetic ganglia from agrin $-/-$ mice (G.F. and J.R.S., unpublished).

Nonsynaptic Functions for Agrin in the CNS

Synaptic differentiation is not the only possible function for agrin in the CNS. Agrin mRNA expression is induced prior to synaptogenesis, being abundant in the developing mammalian nervous system as early as E13 (Stone and Nikolics, 1995). In the developing chick nervous system, agrin is abundant in basal laminae and axon tracts (Halfter *et al.*, 1997). In some immunocytochemical studies, agrin has been observed in cell bodies and proximal dendrites (Cohen *et al.*, 1997) or in axons (Escher *et al.*, 1996) of central neurons. The early expression of agrin, its abundance in basal lamina and axon tracts, its domain structure (including regions homologous to follistatin, EGF, and laminin), and its interactions with NCAM (Halfter *et al.*, 1997), integrins (Martin and Sanes, 1997), and laminin (Denzer *et al.*, 1997) suggest roles in differentiation, cell migration, or axon outgrowth. In the current study, we found no morphological or histological defect except a slight decrease in brain size accompanying the slight decrease in embryo size, in the z^+ agrin mutants. Future studies will be required to determine whether z^- agrin which is still present in these mutants functions in neuronal differentiation, cell migration, or axon outgrowth in the CNS.

Implications for Specificity of Synaptogenic Signals

Our results add to the increasing evidence that each synapse type has evolved its own set of molecular signals for synapse development and organization. On the postsynaptic side, rapsyn, gephyrin, and PDZ domain proteins of the PSD-95 and GRIP families are structurally unrelated molecules that bind to receptors for different neurotransmitters and appear to function directly in localizing these receptors to synapses. There is additional specificity beyond neurotransmitter phenotype, in that rapsyn is required for localizing AChRs at the NMJ but not for localizing neuronal AChRs at synapses in the sympathetic ganglion (Feng *et al.*, 1998). Based on the results presented here, molecules distinct from and possibly unrelated to z^+ agrin function as interneuronal signals for glutamate and GABA synaptic differentiation. Considering the smaller size of the interneuronal synaptic cleft (10–15 nm versus about 50 nm at the NMJ), these signaling molecules may be transmembrane proteins rather than agrin-like extracellular matrix proteins. Several transmembrane proteins that may fulfill this function have been found at glutamatergic (and in some cases GABAergic) synapses: densin-180 (Apperson *et al.*, 1996), neurexins, neuroligin (Irie *et al.*, 1997), cadherins (Yamagata *et al.*, 1995; Uchida *et al.*, 1996; Fannon and Colman, 1996), and cadherin-related neuronal receptors (Kohmura *et al.*, 1998). It will be important to determine whether any of these proteins is required for the *trans*-synaptic signaling that organizes synaptic differentiation in the brain.

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