Mismatched Appositions of Presynaptic and Postsynaptic Components in Isolated Hippocampal Neurons

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To determine whether presynaptic input is necessary for postsynaptic differentiation, we isolated hippocampal neurons in microisland culture and thus deprived pyramidal cells of GABA input and GABAergic neurons of glutamate input. We find that glutamate input is necessary for clustering the AMPA-type glutamate receptor but not for clustering the NMDA receptor or the associated PSD-95 family scaffold in GABAergic cells; GABA input is not necessary for clustering the $GABA_A$ receptor or gephyrin in pyramidal cells. Isolated neurons showed a surprising mismatch of presynaptic and postsynaptic components. For example, in isolated pyramidal neurons, although $GABA_A$ receptor clusters covered <4% of the dendritic surface and presynaptic boutons covered <12%, a full two-thirds of the $GABA_A$ receptor clusters were localized inappropriately opposite the non-GABAergic, presumed glutamatergic, terminals. Furthermore, inhibitory and excitatory postsynaptic components were segregated into separate clusters in isolated cells and apposed to separate boutons of a single axon. Thus, $GABA_A$ receptors were clustered opposite some terminals, whereas NMDA receptors were clustered opposite other terminals of a single axon. These results suggest the involvement of a synaptogenic signal common to glutamate and GABA synapses that permits experimentally induced mismatching of presynaptic and postsynaptic components in isolated neurons, as well as a second specificity-conferring signal that mediates appropriate matching in mixed cultures.

Key words: synaptogenesis; hippocampus; neuron culture; glutamate receptor; GABA receptor; PSD-95; gephyrin; autapse

Synapse formation requires the aggregation of receptors and associated signaling molecules in dendrites in precise apposition to aggregates of synaptic vesicles and release machinery in axons. The clustering of neurotransmitter receptors in the postsynaptic membrane has been well studied at the neuromuscular junction, where acetylcholine receptors cluster at the muscle end plate (for review, see Sanes and Lichtman, 1999). Although acetylcholine receptors can cluster spontaneously on the muscle surface, nerve input induces a redistribution of receptors to generate local clustering and stabilization beneath the nerve terminal. It has been known for some time that similar postsynaptic receptor aggregates are present at CNS synapses (Triller et al., 1985; Somogyi et al., 1989; Craig et al., 1993), but the process by which these aggregates form and the role of presynaptic input are not well understood. In addition, the multiplicity of transmitter systems and the problem of segregating receptors to appropriate postsynaptic sites further complicate receptor clustering in neurons.

Previous studies have shown that presynaptic and postsynaptic specializations for synapses using the neurotransmitters GABA and glutamate are appropriately matched in hippocampal neurons. Thus, AMPA- and NMDA-type glutamate receptors along with the excitatory synapse-associated molecules of the PSD-95 family, GKAP/SAPAP, α-actinin, syn-GAP, and Shank cluster opposite glutamate terminals but not opposite GABA terminals in hippocampal cultures (Craig et al., 1994; Kim et al., 1997; Chen et al., 1998; Kim et al., 1998; Wyszynski et al., 1998; Naisbitt et al., 1999). Immunoelectron microscopy of hippocampal tissue also indicates selective localization of AMPA and NMDA receptors and the PSD-95 family to asymmetric, often spiny, synapses (Nusser et al., 1998a; Petralia et al., 1999; Valtschanoff et al., 1999; Racca et al., 2000; Sans et al., 2000). In contrast, clusters of $GABA_A$ receptor and the inhibitory synapse-associated molecule gephyrin are found at symmetric postsynaptic sites in hippocampal neurons opposite GABA terminals but not opposite glutamate terminals (Fritschy et al., 1992; Craig et al., 1994, 1996; Nusser et al., 1995).

To test the role of specific presynaptic input in generating matching postsynaptic specializations, we analyzed isolated hippocampal neurons grown in microisland culture. Isolation in microcultures allows autaptic connections to form but prevents the pyramidal cells from receiving GABAergic input and the GABAergic neurons from receiving glutamatergic input. We find that most postsynaptic components can form spontaneous clusters. Surprisingly, these receptor clusters are not randomly distributed but selectively localize opposite the chemically inappropriate type of presynaptic terminal.

MATERIALS AND METHODS

Neuronal cultures. Hippocampal neuronal cultures were prepared from 18 d embryonic rats as described in Goslin et al. (1998). For microisland cultures, the substrate was modified according to the method of Segal (1991). The coverslips were coated with 0.2% agarose, dried overnight under UV light, sprayed with a solution of 1% poly-L-lysine in borate buffer by the use of a microatomizer (Thomas Scientific Company), dried again under UV light, and incubated overnight in minimal essential medium (MEM) with 10% horse serum before plating. A large central island of polylysine was created by pipetting on 0.5 μl of solution. This ensured that there were always multiply innervated neurons on the same coverslip to act as a control. Neurons were plated on poly-L-lysine substrates in MEM with 10% horse serum at a density of 2000 cells/cm^2. The coverslips were incubated "face-up" for 6–18 hr to allow cells to attach before flipping over onto a glial monolayer and growing them in serum-free MEM with N2. Microisland cultures were treated with 100 μM 2-amino-5-phosphonovaleric acid (APV) from 7 d after plating until fixation. All analysis of microislands and matched controls shown here was performed after 16–29 d in culture. For the effects of activity blockade on synapse development in multi-innervated cultures, neurons were cultured chronically in the presence of 1 μM tetrodotoxin, 50 μM picrotoxin, 1 μM CNQX, and either 100 μM APV or 10 μM MK-801 from day 2 until they were analyzed at day 16 in culture.

Immunocytochemistry. Cells were fixed in paraformaldehyde or methanol and immunostained as described previously (Craig et al., 1993; Rao and Craig, 1997). Primary antibodies used for immunostaining were as follows: rabbit anti-microtubule-associated protein 2 [anti-MAP2; 266; gift of S. Halpain (Halpain and Greengard, 1990); 1:20,000], mouse anti-

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dephospho-tau (tau-1; Boehringer Mannheim, Indianapolis, IN; 1:400), rabbit anti-synaptophysin [G-95; gift of P. DeCamilli (Navone et al., 1986); 1:8000], rabbit anti-GABA (Sigma, St. Louis, MO; 1:20,000), guinea pig anti-GluR1 [gift of R. Huganir (Craig et al., 1993); 1:1600], mouse anti-glutamic acid decarboxylase (anti-GAD; GAD6; Developmental Studies Hybridoma Bank; 1:2), mouse anti-$GABA_AR$ β2,3 subunit (bd17; Boehringer Mannheim; 1:100), guinea pig anti-$GABA_AR$ γ2 subunit [gift of J.-M. Fritschy (Fritschy and Mohler, 1995); 1:1000], mouse anti-gephyrin (R7a; Boehringer Mannheim; 1:500), guinea pig anti-PSD-95 family [gift of M. Sheng (Kim et al., 1995); 1:300], mouse anti-PSD-95 family (6G6-1C9; Affinity Bioreagents; 1:1000), rabbit anti-GAD (Chemicon, Temecula, CA; 1:2000), mouse anti-NR1 (PharMingen, San Diego, CA; 1:5000), and rabbit anti-NR2A (Upstate Biotechnology, Lake Placid, NY; 1:2000). Secondary antibodies and fluorescent streptavidin were obtained from Vector Laboratories (Burlingame, CA) or Jackson Immunoresearch Laboratories (West Grove, PA). Fluorescent secondaries were all used at 1:200 except FITC–anti-guinea pig secondary that was used at 1:600. When tertiary staining was used, biotin-conjugated secondaries were used at 1:600 followed by Texas Red- or fluorescein-conjugated streptavidin at 1:2000 or aminomethylcoumarin streptavidin at 1:50.

Digital images of fluorescently labeled cells were captured on a Photometrics cooled CCD camera mounted on a Zeiss Axioskop microscope with a 63×, 1.4 numerical aperture lens using Oncor or Metamorph imaging software. Images were prepared for printing with Adobe Photoshop.

Quantitation. To measure mismatching of presynaptic and postsynaptic elements on isolated neurons (Table 1), microisland cultures were double-labeled either for $GABA_AR$ and synaptophysin (see Fig. 4) or for PSD-95 or NR1/NR2A and GAD (see Fig. 5). Pyramidal neurons were identified by morphology, and the number of $GABA_AR$ clusters was determined on the central clearly visualized portions of isolated neuronal processes. Clusters were defined by interactively thresholding the image using a threshold gray level that was 150–200% the intensity on the dendrite. $GABA_AR$ clusters were classified as mismatched if they were apposed to synaptophysin-immunoreactive puncta on an isolated pyramidal neuron. Apposition was measured by generating a binary mask from the thresholded GABA receptor image and widening the regions representing the clusters by 1 pixel all around. This mask was used on the synaptophysin image to count only synaptophysin clusters falling within the GABA receptor domains. For analysis of NMDA receptor or PSD-95 clusters, neurons were identified as GABAergic by positive labeling for GAD. These neurons were then analyzed for the number of NMDA receptor or PSD-95 clusters, and the percent mismatched was defined by the percent apposed to a GAD-labeled bouton (as described above for $GABA_AR$/synaptophysin). As a positive control, the extent of apposition of $GABA_AR$ and GAD puncta was measured in control contiguously cultured cells, whereas the apposition of PSD-95 or NR1 and GAD puncta was measured as a negative control. The percentages reported are the mean ± SEM. The apposition of PSD-95 with GAD after chronic activity blockade in contiguous culture was measured by the use of the same methods.

To measure the extent of separation of two kinds of postsynaptic elements in isolated pyramidal cells (Table 2), microisland cultures were triple-labeled for $GABA_AR$, PSD-95, and synaptophysin (see Fig. 6) or double-labeled for $GABA_AR$ and GluR1 or $GABA_AR$ and NR1/NR2A. Isolated pyramidal cells were identified by morphology. Clusters of each type, $GABA_AR$ or PSD-95/GluR1/NR1/NR2A, were counted by interactive thresholding. The $GABA_AR$ image was then used to generate a binary mask, and all thresholded clusters of excitatory markers in the paired image that fell within the $GABA_AR$ domains were counted as colocalized. As a positive control, the extent of colocalization of NR1 and PSD-95 was measured in control contiguously cultured cells. The percentages reported are the mean ± SEM.

RESULTS

Development of presynaptic specializations in isolated hippocampal neurons

The procedure for preparing microisland cultures was modified from that of Segal and Furshpan (Segal and Furshpan, 1990; Segal, 1991) and adapted to the low-density culture system of Banker and colleagues (Goslin et al., 1998). Neurons were plated onto microislands of permissive substrate on a background of nonpermissive substrate, resulting in the development of many isolated neurons on individual islands. Isolated neurons on microislands developed axons, dendrites, and presynaptic vesicle clusters with the same time course as did multi-innervated cells in contiguous culture (data not shown). MAP2, a dendritic marker, and dephospho-tau, an axonal marker, were segregated appropriately (Fig. 1*a,b*; 19 d in culture). The axonal processes made numerous contacts with the dendrites of isolated cells and often piled up around the edge of the permissive substrate on the islands. Labeling with an antibody to synaptophysin, a synaptic vesicle protein, was used to visualize synaptic vesicle clusters that are morphological indicators of presynaptic specializations (Fletcher et al., 1991). Synaptophysin puncta were usually not as abundant as in contiguous cell culture, although some isolated cells showed a similar density (Fig. 1*c,d*). On average, isolated cells had 56.4 ± 16.3 synaptophysin puncta per 100 μm dendrite at 3 weeks in culture ($n = 31$) compared with 85.3 ± 21.4 on multiply innervated cells ($n = 20$; $p < 0.001$, t test).

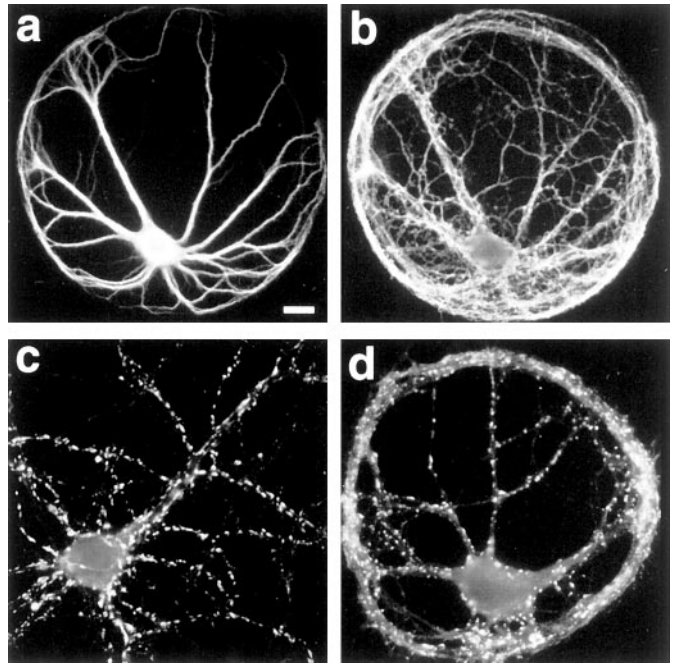

Figure 1. Effects of isolation on neuronal development. *a, b*, A pyramidal cell on a microisland stained with antibodies to MAP2 (*a*) and dephospho-tau (*b*) to label dendrites and axons, respectively. The axons grow profusely over the soma and dendrites, resulting in many contact sites where synapses could form. *c*, Pyramidal cell in contiguous culture labeled with an antibody to synaptophysin to show the density of synaptic vesicle clusters. *d*, Pyramidal island labeled with an antibody to synaptophysin showing a large number of puncta that represent presumptive autaptic sites. Scale bar, 10 μm.

Isolated pyramidal neurons form clusters of the $GABA_A$ receptor in the absence of GABAergic input

Immunostaining for the β2/3 subunits of the $GABA_A$ receptor was evaluated in isolated pyramidal cells to determine whether $GABA_A$ receptor clusters were formed in the absence of contact with GABAergic axons. Cultures were fixed at 18–29 d after plating and immunostained simultaneously with antibodies to GABA, the GluR1 subunit of the AMPA-type glutamate receptor, and the β2/3 subunit of the $GABA_A$ receptor (Fig. 2). Pyramidal neurons were identified by the absence of GABA immunostaining and the presence of GluR1 clusters on dendritic spines rather than the dendrite shaft (Craig et al., 1993). Ninety percent (69 of 77 cells pooled from three separate cultures) of the pyramidal cells on large multicell islands formed clusters of the $GABA_A$ receptor in these mature 3 week cultures. Surprisingly, a similar number (89%, 153 of 171 cells from the same cultures) of the isolated pyramidal cells also formed $GABA_A$ receptor clusters (Fig. 2). Therefore, GABAergic input is not necessary for the formation of $GABA_A$ receptor clusters.

GABAergic neurons deprived of glutamate input do not develop clusters of the AMPA-type glutamate receptor

We also analyzed isolated GABAergic neurons cultured in the absence of glutamatergic innervation (Fig. 3). Isolated and matched multi-innervated neurons were fixed at 18–29 d in culture and immunostained simultaneously with antibodies to GABA, GAD, and the GluR1 subunit of the AMPA-type glutamate receptor (Fig. 3*a–c*). GABAergic neurons were identified by positive

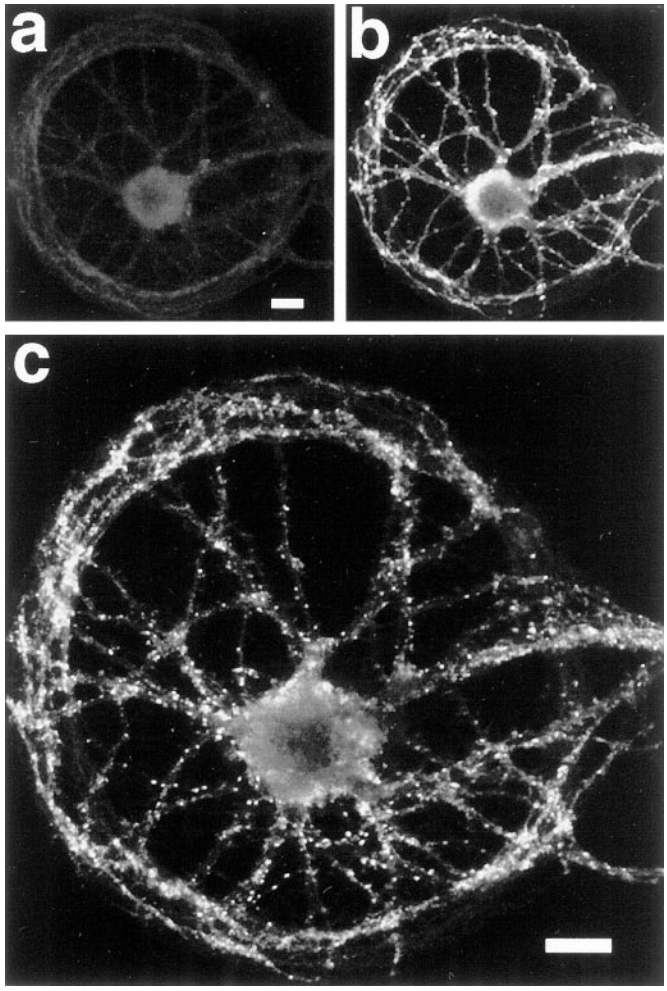

Figure 2. $GABA_A$ receptors can form clusters on pyramidal cells in the absence of GABA input. An isolated pyramidal cell identified by the absence of GABA staining (*a*) and the presence of spiny clusters of the AMPA receptor (*b*) shows distinct clusters when labeled with an antibody to the β2,3 subunit of the $GABA_A$ receptor (*c*). Scale bars: *a, b*, 10 μm; *c*, 10 μm.

immunostaining for GABA and/or the GABA synthetic enzyme GAD. GAD and synaptophysin double-labeling showed previously that GAD is concentrated at all synaptic terminals of isolated GABAergic neurons (Crump et al., 1999). The GluR1 subunit of the AMPA receptor was assessed because it was detected in all neurons in these hippocampal cultures and it coclusters with GluR2/3 (Craig et al., 1993). Whereas 59% (44 of 75 cells from four separate cultures) of the GABAergic cells on multiple-cell islands formed clusters of GluR1, none (0 of 27 cells from the same cultures) of the isolated GABAergic cells formed clusters of GluR1. These neurons that did not exhibit GluR1 clusters still exhibited significant diffuse GluR1 immunoreactivity in the soma and dendrites but not in axons. Therefore, glutamatergic innervation of these neurons is necessary to induce formation of AMPA receptor clusters.

Further analysis of isolated GABAergic neurons for other excitatory postsynaptic components yielded different results. PDZ domain proteins of the PSD-95 family (PSD-95/SAP90, SAP102, SAP97, and chapsyn-110/PSD-93) are ubiquitous and early components of excitatory postsynaptic specializations in this culture system (Rao et al., 1998) and *in vivo* (Sans et al., 2000). Using antibodies raised against PSD-95 that react with multiple PSD-95 family members, we found that isolated GABAergic cells did form clusters of the PSD-95 family proteins (Fig. 3*d*). Furthermore, the NMDA receptor subunits NR1 and NR2A also formed clusters on the isolated GABAergic cells (Fig. 3*e*; data not shown). The detection of clusters of the PSD-95 family and NR1/NR2A further supports the hypothesis, based on previous developmental observations (Rao et al., 1998), that glutamatergic innervation is not necessary to induce aggregates of these excitatory postsynaptic components.

Postsynaptic components are mismatched with presynaptic components in isolated neuron cultures

Comparing the distribution of these "spontaneously" formed clusters of postsynaptic components with that of presynaptic components in isolated cells, we found that postsynaptic clusters were not randomly distributed on the postsynaptic surface but were specifically apposed to presynaptic aggregates. In isolated pyramidal cells identified by morphology, we evaluated the relationship between $GABA_A$ receptor clusters and presumptive glutamatergic presynaptic specializations revealed by synaptic vesicle staining (Fig. 4*a*; Table 1). Surprisingly, a full two-thirds of the $GABA_A$ receptor β2/3 subunit clusters were apposed to presumptive glutamatergic presynaptic specializations indicated by synaptophysin staining. Each of these markers covered a small percentage of the total somatodendritic area measured ($GABA_A$ receptor, 3.7%; synaptophysin, 11.7%), indicating that the observed apposition was not random. In fact, the extent of apposition of the $GABA_A$ receptor clusters to presumptive glutamate terminals in the islands is not significantly different from the extent of apposition of $GABA_A$ receptor clusters to GAD puncta in control contiguous cultures (Table 1). Both mismatched and nonsynaptic $GABA_A$ receptor clusters in isolated cells were at the cell surface, because both were observed when the primary antibody was applied before cell permeabilization (data not shown). In additional experiments, we triple-stained microcultures with antibodies to GAD, synaptophysin and the γ2 subunit of the $GABA_A$ receptor at 3 weeks in culture. In multicell islands exhibiting GAD-positive puncta, the $GABA_A$ receptor γ2 clusters were selectively localized opposite the GAD puncta that indicate GABAergic presynaptic terminals, which were also immunoreactive for synaptophysin (Fig. 4*b*). However, even isolated neurons that were clearly GAD-negative showed similar apposition of $GABA_A$ receptor clusters with synaptophysin puncta that indicate non-GABAergic terminals (Fig. 4*c*). Double staining with synaptophysin and the inhibitory postsynaptic molecule gephyrin showed that gephyrin also formed clusters apposed to synaptophysin puncta in cells identified by morphology as pyramidal (Fig. 4*d*). Thus, in these isolated pyramidal cells, the inhibitory postsynaptic components are often specifically apposed to an excitatory presynaptic specialization. Note that excitatory postsynaptic components are also clustered on these isolated pyramidal cells (e.g., Fig. 2*b*; AMPAR) at presumptive autapses.

Furthermore, we compared the distribution of $GABA_A$ receptor and synaptophysin clusters in isolated cells from another type of hippocampal culture system that has been extensively used for physiological analyses (Segal, 1991; Gomperts et al., 1998). In this culture, postnatal neurons are grown directly on a glial bed in a medium containing serum. In this system also, isolated pyramidal cells (at 15 d in culture) had $GABA_A$ receptor β2/3 subunit clusters that were apposed to presumptive glutamatergic presynaptic specializations as indicated by synaptophysin staining (data not shown).

Do mismatched synapses of the opposite orientation (excitatory postsynaptic components apposed to inhibitory presynaptic specializations) also form? GABAergic cells in this system also form presumptive autaptic sites, as determined by the presence of matched GAD and GABAR puncta on isolated cells (Fig. 5*e*). To examine potential mismatching, we measured the extent of apposition of two excitatory postsynaptic components, the NMDA receptor and the PSD-95 family, to puncta of the inhibitory presynaptic component GAD (Fig. 5*a–d*; Table 1). Both NMDA receptor NR1 and NR2A subunits and PSD-95 family proteins selectively clustered opposite GAD-positive puncta in these isolated GABAergic neurons. In matched control contiguous cultures, 9.6% of the clusters of PSD-95 family proteins were apposed to GAD puncta. Analysis of appositions of the same two markers using the same method in isolated neurons revealed 48.7% apposition, significantly different from the number of appositions in

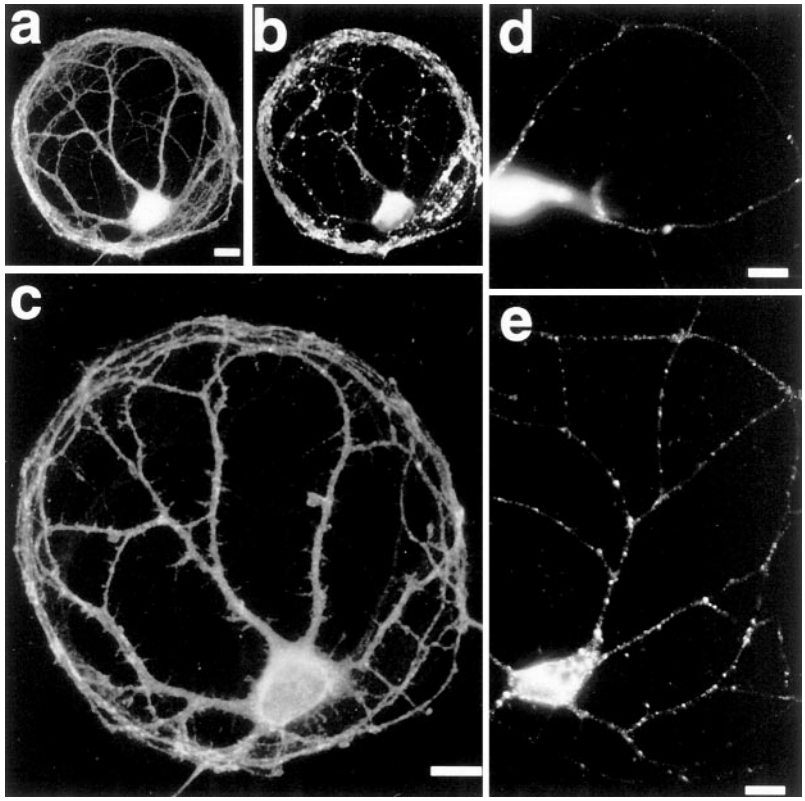

Figure 3. AMPA receptors are not clustered on GABAergic cells in the absence of glutamate input, but other components of excitatory postsynaptic sites are clustered. *a–c*, An isolated GABAergic cell identified by positive labeling for GABA (*a*) and GAD (*b*) shows no clusters when labeled with an antibody to the GluR1 subunit of the AMPA receptor (*c*). GluR1 immunoreactivity is present in the cell body and dendrites but excluded from axons (note the thin GABA-positive processes that do not label for GluR1). *d*, An isolated GABAergic cell identified by positive labeling for GAD (data not shown) bears distinct clusters of PSD-95. *e*, An isolated GABAergic cell identified by positive labeling for GAD (data not shown) shows punctate labeling for the NR1 subunit of the NMDA receptor. Scale bars: *a, b*, 10 μm; *c–e*, 10 μm.

contiguous culture (*t* test, $p < 0.001$; Table 1). Puncta of NR1 were also found to be frequently (43.1%) apposed to GABAergic terminals in these isolated cells, whereas the number of such mismatched appositions in control contiguous cultures was small (4.8%). Thus, in isolated neurons mismatched synapses of both types are common, inhibitory postsynaptic components apposed to excitatory presynaptic specializations and excitatory postsynaptic components apposed to inhibitory presynaptic specializations.

A similar mismatch of $GABA_A$ receptor clusters and non-GABAergic terminals was observed in isolated 8-d-old cells. This is a time when $GABA_A$ receptor clusters first form in this culture system, suggesting that mismatch may be an early feature of synaptogenesis. However, we have compared the level of mismatch of PSD-95 and GAD in control contiguous cultures at early (6 d) and late (19 d) developmental time points and found no significant difference. This observation suggests that either mismatching is not a common early stage in the development of synapses in contiguous culture or mismatch may be a transient feature that cannot be observed without dynamic imaging during synaptogenesis.

The low level of mismatched appositions that is seen in control contiguous culture could be interpreted as an artifact of our measurements, in that clusters that are close together but not apposed were counted as apposed because of the resolution of the method. However, many of these appositions were present in areas with low cluster density, arguing against such a crowding artifact. Furthermore, at these mismatched appositions the clusters appeared to be shaped similarly to and apposed as extensively as correctly matched appositions, suggesting that this estimate represents the baseline of overlap of incorrectly matched presynaptic and postsynaptic aggregates.

Inhibitory and excitatory postsynaptic components are segregated but both apposed to presynaptic specializations in isolated cells

The apposition of both glutamatergic and GABAergic postsynaptic components to presynaptic specializations in isolated cells, even presynaptic specializations of the inappropriate transmitter phenotype, suggested the hypothesis that all postsynaptic molecules might cluster at a single mixed type of postsynaptic element in these cells. To evaluate whether such mixed elements were formed, in isolated pyramidal cells we compared the distribution of the $GABA_A$ receptor clusters with that of GluR1, NR1/NR2A, PSD-95, or GKAP. Double-label immunocytochemistry with antibodies to $GABA_A$ receptor subunits and any of these proteins (PSD-95, NR1, and GluR1 labels shown in Fig. 6; data not shown for NR2A and GKAP) showed that the two types of clusters were primarily segregated. Quantitative analysis of these double labels confirmed minimal (<7%) colocalization of $GABA_A$ receptor with GluR1, NR1, or the PSD-95 family in isolated neurons, compared with a positive control of 86% colocalization of NR1 and the PSD-95 family in contiguous culture using the same quantitation method (Table 2). Thus, the inhibitory postsynaptic receptor was present in presumptive inhibitory postsynaptic specializations distinct from the excitatory postsynaptic specializations. Triple-label immunostaining for the $GABA_A$ receptor (β2/3 or γ2 subunits), PSD-95 family or NR1, and synaptophysin showed that both kinds of postsynaptic aggregate, containing either $GABA_A$ receptor or NR1/PSD-95 family proteins, were apposed to separate presynaptic specializations in pyramidal cells (Fig. 6). Thus, in these isolated pyramidal cells, when visualizing two glutamatergic terminals side by side on a single axon, one is apposed to a cluster of NMDA receptor or the PSD-95 family and the other is apposed to a cluster of $GABA_A$ receptor. The mismatched inhibitory postsynaptic receptor clusters apposed to excitatory presynaptic terminals were sometimes nearly as numerous as the matched excitatory postsynaptic receptor clusters apposed to excitatory presynaptic terminals (e.g., Fig. 6*b*). These experiments indicate that a single type of presynaptic element can be apposed separately to inhibitory or excitatory postsynaptic components. In the regular contiguous culture, however, this kind of mismatch is uncommon (see above) indicating that additional signaling events in the contiguous culture inhibit the mismatch and favor the appropriate pairing. One obvious feature of the appropriate pairing of presynaptic and postsynaptic components that might result in a preference for it is the presynaptic release and postsynaptic binding of neurotransmitter to its receptor, which would lead to receptor activation only at a correctly matched presynaptic and postsynaptic apposition.

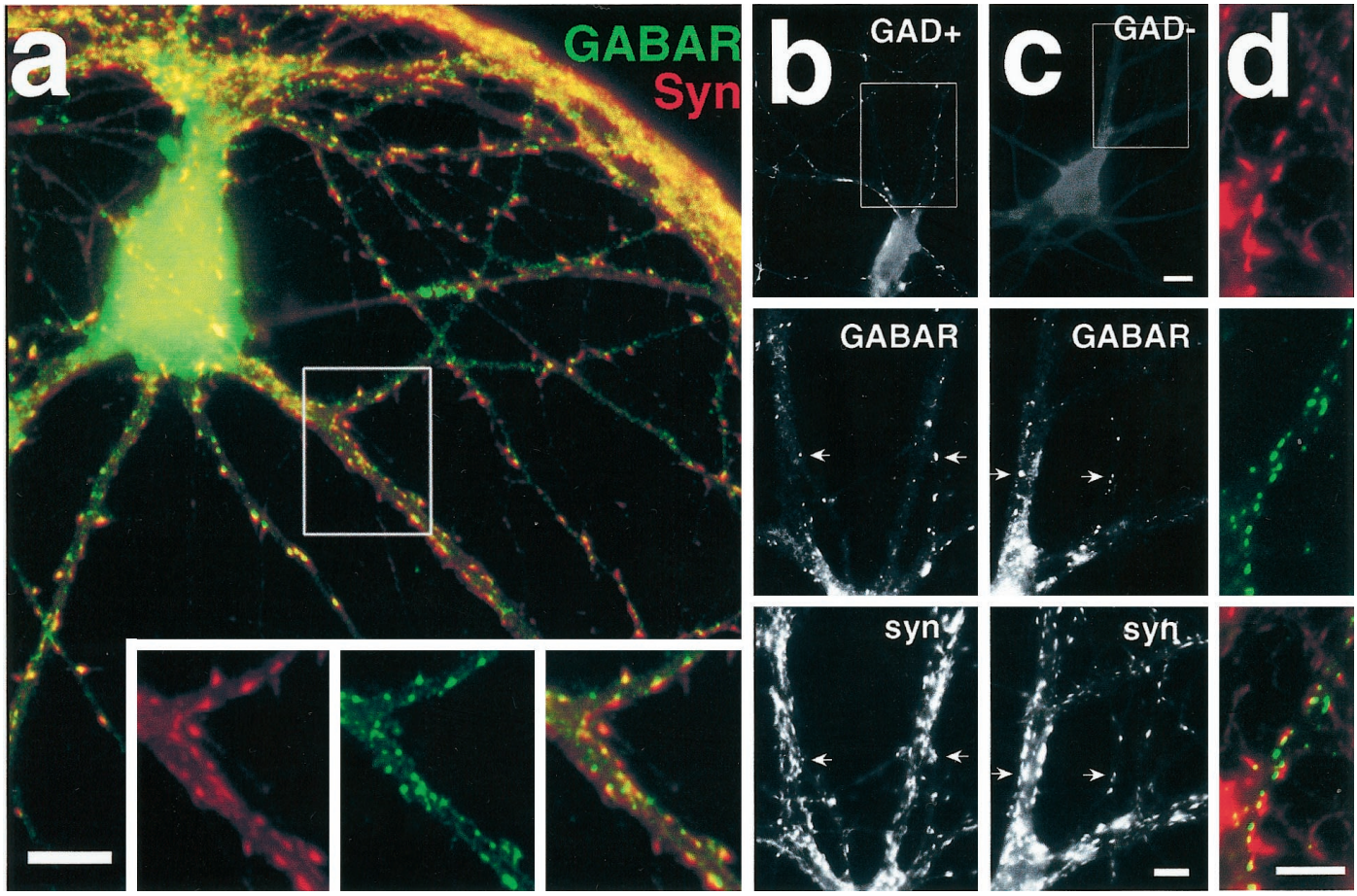

Figure 4. Mismatch of presynaptic and postsynaptic elements in pyramidal microislands. *a*, An isolated pyramidal cell immunolabeled with antibodies to the β2,3 subunit of the $GABA_A$ receptor (*green*) and synaptophysin (*red*) showing many appositions of the two (*yellow*). *Inset*, The *boxed region* in *a* shown at double magnification in separate *red* and *green panels* as well as the *red/green overlay*. *b, c*, Comparison of triple-label immunolabeling for GAD (*GAD*), γ2 subunit of the $GABA_A$ receptor (*GABAR*), and synaptophysin (*syn*) in an innervated cell (*b*) and an isolated one (*c*). Immunolabeling for GAD (*top panels*) shows GABAergic presynaptic specializations in the innervated cell in *b*, whereas the isolated pyramidal cell in *c* has none. The *boxed regions* are shown enlarged below in *middle panels* (GABAR) and in *bottom panels* (*syn*). Both innervated and isolated cells have clusters of the γ2 subunit of the $GABA_A$ receptor apposed to clusters of synaptic vesicles defined by puncta of synaptophysin immunolabel (*arrows*). *d*, Region of an isolated pyramidal cell immunolabeled with antibodies to gephyrin (*green, top panel*) and synaptophysin (*red, middle panel*). Many of the larger gephyrin clusters are apposed to synaptophysin (*yellow*, in the *bottom overlay panel*). Scale bars: *a*, 10 μm; *inset* (shown in *a*), 20 μm; *b, c*, GAD panels, 10 μm; *b, c*, GABAR and synaptophysin panels, 5 μm; *d*, 5 μm.

Appropriate matching of presynaptic and postsynaptic components occurs in contiguous culture even with chronic activity blockade

To test the role of receptor activation in the matching of presynaptic and postsynaptic components, contiguous cultures were grown chronically in the presence or absence of an activity blockade cocktail containing tetrodotoxin and antagonists of AMPA, NMDA, and $GABA_A$ receptors. Neurons were fixed at 16 d and immunostained for GAD and PSD-95, and the number of appositions was compared in the control cultures and after activity blockade, resulting in no obvious difference (Fig. 7). The number of PSD-95 clusters scored as apposed to GAD was not significantly different when the two conditions were compared (6.9 ± 0.9% for control cultures vs 6 ± 0.9% with blockade; $n = 10$ cells each per treatment from two separate cultures; t test, $p > 0.05$). Thus the release of transmitter and activation of the appropriate receptor

Table 1. Mismatch of synaptic markers on isolated neurons

Marker 1	Marker 2	Number of clusters apposed/ Total number of clusters	% Apposed	Number of cells	Number of clusters
Isolated neurons					
GABAR	Synaptophysin	23.9 ± 3.7/35.3 ± 4.4	66.6 ± 4.3	15	530
NR1	GAD	15.4 ± 2.7/41.4 ± 7.6	43.1 ± 8.2	11	455
PSD-95	GAD	16.3 ± 5.6/29.8 ± 7.0	48.7 ± 8.1	12	357
Innervated neurons					
GABAR	GAD	26.8 ± 2.5/40.1 ± 3.4	67.9 ± 3.3	16	641
GABAR	Synaptophysin	31.4 ± 3.2/51.5 ± 4.6	60.1 ± 2.9	19	979
PSD-95	GAD	4.3 ± 1.3/41 ± 4.8	9.6 ± 2.7	19	1045
NR1	GAD	5.0 ± 1.1/100.9 ± 13.7	4.8 ± 0.9	10	1009

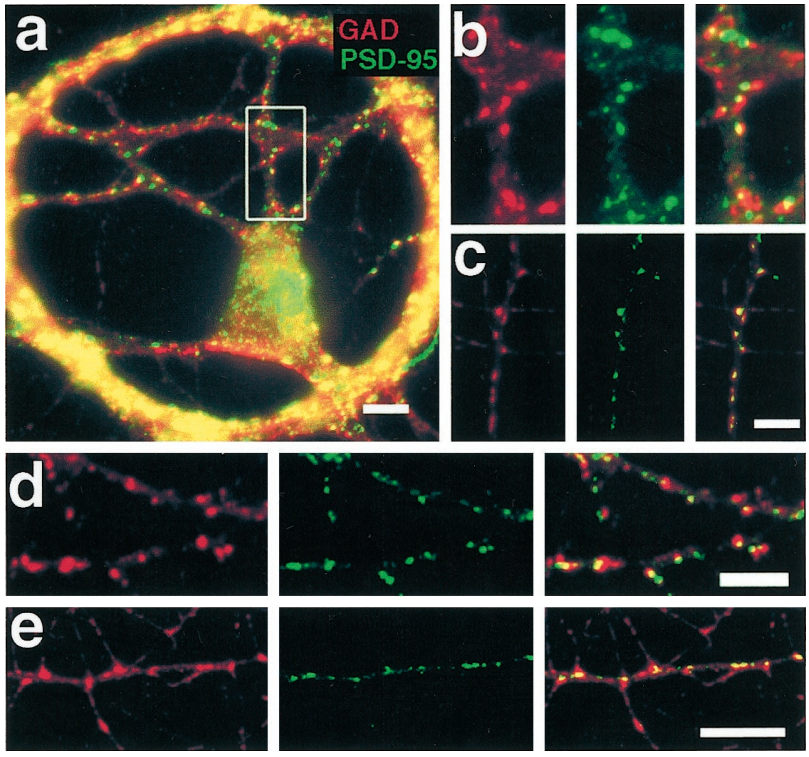

Figure 5. Mismatch of presynaptic and postsynaptic elements in GABAergic microislands. *a*, An isolated GABAergic cell immunolabeled with antibodies to PSD-95 (*green*) and GAD (*red*) shows a large degree of apposition of the two (*yellow*). *b, c*, A twofold magnification of the *boxed region* in *a* (*b*) and of a similar region from another isolated cell (*c*) is shown with PSD-95 in *green* (*left panel*), GAD in *red* (*middle panel*), and in the *red*/*green overlay* (*right panel*). *d*, Similar appositions are seen in an isolated GABAergic cell double-labeled for GAD (*red*) and the NR2A subunit of the NMDA receptor (*green*). *e*, Extensive apposition in an isolated GABAergic cell double-labeled for GAD (*red*) and the β2,3 subunit of the $GABA_A$ receptor (*green*) is shown. Scale bars: *a*, 10 μm; *d, e*, 5 μm.

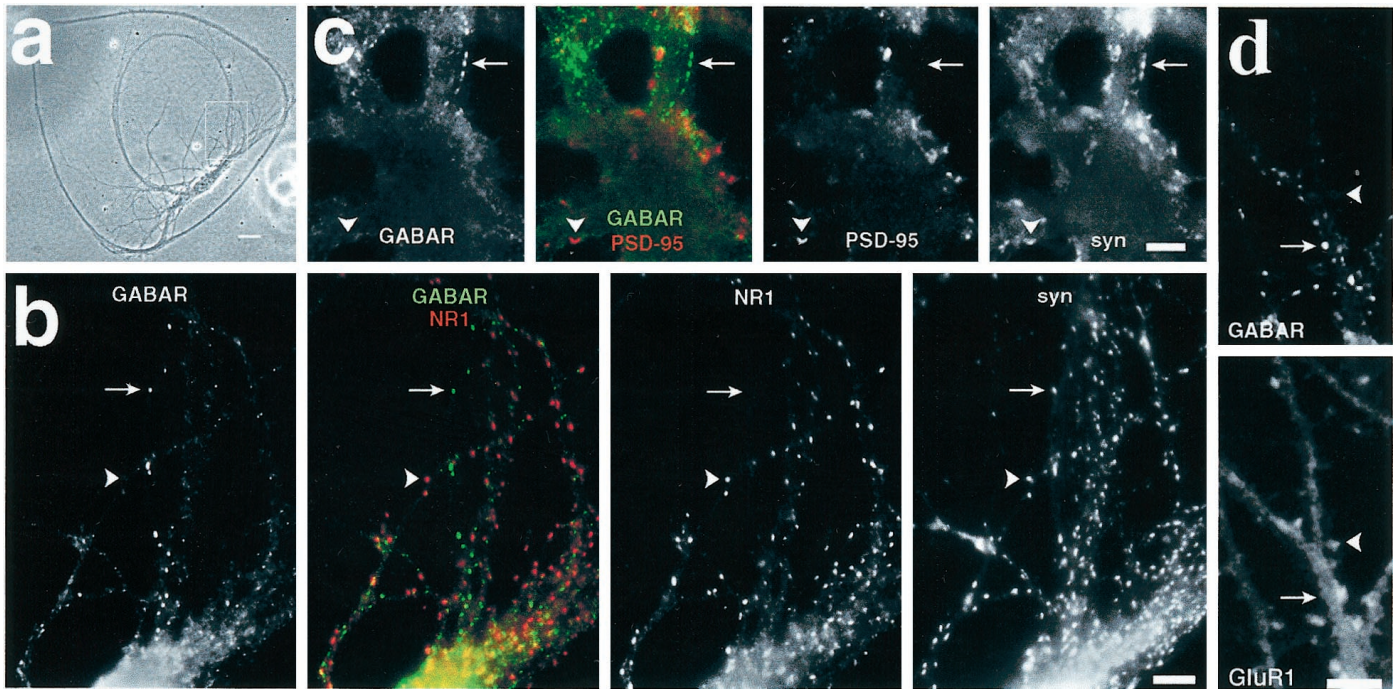

Figure 6. Segregation of inhibitory and excitatory postsynaptic specializations in isolated pyramidal cells. *a*, Phase image of an isolated pyramidal cell. Immunolabeling in the *boxed region* is shown at higher magnification in *b*. *b*, An isolated pyramidal cell immunolabeled with antibodies to the γ2 subunit of the $GABA_A$ receptor (*GABAR*), the NR1 subunit of the NMDA receptor (*NR1*), and synaptophysin (*syn*). An *arrow* indicates $GABA_A$ receptor clusters at synaptic sites without NR1. An *arrowhead* indicates NR1 clusters at synaptic sites without $GABA_A$ receptor. *c*, An isolated pyramidal cell immunolabeled with antibodies to the β2,3 subunit of the $GABA_A$ receptor (*GABAR*), PSD-95 (*PSD-95*), and synaptophysin (*syn*). An *arrow* indicates $GABA_A$ receptor clusters at synaptic sites without PSD-95. An *arrowhead* indicates PSD-95 clusters at synaptic sites without $GABA_A$ receptor. *d*, An isolated pyramidal cell immunolabeled with antibodies to the β2,3 subunit of the $GABA_A$ receptor (*GABAR*) and the GluR1 subunit of the AMPA receptor (*GluR1*). An *arrow* indicates $GABA_A$ receptor clusters at synaptic sites without GluR1. An *arrowhead* indicates GluR1 clusters at synaptic sites without $GABA_A$ receptor. Scale bars: *a*, 20 μm; *b–d*, 5 μm.

may not be required to generate appropriately matched presynaptic and postsynaptic elements.

DISCUSSION

In this report we examined the question of how central neurons generate functionally matched presynaptic and postsynaptic elements. By growing single neurons in isolation, we show that AMPA-type glutamate receptors will not form clusters in the absence of glutamate innervation but that $GABA_A$ receptors will form clusters in the absence of GABA innervation (Fig. 8). Surprisingly, mismatched synapses of both orientations were observed on isolated neurons; 66% of $GABA_A$ receptor clusters were ap-

Table 2. Lack of colocalization of inhibitory and excitatory postsynaptic markers on isolated neurons

Marker 1	Marker 2	Number of colocalized clusters/ Total number of clusters	% Colocalized	Number of cells	Number of clusters
Isolated neurons					
GABAR	NR1	1.9 ± 0.7/42.3 ± 9.1	4.1 ± 1.4	7	296
GABAR	GluR1	0.3 ± 0.2/21.0 ± 3.2	1.7 ± 1.2	10	210
GABAR	PSD-95	2.0 ± 0.8/25.8 ± 3.1	6.5 ± 2.2	17	439
Control neurons					
NR1	PSD-95	56.3 ± 10.8/69.1 ± 15.9	85.8 ± 3.1	10	691

posed to presumptive glutamatergic terminals on isolated pyramidal neurons, and 49% of PSD-95 clusters or 43% of NR1 clusters were apposed to GABAergic terminals on isolated GABAergic neurons. Additional synapses on the same neurons had appropriately matched presynaptic and postsynaptic elements, but components of glutamatergic and GABAergic postsynaptic sites were rarely colocalized. The experimentally induced mismatching of these elements suggests a common signal involved in the alignment of presynaptic and postsynaptic components during the formation of excitatory and inhibitory synapses.

Mechanisms of AMPA receptor clustering

We report that isolated GABAergic cells in hippocampal cultures cannot form AMPA receptor clusters in the absence of glutamatergic input. Similar results were reported by O'Brien et al. (1997) for subsets of isolated spinal cord neurons in culture. These data indicate that presynaptic contact is necessary for AMPA receptor cluster formation and imply that glutamatergic terminals release a specific molecular signal necessary for the clustering of AMPA receptors on apposing dendrites. However, although contact with a glutamatergic axon is necessary, it is not sufficient to induce AMPA receptor clustering because AMPA receptors cluster opposite some but not all glutamatergic terminals in these cultures and *in vivo* (Rao and Craig, 1997; Gomperts et al., 1998; Nusser et al., 1998a; Rao et al., 1998; Petralia et al., 1999; Takumi et al., 1999). Thus, AMPA receptor clustering requires an inductive signal from the glutamatergic terminal, perhaps neuronal activity-regulated pentraxin (Narp) (O'Brien et al., 1999) and, at least in pyramidal cells, additional signals that may be related to activity (Isaac et al., 1995; Liao et al., 1995; Shi et al., 1999).

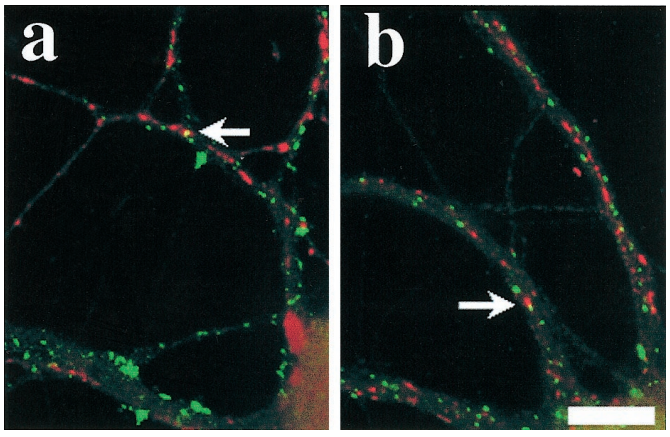

Figure 7. Blockade of postsynaptic receptors does not affect the degree of mismatch. Contiguous cultures grown under conditions of spontaneous activity (*a*) or with chronic blockade of AMPA, NMDA, and $GABA_A$ receptors as well as of voltage-gated sodium channels (*b*) were immunostained for GAD as a presynaptic marker of GABAergic synapses (*red*) and PSD-95 as a postsynaptic marker of glutamatergic synapses (*green*). Mismatched presynaptic and postsynaptic appositions (*arrows*) are rare under both conditions. Scale bar, 10 μm.

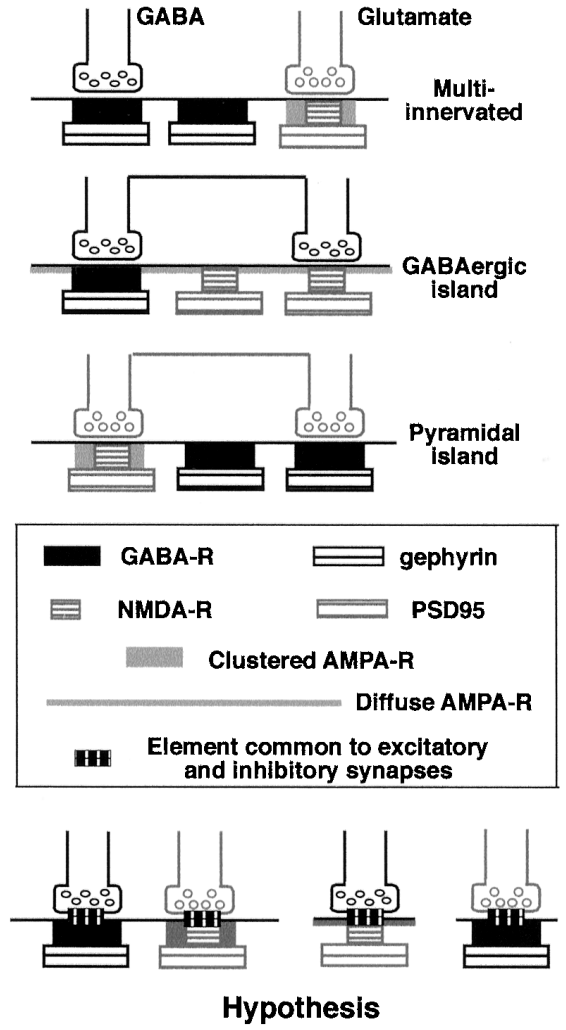

Figure 8. Summary of results leading to the hypothesis that a common signal must exist at excitatory and inhibitory synaptic sites that permits the observed level of synaptic mismatch. In multi-innervated cells, GABA-containing presynaptic specializations are specifically apposed to postsynaptic specializations containing clusters of GABA receptor as well as of gephyrin and other scaffolding molecules for inhibitory synapses, and glutamate-containing presynaptic specializations are apposed to postsynaptic clusters of AMPA- and NMDA-type glutamate receptors as well as scaffolding proteins such as PSD-95 family members. In contrast, in isolated GABAergic cells GABA-containing presynaptic specializations also become apposed to clusters of the NMDA receptor and PSD-95, and in isolated pyramidal neurons glutamatergic presynaptic specializations also become apposed to clusters of the GABA receptor and gephyrin scaffolds. The existence of such mismatched appositions suggests the hypothesis that there is an element common to GABA and glutamate synapses that permits mismatch.

Spontaneous formation of both presynaptic and postsynaptic elements

Studies of the neuromuscular synapse show that acetylcholine receptors form spontaneous clusters on cultured myotubes without neural input (for review, see Sanes and Lichtman, 1999). Postsynaptic membrane specializations resembling PSDs unapposed to presynaptic elements are observed in developing (Hinds and Hinds, 1976; Blue and Parnavelas, 1983) and adult CNS tissue (Desmond and Levy, 1998). In culture, uninnervated cortical neurons have $GABA_A$ response "hot spots" (Frosch and Dichter, 1992), and early in development hippocampal neurons cluster NMDA receptors and associated proteins in the absence of cell–cell contact (Rao et al., 1998). Cholinergic cultures of purified motoneurons form spontaneous extrasynaptic clusters of glycine and $GABA_A$ receptors (Levi et al., 1999). In this study, we report that $GABA_A$ receptors cluster on pyramidal cells in the absence of GABA input and that the NMDA receptor and PSD-95 cluster on GABAergic neurons in the absence of glutamatergic input. Some of these clusters of postsynaptic markers were apposed to mismatched presynaptic elements, but a significant number (33–57%; see Table 1) were not apposed to any presynaptic element. All these lines of evidence suggest that postsynaptic proteins can aggregate to form multimolecular complexes in the absence of input; the developmental sequence suggests that these aggregates can be precursors to the postsynaptic specialization.

Other studies suggest that axons form aggregates of synaptic vesicles and associated release machinery before contacting the somatodendritic surface. Nerve growth cones are known to be capable of spontaneous and evoked release of neurotransmitter (Hume et al., 1983; Young and Poo, 1983; Sun and Poo, 1987). Clusters of synaptic vesicles are present both at the growth cone and along the axons of neurons in culture (Kraszewski et al., 1995) and are capable of neurotransmitter release and recycling (Antonov et al., 1999; Zakharenko et al., 1999). On contact with the somatodendritic domain, axons develop larger and more stable clusters of synaptic vesicles that are of the same size as those seen in mature cultures and may be considered presynaptic specializations (Kraszewski et al., 1995; Ahmari et al., 2000). However, these developing presynaptic specializations are frequently not matched to any postsynaptic component when assessed with a panel of currently known molecular markers (AMPA receptor, NMDA receptor, $GABA_A$ receptor, gephyrin, PSD-95, and GKAP; data not shown) (see also Friedman et al., 2000).

Taken together, these findings suggest a sequence of development in which both presynaptic and postsynaptic precursors can form independently of each other; i.e., neither component is necessary for formation of the other, but both can form spontaneously and later become aligned to form a functional synapse.

Mismatching of synaptic elements

A novel finding in this study is the abundance of appositions of chemically distinct presynaptic and postsynaptic components in isolated cells. We suggest that these mismatches indicate the existence of a synaptogenic signal common to both inhibitory and excitatory synapses (Fig. 8). The observation that mismatched appositions are rarely observed in contiguous cell culture suggests that there is an additional specificity-conferring signal, present in contiguous culture but lacking in isolated neurons, to ensure that appropriate pairing is normally preferred.

A major question raised by this study is the molecular nature of the common cue and the specificity-conferring signal(s). An obvious and attractive candidate for a specificity-conferring signal is the neurotransmitter: it would be released presynaptically and bind postsynaptically to activate only the appropriate receptor. However, we could find no evidence of such an activity-dependent cue (Fig. 7), suggesting that the specificity cue is not the transmitter itself but another molecular signal. Candidate trans-synaptic signaling proteins include cadherins (Fannon and Colman, 1996), cadherin-related neuronal receptors (Kohmura et al., 1998), neurexins–neuroligins (Irie et al., 1997; Missler et al., 1998; Scheiffele et al., 2000), ephrins and Eph receptors (Torres et al., 1998), densin-180 (Apperson et al., 1996), and Narp (O'Brien et al., 1999). N-cadherin is a particularly good candidate for a common adhesive signal. It is concentrated at both GABA and glutamate synapses early in development in hippocampal cultures, but only at glutamate synapses later in development (Benson and Tanaka, 1998). The cadherin-related neuronal receptors are a large gene family, and neurexins–neuroligins are gene families with many alternatively spliced products; both would have the potential diversity to serve as specificity-determining components. Alternatively, some protein families, such as the ephrins and Eph receptors, could serve simultaneously as common and specificity-conferring components under different conditions, because of the abilities of different family members to form interactions of differing affinities (Gale et al., 1996).

What is the functional importance of these mismatched synapses? One possibility is that terminals of the same axon that innervate two different kinds of postsynaptic specialization could activate both. Release of two fast transmitters and responses to both have been recorded at individual synapses (Jonas et al., 1998; Jo and Schlichter, 1999; Tsen et al., 2000). In our culture system, isolated pyramidal neurons had no GABA or GAD immunoreactivity and so are unlikely to release GABA, and there is no nearby source of GABA from other synapses or glia. Isolated pyramidal cells could release unknown factors that activate $GABA_A$ receptors, or isolated GABAergic neurons could corelease glutamate with GABA. However, in recordings from similar isolated hippocampal cells in culture, cells showed either an inhibitory or excitatory autaptic response but not both in the same cell (Bekkers and Stevens, 1991; Segal, 1991). Thus it is unlikely that mismatched appositions in microisland cultures are classically functional synapses.

Some specialized synapse types seen *in vivo* may use both GABAergic and glutamatergic signaling without neurotransmitter corelease from the same terminal. In the cerebellum, $\alpha 6$, $\beta 2/3$, and $\gamma 2$ subunits of the $GABA_A$ receptor are present at the postsynaptic membrane of glutamatergic synapses between mossy fibers and granule cells as well as at the inhibitory Golgi synapses (Nusser et al., 1996, 1998b). These $\alpha 6$ subunit-containing $GABA_A$ receptors at mossy fiber synapses are suggested to mediate inhibition via GABA spillover from nearby synapses (Nusser et al., 1996; Rossi and Hamann, 1998). Similarly, group 1 metabotropic glutamate receptors have been localized at GABAergic postsynaptic specializations in monkey pallidus (Hanson and Smith, 1999). Many synapses *in vivo* have not been well characterized regarding the possibility of apposition of chemically distinct presynaptic and postsynaptic components, and there may be other instances of such unorthodox physiologically relevant appositions. Mismatched synapses in our culture system may reflect an experimentally induced overabundance of a pairing that is physiologically relevant in other situations.

Recently, the localization and activity of GAD at presynaptic terminals were found to be necessary for postsynaptic glutamate receptor accumulation at the glutamatergic neuromuscular synapse in *Drosophila* (Featherstone et al., 2000). We hypothesize that the experimentally induced mismatched appositions described here reveal a pathway common to glutamate and GABA synapses that may similarly be important in central synapse formation (Fig. 8). We suggest that such a common pathway would mediate formation of short-lived mismatched appositions during the phase of normal synapse formation. Another scenario in which such a common pathway may be particularly important is in the process of reinnervation in response to injury, in which the formation of multiple transient appositions may aid in the repair process.

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