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Gephyrin-Independent Clustering of Postsynaptic $GABA_A$ Receptor Subtypes

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Gephyrin has been shown to be essential for the synaptic localization of the inhibitory glycine receptor and major $GABA_A$ receptor ($GABA_AR$) subtypes. However, in retina certain $GABA_AR$ subunits are found at synaptic sites in the absence of gephyrin. Here, we quantitatively analyzed $GABA_AR$ α1, α2, α3, α5, β2/3, and γ2 subunit immunoreactivities in spinal cord sections derived from wild-type and gephyrin-deficient (*geph −/−*) mice. The punctate staining of $GABA_AR$ α1 and α5 subunits was unaltered in *geph −/−* mice, whereas the numbers of α2-, α3-, β2/3-, and γ2-subunit-immunoreactive synaptic sites were significantly or even strikingly reduced in the mutant animals. Immunostaining with an antibody specific for the vesicular inhibitory amino acid transporter revealed that the number of inhibitory presynaptic terminals is unaltered upon gephyrin deficiency. These data show that in addition to gephyrin other clustering proteins must exist that mediate the synaptic localization of selected $GABA_AR$ subtypes.

INTRODUCTION

A high density of neurotransmitter receptors in the postsynaptic membrane is essential for fast synaptic transmission between neurons. The targeting of such receptors to and their clustering at developing postsynaptic sites involve receptor-associated proteins and cytoskeletal elements that are highly concentrated at postsynaptic densities (PSDs)[2] (Sheng, 1996; Craven and Bredt, 1998). At excitatory PSDs, PDZ-domain-mediated protein interactions have been shown to generate a scaffold for different structural proteins and regulatory enzymes involved in synaptic signaling (Sheng, 1996; Craven and Bredt, 1998), whereas at inhibitory synapses the tubulin-binding protein gephyrin is known to play an essential role in the synaptic localization of both glycine receptors (GlyRs) and $GABA_A$ receptors ($GABA_ARs$) (reviewed in Kneussel and Betz, 2000a; Sassoe-Pognetto and Fritschy, 2000).

Gephyrin, a 93-kDa polypeptide that was originally identified by copurification with the mammalian GlyR (Pfeiffer *et al.*, 1982; Schmitt *et al.*, 1987), directly interacts with both the GlyR β subunit (Meyer *et al.*, 1995; Kneussel *et al.*, 1999a) and polymerized tubulin (Kirsch *et al.*, 1991), thus serving as a GlyR–cytoskeleton linker (Kirsch and Betz, 1995). Immunoelectron microscopy on neurons in the spinal cord, the cochlear nucleus, and the retina has shown that gephyrin colocalizes with GlyRs precisely at the postsynaptic membrane (Triller *et al.*, 1985; Altschuler *et al.*, 1986; Sassoe-Pognetto *et al.*, 1997). Gephyrin is also highly enriched at most GABAergic synapses (Triller *et al.*, 1987; Bohlhalter *et al.*, 1994; Cabot *et al.*, 1995; Sassoe-Pognetto *et al.*, 1995; Craig *et al.*, 1996; Todd *et al.*, 1996; Giustetto *et al.*, 1998).

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[2] Abbreviations used: CNS, central nervous system; GABA, γ-aminobutyric acid; geph, gephyrin; GlyR, glycine receptor; $GABA_AR$, $GABA_A$ receptor; PSD, postsynaptic density; PDZ, postsynaptic density, discs large, zonula adherens; VIAAT, vesicular inhibitory amino acid transporter.

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Consistent with a general function in inhibitory neurotransmitter receptor clustering and anchoring, the loss of gephyrin expression resulting from either antisense depletion (Kirsch *et al.*, 1993; Essrich *et al.*, 1998) or gene knockout in mice (Feng *et al.*, 1998) prevents the formation of all clusters of the GlyR (Feng *et al.*, 1998; Fischer *et al.*, 2000) and of $GABA_A$Rs containing the abundant $\alpha2$ and/or $\gamma2$ subunits (Essrich *et al.*, 1998; Kneussel *et al.*, 1999b). However, in contrast to results obtained for the GlyR, gephyrin does not copurify with $GABA_A$Rs (Meyer *et al.*, 1995), and transfection experiments have failed to provide evidence for a direct interaction of gephyrin with major $GABA_A$R subunits (Kirsch *et al.*, 1996). Therefore specific $GABA_A$R–gephyrin linker proteins have been proposed to exist (Essrich *et al.*, 1998; Kneussel *et al.*, 1999b). An attractive candidate for such a linker protein has been the putative $GABA_A$R clustering protein GABARAP (for $GABA_A$R-associated protein; Wang *et al.*, 1999). Indeed, GABARAP interacts with gephyrin but is not found at gephyrin-positive GABAergic synapses (Kneussel *et al.*, 2000). The identity of the protein(s) immobilizing $GABA_A$Rs on postsynaptic gephyrin clusters thus remains enigmatic (Kirsch and Betz, 1998; Kneussel and Betz, 2000b).

Further complexity of the processes that localize inhibitory neurotransmitter receptors at postsynaptic sites emerges from $GABA_A$Rs comprising a highly heterogeneous set of membrane proteins that are differentially expressed in the mammalian CNS (Wisden *et al.*, 1992; Laurie *et al.*, 1992a,b; Fritschy and Möhler, 1995). Studies on $GABA_A$R clustering in various regions of the CNS of wild-type and gephyrin-deficient (*geph* $-/-$) mice have disclosed significant differences in the synaptic localization of the major $GABA_A$R subunits $\alpha2$ and $\gamma2$. In particular, in *geph* $-/-$ hippocampal cultures and spinal cord, an almost complete loss of punctate staining has been reported (Kneussel *et al.*, 1999b). In contrast, in organotypic retinal cultures $GABA_A$R clusters containing the subunits $\alpha2$, $\alpha3$, and/or $\gamma2$ develop in the absence of gephyrin (Fischer *et al.*, 2000). These observations point to a differential regulation of $GABA_A$R clustering that might involve receptor subtype- and neuron-specific mechanisms which act independently of gephyrin deposition at developing postsynaptic sites. Consistent with this view, in differentiating spinal neurons the formation of a gephyrin scaffold has been found to precede (Kirsch *et al.*, 1993; Bechade *et al.*, 1996) but not to be sufficient for (Levi *et al.*, 1999) GlyR and $GABA_A$R clustering.

In this study, we compared the synaptic localization of the $GABA_A$R subunits $\alpha1$, $\alpha2$, $\alpha3$, $\alpha5$, $\beta2/3$, and $\gamma2$ in spinal cord sections derived from wild-type and gephyrin-deficient mice. We report that $GABA_A$Rs containing the $\alpha1$ and $\alpha5$ subunits are fully synaptically localized in gephyrin-deficient animals whereas the number of $\alpha2$-, $\alpha3$-, $\beta2/3$-, and $\gamma2$-subunit-immunoreactive puncta is significantly or dramatically reduced. This reduction cannot be attributed to a loss of inhibitory nerve terminals, since their density as revealed by staining with an antibody specific for the vesicular inhibitory amino acid transporter (VIAAT) is not altered in the spinal cord of the mutant animals. Our results unravel novel subunit-specific differences in $GABA_A$R cluster formation and support the notion (Kirsch and Betz, 1998; Levi *et al.*, 1999; Kneussel *et al.*, 2000b; Fischer *et al.*, 2000) that additional $GABA_A$R clustering proteins must exist.

RESULTS

In order to evaluate whether gephyrin is required for the postsynaptic localization of non-$\alpha2$ and non-$\gamma2$ $GABA_A$Rs, we examined spinal cord sections from E19.5 wild-type and *geph* $-/-$ mouse embryos by immunocytochemistry using antibodies specific for the $GABA_A$R subunits $\alpha1$, $\alpha3$, $\alpha5$, and $\beta2/3$ and compared these results with those obtained in parallel stainings of the $\alpha2$ and $\gamma2$ subunits. In addition, the distribution of inhibitory presynaptic terminals was evaluated by using a VIAAT-specific antibody that decorates glycine- and GABA-containing synaptic vesicles (Dumoulin *et al.*, 1999).

The Density of Inhibitory Presynaptic Terminals Is Not Reduced in Spinal Cord of geph −/− Mice, and Inhibitory Terminals Are Not Misrouted to Other Postsynaptic Sites upon the Loss of Most Inhibitory Receptor Clusters

Gephyrin-deficient mice display a lethal phenotype (Feng *et al.*, 1998) and show a loss of postsynaptic clustering for the majority of inhibitory neurotransmitter receptors in the CNS (Feng *et al.*, 1998; Kneussel *et al.*, 1999b; Fischer *et al.*, 2000). To exclude the possibility that this might reflect reduced inhibitory innervation, we determined the density of inhibitory presynaptic terminals in spinal cord sections from E19.5 *geph* $-/-$ mice by VIAAT staining and compared these values with those found in age-matched wild-type tissue (Fig. 1A). The resulting density values of 193.1 ± 4.7 VIAAT-immunoreactive puncta per 50 μm^2 for *geph* $-/-$ tissue and of 190.1 ± 4.2 such puncta per 50 μm^2 in wild-type

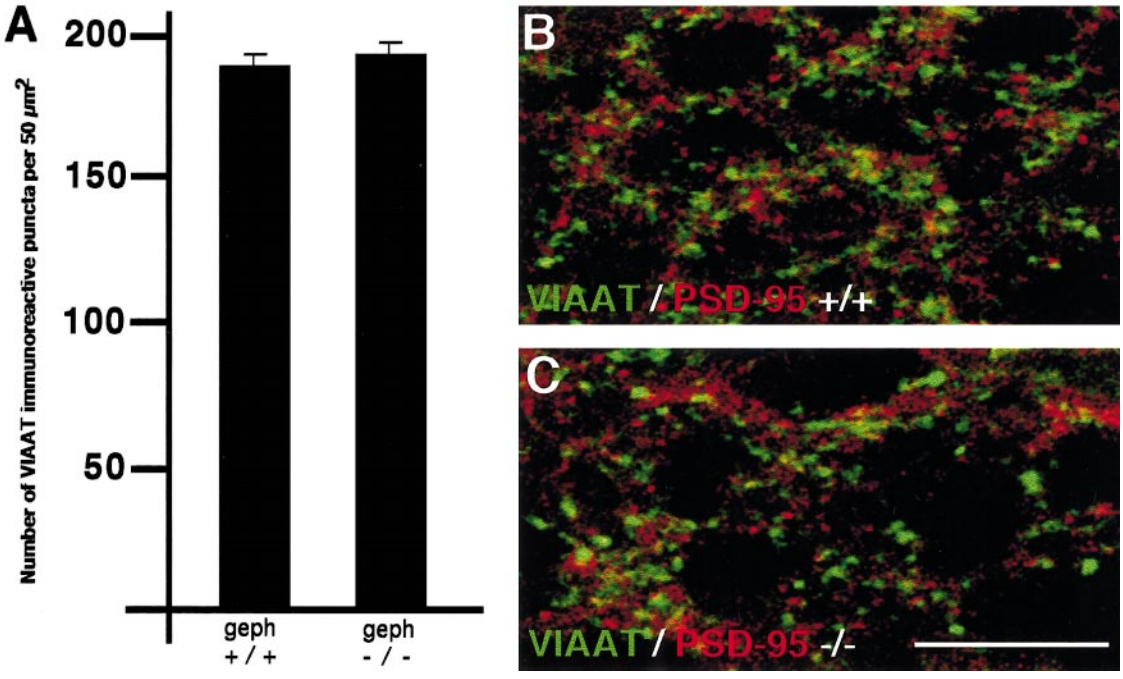

FIG. 1. Analysis of inhibitory presynaptic terminal density and localization by VIAAT immunolabeling. (A) Comparison of the density of VIAAT-immunoreactive terminals in spinal cord sections derived from wild-type and *geph* −/− mice. Values represent total numbers of VIAAT-positive puncta per 50 μm^2 as determined in different experiments [$n(+/+)$ = 8555 puncta, $n(-/-)$ = 8688 puncta]. (B, C) Double-immunostaining of VIAAT-positive inhibitory terminals and the postsynaptic density protein PSD-95, exclusively located at excitatory postsynaptic sites. VIAAT-positive inhibitory terminals do not colocalize with PSD-95 in wild-type (+/+) spinal cord tissue (B). Note that despite a major loss of postsynaptic inhibitory neurotransmitter receptors in gephyrin knockout mice (−/−), VIAAT-positive inhibitory terminals are not misrouted to excitatory postsynaptic sites, as represented by separated immunoreactivities (C). Scale bar, 10 μm.

sections indicate that the density of inhibitory presynaptic terminals is not altered upon inactivation of the gephyrin gene.

In cultured autaptic hippocampal neurons, excitatory nerve terminals have been reported to be incorrectly apposed to gephyrin-positive inhibitory postsynaptic membrane specializations (Rao *et al.*, 2000). To examine whether inhibitory nerve terminals might be misrouted to excitatory postsynaptic sites in gephyrin-deficient mice, we also performed double-immunolabeling of spinal cord sections for both VIAAT and PSD-95 (for postsynaptic density protein, 95 kDa), an established marker of glutamatergic postsynaptic densities (Kennedy, 1997). In both wild-type and *geph* −/− animals, individual immunoreactivities were found at distinct non-overlapping sites (Figs. 1B and 1C). We therefore conclude that the loss of the majority of postsynaptic inhibitory receptor clusters in gephyrin-deficient animals does not result in misrouting of inhibitory nerve terminals, as seen with autaptic glutamatergic hippocampal neurons *in vitro*.

The Number of Immunoreactive Puncta of the $GABA_AR$ Subunits α1 and α5 Is Not Reduced in Spinal Cord of geph −/− Mice

Immunoreactive puncta of the $GABA_A$ receptor subunits α1 and α5 were compared using E19.5 spinal cord sections prepared from wild-type and *geph* −/− mice. Both subunits show distinct expression patterns in certain layers of the dorsal horn of rodent spinal cord (Bohlhalter *et al.*, 1996). In several experiments, no differences in synaptic immunostaining for the α1 and α5 subunits were detected when comparing wild-type and *geph* −/− sections (Fig. 2). Quantification of the numbers of synaptic puncta per 50-μm^2 section area for the individual genotypes revealed density values of 109.8 ± 4.9 for the α1 subunit in *geph* −/− tissue compared to 108.2 ± 4.9 in wild-type tissue (Table 1). For the α5 subunit, 206.0 ± 13.1 puncta per 50-μm^2 section area were obtained in *geph* −/− tissue compared to 191.0 ± 6.7 puncta in wild-type tissue (Table 1). This corresponds to relative receptor cluster densi-

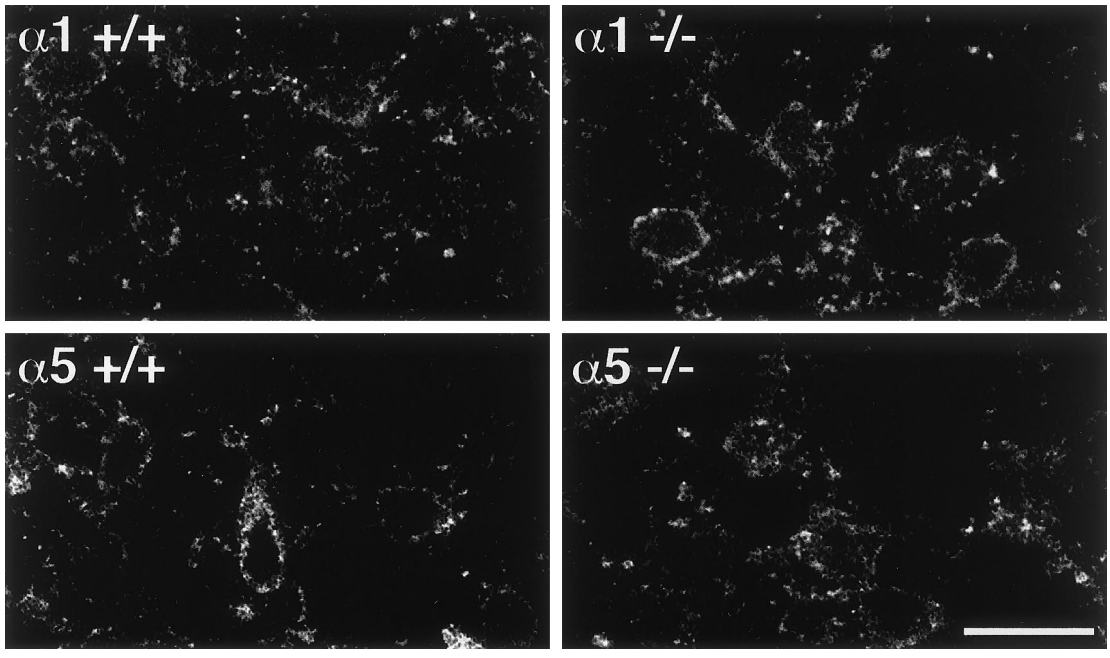

FIG. 2. Immunostaining of postsynaptic $GABA_AR$ clusters in spinal cord sections of wild-type and *geph* −/− mice. Sections were stained with α1- and α5-specific antibodies. Note that the punctate staining of sections derived from the homozygous mutants is not altered. Scale bar, 20 μm.

ties in *geph* −/− animals of 101.5 ± 4.5 and 107.9 ± 6.8% of the wild-type values for α1 and α5, respectively. We therefore conclude that the $GABA_A$ receptor isoforms containing the α1 and/or α5 subunits do not depend on the receptor-anchoring protein gephyrin for clustering at putative postsynaptic sites.

TABLE 1

Quantification of Punctate $GABA_AR$ and NMDAR1 Subunit Immunoreactivities in Spinal Cord Sections Derived from Wild-type and *geph* −/− Mice

Subunit immunoreactivity	Number of puncta/50 μm^2		Relative synapse density (% wild type)
	geph +/+	*geph* −/−	
$GABA_AR$ α1	108.2 ± 4.9	109.8 ± 4.9	101.5 ± 4.5
$GABA_AR$ α2	221.4 ± 6.1	34.3 ± 3.1*	15.5 ± 1.4*
$GABA_AR$ α3	239.4 ± 12.3	181.0 ± 10.1*	75.6 ± 4.2*
$GABA_AR$ α5	191.0 ± 6.7	206.0 ± 13.1	107.9 ± 6.8
$GABA_AR$ β2/3	137.2 ± 10.7	52.4 ± 6.0*	38.2 ± 4.4*
$GABA_AR$ γ2	257.5 ± 9.5	38.0 ± 4.1*	14.8 ± 1.6*
NMDAR 1	390.3 ± 12.6	396.8 ± 16.4	101.7 ± 4.2

Note. Values represent numbers of immunoreactive puncta per 50 μm^2 as determined in different (n = 3) experiments. For the subunits α1 and α5, which display restricted expression in certain spinal cord layers (Bohlhalter *et al.*, 1996), areas with the highest abundance were used for analysis. Data are normalized to the mean values (100%) obtained with wild-type samples. SEM (standard error of the mean) values are indicated (*n:* α1 = 1090, α2 = 1532, α3 = 2102, α5 = 1191, β2/3 = 948, γ2 = 1735, NR1 = 4326). Asterisks indicate statistically significant differences between the two genotypes (P values <0.01).

Previously, we showed that the distribution of several markers of glutamatergic postsynaptic sites, including the AMPA receptor GluR1 and GluR2/3 subunits, the postsynaptic density protein PSD-95, and the principal NMDA receptor subunit NMDAR1, is not detectably altered in *geph* −/− neurons (Feng *et al.*, 1998; Kneussel *et al.*, 1999b). For quantitative evaluations, spinal cord sections stained for the NMDAR1 subunit were also examined in this study. This revealed an average density of 396.8 ± 16.4 puncta per 50-μm^2 section area in *geph* −/− tissue compared to 390.3 ± 12.6 puncta in wild-type tissue (Table 1) and corresponds to a relative receptor cluster density of 101.7 ± 4.2% in *geph* −/− animals. These data corroborate our previous conclusions (Feng *et al.*, 1998; Kneussel *et al.*, 1999b) and show that the average density of NMDA receptor clusters in E19.5 spinal cord sections is significantly higher than that of $GABA_AR$ clusters.

Clustering of $GABA_A$Rs Containing the α3 and/or β2/3 Subunits Is Impaired in Spinal Cord of geph −/− Mice

In spinal cord, the α3 subunit is abundantly expressed in all layers of the dorsal horn (Bohlhalter *et al.*, 1996). Upon staining with an α3-subunit-specific antibody, the number of immunoreactive puncta was significantly lower in *geph* −/− mice than in wild-type tissue (Fig. 3).

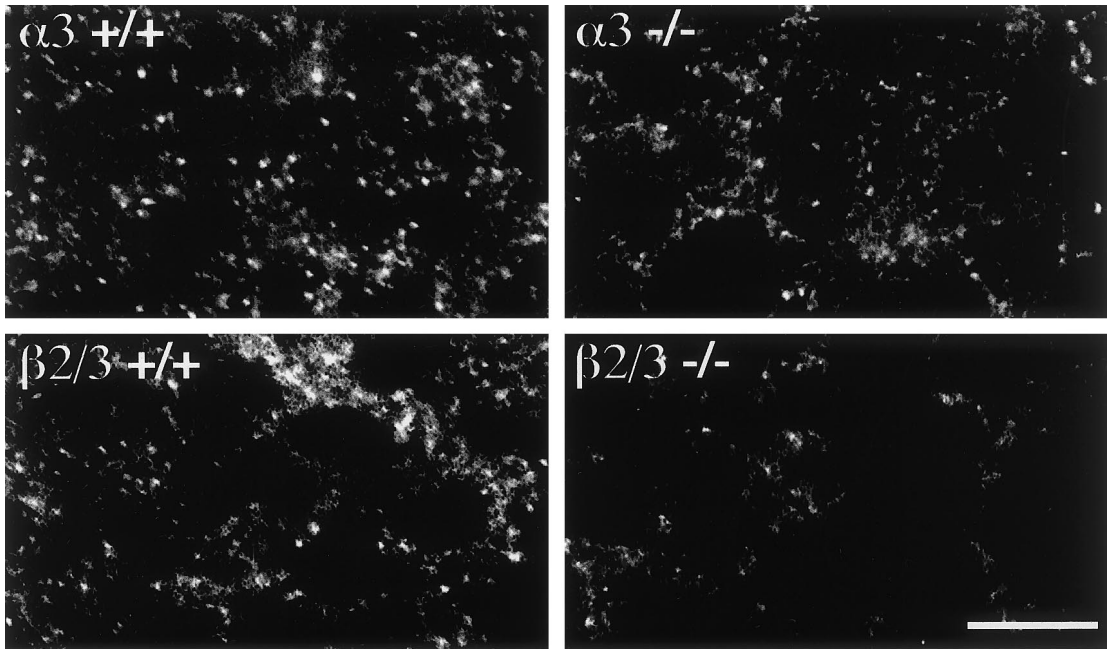

FIG. 3. Immunostaining of postsynaptic GABA$_A$R receptor clusters in spinal cord sections of wild-type and *geph* −/− mice. Sections were stained with α3- and β2/3-specific antibodies. The punctate staining of sections derived from homozygous mutants was partially reduced for α3 and strongly reduced for β2/3. Scale bar, 20 μm.

Quantification in tissue sections of the individual genotypes revealed density values of 181.0 ± 10.1 puncta per 50 μm^2 for *geph* −/− tissue compared to 239.4 ± 12.3 puncta found per 50 μm^2 in wild-type sections (Table 1). This represents an average reduction in GABA$_A$R α3 subunit cluster density to 75.6 ± 4.2% of that in wild-type tissue. When an antibody recognizing the GABA$_A$ receptor subunits β2/3 was used for immunostaining, an even more drastic alteration in synaptic staining was obtained. The number of β2/3-subunit-immunoreactive puncta, whose wide distribution in the cervical spinal cord resembles that of the γ2 subunit (Bohlhalter *et al.*, 1996), was strikingly lowered in *geph* −/− tissue (Fig. 3). Determination of the number of puncta per 50-μm^2 area in the individual genotypes revealed density values of 52.4 ± 6.0 puncta per 50 μm^2 for *geph* −/− tissue compared to 137.2 ± 10.7 puncta found per 50 μm^2 in wild-type tissue (Table 1). This corresponds to a reduction of GABA$_A$R β2/3 subunit cluster density to 38.2 ± 4.4% of the wild-type value (Table 1). Together, these data indicate that gephyrin is required for the clustering of certain but not all GABA$_A$ receptors containing the α3 and/or the β2/3 subunits.

Previously, we showed that cultured hippocampal neurons from *geph* −/− mice fail to accumulate GABA$_A$R α2 and γ2 subunits at developing postsynaptic sites (Kneussel *et al.*, 1999b). To allow a direct comparison of the quantitative data described above with the previously observed lack of α2 and γ2 subunit clustering in these cultured neurons, E19.5 spinal cord sections were also stained in parallel with α2- and γ2-subunit-specific antibodies. For the α2 subunit, determination of the number of puncta per 50-μm^2 area in the individual genotypes revealed density values of 34.3 ± 3.1 puncta per 50 μm^2 for *geph* −/− tissue compared to 221.4 ± 6.1 puncta found per 50 μm^2 in wild-type tissue (Table 1). This corresponds to a reduction of GABA$_A$R α2 subunit cluster density to 15.5 ± 1.4% of the wild-type value (Table 1). Similarly, for the γ2 subunit, density values of 38.0 ± 4.1 puncta per 50 μm^2 were obtained in *geph* −/− tissue compared to 257.5 ± 9.5 puncta found per 50 μm^2 in wild-type tissue (Table 1), indicating a reduction of GABA$_A$R γ2 subunit cluster density to 14.8 ± 1.6% of the wild-type value (Table 1). We therefore conclude that in spinal cord, both α2- and γ2-subunit-containing GABA$_A$Rs also require gephyrin for correct synaptic localization.

Expression Levels of GABA$_A$R α3 and β2/3 Subunits Are Unaltered in geph −/− Mice

To determine whether the reductions in cluster density observed for the GABA$_A$R subunits α3 and β2/3

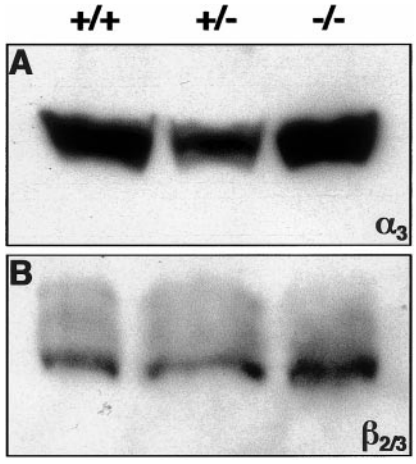

FIG. 4. Western blot analysis of the $GABA_AR$ subunits $\alpha3$ and $\beta2/3$ in brain and spinal cord membrane fractions prepared from wild-type, *geph* $+/-$, and *geph* $-/-$ animals. Equal amounts of protein (40 or 25 μg/lane, respectively) were loaded and probed with the indicated antisera. Note that the expression levels of all $GABA_AR$ subunits tested were not significantly different in the individual genotypes.

were due to changes in subunit expression levels, we performed Western blot analysis on pooled extracts of brain and spinal cord prepared from E19.5 wild-type as well as from heterozygous and homozygous gephyrin-deficient mice. The immunoreactivities of these $GABA_AR$ subunits were unaltered in all genotypes examined (Fig. 4), indicating that the rate of synthesis of these membrane proteins does not change upon gephyrin deficiency. These data are consistent with previous Western blot data showing unaltered expression levels of the $GABA_AR$ subunits $\alpha2$ and $\gamma2$ in wild-type and *geph* $-/-$ mice (Kneussel *et al.*, 1999b).

Remaining α3- and β2/3-Subunit-Immunoreactive Puncta in geph −/− Tissue Are Localized at Synaptic Sites

To reveal whether the remaining $GABA_AR$ clusters in *geph* $-/-$ tissue were found at sites of presynaptic terminal contact, we performed double-labeling of sections stained for the $GABA_AR$ $\alpha3$ or $\beta2/3$ subunits with an antibody against the vesicular inhibitory amino acid transporter (VIAAT, also named VGAT) (Sagne *et al.*, 1997; McIntire *et al.*, 1997). VIAAT represents an established marker of both glycinergic and GABAergic presynaptic boutons (Chaudhry *et al.*, 1998; Dumoulin *et al.*, 1999). As shown in Figs. 5A–5D, in *geph* $-/-$ mice the vast majority of the remaining $\alpha3$ and $\beta2/3$ clusters were apposed to sites of VIAAT immunoreactivity. Quantification of the puncta positive for $GABA_AR$ $\alpha3$ and $\beta2/3$ immunoreactivities that colocalized with presynaptic VIAAT-positive terminals revealed average colocalization indices of 91.5 $\pm$ 3.3% for the $\alpha3$ subunit and of 93.5 $\pm$ 1.4% for the $\beta2/3$ subunits, respectively (Fig. 5E). Thus, certain $GABA_AR$s containing $\alpha3$ and/or $\beta2/3$ subunits do not require gephyrin for synaptic localization.

DISCUSSION

The major finding of this study is that the number of $GABA_A$ receptor subunit $\alpha1$- and $\alpha5$-immunoreactive puncta is unaltered in spinal cord tissue derived from gephyrin-deficient mice. This indicates that in spinal neurons gephyrin is not required for the synaptic accumulation of receptors containing the $\alpha1$ and $\alpha5$ subunits. Our result contrasts with different studies with hippocampal, spinal, and retinal neurons (Essrich *et al.*, 1998; Kneussel *et al.*, 1999b; Fischer *et al.*, 2000), in which gephyrin was found to be essential for the clustering of the abundant $GABA_A$ receptor $\alpha2$, $\alpha3$, and $\gamma2$ subunits. In hippocampal neurons, gephyrin deficiency fully prevented the synaptic localization of $\alpha2$ and $\gamma2$ subunit immunoreactivities (Essrich *et al.*, 1998; Kneussel *et al.*, 1999b). Our quantitative immunofluorescence data from E19.5 spinal cord sections largely corroborate this conclusion; an 85% reduction in both $\alpha2$- and $\gamma2$-subunit-immunoreactive puncta was seen. As previous studies reported differences in the number of postsynaptic receptor clusters in comparisons of retinal neurons *in vitro* and *in vivo* (Fischer *et al.*, 2000), the lower incidence of $\alpha2$ and $\gamma2$ subunit clusters in cultured hippocampal neurons than in spinal cord sections might reflect effects of the tissue culture conditions.

In organotypic retinal cultures prepared from *geph* $-/-$ mice, a strong reduction but no complete loss of $\alpha2$-, $\alpha3$-, and $\gamma2$-positive clusters has been noted (Fischer *et al.*, 2000). Consistent with these earlier observations, the punctate staining of $GABA_A$ receptors containing the $\alpha3$ and/or $\beta2/3$ subunits was partially reduced in the spinal cord of gephyrin-deficient mice. To determine whether the residual $GABA_AR$ hotspots in *geph* $-/-$ mice were still synaptically localized, we double-labeled sections for both $\alpha3$ or $\beta2/3$ and the presynaptic inhibitory marker VIAAT. Notably, the majority of the $\alpha3$- and $\beta2/3$-subunit-positive puncta seen in the mutant mice precisely colocalized with VIAAT immunoreactivity, showing that the residual clusters were apposed to inhibitory nerve endings. This observation further strengthens the view that clustering pro-

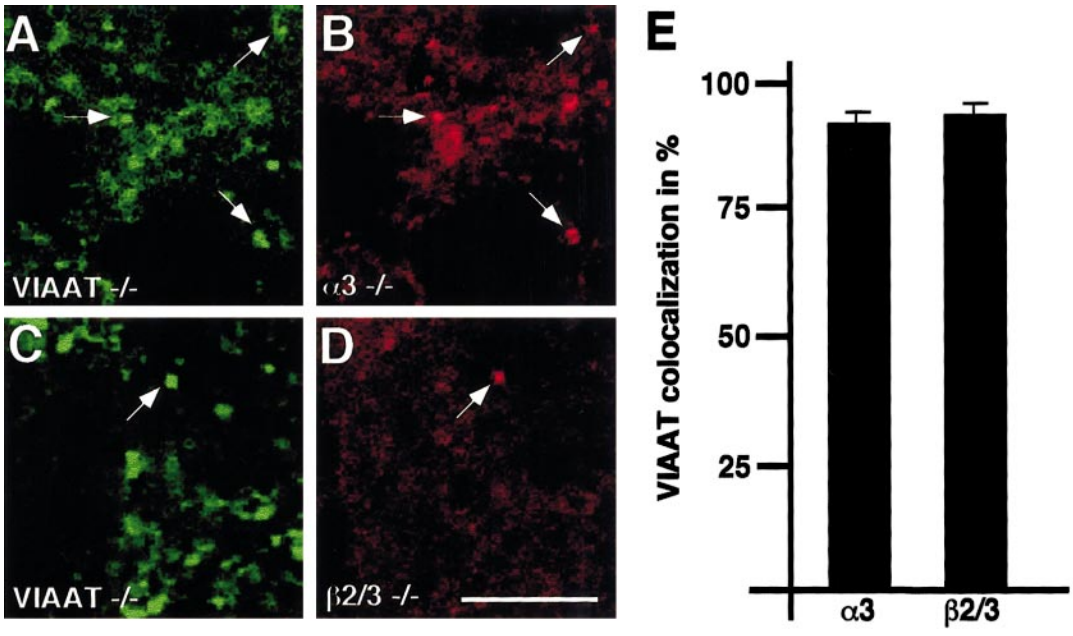

FIG. 5. $GABA_AR$ clusters and VIAAT-immunoreactive presynaptic terminals. Double-immunostaining of VIAAT-positive terminals (A, C) and $GABA_AR$ $\alpha3$ (B) or $\beta2/3$ (D) subunits in spinal cord sections of *geph* −/− mice. Most of the remaining $GABA_AR$ receptor clusters (red) containing $\alpha3$ and/or $\beta2/3$ subunits colocalize with presynaptic VIAAT (green) immunoreactivity, indicating that these receptor clusters are present at synaptic sites in the absence of gephyrin (arrows). Scale bar, 5 μm. (E) Quantitative evaluation of $GABA_AR$ subunit-immunoreactive puncta colocalizing with VIAAT immunoreactivity. The relative densities of double-immunoreactive hotspots were determined in 20 section areas of 50 μm^2 each. Note that >90% of both the $\alpha3$- and $\beta2/3$-subunit-immunoreactive sites are still apposed to presynaptic terminals. For $\alpha3$, $n = 1401$; for $\beta2/3$, $n = 729$.

teins other than gephyrin contribute to the synaptic localization of $GABA_AR$s. The partial impairment of $\alpha3$ and $\beta2/3$ subunit clustering revealed by our quantitative analysis might reflect the fact that more than a single subunit within the receptor pentamer is anchored to the subsynaptic gephyrin scaffold and/or cytoskeletal elements via specific adaptor proteins (Kneussel and Betz, 2000b). Consequently, only those $GABA_AR$ clusters whose synaptic localization solely depends on gephyrin should be lost upon gephyrin depletion. Since all these $GABA_AR$ clusters have been shown to represent synaptically localized receptors (Bohlhalter *et al.*, 1994; Essrich *et al.*, 1998; Kneussel *et al.*, 1999b; this study), gephyrin clearly is important for the clustering of most $GABA_AR$s at postsynaptic membrane specializations in the mammalian CNS.

The most important conclusion of this paper, however, is that some $GABA_AR$ subtypes must use largely different localization mechanisms, since the densities of both the $\alpha1$ and $\alpha5$ subunits were found here to be essentially unaltered in *geph* −/− tissue. We therefore propose that additional gene products, which mediate the clustering of these subunits, must exist. Notably, the $\alpha1$ and $\alpha5$ subunits have been suggested to form functional heterooligomeric receptors *in vivo* with the $\gamma2$ subunit (McKernan and Whiting, 1996), a $GABA_AR$ protein whose synaptic localization is abolished upon gephyrin depletion (Essrich *et al.*, 1998; Kneussel *et al.*, 1999b; this study). Our data argue against this interpretation, since $\gamma2$-immunoreactive puncta are drastically reduced in the spinal cord of *geph* −/− mice. In conclusion, the data presented here support our previous suggestion (Kirsch and Betz, 1998; Kneussel and Betz, 2000b; Fischer *et al.*, 2000) that yet unknown proteins distinct from gephyrin mediate the formation of those $GABA_AR$ clusters which persist in gephyrin-deficient mice.

The selective loss of some $GABA_AR$ subtype clusters in the absence of gephyrin expression cannot be attributed to impaired axonal growth of distinct subtypes of GABAergic interneurons. The density of inhibitory presynaptic terminals as defined by VIAAT immunoreactivity was unaltered in the spinal cord of gephyrin-deficient mice. This finding is remarkable since all GlyRs and most $GABA_AR$ subtypes fail to accumulate synaptically in *geph* −/− animals. We therefore con-

clude that the loss of inhibitory postsynaptic receptor densities does not cause changes in the number of the respective presynaptic terminals. Since previous studies on autaptic hippocampal neurons in culture reported mismatched appositions of $GABA_AR$ clusters opposite of glutamatergic terminals (Rao *et al.*, 2000), we further performed double-immunostainings for VIAAT and PSD-95 on sections derived from wild-type and *geph* $-/-$ mice to unravel eventual appositions of the inhibitory nerve endings to excitatory postsynaptic membrane specializations. In both genotypes, the immunoreactivities of both markers were found at distinct nonoverlapping sites, suggesting a clear separation of inhibitory and excitatory synaptic compartments.

An interesting aspect of our quantitative analysis of VIAAT-immunoreactive puncta concerns their absolute density values, which are similar to or even lower than those of the $GABA_AR$ $\alpha2$, $\alpha5$, and $\gamma2$ subunit clusters (Table 1). In previous studies (Kneussel *et al.*, 1999b, 2000), only about 65% of the $GABA_AR$ clusters in wild-type neurons were found to be located at synaptic sites. This includes the $GABA_AR$ $\alpha2$ and $\gamma2$ subunit clusters in cultured hippocampal neurons (Kneussel *et al.*, 1999b), of which 65.9 $\pm$ 6.9% for $\alpha2$ and 66.4 $\pm$ 6.6% for $\gamma2$ colocalized with presynaptic synaptophysin immunoreactivity. Thus, about 35% of the receptor clusters included in such quantifications might represent nonsynaptic receptor clusters. In addition, GlyR-positive sites that also are abundant in spinal cord and opposed to VIAAT-positive presynaptic terminals (Triller *et al.*, 1985; Bohlhalter *et al.*, 1994; Feng *et al.*, 1998) have not been considered in this study. However, different reports indicate the existence of mixed synapses using both GABA and glycine as inhibitory transmitters in spinal cord (Triller *et al.*, 1987; Bohlhalter *et al.*, 1994; Jonas *et al.*, 1998; Dumoulin *et al.*, 2000). A recent quantitative analysis of GlyR- and $GABA_AR$-containing synapses in cultured spinal neurons reports colocalization values as high as 66.2 $\pm$ 2.5% after 11 days of *in vitro* differentiation (Dumoulin *et al.*, 2000). Thus, the overall number of presynaptic VIAAT-positive nerve endings cannot be expected to be identical to the sum of all GlyR and $GABA_AR$ subunit clusters. In addition, differences in antibody affinities and stages of development and/or maturity of the presynaptic nerve endings may contribute to the remaining inconsistencies. For example, at E19.5 a considerable fraction of the contacting terminals might not contain sufficient vesicles to be recognized by the VIAAT antibody in our light microscopical analysis.

In conclusion, the data presented confirm our previous notion that gephyrin is indeed essential for the clustering of the majority but not all $GABA_AR$s and extend this idea by the identification of two additional $GABA_AR$ subunits, $\alpha1$ and $\alpha5$, which are synaptically localized in the absence of gephyrin. Future studies will be required to identify the clustering proteins which localize $\alpha1$- and $\alpha5$-subunit-containing $GABA_AR$s at developing inhibitory postsynaptic membrane specializations.

EXPERIMENTAL METHODS

Antibodies

$GABA_AR$ immunoreactivities were visualized using primary antibodies specific for the $\alpha1$ (1:20,000), the $\alpha2$ (1:3000), the $\alpha3$ (1:6000), the $\alpha5$ (1:4000), the $\gamma2$ (1:2000) (Bohlhalter *et al.*, 1996; kindly provided by Hans Möhler and Jean Marc Fritschy), and the $\beta2/3$ (1:100) (Roche Diagnostics, Mannheim, Germany) subunits, respectively. Inhibitory presynaptic terminals were detected using a polyclonal antibody to the synaptic vesicle inhibitory amino acid transporter (VIAAT; 1:200) (Dumoulin *et al.*, 1999). Glutamatergic postsynaptic densities were stained using an antibody specific for PSD-95 (1:250, Affinity BioReagents, Grünberg, Germany). As secondary antibodies, Alexa 488 and Alexa 594 (Molecular Probes, Leiden, The Netherlands) were used.

Immunocytochemistry

To obtain spinal cord sections from wild-type and *geph* $-/-$ mice (Feng *et al.*, 1998; Kneussel *et al.*, 1999b), tissue dissected from E19.5 mice was cut into blocks of 5 mm side length and fixed in 4% (w/v) paraformaldehyde for 10 min followed by a short wash in phosphate-buffered saline (PBS). To prevent the formation of crystals upon freezing, the sections were incubated in increasing concentrations of sucrose solution [10% (w/v), 20% (w/v), 30% (w/v) +0.01% (w/v) sodium azide, respectively] at 4°C for 1 h each. Cryostat sections (10–15 μm) were refixed for 5 min in 4% (w/v) paraformaldehyde and processed for immunocytochemistry as described (Kneussel *et al.*, 1999b). Confocal microscopy was performed using a Leica TCS-SP confocal laser-scanning microscope equipped with Leica-TCS-NT version 1.6.551 image software. Images were taken from dorsal horn regions of the spinal cord.

Western Blot Analysis

Mouse homogenates were prepared as previously described (Kneussel *et al.*, 1999b). Forty micrograms of

total protein per lane was separated by 10% SDS–PAGE. For visualization the α3-specific antibody was diluted 1:1000; the β2/3-specific antibody was diluted 1:100. After washing, bound Igs were visualized with horseradish peroxidase-conjugated secondary antibodies using the ECL system (Pierce, Rockford, IL).

Data Analysis

Punctate immunofluorescence was analyzed as described previously (Fischer *et al.*, 2000). Confocal images, taken with a 63× objective, were printed and immunofluorescent puncta transferred onto tracing paper. The number of puncta per 50 μm^2 was counted from different sections (n = 3–5). Between 948 and 2102 individual puncta were analyzed for each $GABA_AR$ subunit in wild-type and *geph* −/− samples. For the subunits α1 and α5, which display a restricted expression in certain spinal cord layers (Bohlhalter *et al.*, 1996), areas of highest receptor abundance were used for quantitative evaluation. Remaining $GABA_AR$ α3- and β2/3-immunoreactive puncta were separately transferred onto tracing paper, and 778–1525 individual puncta were analyzed for colocalization in a total area of 1000 μm^2 (Kirsch and Betz, 1998). For the quantification of the density of inhibitory presynaptic terminals represented by VIAAT immunoreactivity, 17,243 puncta from both genotypes were analyzed. Statistical significance was evaluated using the KaleidaGraph program (Synergy Software, Reading, PA) and Student's t test.

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