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Loss of Postsynaptic $GABA_A$ Receptor Clustering in Gephyrin-Deficient Mice

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The tubulin-binding protein gephyrin, which anchors the inhibitory glycine receptor (GlyR) at postsynaptic sites, decorates GABAergic postsynaptic membranes in various brain regions, and postsynaptic gephyrin clusters are absent from cortical cultures of mice deficient for the $GABA_A$ receptor $\gamma 2$ subunit. Here, we investigated the postsynaptic clustering of $GABA_A$ receptors in gephyrin knock-out (*geph* $-/-$) mice. Both in brain sections and cultured hippocampal neurons derived from *geph* $-/-$ mice, synaptic $GABA_A$ receptor clusters containing either the $\gamma 2$ or the $\alpha 2$ subunit were absent, whereas glutamate receptor subunits were normally localized at postsynaptic sites. Western blot analysis and electrophysiological recording revealed that normal levels of functional $GABA_A$ receptors are expressed in *geph* $-/-$ neurons, however the pool size of intracellular $GABA_A$ receptors appeared increased in the mutant cells. Thus, gephyrin is required for the synaptic localization of GlyRs and $GABA_A$ receptors containing the $\gamma 2$ and/or $\alpha 2$ subunits but not for the targeting of these receptors to the neuronal plasma membrane. In addition, gephyrin may be important for efficient membrane insertion and/or metabolic stabilization of inhibitory receptors at developing postsynaptic sites.

Key words: $GABA_A$ receptor; gephyrin; receptor clustering; knock-out mice; hippocampal cultures; NMDA receptor; AMPA receptor; PSD-95

Neurotransmission in the nervous system depends on voltage-gated and ligand-gated ion channels that are highly concentrated at specific synaptic sites. Intricate mechanisms must therefore exist that regulate the expression and synaptic accumulation of these membrane proteins. Different lines of evidence show that the selective synaptic localization of ion channels in the CNS (for review, see Kirsch and Kröger, 1996; Sheng, 1996) and the neuromuscular junction (for review, see Froehner, 1993) requires interactions with associated cytosolic proteins that serve as membrane–cytoskeleton linkers.

The tubulin-binding protein gephyrin (Prior et al., 1992), identified by copurification with the inhibitory glycine receptor (GlyR) (Pfeiffer et al., 1982; Graham et al., 1985; Schmitt et al., 1987), is thought to serve as an anchor molecule that immobilizes GlyRs on the subsynaptic cytoskeleton (Kirsch and Betz, 1995). Gephyrin binds to the GlyR via an amphipatic sequence in the large cytosolic loop of its β-subunit (Meyer et al., 1995; Kneussel et al., 1999), displays a high affinity for polymerized tubulin (Kirsch et al., 1991), and precedes the postsynaptic localization of GlyRs at sites of axosomatodendritic contact (Kirsch et al., 1993b; Bechade et al., 1996). Notably, depletion of gephyrin either by antisense treatment (Kirsch et al., 1993b) or gene targeting (Feng et al., 1998) prevents the synaptic accumulation of GlyRs. Gephyrin therefore is thought to orchestrate the development of glycinergic postsynaptic membrane specializations.

In situ hybridization and immunocytochemistry have shown that gephyrin is widely expressed throughout the CNS (Triller et al., 1985; Altschuler et al., 1986; Kirsch and Betz, 1993; Kirsch et al., 1993a). Gephyrin transcripts are also found in non-neuronal tissues (Prior et al., 1992), where gephyrin is essential for the biosynthesis of the molybdenum cofactor (moco) (Feng et al., 1998). In addition, gephyrin has been found at GABAergic synapses in different regions of the CNS, i.e., spinal cord (Triller et al., 1987; Bohlhalter et al., 1994; Cabot et al., 1995; Todd et al., 1996), retina (Sassoe-Pognetto et al., 1995, 1997), and olfactory bulb (Giusetto et al., 1998), as well as in cultured hippocampal (Craig et al., 1996) and cortical (Essrich et al., 1998) neurons. Recently, experiments supporting a crucial role of gephyrin in $GABA_A$ receptor clustering have been reported (Betz, 1998). In mice deficient for the $GABA_A$ receptor $\gamma 2$ subunit, gephyrin clusters were strongly reduced, and antisense depletion of gephyrin in hippocampal neurons produced a reduction of punctate synaptic staining for the $GABA_A$ receptor $\gamma 2$ subunit (Essrich et al., 1998). Also, after coexpression in transfected HEK 293 cells $GABA_A$ receptor $\beta 3$ subunit immunoreactivity has been found to largely colocalize with gephyrin aggregates (Kirsch et al., 1995). By analyzing the clustering of two highly abundant $GABA_A$ receptor subunits ($\alpha 2$ and $\gamma 2$) displaying strong synaptic localization, we now provide direct genetic proof, using gephyrin-deficient (*geph* $-/-$) mice, that gephyrin is essential for the postsynaptic localization of these $GABA_A$ receptor proteins. Our data in addition suggest that gephyrin may be important for inserting and/or stabilizing inhibitory receptors at developing synapses.

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MATERIALS AND METHODS

Antibodies. Double labeling studies were performed using primary antibodies to the $GABA_A$ receptor subunits $\alpha 2$ (1:3000) and $\gamma 2$ (1:2000) (Fritschy and Möhler, 1995), gephyrin (1:250; Dianova, Hamburg, Germany), the NMDA receptor subunit NR1 (1:150; Chemicon, Hofheim, Germany), the AMPA receptor subunits GluR2/GluR3 (1:100; Chemicon), PSD-95/SAP90 (1:250; Affinity BioReagents, Grünberg, Germany), and synaptophysin (1:100; Dako, Hamburg, Germany).

Animals. The creation and phenotype of *geph* −/− mice have been described previously (Feng et al., 1998). The animals used here were in a 129/Ola × C57BL6 mixed background. Animals were housed in a special pathogen-free unit and kept under optimal hygiene conditions. Time matings between heterozygous mice were set up in the late afternoon followed by vaginal plug check on the next morning. The time of a detected plug was considered as embryonic day 0.5 (E0.5).

Hippocampal cultures. Astrocyte feederlayers were prepared and cultured in MEM and 10% horse serum supplemented with 0.6% (w/v) glucose and 2 mM glutamine as described (Banker and Goslin, 1998). The medium of these astrocyte cultures was replaced with serum-free Neurobasal/B27 (Life Technologies, Eggenstein, Germany) medium 1 d before hippocampus dissection. Hippocampal cultures were prepared as described (Banker and Goslin, 1998) with minor modifications. Hippocampi from single E16.5 mouse embryos derived from heterozygous intercrosses were collected in Ca^{2+}–Mg^{2+}-free HBSS containing 10 mM HEPES, pH 7.2. Genotyping was done by PCR on tail tissue (Feng et al., 1998). After addition of 0.05% (w/v) trypsin, the tissue was incubated for 8 min at 37°C. After trypsin removal, the tissue was washed with 15 ml HBSS–HEPES, pH 7.2, and the hippocampi were triturated in plating medium (serum-free Neurobasal/B27). Cells were seeded on poly-L-lysine-coated glass coverslips containing paraffin dots (to support them above the glia) at a density of 60,000 cells/well. Cells were allowed to attach for 3–5 hr before transfer to wells containing an astrocyte monolayer. Cocultures were treated with cytosine-β-D-arabinofuranoside (Sigma, Deisenhofen, Germany) on day 3 *in vitro* (DIV 3) to prevent glial proliferation. One-third of the medium was exchanged once weekly. Cells were cultured for 21 d before being processed for immunostaining.

Immunochemistry and confocal microscopy. Coverslips carrying hippocampal neurons were fixed in 95% (v/v) methanol and 5% (v/v) acetic acid for 5 min and air-dried. Cells were then permeabilized in 0.2% (w/v) Triton X-100 for 5 min followed by incubation in 5% (v/v) goat serum for 20 min before processing for immunofluorescence. To obtain spinal cord sections, tissue of E19.5 mice was cut in blocks of 5 mm and fixed in 4% (w/v) paraformaldehyde for 10 min followed by a short wash in PBS. To prevent the formation of crystals after freezing, the sections were incubated in increasing concentrations [10% (w/v), 20% (w/v), or 30% (w/v) plus 0.01% (w/v) sodium azide] of sucrose solution at 4°C for 1 hr, each. Cryostat sections were refixed for 5 min in 4% (w/v) paraformaldehyde and processed for immunofluorescence. Confocal microscopy was performed using a confocal laser-scanning microscope Leica TCS-SP equipped with the image software Leica-TCS-NT version 1.6.551.

Brain membrane preparation and Western blotting. Mouse brain was homogenized in 2 ml of ice-cold PBS, containing 1 mM phenylmethylsulfonyl fluoride (Boehringer Mannheim, Mannheim, Germany) and Complete Mini protease inhibitor (Boehringer), and the homogenate was centrifuged at 1000 × *g* for 10 min at 4°C. After a second centrifugation at 10,000 × *g* for 15 min at 4°C, the resulting high-speed pellet (P2) was resuspended in PBS, containing protease inhibitors, as above. Protein concentrations were determined using a protein assay system (Bio-Rad, München, Germany). Forty micrograms of total protein per lane were separated by 10% SDS-PAGE and transferred to a nitrocellulose membrane (Schleicher & Schüll, Dassel, Germany). The membrane was blocked with 3% (w/v) nonfat dry milk powder in Tris-buffered saline, pH 8.0, for 20 min followed by a 12 hr incubation with antibodies, as indicated. After washing, bound Igs were visualized with horseradish peroxidase-conjugated secondary antibodies using the ECL system (Pierce, Rockford, IL).

Electrophysiological recording of agonist-evoked currents. Agonist-induced currents in cultured hippocampal neurons were recorded from neuronal somata in the whole-cell configuration of the patch-clamp technique (Hamill et al., 1981) at room temperature (20–25°C). Cultured cells at DIV 12–21 were viewed with an inverted microscope (Zeiss, Jena, Germany) and clamped at a holding potential of −70 mV. Whole-cell current recordings were obtained with an EPC-9 (Heka, Lambrecht, Germany) amplifier linked to an Atari STE computer controlled by Heka software, sampled at 20 Hz, and stored on disk as described previously (Laube et al., 1995). Electrodes were pulled from borosilicate glass capillaries (Hilgenberg, Malsfeld, Germany) with a Zeitz DMZ Universal Puller (Zeitz Instruments, Augsburg, Germany) to yield tip resistances of 3–6 MΩ. Series resistances after whole-cell formation (15–40 MΩ) were monitored regularly throughout recordings and compensated up to 80%. Pipettes were filled with a solution containing (in mM): 120 CsCl, 20 tetraethylammonium chloride, 1 $CaCl_2$, 2 $MgCl_2$, 11 EGTA, and 10 HEPES, pH 7.2. The bathing Ringer solution consisted of (in mM): 137 NaCl, 5.4 KCl, 1.8 $CaCl_2$, and 5 HEPES, pH 7.4. Currents through the respective receptors were elicited by direct application of agonists via a DAD-12 (Adams and List, Westbury, NY) drug application system. All drugs were purchased from Sigma with the exception of 6-cyano-7-nitroquinoxaline-2,3-dione, disodium salt and 2-amino-5-phosphonopentanoic acid, which were from Tocris Cookson (Bristol, UK).

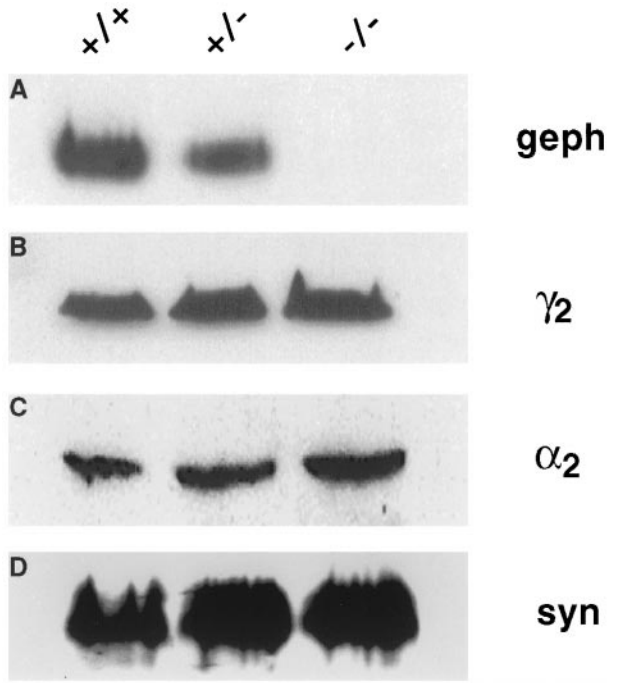


Figure 1. Western blot analysis of gephyrin, $GABA_A$ receptor subunits $\alpha 2$ and $\gamma 2$, and synaptophysin, in membranes isolated from *geph* +/+, *geph* +/−, and *geph* −/− brain. Proteins (40 μg/lane) were probed with the indicated antisera. Gephyrin expression was reduced to ~50% in heterozygotes (+/−) and abolished in homozygotes (−/−). In contrast, the expression levels of the $GABA_A$ receptor subunits $\alpha 2$ and $\gamma 2$ as well as of synaptophysin were not significantly different in the three genotypes.

RESULTS

Expression of $GABA_A$ receptor subunits in *geph* −/− mice

Different knock-out experiments have shown that the genetic inactivation of certain genes can alter the expression levels of related proteins (Jones et al., 1997). We therefore performed Western blot analysis on brain extracts prepared from E19.5 wild-type (+/+), heterozygous (+/−), as well as homozygous (−/−) *geph* −/− mouse embryos (Feng et al., 1998) to determine whether expression levels of the $GABA_A$ receptor subunits $\alpha 2$ and $\gamma 2$ differ between these three genotypes. The immunoreactivities of these $GABA_A$ receptor subunits and of the presynaptic marker protein synaptophysin (Wiedenmann and Franke, 1985) were not altered in the heterozygous and homozygous genotypes, whereas the intensity of the gephyrin band was reduced to ~50% in +/−, and totally abolished in −/− mice, respectively (Fig. 1). Thus, $GABA_A$ receptor synthesis appears to occur independently of gephyrin gene expression.

Loss of postsynaptic $GABA_A$ receptor clusters in spinal cord of *geph* −/− animals

The synaptic localization of the $GABA_A$ receptor subunits $\alpha 2$ and $\gamma 2$ was first investigated using spinal cord sections prepared from E19.5 *geph* −/− mice. In several staining experiments comparing wild-type (+/+) and homozygous (−/−) genotypes, we consistently found a loss of immunoreactive synaptic punctae for both $GABA_A$ receptor subunits $\alpha 2$ and $\gamma 2$. Staining of wild-type sections revealed intense punctate staining for $\alpha 2$ and $\gamma 2$ immunoreactivities, whereas background staining was observed for both antigens in *geph* −/− sections (Fig. 2*A–D*). This indicates that $GABA_A$ receptor clustering is impaired in the mutant mice and closely resembles observations made for inhibitory GlyR localization (Feng et al., 1998).

Blocking neuronal activity has been shown to increase synaptic levels of AMPA and/or NMDA receptors, and, conversely, increased activity levels have been reported to decrease levels of both receptor proteins (Craig, 1998). Because we found a significant reduction of synaptically localized $GABA_A$ receptor $\alpha 2$ and $\gamma 2$ subunits in *geph* −/− mice, we also analyzed the distribution of different glutamate receptor subunits and the NMDA receptor anchoring protein PSD-95/SAP90 (Kennedy, 1997) to unravel possible secondary effects that might result from alterations in the number of excitatory synapses. After staining with antibodies specific for the NMDA receptor subunit NR1, the AMPA receptor subunits GluR2/GluR3, or PSD-95/SAP90, sections from both mutant animals and controls revealed similar sizes and distributions of immunoreactive clusters containing these proteins (Fig. 2), suggesting that the loss of clustering of the abundant $GABA_A$ receptor isoforms $\alpha 2$ and $\gamma 2$ has no major influence in the number and morphology of glutamatergic synapses in spinal cord. However, in some sections stained for the NR1 subunit, the number of NMDA receptor clusters appeared to be slightly lower in the homozygous mutants, although expression levels of glutamate receptors and PSD-95/SAP90, as revealed by Western blotting, were not detectably altered among the three genotypes (data not shown).

Loss of synaptic $GABA_A$ receptor clusters in hippocampal primary neurons of *geph* −/− animals

Because *geph* −/− mice die within the first day after birth (Feng et al., 1998), whereas most $GABA_A$ receptors are expressed postnatally (Laurie et al., 1992), the subcellular localization of the $GABA_A$ receptor subunits $\alpha 2$ and $\gamma 2$ from *geph* −/− mice was investigated in more detail in primary hippocampal cultures prepared from E16.5 *geph* −/− animals after an additional differentiation period of 21 DIV (Fig. 3*A*). At least seven embryos of each genotype were used in these staining experiments; all cultures were counterstained with a synaptophysin antibody. At least one culture from each embryo was stained for gephyrin to confirm the genotyping results obtained by PCR reaction. As expected, *geph* −/− neurons did not display any gephyrin immunoreactivity. In *geph* +/+ neurons, for both the $GABA_A$ receptor $\alpha 2$ and $\gamma 2$ subunits, a punctate staining was obtained (Fig. 3*A*) that was predominantly membranous as revealed by confocal sectioning. In the $\alpha 2$-staining experiments, 65.9 + 6.9% of the punctate structures colocalized with synaptophysin (see yellow overlap) (Fig. 3*B*), whereas 66.4 + 6.6% of the punctae ($n = 7$ cells/each with ~250–400 punctae per cell) were colocalized in cultures stained with the $\gamma 2$-specific antibody (see yellow overlap) (Fig. 3*B*). *Geph* +/− neurons did not significantly differ from wild-type cultures (+/+). In contrast, inspection of *geph* −/− neurons

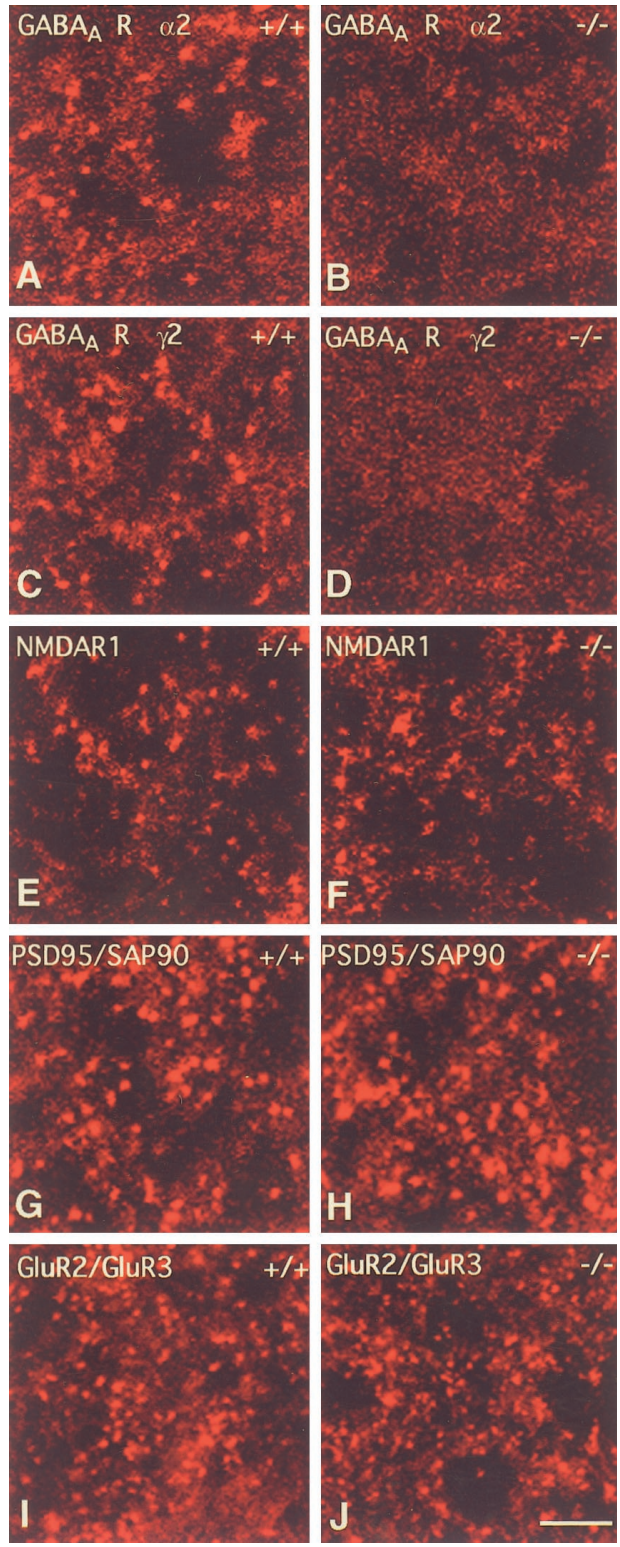
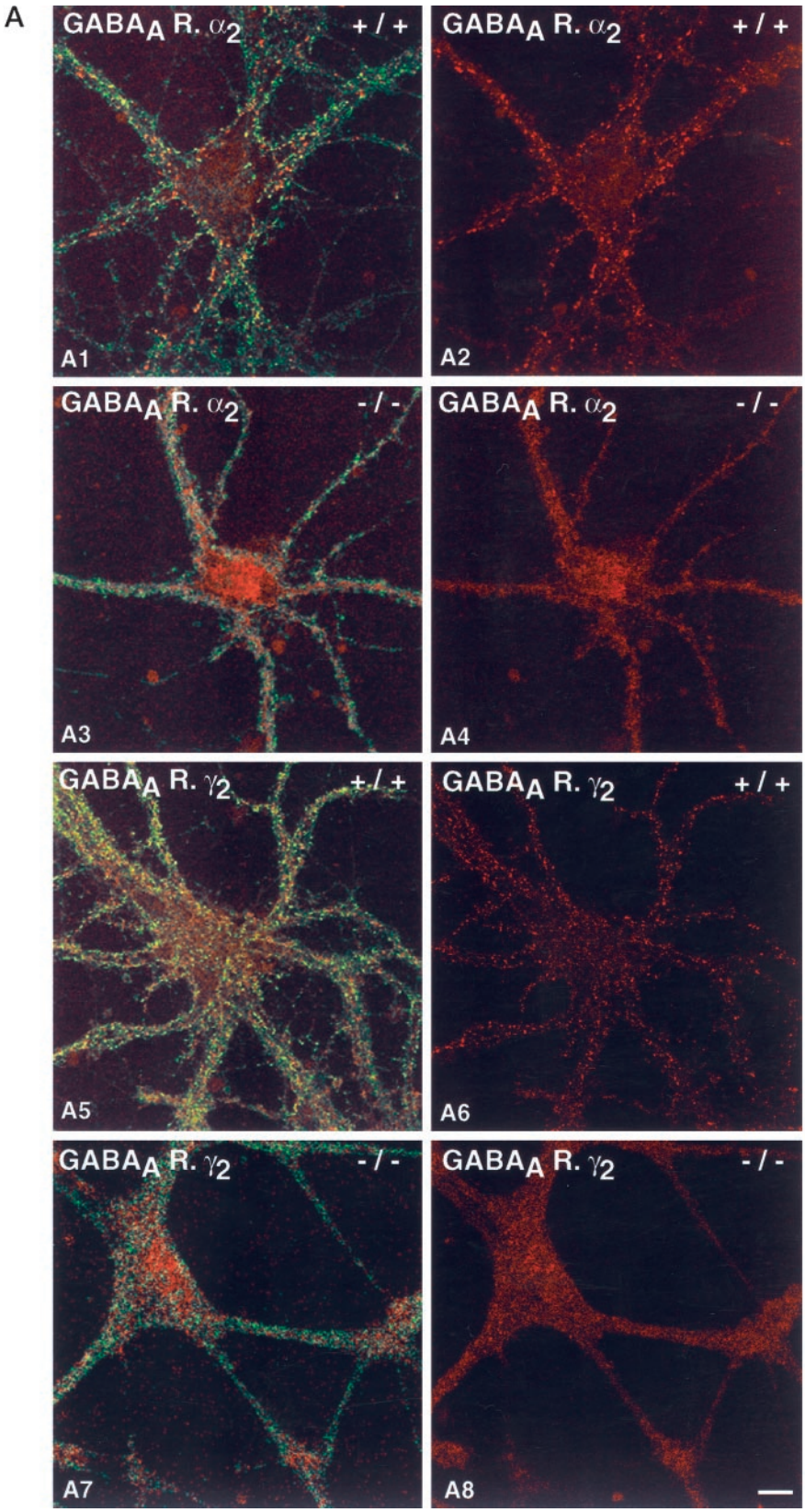


Figure 2. Postsynaptic receptor immunoreactivities in spinal cord sections of wild-type and *geph* −/− mice. All staining experiments were performed three times with antibodies indicated. The punctate staining of sections from homozygous mutants (−/−) for both $GABA_A$ receptor subunits $\alpha 2$ and $\gamma 2$ was reduced to background levels (*B*, *D*) as compared to wild-type sections (*A*, *C*), indicating a loss of $GABA_A$ receptor clustering. In contrast, NR1 (*E*, *F*), the PSD-95/SAP90 protein (*G*, *H*), and GluR2/3 (*I*, *J*) showed similar punctate staining in sections of both wild-type and *geph* −/− mice. Scale bar, 5 μm.



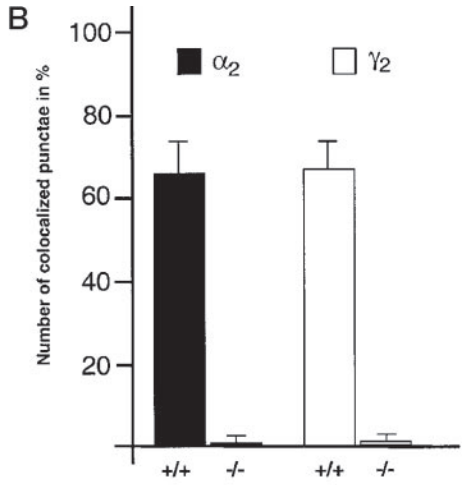


Figure 3. Immunochemistry of cultured hippocampal neurons prepared from *geph* −/− mice double-stained for the indicated proteins (*red*) and synaptophysin (*green*). *A,* After 21 DIV, neurons prepared from wild-type and knock-out animals were double-stained with antisera specific for the subunits α2 (*red*) and or γ2 (*red*), and synaptophysin (*green*), respectively. Note the loss of synaptically clustered $GABA_A$ receptors containing the α2 (*A1–A4*) and γ2 (*A5–A8*) subunits in neurons from *geph* −/− mice. These mutant neurons consistently showed a significant increase in intracellular $GABA_A$ receptor subunit immunoreactivity (*A3* and *A4*, *A7* and *A8*), as revealed by confocal microscopy. Furthermore, the number of yellow punctae, indicative of colocalization, was lost (*A3, A7*). Scale bar, 20 μm. *B*, Quantification of $GABA_A$ receptor subunits and synaptophysin colocalization in cultured hippocampal neurons from *geph* +/+ and *geph* −/− mice. After 21 DIV, neurons were stained as in *A*, and the number of yellow punctae per 100 μm dendrite length was counted. For both genotypes, each bar corresponds to counts performed on 7–10 cells.

revealed an almost complete loss of membranous punctate staining (<5%) for both the α2 and γ2 subunits, whereas the distribution of presynaptic terminals as revealed by synaptophysin immunoreactivity was unaltered (Fig. 3*A*). Control experiments revealed that, as in spinal cord sections, the distribution of excitatory synapses was not detectably altered in the *geph* −/− neurons. The punctate staining of NR1, GluR2/GluR3, and PSD-95/SAP90 immunoreactivities was indistiguishable in neurons from

wild-type and knock-out mice (Fig. 4*A–F*). We therefore conclude that the absence of gephyrin causes a loss of synaptically localized $GABA_A$ receptor subunits, but does not affect the formation of glutamatergic postsynaptic membrane specializations.

$GABA_A$ receptor α2 and γ2 subunits appear in intracellular microclusters after gephyrin deletion

An important difference in the distribution of $GABA_A$ receptors between control and *geph* −/− mice became apparent on closer inspection. Consistently, the cytoplasm of −/− neurons contained significant α2- and γ2-immunoreactive staining throughout soma and dendrites, which did not colocalize with presynaptic terminals (Fig. 3*A*). At higher magnification, it was obvious that the majority of immunoreactive punctae for both α2 and γ2 were significantly smaller as compared to wild-type (+/+) neurons, whereas their number was increased (Fig. 5*A,B*). Moreover, confocal sectioning revealed that these small $GABA_A$ receptor punctae were localized in the cytoplasm. The number of these intracellular "microclusters" was quantified using confocal sections of dendritic terminals from both wild-type (+/+) and homozygous mutant (−/−) neurons. Average numbers of $8/\mu m^2 + 0.55$ ($n = 5$) for wild-type (+/+), and of $25.5/\mu m^2 + 1.45$ ($n = 5$) for *geph* −/− neurons (−/−) were obtained (Fig. 5*C*). These data indicate that the absence of gephyrin does not impair the translocation of $GABA_A$ receptors to dendritic compartments, but apparently increases the intracellular pool of $GABA_A$ receptor protein.

GABAergic and glycinergic currents are decreased in *geph* −/− neurons

To examine whether the increased formation of intracellular $GABA_A$ receptor microclusters might reflect a reduced number of receptors in the plasma membrane of *geph* −/− mice, we recorded GABAergic currents from cultured hippocampal neurons in the whole-cell current mode. At least seven individual neurons per genotype were examined for GABA responses. All cells tested responded to GABA with a large inward current; however, there was considerable variability between individual whole-cell currents. We therefore normalized the GABAergic responses to the robust NMDA receptor currents detected with all neurons present in the cultures. As shown in Figure 6*A*, this revealed that the normalized $GABA_A$ receptor-mediated responses of neurons from *geph* −/− mice were significantly reduced in amplitude as compared to those of wild-type (+/+) cells (Student's *t* test, $p = 0.039$). Similarly, the glycine responses detectable in ~50% of the cells were slightly smaller in amplitude for mutant *geph* −/− neurons (−/−) than for wild-type (+/+) cells (Fig. 6*B*). These data indicate that diffusely distributed $GABA_A$ receptors are present in the plasma membrane of *geph* −/− neurons despite an increased occurrence of intracellular microclusters. Notably, however, the agonist responses of the inhibitory amino acid receptors that are clustered by gephyrin appeared to be reduced in the *geph* −/− cells. In contrast, the same neurons showed no significant differences in NMDA receptor and AMPA receptor-mediated responses (Fig. 6*C,* and data not shown), regardless of whether they were prepared from *geph* +/+ or *geph* −/− mice. This corroborates the above conclusion that excitatory circuits are not affected in gephyrin-deficient mice.

DISCUSSION

In this study, we demonstrate that the $GABA_A$ receptor subunits α2 and γ2 are not synaptically localized in *geph* −/− mice. Our data corroborate and extend the findings of Essrich et al. (1998), in which gephyrin depletion by antisense oligonucleotides was shown to cause a reduction of synaptically localized $GABA_A$ receptor subunits. By analyzing the punctate synaptic staining of the $GABA_A$ receptor subunits α2 and γ2 in spinal cord sections and cultured hippocampal neurons from *geph* −/− mice, we now provide direct genetic proof that gephyrin is indeed essential for the postsynaptic localization of $GABA_A$ receptors. In cultured *geph* +/+ neurons, ~60% of the $GABA_A$ receptor α2 and γ2 subunit immunoreactivities were found to be synaptically localized, whereas very little colocalization (<5%) was found in *geph* −/− neurons. This could not be attributed to a lack of $GABA_A$ receptor protein, since Western blot analysis revealed normal expression of both $GABA_A$ receptor subunits and synaptophysin; these proteins were found at equal levels in all genotypes analyzed, whereas gephyrin immunoreactivity was reduced in heterozygotes (+/−) and completely lost in homozygotic animals (−/−) (Feng et al., 1998). We therefore conclude that the observed loss of $GABA_A$ receptor clustering is a consequence of gephyrin deletion.

The clustering of different neurotransmitter receptors including GlyRs and glutamate receptors has been shown to be activity-dependent (Kirsch and Betz, 1998; for review, see Craig, 1998). To exclude that alterations in circuitry resulting from gephyrin deletion may be responsible for the observed absence of synaptic $GABA_A$ receptor clusters in *geph* −/− mice, we also investigated the distribution of the NMDA receptor subunit NR1, the AMPA receptor subunits GluR2/GluR3, and the NMDA receptor-associated protein PSD-95/SAP90 in both hippocampal cultures and spinal cord. We did not find any significant effect of the *geph* −/− genotype on the localization of the receptor subunits GluR2/GluR3 or PSD-95/SAP90; however, in some *geph* −/− spinal cord sections, NR1 immunoreactivity seemed to be slightly decreased as compared to controls, an observation that was not confirmed with cultured hippocampal neurons. Electrophysiological recordings in the whole-cell current mode revealed that GABAergic currents were still detectable in all *geph* −/− neurons analyzed (Fig. 6*A*), indicating that despite the loss of postsynaptic receptor clusters, functional $GABA_A$ receptors are present in the somatodendritic plasma membrane. Similarly, all cells displayed NMDA receptor and AMPA receptor-mediated currents, whereas glycine responses where detected in ~50% of the neurons analyzed.

Closer inspection of the cultured hippocampal *geph* −/− neurons revealed that $GABA_A$ receptor α2 and γ2 subunit immunoreactivities were not diffusely distributed but found in intracellular aggregates, which were significantly smaller than the synaptic receptor clusters that colocalized with synaptophysin in wild-type neurons. These microclusters were also detected in neurons from *geph* +/+ mice, however, at an approximately threefold lower frequency. Their size and localization is consistent with these structures representing $GABA_A$ receptor-containing vesicles that correspond to either post-Golgi vesicles in transit to the cell surface or, alternatively, an early endosomal compartment. The increased size of this intracellular $GABA_A$ receptor pool suggests that either the rate of $GABA_A$ receptor incorporation into the plasma membrane is reduced, or alternatively receptor endocytosis and degradation is enhanced in the absence of gephyrin. Both mechanisms should decrease the number of functional $GABA_A$ receptors on the cell surface. Indeed, normalization of whole-cell GABA currents to the robust NMDA response showed that $GABA_A$ receptor densities were significantly decreased in *geph* −/− neurons. In contrast, the glutamatergic currents elicited by glutamate, AMPA, or NMDA were not

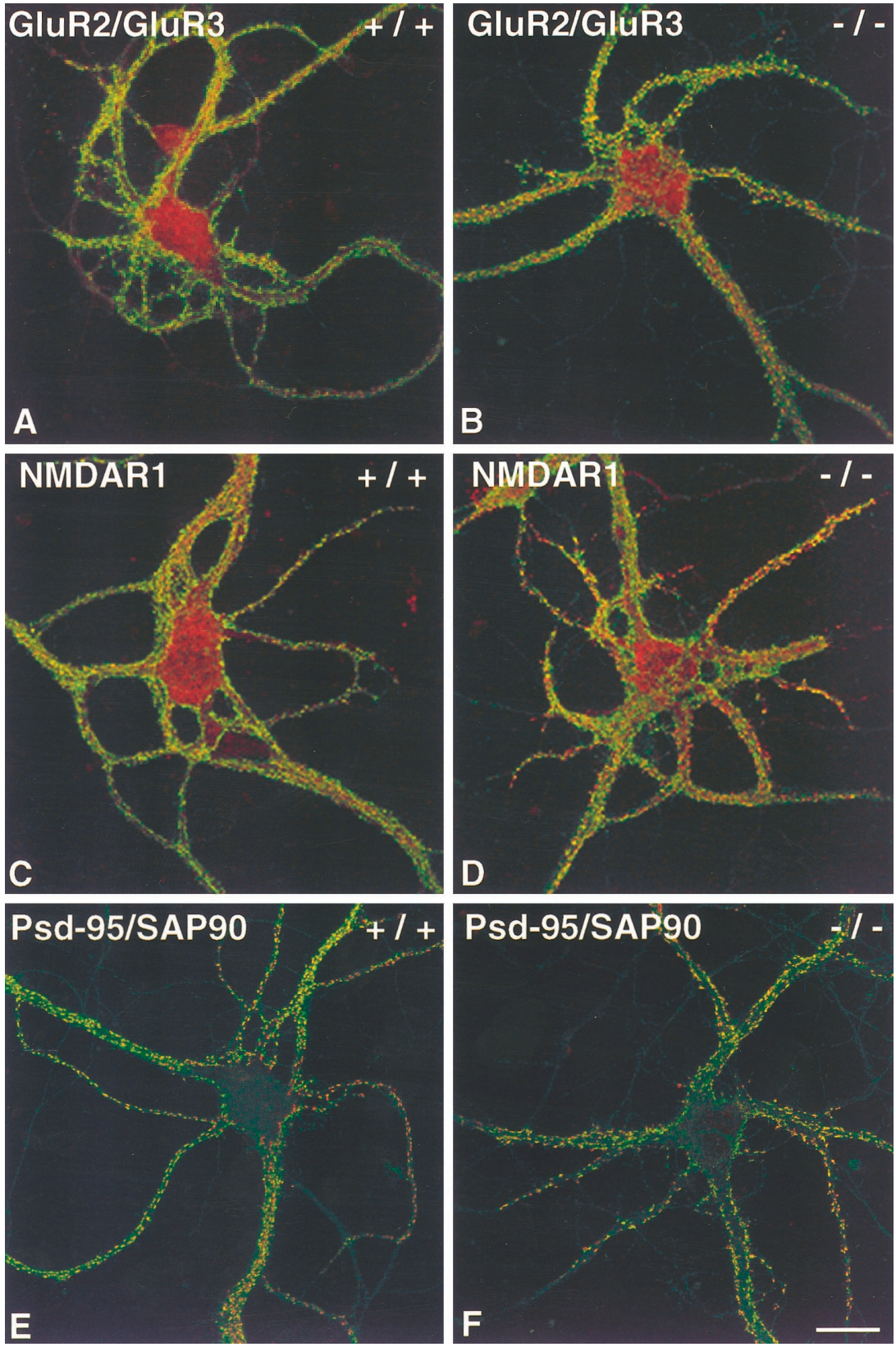


Figure 4. Immunochemistry of cultured hippocampal neurons prepared from *geph* +/+ and *geph* −/− mice double-stained for the indicated proteins (*red*) in combination with synaptophysin (*green*). The synaptic colocalization of the AMPA receptor GluR2/GluR3 subunits (*A, B*), the NMDA receptor NR1 subunit (*C, D*), and of the postsynaptic density protein PSD-95/SAP90 (*E, F*) is not altered in *geph* −/− mice. Scale bar, 20 μm.

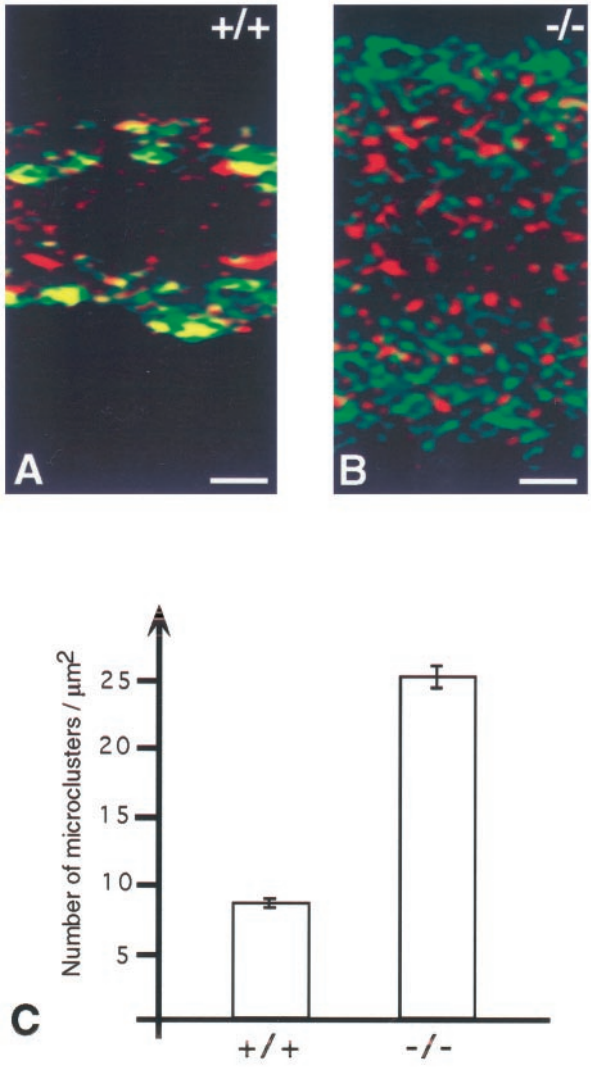


Figure 5. High-power magnification of confocal sections from dendrites of cultured hippocampal neurons derived from *geph* +/+ (*A*) and *geph* −/− (*B*) mice. Neurons were double-stained with antisera specific for the $GABA_A$ receptor subunit γ2 and synaptophysin. The number of intracellular microclusters per square micrometer was significantly increased, and synaptically localized punctae are not detectable, in neurons from *geph* −/− mice. Scale bar, 2 μm. *C,* Quantification of intracellular microclusters by confocal microscopy. Neurons derived from *geph* −/− mice show an approximately threefold increase in the number of microclusters per square micrometer.

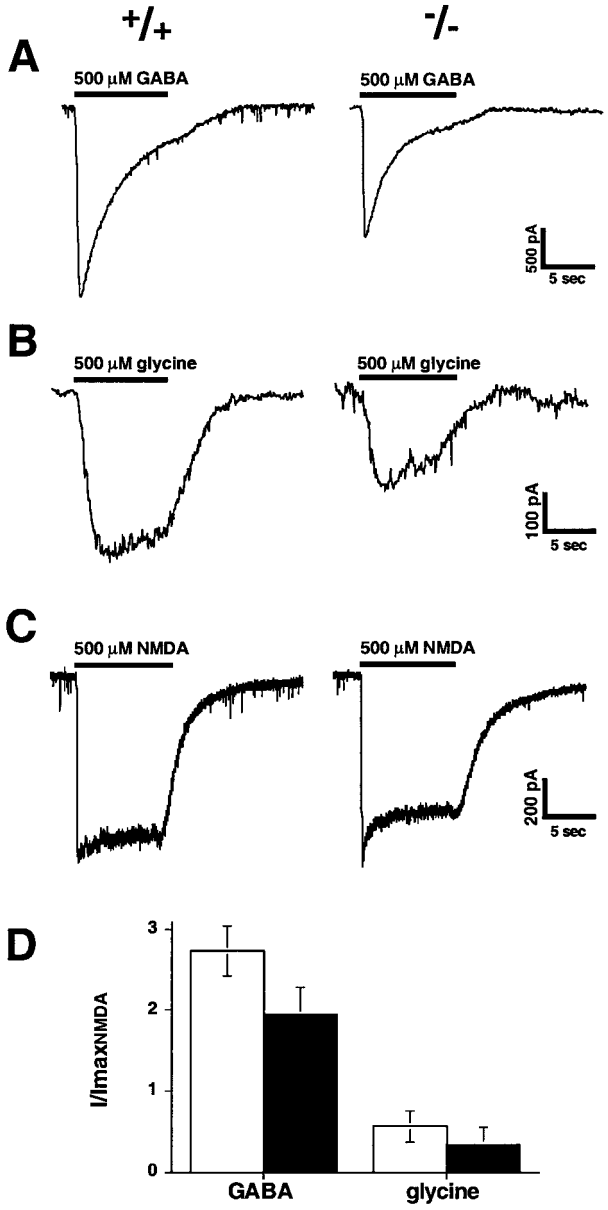


Figure 6. Agonist-induced whole-cell currents of hippocampal neurons from control and *geph* −/− mice recorded after 21 DIV. *A–C*, Membrane currents elicited by 500 μM GABA (*A*), glycine (*B*), and NMDA in the presence of 10 μM glycine (*C*) were recorded in the voltage-clamp mode at a holding potential of −70 mV. Agonists were applied to single neurons at 1 min intervals for the duration indicated by the *horizontal bar*. All cells tested responded to GABA and NMDA; however, only 50% of the neurons showed a glycine response. *D,* Maximal agonist-inducible whole-cell currents in control and *geph* −/− mice normalized relative to the robust NMDA receptor-mediated current. Statistical analysis by the unpaired Student's *t* test of the +/+ (*white*) and −/− (*black*) current values indicated that for GABA, the differences between the two sets of animals were significant ($p = 0.039$). Results are expressed as means + SEM of seven determinations.

significantly different between wild-type and homozygous mutant cells. The selective reduction of GABA currents is unlikely to reflect changes in receptor subunit composition of the mutant cells, because expression of the α2 and the γ2 subunits, which constitute the predominant $GABA_A$ receptor polypeptides in both developing and adult hippocampus (Laurie et al., 1992), was not altered in the CNS of *geph* −/− mice.

Different lines of evidence suggest that $GABA_A$ receptors do not depend on gephyrin for incorporation into the neuronal plasma membrane. First, recombinant $GABA_A$ receptors are readily assembled and inserted into the plasma membrane of *Xenopus* oocytes and mammalian cells that express only low levels of gephyrin (Schofield et al., 1987; Pritchett et al., 1989; Meyer et al., 1995). Second, both glycine receptors and mutant $GABA_A$ receptors are retained in the cytoplasm of transfected cells after overexpression of gephyrin (Meyer et al., 1995; Kirsch et al., 1996). Also, in developing neurons, membrane apposition of gephyrin precedes receptor clustering at synaptic sites (Kirsch et al., 1993; Bechade et al., 1996; Craig et al., 1996). We therefore interpret the increased accumulation of intracellular gephyrin microclusters in *geph* −/− neurons as a consequence of enhanced

receptor endocytosis rather than decreased plasma membrane insertion. Thus, $GABA_A$ receptor clustering by gephyrin may prolong the half-life of these membrane proteins by recruiting them to the developing postsynaptic membrane, and thus protecting them against internalization. Indeed, for the closely related GlyR, pharmacological disruption of postsynaptic clustering by the selective antagonist strychnine has also been found to drastically increase receptor endocytosis in cultured spinal neurons (Kirsch and Betz, 1998; Levi et al., 1998). We therefore propose that gephyrin is important in stabilizing inhibitory amino acid receptors against endocytosis and subsequent degradation at developing inhibitory postsynaptic sites.

Gephyrin, which anchors GlyRs to the underlying cytoskeleton, is found at GABAergic postsynaptic membranes in many brain regions. However, presently evidence for a direct interaction between $GABA_A$ receptor subunits and gephyrin is scarce. Gephyrin fails to copurify with $GABA_A$ receptors after affinity chromatography (Meyer et al., 1995), which contrasts its tight association with the GlyR in different mammalian species (Pfeiffer et al., 1982; Graham et al., 1985; Becker et al., 1986; Schmitt et al., 1987). Coexpression studies in embryonic kidney cells have shown that of different $GABA_A$ receptor proteins tested, only the β3 subunit colocalizes to a significant extent with gephyrin (Kirsch et al., 1995); this may, however, reflect indirect interactions between these proteins. Similarly, the data presented here exclusively show that, in the absence of gephyrin, $GABA_A$ receptor α2 and γ2 subunit clustering is abolished. Thus, it remains unsolved whether gephyrin alone is sufficient for the synaptic localization of the $GABA_A$ receptors or whether additional proteins are required. The abundant $GABA_A$ receptor subunit γ2 has recently been reported to bind GABARAP, a protein that shows homology to microtubule-associated proteins (MAPs) (Wang et al., 1999). Notably, the tubulin-binding properties of gephyrin closely resemble those of microtubule-binding proteins (Kirsch et al., 1991), and a cDNA encoding the microtubule-associated protein MAP1B (or MAP5) has been isolated in attempts to clone gephyrin (Rienitz et al., 1989; Kirsch et al., 1990). Moreover, a recent report by Hanley et al. (1999) demonstrates an interaction of the $GABA_C$ receptor subunit ρ1 with MAP1B. Thus, GABARAP and/or related proteins may coexist with gephyrin in a tubulin-bound receptor clustering complex. Such complexes may stabilize $GABA_A$ receptors at the newly formed postsynaptic membrane against intracellular degradation by preventing clathrin coating, and thus endocytosis, of the respective plasma membrane domain. Future studies should show whether GlyR and $GABA_A$ receptor turnover is altered in gephyrin-deficient mice, thereby extending the synaptic roles of gephyrin from inhibitory receptor clustering and anchoring to synapse stabilization (Changeux and Danchin, 1976).

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