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Morphologically identified glycinergic synapses in the hippocampus

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Inhibitory transmission in the hippocampus is predominantly GABAergic, but electrophysiological data evidenced strychnine-sensitive glycine-induced currents. However, synaptic currents have not been reported. Here, we describe, for the first time, the presence of GlyR clusters in several areas of the hippocampus as well as in cultured hippocampal neurons. In contrast with spinal cord, hippocampal GlyRs contain α2 but no α1 subunit. Optical and electron microscopy indicates that GlyRs can be synaptic as well as extrasynaptic. Synaptic GlyRs were apposed to glycinergic boutons characterized by the expression of the vesicular and the plasma membrane transporters of glycine (VIAAT and GlyT2, respectively). Double labeling with calcium-binding proteins showed that GlyT2 could be detected in boutons innervating both excitatory cells (soma and dendrites) and interneurons. Finally, GlyR clusters could be detected at synaptic sites with the GABAA receptor γ2 subunit and gephyrin, suggesting that mixed GABA/glycine synapses might exist in the hippocampus.

Introduction

Inhibitory synapses in the hippocampus control the principal cell network. Most, if not all, inhibitory hippocampal synapses are thought to be GABAergic (Freund and Buzsaki, 1996), while in brain stem and spinal cord inhibitory synapses are GABAergic and/or glycinergic (Aprison and Daly, 1978; Araki et al., 1988). However, synaptosomes from adult rat hippocampus contain glycine in concentrations similar to GABA (Burger et al., 1991; Engblom et al., 1996). Furthermore, in situ hybridization analysis showed that mRNAs for GlyR β, α2, and to a lesser extent α3 subunits are expressed in pyramidal and granular cell layers (Malosio et al., 1991; Racca et al., 1998; Sato et al., 1991, 1992). Immunochemistry with glycine antiserum has revealed the staining of a few axons (van den Pol and Gorcs, 1988) and of certain neurons in the pyramidal cell layer (Pourcho et al., 1992). In addition, electrophysiological studies in the hippocampus have identified two types of strychnine-sensitive glycine-gated channels (Chattipakorn and McMahon, 2002, 2003; Ito and Cherubini, 1991; Thio et al., 2003). The incomplete blockade of the responses to glycine by picrotoxin indicated that hippocampal glycine receptors (GlyRs) are composed both of homomeric α subunits and heteromeric αβ subunits (Chattipakorn and McMahon, 2002; Pribilla et al., 1992; Yoon et al., 1998). As shown in spinal cord neurons, the GlyR β subunit is required for interaction with the synaptic anchoring protein gephyrin (Meyer et al., 1995). Because gephyrin is also present in hippocampal inhibitory synapses (Sassoe-Pognetto et al., 2000), clusters of β-subunit-containing GlyRs are expected to be located at synapses. Yet, until now, only diffuse GlyR immunoreactivity (IR) has been detected in the hippocampus (Chattipakorn and McMahon, 2002; Kirsch and Betz, 1993).

In this work, we have further investigated in the hippocampus the synaptic expression of GlyR and other glycinergic synapse components such as the neuronal plasma membrane glycine transporter (GLYT2, Poyatos et al., 1997) or the vesicular inhibitory amino acid transporter (VIAAT, Dumoulin et al., 1999) and gephyrin, which are also present at GABAergic synapses.

Results

Clusters of glycine receptors are widely expressed in the hippocampus

The monoclonal antibody mAb4a, which is known to recognize all GlyR α subunits (Pfeiffer et al., 1984), and a polyclonal antibody directed against the GlyR α1–2 subunits were first used for Western blot experiments (Fig. 1A). As expected, these antibodies recognized proteins with the same apparent molecular mass (48–49 kDa) in both hippocampus and spinal cord extracts, but the signal was about 7-fold less intense in the latter.

Previous immunohistochemical analysis of GlyR expression using mAb4a suggested that only diffuse GlyR immunoreactivity (IR) could be detected in the hippocampus (Chattipakorn and

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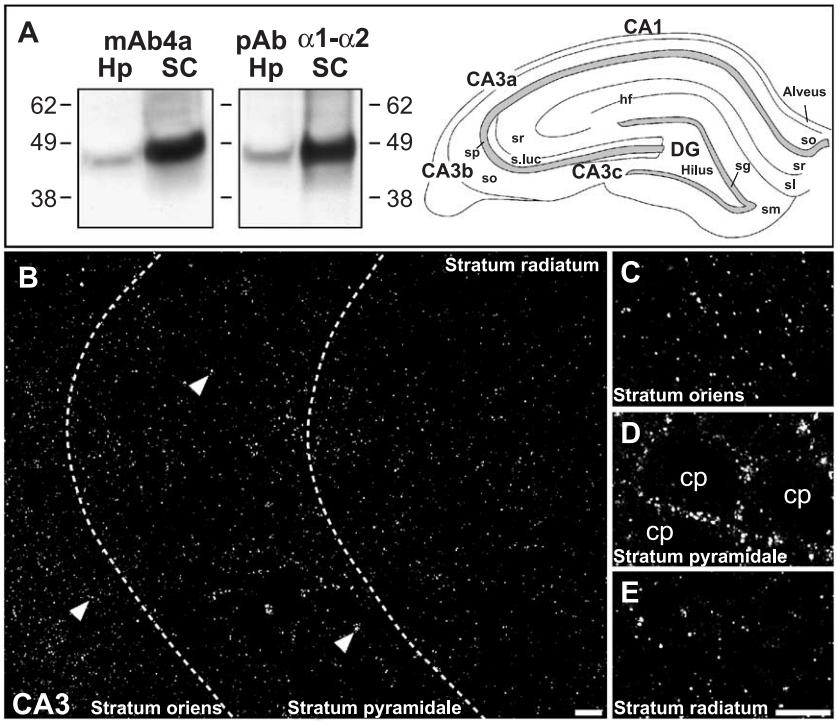


Fig. 1. Widespread expression of clustered GlyRs in the hippocampus. (A) Western blot analysis of GlyRs in hippocampal (Hp) and spinal cord (Sc) extracts of adult rats using either mAb4a (recognizing all GlyR α subunits) or pAb α1–2 antibody (recognizing GlyRα1 and α2 subunits). Importantly, under nonreducing conditions, a slow migrating band (≈90 kDa) was detected with both antibodies in both extracts. This band most probably represents dimers of GlyR α subunits as it completely disappears under reducing conditions (not shown). Left corner: schematic representation of a coronal section through the dorsal hippocampus. CA1–CA3a–CA3b–CA3c: fields of the hippocampus proper (cornu ammonis), hippocampal strata: So, stratum oriens; Sp, stratum pyramidale; Sr, stratum radiatum; S.luc, stratum lucidum. Hf: hippocampal fissure. DG: dentate gyrus, dentate gyrus strata: Sm: stratum moleculare, Sg: stratum granulosum, hilus. (B) Clustered GlyR immunoreactivity (IR, arrowheads) can be detected throughout the hippocampus using mAb4a and methanol fixation analyzed by confocal microscopy (one confocal slice in CA3b region). (C–E) Projections of four confocal slices show clustered GlyR-IR in various strata of the CA3b region and underline the neuronal somata (D). Scale bars, 10 μm.

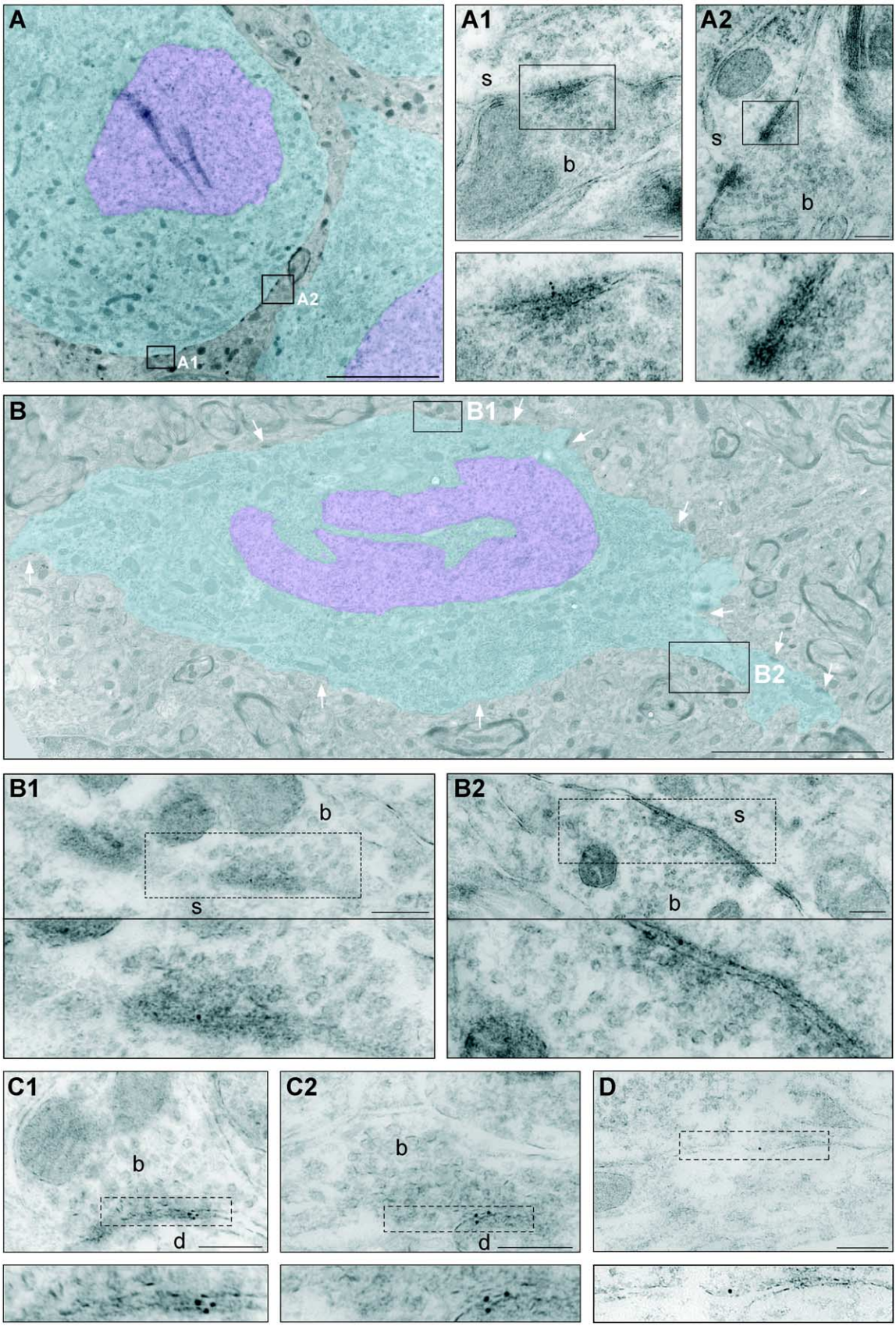
McMahon, 2002; Kirsch and Betz, 1993). However, in these studies, the experimental procedures were not those known to optimally detect clustered and diffuse GlyRs with mAb4a (Dumoulin et al., 2000). Therefore, we re-investigated the expression of GlyRs using immunocytochemistry and improved labeling conditions (see Experimental methods) in adult rat hippocampus and in cultured neurons. With these procedures, immunoreactive clusters of GlyR were detected throughout the hippocampus (Figs. 1B–E) as well as in cultured hippocampal neurons. In the pyramidal cell layer, a high proportion of pyramidal soma was surrounded by GlyR clusters (Fig. 1D). In the CA3 region, the surface density of GlyR clusters was higher in layers containing the somata of pyramidal cells than in the dendritic layer (Table 1). This preferential localization ($P < 0.005$, Kolmogorov–

Table 1
Cluster density of GlyRs and GABARs γ2 per 10000 μm^2 in hippocampus and spinal cord

Receptors	Strata	Hippocampus			Spinal cord
		CA1	CA3	GD	
GlyRs	So	234 ± 10.6	137 ± 2.7	–	
	Sp	175 ± 6.4	188 ± 4.8	–	
	Sr	191 ± 2.8	148 ± 3.9	–	
	Sm	–	–	212 ± 3.8	
	Sg	–	–	236 ± 7.8	
	Hilus	–	–	204 ± 6.4	
	Mean	196 ± 4.1	161 ± 3.4	214 ± 3.8	1214 ± 13.7
		(*n* = 8563)	(*n* = 7029)	(*n* = 9363)	(*n* = 53102)
GABAR γ2	Mean	632 ± 63.6	409 ± 14.5	523 ± 24.1	974 ± 27.4
		(*n* = 27658)	(*n* = 17882)	(*n* = 22892)	(*n* = 42619)

Hippocampal slices were immunolabeled with mouse anti-GlyR (mAb4a) or guinea pig anti-GABAR γ2. Cluster densities (Mean ± SEM) were evaluated on confocal section of hippocampal or spinal slices. The total number of counted clusters is indicated in brackets. Spinal GlyR and GABAR clusters were counted in the ventral horn of cervical spinal slices.

CA1–CA3, fields of the hippocampus (cornu ammonis); So, stratum oriens; Sp, stratum pyramidale; Sr, stratum radiatum; DG, dentate gyrus; Sm, stratum moleculare; Sg, stratum granulosum.


A
A1
A2
s
b
B
B1
B2
C1
C2
D
d

Smirnov) is consistent with the perisomatic localization of inhibitory synapses (Megias et al., 2001). In agreement with the Western blot data, we found that the density of GlyR clusters in the pyramidal cell or granule cell layer in hippocampus was 5- to 9-fold lower than on soma of the spinal cord motoneurons (Table 1). However, it should be noted that the density of GABAR γ2 clusters was only 2- to 3-fold higher than for GlyR ($P < 0.005$, Kolmogorov–Smirnov, Table 1). Therefore, these analyses show that GlyRs are widely expressed in the hippocampus where they constitute a significant proportion of inhibitory receptors. In contrast, to identify the different subunits expressed in the hippocampus, we performed immunolabeling using mAb2b, which is specific for GlyR α1 subunit. Whereas mAb2b strongly labeled spinal cord sections, it failed to produce any signal in the hippocampus (not shown). Together with the Western blot results (Fig. 1A), these data further indicate that hippocampal clusters of GlyRs contained at least α2 but no α1 subunit.

Clusters of glycine receptors are located at the synapse in the hippocampus

We next used electron microscopy (EM) to investigate the possible localization of GlyR clusters at the synapse. The number of particles associated with the plasma membrane was always low, but the density of gold particles at synapses was 700-fold higher than background labeling. Interestingly, GlyR-IR was found at somatic (Figs. 2A–B) and dendritic synapses (Figs. 2C1–C2) as well as at membrane-associated extrasynaptic locations (Fig. 2D). In addition, presynaptic boutons apposed to GlyR-IR contained pleiomorphic vesicles (Figs. 2B–C) that are characteristic of inhibitory boutons (Peters et al., 1991). In the pyramidal cell layer, synaptic GlyR-IR could be detected on the soma of cells on which no asymmetric synapses could be found and which have rounded nuclei (Fig. 2A). These cells are most probably principal excitatory neurons. We analyzed somatic sections of two principal cells and found that around 13% of the perisomatic synapses express GlyR-IR. If the analyzed sections are representative of the entire neuron, this suggests that each pyramidal cell may be contacted by 90 glycinergic synapses. In the same layer, we also observed synaptic GlyR-IR on cells with invaginated nuclei that are characteristic of interneurons (Fig. 2B). At the post-synaptic side, 1.3 ± 0.2 immunogold particles were detected preferentially at the edge (Figs. 2B1, B2, and C1) of synaptic complexes ($n = 10$ synapses). With the same labeling procedures, immunogold particles labeled a larger proportion of the post-synaptic membrane in glycinergic synapses of the spinal cord (not shown and see Colin et al., 1998) with more GlyR-IR particles per synapse (7.05 ± 1.32; $n = 21$). This shows that, besides being less numerous than in the spinal cord, labeled hippocampal synapses contain less GlyR.

The synaptic localization of GlyRs was further investigated by optical microscopy of the CA3b region using double labeling experiments with mAb4a and antibodies specific for VIAAT to label inhibitory (GABAergic and glycinergic) boutons. With this labeling, 30 ± 2% ($n = 22$, 23 127 clusters) of the GlyR clusters were apposed to VIAAT-IR boutons (Fig. 3). Such a low percentage of co-localization could be due to an impaired detection of inhibitory terminals by the anti-VIAAT antibody. However, with the same protocol in the spinal cord, 90.4 ± 1.4% of GlyR ($n = 29\,591$ clusters) was associated with VIAAT-IR. Therefore, the proportion of extrasynaptic GlyR clusters observed in the hippocampus is probably not over-estimated. In addition, comparable proportions were obtained when the presynaptic terminals were identified with an antibody directed against bassoon (Richter et al., 1999), which is a major scaffolding protein of the complex cytomatrix assembled at the active zone. This indicates that 70% of GlyR clusters were indeed extrasynaptic and were not apposed to excitatory synapses. Reciprocally, 17 ± 1% ($n = 21$, 29 899 clusters) of the VIAAT-positive inhibitory synapses were apposed to GlyR clusters.

We further characterized the presynaptic terminals facing GlyR-IR using an antibody specific for the neuronal membrane glycine transporter GlyT2 (Zafra et al., 1995) to label putative glycinergic boutons (Figs. 3 and 4). GlyT2-positive terminals were detected throughout the hippocampus. In CA3, 40 ± 3% ($n = 18$, 4297 clusters) of the GlyR clusters were apposed to GlyT2-positive boutons. GlyR clusters could be detected apposed to boutons expressing only GlyT2 (Fig. 3, open arrowheads) or only VIAAT (Fig. 3, closed arrowheads) or both (Fig. 3, open–closed arrowheads). Fig. 3 shows that 35 ± 2% of VIAAT-IR boutons also displayed GlyT2-IR ($n = 30$, 42 648 clusters) and, reciprocally, some GlyT2-IR puncta (open arrowheads) did not display VIAAT-IR (55 ± 2%, $n = 30$, 45 047 clusters). These puncta might either be co-localized with another isoform of VIAAT not recognized by our antibody (Dumoulin et al., 1999), or intracellular (Geerlings et al., 2001, 2002). Together with electron microscopy, these immunocytochemical data support the notion that genuine glycinergic synapses exist in the adult hippocampus.

Several subsets of interneurons are glycinergic

We next investigated the identity of putative glycinergic interneurons by double immunolabeling of GlyT2 and parvalbumin (PV), calbindin (CB), calretinin (CR), neuropeptide Y (NPY), or NO synthase (NOS), which are specific markers of hippocampal interneuron populations (Fig. 4). PV-containing interneurons are known to innervate perisomatic regions and the initial segment of principal cells (Freund and Buzsaki, 1996; Kosaka et al., 1987), whereas CB- and NPY-containing inter-

Fig. 2. Synaptic localization of GlyR immunoreactivity (mAb4a) in CA3. In the stratum pyramidale somatic GlyR-IR-containing synapses can be detected in different cell types. (A) The cell displays a round nucleus (light pink) and no excitatory synapses were detected on the soma (light blue), suggesting that it is an excitatory pyramidal neuron. (A1–A2) Higher magnifications of fields displaying GlyR-containing synapses detected in A. Note that few gold particles are detected always on the somatic membrane (s) facing a presynaptic bouton (b) filled with pleiomorphic vesicles that are characteristic of inhibitory synapses. (B) The cell displays a thick perinuclear cytoplasm containing mitochondria, and large amounts of RER. Moreover, the cell displays a characteristic invaginated nucleus, strongly suggesting that this cell is an interneuron. White arrows indicate synapses. (B1–B2) Higher magnifications of fields that exhibit GlyR-containing synapses. Note that the gold particles are preferentially located at the edge of the synapses. (C1–C2) GlyR-containing synapses were also detected in dendrites (d). (D) Extrasynaptic membrane-associated gold particles demonstrate an extrasynaptic localization of GlyR in the hippocampus. Panels in the lower part of the figure are magnifications of the boxed windows. Scale bars, 200 nm.

neurons innervate proximal and distal dendrites of principal cells, respectively (Fig. 4A; Freund and Buzsaki, 1996; Miettinen and Freund, 1992). Consistently with the observed somatic and dendritic localization of GlyR-containing synapses, we found that, respectively, 26.8%, 31.7%, and 47.1% of the GLYT2-positive terminals in CA3 also displayed PV, CB, and NPY-IR (Table 2). CR-positive interneurons selectively innervate other interneurons. We found that 9.1% of CR-positive boutons also display GlyT2-IR (Table 2). In the hippocampus, neuronal NO synthase (nNOS) IR is restricted to interneurons (Dinerman et al., 1994; Freund and Buzsaki, 1996). Table 2 shows that GLYT2-IR strongly co-localizes with nNOS-IR. This shows that GlyT2 could be detected in all subsets of interneurons that we have studied. However, due to the weak staining of the somata, it was not possible to determine the proportion of interneurons expressing GlyT2.

Reciprocally, we found that 17.5% of the PV-positive terminals and up to 14.9% of the CB-positive terminals also displayed GlyT2-IR (Table 2). These results show that glycinergic afferences account for a significant proportion of inhibitory terminals in the hippocampus.

Mixed GABA/Glycine synapses

GABA is the major inhibitory neurotransmitter in the hippocampus. Because a large proportion of GlyR clusters were apposed to VIAAT-positive boutons, we inferred that some of these clusters could be co-localized with GABAA receptors (GABAARs). This was investigated using co-detection of GlyRs and GABAAR γ2 subunits in brain slices (Fig. 5A) and in cultured hippocampal neurons (Fig. 5B). In CA3, 41 ± 1% of the GlyR clusters (n = 54, 64936) co-localized with GABAAR γ2 immunoreactivity. About half (48 ± 5%) of the GlyR clusters co-localizing with GABAAR γ2 were not apposed to VIAAT-positive boutons. Therefore, unless an undetected form of VIAAT exists, GlyRs and GABAAR γ2 subunit-containing clusters co-localize not only at synaptic but also at extrasynaptic sites. The same observation was made in cultured neurons (Fig. 5B), where antibody penetration is optimal. Importantly, only 29.1 ± 1.1%, (n = 89 666 clusters) of total hippocampal GABAAR γ2 clusters were associated with GlyR clusters. This implies that only a small fraction (less than 15%) of the total GABAR γ2 is extrasynaptic.

We next investigated by triple labeling experiments the association of GlyR with gephyrin and VIAAT (Fig. 6). We found that 65 ± 1% (n = 15; 6327 clusters) of the GlyR clusters co-localized with gephyrin-IR. Interestingly, 62 ± 1% of the GlyR clusters that were apposed to VIAAT-IR also co-localized with gephyrin-IR. Reciprocally, most clusters of gephyrin were not co-localized with GlyR (84 ± 1%). This is in agreement with the large number of inhibitory synapses devoid of GlyR. These results demonstrate that in the hippocampus, GlyR may co-localize with the GABAAR γ2 subunit and gephyrin.

Discussion

The major finding of this study is the existence of glycinergic synapses in the hippocampus. Clusters of GlyR were detected not only at extrasynaptic sites but also at synaptic sites in association with all the classical markers of glycinergic synapses such as GLYT2, VIAAT, and gephyrin. GABAAR γ2 subunits and GlyRs were frequently co-detected at the same loci facing inhibitory terminals, suggesting that mixed GABA/glycine synapses do exist in the hippocampus. GABAAR and GlyR clusters were also associated at extrasynaptic sites. Such extrasynaptic localization of GABAR has already been described in hippocampal slices (Banks and Pearce, 2000; Nusser et al., 1995) as well as in cultured hippocampal neurons (Danglot et al., 2003; Kannenberg et al., 1999). Extrasynaptic clusters may represent kinetic intermediates of receptors floating in the plasma membrane, “en route” to the postsynaptic sites (Danglot et al., 2003; Meier et al., 2000, 2001). Yet, in the case of hippocampal neurons, this would have to be demonstrated using direct physical methods. Interestingly, our data (Fig. 5) indicate that GlyR and GABAR are also intermingled in extrasynaptic clusters, suggesting that they exist as genuine entities.

The clustered labeling pattern of GlyR-IR that we have observed is in apparent contradiction with the diffuse cytoplasmic staining observed earlier with the same mAb4a antibody (Chattipakorn and McMahon, 2002; Kirsch and Betz, 1993). This discrepancy is most probably due to differences in fixation procedures and labeling protocols. Aldehyde fixation used in these studies did not permit optimal detection of GlyR clusters even in spinal cord or brain stem. In contrast, an alcohol treatment before incubation with the primary antibody allowed reliable detection of clusters. Moreover, this clustered pattern is consistent with the EM data showing expression of GlyR-IR at the edge of the inhibitory post-synaptic element as well as at extrasynaptic sites. Thus, our results unambiguously demonstrate the expression of GlyR at synapses in the hippocampus.

During the development of the spinal cord, the subunit composition of GlyR switches from a homomeric receptor composed of five α2 subunits to a heteromeric receptor composed of three α1 and two β subunits (Legendre, 2001). We have now shown that in agreement with in situ hybridization and RT-PCR analysis (Malosio et al., 1991; Sato et al., 1991, 1992; Thio et al., 2003), the GlyRs in the hippocampus are composed of α2 but not α1 subunits. Electrophysiological studies (Chattipakorn and McMahon, 2002) suggested that homomeric receptors as well as heteromeric GlyRs containing β subunits exist in the hippocampus, in agreement with the detection of GlyRβ subunit transcripts (Racca et al., 1998). Expression of the α2 subunit in adult has also been documented in the auditory brainstem (Piechotta et al., 2001), suggesting that the switch initially described in the spinal cord is not imperative in other structures. The presence of α3 and α4 subunits cannot be excluded, but their mRNAs are faintly expressed in rat brain (Becker et al., 1993; Malosio et al., 1991).

We have now found that most, if not all, subsets of hippocampal interneurons express GlyT2 in part of their terminals. Using GlyT2/GAD double labeling immunohistochemistry (data not shown), somatic GlyT2 labeling was weak in the somata and did not allow clear discrimination between glycinergic and GABAergic interneurons. However, single GlyT2 (88.4 ± 0.43%)- and mixed GlyT2/GAD65 (11.6 ± 0.43%)-immunoreactive terminals were identified. This indicates that pure glycinergic and mixed GABA/glycinergic terminals are present in the hippocampus, which is consistent with the existence of mixed microdomains of GlyR and GABAAR. GlyT2-IR has been detected in boutons innervating both excitatory cells (soma and dendrites) and interneurons. Importantly, these cytochemical data indicating a

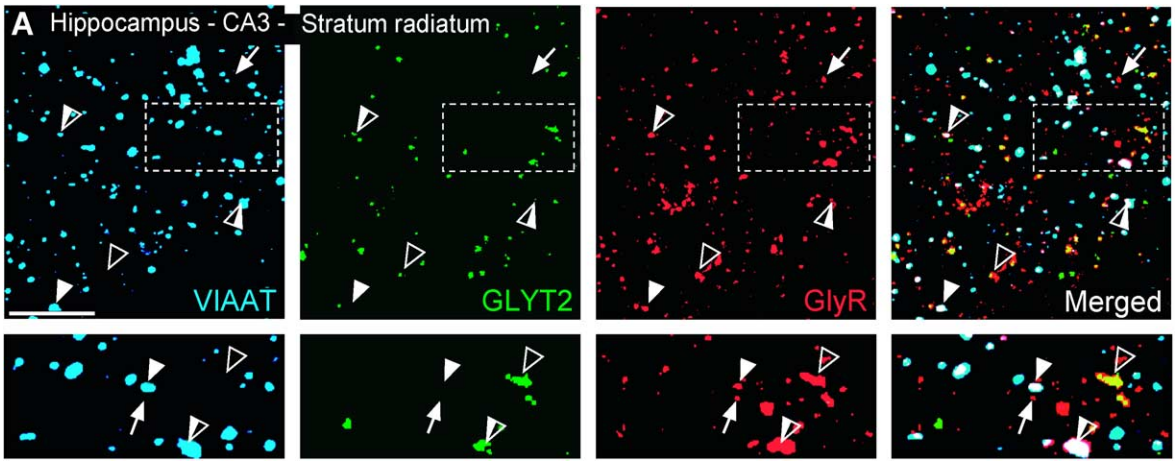


Fig. 3. Synaptic GlyR clusters facing glycinergic presynaptic proteins in the CA3 region of adult rat hippocampus. Shown are triple detections of GlyR (red), VIAAT (blue), and GLYT2 (green). Examples of GlyRs apposed to VIAAT-IR (closed arrowheads), or GLYT2-IR (open arrowheads), or both (double open–closed arrowheads). Arrows indicate GlyRs not co-localized with VIAAT- or GLYT2-IR.

possible glycinergic afference in the hippocampus are consistent with EM data showing that synaptic GlyR can be detected in the somato-dendritic compartment and on interneurons. In addition, detection of GlyR at GlyT2-immunoreactive synapses supports the existence of genuine glycinergic transmission. Paradoxically, and despite extensive studies of hippocampal inhibitory interneuron physiology, endogenous glycinergic inhibitory post-synaptic potentials could not be detected in the hippocampus (Chattipakorn and McMahon, 2002; Mori et al., 2002). One explanation would be that presynaptic afferences cannot be easily activated or are extrinsic. Our study does not rule out a possible extrinsic glycinergic innervation of the hippocampus but unambiguously demonstrates that intrinsic GLYT2-positive terminals are present. Alternatively, one may hypothesize that the glycinergic afferences are only stimulated when the protocol is atypical. This is the case in the cerebellum where Golgi cells receive glycinergic inhibition from Lugaro cells only when the latter is activated by serotonin (Dieudonne and Dumoulin, 2000). Another non-exclusive hypothesis to explain that glycinergic synaptic current has not yet been observed is the relatively low content of GlyR in the hippocampus compared to GABAAR. With immunofluorescence, we have observed that the density of GlyR was 2- to 3-fold lower than

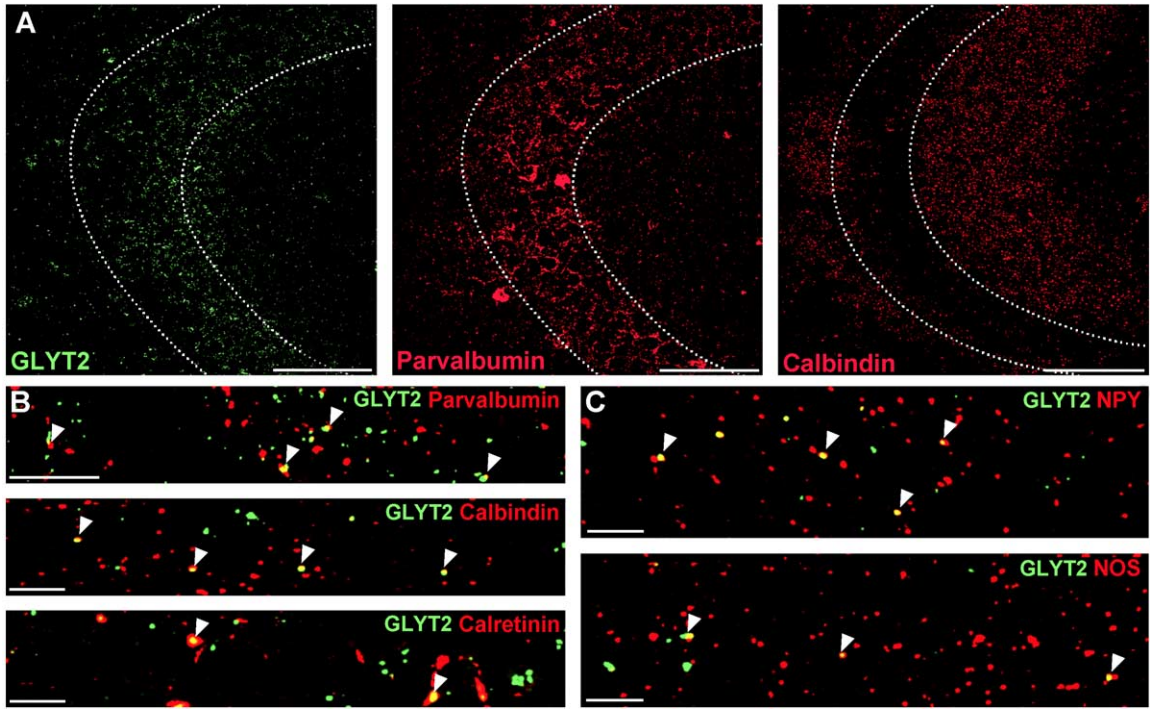


Fig. 4. Several subsets of hippocampal interneurons express GlyT2. (A) Widespread expression of GlyT2 in stratum oriens, pyramidal, and radiatum. In CA3 puncta of GlyT2-IR co-localize with parvalbumin (PV) and calbindin (CB) IR. Dotted lines indicate pyramidal cell layer limits. PV-containing terminals innervate perisomatic regions and the initial segment of principal cells whereas CB-containing terminals innervate dendrites of principal cells. (B) GlyT2-positive boutons (green) also express other neurochemical markers (Red). Arrowheads show co-localization. Scale bars, 10 μm.

Table 2
Proportion of GLYT2-positive terminal among several inhibitory terminal populations

Regions	Percent of GLYT2-positive puncta labeled with:				
	Parvalbumin	Calbindin	Calretinin	NPY	NOS
CA1	37.8 ± 2.1	19.1 ± 2.1	10.3 ± 0.9	31.1 ± 2.3	26.4 ± 1.6
CA3b	26.8 ± 1.1	31.7 ± 1.7	4.2 ± 0.2	47.1 ± 2.7	29.8 ± 1.7
CA3c	42.9 ± 1.5	17.8 ± 1.5	3.1 ± 1.0	11.8 ± 1.3	12.6 ± 1.3
GD	38.2 ± 1.9	27.6 ± 2.4	3.6 ± 0.2	37.1 ± 2.6	1.7 ± 0.4

Marker	Percentage of marker-positive puncta displaying GLYT2 immunoreactivity			
	CA1	CA3b	CA3c	GD
Parvalbumin	7.9 ± 0.4	17.5 ± 1.3	19.9 ± 1.0	9.6 ± 0.5
Calbindin	14.9 ± 2.0	1.4 ± 0.2	1.2 ± 0.2	11.0 ± 1.1
Calretinin	10.5 ± 3.2	9.1 ± 0.2	6.9 ± 3.3	6.7 ± 0.42
NPY	0.17 ± 0.0	0.52 ± 0.0	0.25 ± 0.0	0.19 ± 0.0
NOS	0.81 ± 0.1	0.42 ± 0.1	0.29 ± 0.0	0.14 ± 0.0

Hippocampal slices were immunolabeled with guinea pig anti-GLYT2 and rabbit anti-neurochemical marker (see Experimental methods). Glycinergic terminals were identified by GLYT2 labeling. CA1–CA3b–CA3c, fields of the hippocampus (cornu ammonis); DG, dentate gyrus.
(1) Values (mean ± SEM) represent the percentage of GLYT2 clusters that also colocalize with the neurochemical marker (i.e. for example, 37.8% of all GLYT2 clusters in CA1 are also labeled with anti-parvalbumin).
(2) Values represent the percentage of marker-positive clusters that also colocalize with the GLYT2 immunoreactivity (i.e., 7.9% of all parvalbumin clusters in CA1 are also labeled with anti-GLYT2).

for GABAAR. This is in agreement with EM data because 1.3 ± 0.2 GlyR-IR particles were detected per synapse whereas 4.1 ± 1.7 particles of GABAAR-IR have been reported in the hippocampus (Nusser et al., 1996).

In the hippocampus, taurine and β-alanine are endogenous agonists of GlyR, but evoked synaptic currents in CA3 pyramidal cells appear to be mediated entirely by GABA (Mori et al., 2002). It was suggested that at ambient levels, taurine and β-alanine could act at GlyRs to modulate neuronal processing. In agreement with these observations, we have now detected a large proportion of GlyR clusters at extrasynaptic sites. Indeed, GlyRs may be activated by taurine and β-alanine spilled from glial cells and thus also be involved in a paracrine function (see for instance Flint et al., 1998; Furuya et al., 2000; Sanes and Hafidi, 1996). Interestingly, it has been recently demonstrated (Mangin et al., 2003) that kinetic properties of α2 homomeric GlyRs are compatible with non-synaptic liberation of agonist. In contrast, heteromeric GlyRs might account for synaptic GlyRs because they can accumulate at postsynaptic loci through interactions with gephyrin via the β-subunit. These GlyRs may or may not be associated with GABAAR, indicating that inhibition of the hippocampal network is probably more complex than expected. This implies that, whereas GABAR is known to be mainly synaptic, hippocampal GlyR may have beyond its presumed synaptic role another function such as the maintenance of inhibitory tone (Mori et al., 2002), which could explain its main extrasynaptic localization.

In light of these results, it would be worthwhile reconsidering the role of glycine and GlyRs in the regulation of excitability in the hippocampus during normal and pathological processes. For instance, recent studies have demonstrated that strychnine-sensitive GlyRs could depress hyperexcitability in rat dentate gyrus (Chattipakorn and McMahon, 2003) and that GlyR agonists could serve as potential anticonvulsants in the hippocampus (Kirchner et al., 2003). Our results now suggest the possible involvement of glycinergic inhibition in the genesis and control of epileptic status.

Experimental methods

Animals

Four-week-old Sprague–Dawley rats weighing 125–149 g (Janvier, France) were anesthetized with pentobarbital (60 mg/kg, ip) then intracardially perfused with 4% paraformaldehyde (PFA) in PBS or with 4% PFA and 0.05% glutaraldehyde in PBS for optical and electron microscopy, respectively.

Optical immunocytochemistry

Tissues were postfixed for 1 h in 4% PFA and cryoprotected in PBS-sucrose 30% (w/v) overnight. Cryostat sections (30 μm) were fixed with 4% PFA-4% sucrose in PBS (20 min, room temperature) and permeabilized in PBS + 0.1% Triton, 0.1% gelatin (PBSTg) for 4 min. For GlyR labeling with mAb4a, sections were subsequently immersed in methanol for 10 min at −20°C. Sections were then incubated for 2 days at 4°C with the primary antibodies in PBSTg and with the secondary antibodies for 3 h, followed by fluorescent streptavidin when necessary. Controls without primary antibody were always negative.

For GABAAR labeling, unfixed brains were rapidly frozen and cryostat sections were fixed in 0.5% PFA and 5% Kryofix (Merck), and irradiated in a microwave oven (45 s; 650 W) (Fritschy et al., 1998). They were then processed for staining.

Cultured neurons were prepared as described (Danglot et al., 2003) and used at 14 and 21 days in vitro. For GABAAR, fixation and labeling conditions were as described (Danglot et al., 2003). For GlyRs, living cells were fixed for 2 min in methanol at −20°C before proceeding to the labeling procedure.

Electron microscopy

The brain and control cervical spinal cord vibratome sections (200 μm) were cryoprotected using graded PBS-glycerol (10%, 20%, and 30%). Sections were rapidly frozen in liquid propane at

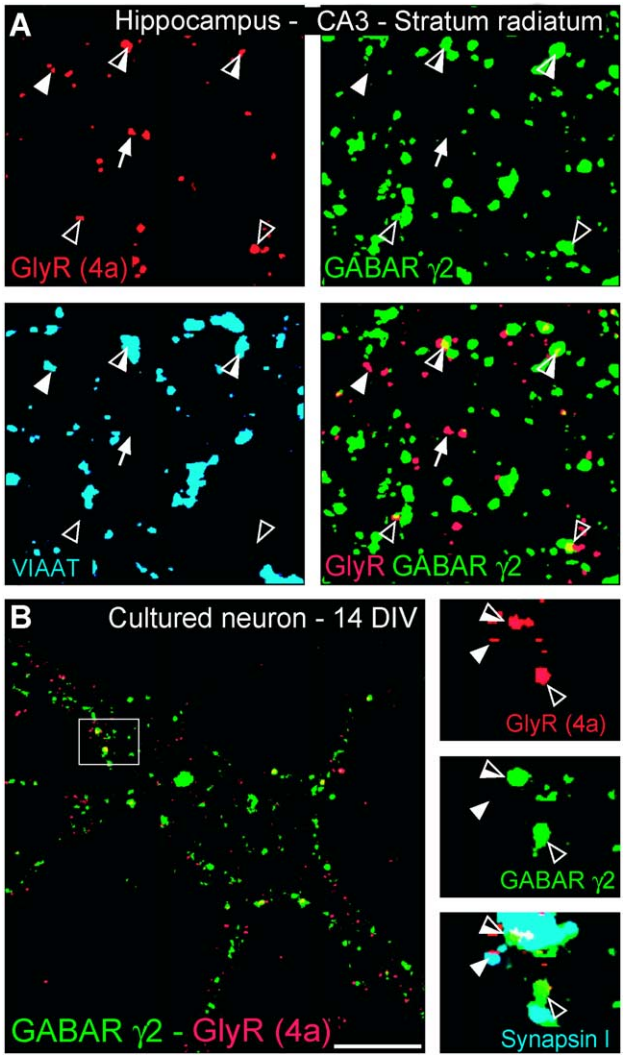


Fig. 5. Co-localization of synaptic and extrasynaptic clusters of GlyRs and GABAR γ2. Triple detection of GABAAR γ2 subunits (green), GlyRs (red), and synaptic markers (blue) that are VIAAT in the CA3 region (A) and Synapsin in cultured hippocampal neurons (B). Shown are examples of synaptic (double open-closed arrowheads) and extrasynaptic (open arrowheads) co-localization of GlyR and GABAAR γ2 subunit clusters. Closed arrowheads and arrows show, respectively, synaptic and extrasynaptic GlyR clusters not associated with GABAAR γ2 subunit clusters. Scale bars, 10 μm.

−180°C, cryosubstituted in methanol, and infiltrated with HM20 resin (cryosubstitution unit, AFS; Reichert). Ultrathin sections were treated with 5% BSA and incubated in primary antibody overnight at 4°C and then with 10 nm gold-coupled anti-mouse antibody (British BioCell International, Cardiff). Sections were postfixed in 1% glutaraldehyde in PBS and then contrasted with uranyl acetate and lead citrate before examination (Philips Technaï 12).

Antibodies

Polyclonal: rabbit anti-synapsin (0.1 μg/ml, Novus Biological, Littleton, CO); rabbit anti-bassoon (1/1000, provided by E. Gundelfinger, Magdeburg); rabbit anti-VIAAT (1:500 provided by B. Gasnier, Paris); guinea pig anti-GLYT2 (1:2000, Chemicon Int, Temecula). Monoclonal: mAb7a anti-gephyrin (Alexis Biochemicals, San Diego, CA; 1 μg/ml). For double staining with mAb7a and mAb4a, mAb7a was coupled to Alexa 488, and mAb4a was revealed using an anti-mouse Fab to avoid cross-reactivity. For staining of GABAARs, we used a guinea pig anti-GABAAR γ2 (1:2000; given by JM. Fritschy, Zurich; Fritschy and Mohler, 1995). Anti-parvalbumin, calbindin, and calretin were from SWant (1:1000, 1:10 000, and 1:500). Anti-neuropeptide Y was from Sigma (1:8000) and anti-nNOS was from Transduction Lab. (1:200). Triple detection experiments were performed with antibodies labeled with Cy3, FITC, and Cy5 (Jackson ImmunoResearch Laboratories, 1:500).

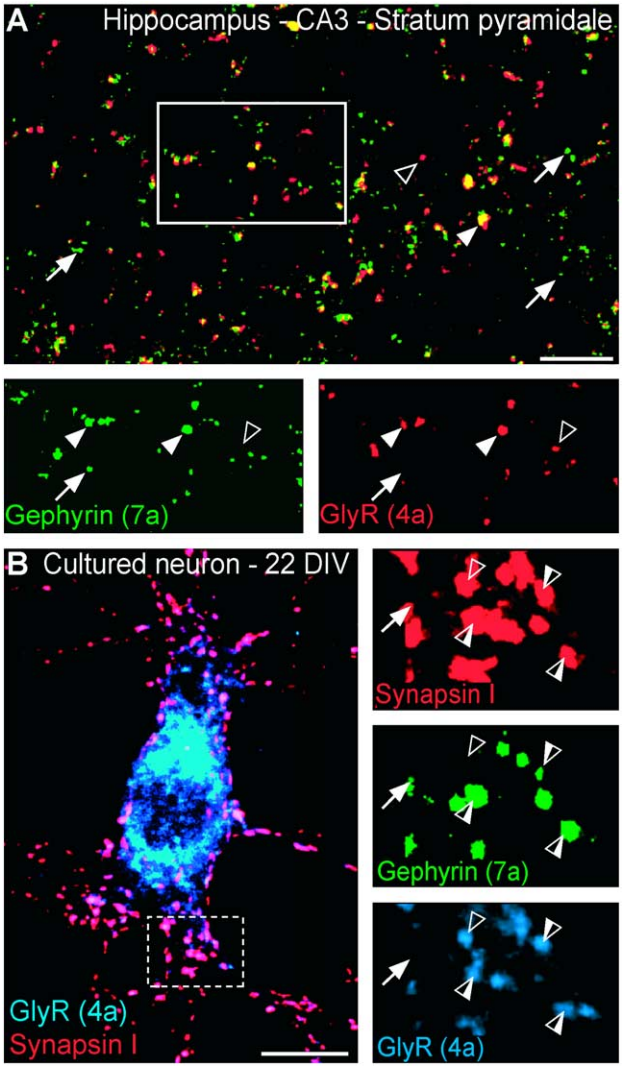


Fig. 6. Clusters of GlyRs and gephyrin co-localize at synaptic and extrasynaptic sites. (A) Double detection of gephyrin and GlyRs in the CA3 region. GlyR clusters can be detected co-localized (closed arrowheads) or not (open arrowheads) with gephyrin-IR. However, a major part of gephyrin is devoid of GlyRs (arrows). (B) Triple detection of gephyrin (green), GlyR (red), and synapsin (blue) in hippocampal cultured neurons. Most GlyR clusters associated with gephyrin are synaptic (double open–closed arrowheads), but all GlyRs are not systematically apposed to synaptic sites (open arrowhead). Scale bars, 10 μm.

Quantitative analysis

Fluorescence images were acquired by sequential scanning using a Leica confocal microscope TCSP2. Typically, stacks of 10–20 sections (1024 × 1024 pixels) with an interval of 0.32 μm were scanned three times.

Quantifications were performed using Metamorph software. Images were subjected to the same user-defined intensity threshold to select clusters. Apposition was determined by first generating a binary mask from the thresholded VIAAT image and widening the regions of the puncta by one pixel all around. GlyR clusters were classified as synaptic if clusters in the thresholded GlyR images had any pixel overlapping with the binarized-dilated VIAAT mask.

All results given are means ± SEM, referring to the number of slices analyzed. The total number of clusters is indicated in the case of multiple labeling.

Western blot analysis

Hippocampi and spinal cords were quickly dissected and homogenized in ice-cold 25 mM Tris–HCl pH 7.4, 0.32 M sucrose buffer using a Dounce homogenizer. Homogenates were centrifuged at 800 × *g* for 10 min, and post-nuclear supernatant was centrifuged at 15 000 × *g* for 40 min. The pellet was further processed for Western blot analysis. Twenty-five micrograms of protein was analyzed on 4–12% Bis/Tris polyacrylamide gel (NuPAGE Invitrogen). β-Mercaptoethanol (480 mM) was added when working under reducing conditions. Rabbit polyclonal antibody anti-GlyR α1–α2 (Zymed) and mAb4a (Alexis) were used, respectively, at 0.4 and 0.6 μg/ml. Primary antibodies were then revealed with a goat anti-rabbit (1:15,000) or a rabbit anti-mouse (1:5000) antibody conjugated to horseradish peroxidase (Jackson Lab.) followed by chemiluminescent detection on Hyperfilm using ECL-PLUS (Amersham). Importantly, under non-reducing conditions, some extracts allowed the detection of slow migrating species (≈90 kDa) with both antibodies (monoclonal and polyclonal). The existence of a high molecular weight band in SDS-PAGE of receptor subunits is not specific to the glycine receptor. This band may correspond to aggregates of the GlyR alpha subunit formed during solubilization as described for GABA receptor (Gorrie et al., 1997) and AchR α7 (Rakhilin et al., 1999). According to these authors, aggregates are absent in vivo and form during solubilization. Subunit aggregates can be prevented by subunit alkylation (NEM or iodoacetamide) or disrupted by reducing conditions (β-mercaptoethanol or DTT). The upper band is reportedly not observed under these conditions (Becker et al., 1989; Hoch et al., 1989). To clear up any doubt, we did Western blot with spinal cord and hippocampal extract under reducing conditions, and found that the upper band disappeared.

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