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Synaptic and non-synaptic localization of GABA_A receptors containing the $\alpha 5$ subunit in the rat brain

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Abstract

The $\alpha 5$ subunit of the GABA_A receptors (GABA_ARs) has a restricted expression in the brain. Maximum expression of this subunit occurs in the hippocampus, cerebral cortex and olfactory bulb. Hippocampal pyramidal cells show high expression of $\alpha 5$ subunit-containing GABA_ARs ($\alpha 5$ -GABA_ARs) both in culture and in the intact brain. A large pool of $\alpha 5$ -GABA_ARs is extrasynaptic and it has been proposed to be involved in the tonic GABAergic inhibition of the hippocampus. Nevertheless, there are no studies on the localization of the $\alpha 5$ -GABA_ARs at the electron microscope (EM) level. By using both immunofluorescence of cultured hippocampal pyramidal cells and EM postembedding immunogold of the intact hippocampus we show that, in addition to the extrasynaptic pool, there is a pool of $\alpha 5$ -GABA_ARs that concentrates at the GABAergic synapses in dendrites of hippocampal pyramidal cells. The results suggest that the synaptic $\alpha 5$ -GABA_ARs might play a role in the phasic GABAergic inhibition of pyramidal neurons in hippocampus and cerebral cortex.

Keywords

hippocampus; immunocytochemistry; immunogold; electron microscopy; tonic inhibition

INTRODUCTION

The $\alpha 5$ subunit of the GABA_A receptors (GABA_ARs) is preferentially expressed in the hippocampus, particularly in the dendrites of the pyramidal cells of the CA1 region (Wisden et al. 1992; Fritschy and Möhler 1995; Sperk et al. 1997; Wainwright et al. 2000; Christie et al. 2002b). The $\alpha 5$ subunit mainly combines with the $\beta 3$ and $\gamma 2$ subunits forming $\alpha 5\beta 3\gamma 2$ heteropentameric GABA_ARs (Sur et al. 1998). These receptors show lower affinity for zolpidem and higher affinity for L655,708, RY080 and RY023 ligands than the GABA_ARs containing other α subunits (Pritchett and Seeburg 1990; Liu et al. 1996; Quirk et al. 1996; Skolnick et al. 1997). About 20% of the GABA_ARs in the hippocampus contain the $\alpha 5$ subunit (Fritschy et al. 1997; Sur et al. 1998). In a pilocarpine rat model of temporal lobe epilepsy, the $\alpha 5$ subunit is down-regulated in the CA1 and CA2 regions of the hippocampus (Houser and Esclapez 2003, Scimemi et al. 2005).

Various lines of evidence indicate that the $\alpha 5$ subunit is involved in hippocampal-dependent learning and memory: A homozygous $\alpha 5$ mouse knockout showed decreased amplitude of the

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IPSCs in the CA1 region of the hippocampus and enhanced hippocampal learning and memory (Collinson et al. 2002). Mutant mice with the point mutation $\alpha 5(H105R)$, which renders the $\alpha 5$ subunit-containing GABA_AR ($\alpha 5$ -GABA_AR) diazepam insensitive, resulted in a selective reduction of $\alpha 5$ expression in the hippocampus and facilitation of hippocampal-dependent learning (Kelly et al. 2002; Crestani et al. 2002). Selective inverse agonists for $\alpha 5$ -GABA_ARs enhance memory in animal models (Chambers et al. 2003; Maubach 2003) and pharmaceutical companies are thought to be developing some of these compounds as cognition enhancers for clinical use (Chambers et al. 2004; Sternfeld et al. 2004).

Hippocampal pyramidal neurons show a small tonic inhibitory current that is thought to correspond to extrasynaptic receptors (Banks and Pearce 2000; Petrini et al. 2004; Mody 2001). Immunofluorescence experiments have shown that in hippocampal pyramidal neurons, the $\alpha 5$ -GABA_ARs are largely extrasynaptic and that they might not be present at GABAergic synapses (Brunig et al. 2002; Crestani et al. 2002). It has been proposed that the extrasynaptic $\alpha 5$ -GABA_ARs are involved in the tonic GABAergic inhibitory current (Caraiscos et al. 2004). Nevertheless, these experiments were done in neuronal cultures at elevated concentration of GABA, after treatment of the cultures with the GABA transaminase inhibitor vigabatrin. Others have shown that under baseline conditions of GABA, tonic currents in hippocampal pyramidal neurons are not mediated by $\alpha 5$ -GABA_ARs (Scimemi et al. 2005).

By using immunofluorescence methods, we have recently reported the presence of $\alpha 5$ -GABA_ARs not only extrasynaptically but also in GABAergic synapses in cultured hippocampal pyramidal neurons (Christie and De Blas 2002). These results challenge the notion that in pyramidal neurons, the $\alpha 5$ -GABA_ARs are exclusively extrasynaptic. Nevertheless, there is the possibility that the localization of $\alpha 5$ -GABA_ARs in GABAergic synapses observed in culture does not occur in the intact hippocampus. In the present communication, by using postembedding electron microscopy (EM) immunogold methods, we show that $\alpha 5$ -GABA_ARs are localized not only extrasynaptically but also in the GABAergic synapses, on the dendrites of pyramidal neurons in the CA1 region of the intact hippocampus. The localization of $\alpha 5$ -GABA_ARs in GABAergic synapses also occurs in the rat cerebral cortex. We also show that $\alpha 5$ -GABA_ARs are present in a subcellular fraction derived from the rat hippocampus that is enriched in postsynaptic densities. These results support the notion that $\alpha 5$ -GABA_ARs are not exclusively localized extrasynaptically in a diffuse way, as proposed by others (Brunig et al. 2002; Crestani et al. 2002). They are also present at GABAergic synapses both in culture and in the intact brain.

MATERIALS AND METHODS

All the animal protocols have been approved by the Institutional Animal Care and Use Committee and follow the National Institutes of Health guidelines.

Antibodies

A rabbit (Rb) anti-rat $\alpha 5$ was raised in our laboratory to amino acids 1-13 (QMPTSSVQDETND). This peptide was covalently linked, via a C-terminal cysteine, to keyhole limpet hemocyanin (KLH). A New Zealand rabbit was injected subcutaneously with a 1:1 emulsion of KLH-coupled peptide in complete Freund's adjuvant (for the first immunization) and in incomplete Freund's adjuvant (for all subsequent immunizations) once per month. The antibody titer in the sera was monitored by ELISA. Sera were collected after four months of immunizations and were affinity-purified on immobilized peptide. The guinea pig (GP) anti-rat $\gamma 2$ (to amino acids 1-15 QKSDDDYEDYASNKT) and the rabbit anti-rat $\alpha 1$ (to amino acids 1-5 QPSQDELKDNTTVFT) GABA_AR subunit antibodies were raised in our laboratory and affinity-purified on the corresponding immobilized peptide antigen. The mouse monoclonal (mAb) anti- $\beta 2/3$ GABA_AR antibody (62-3G1) was raised in our laboratory to the

affinity-purified GABA_AR (De Blas et al, 1988; Vitorica et al, 1988). It recognizes an N-terminal epitope that is common to the rat β 2 and β 3 subunits but not present in the β 1 subunit (Ewert et al, 1992). All the anti-GABA_AR antibodies raised in our laboratory have been thoroughly characterized. Specificity tests of the GABA_AR antibodies included ELISA, immunoblotting, subunit-specific staining in host-transfected cell lines, light microscopy, EM immunocytochemistry, and displacement of immunoreactivity in these assays by specific peptides or immunizing protein antigen. These GABA_AR antibodies have been used in several studies (i.e. De Blas et al, 1988; Vitorica et al, 1988; Miralles et al, 1999; Christie et al, 2002a,b, Christie and De Blas 2002, 2003; Riquelme et al. 2002; Charych et al. 2004a,b; Li et al. 2005a). The Rb anti-rat gephyrin antibody to the C-terminus, amino acids 720-736 VELHKGEVVDVMVIGRL, was a gift from Dr. Ben A. Bahr (University of Connecticut, Storrs). This antiserum recognizes the ~93 kDa gephyrin isoforms on immunoblots of rat brain (Kawasaki et al. 1997). The monoclonal mouse anti-gephyrin (mAb 7a) to purified gephyrin (Kirsch and Betz 1995) was purchased from Cedarlane (Accurate Chemical & Scientific Corp., Westbury, NY, Catalog number CL 281205030; lot number 7/96/2). This antibody has been thoroughly characterized and widely used in the literature to localize gephyrin by light microscopy and EM immunocytochemistry in the rat brain and in neuronal cultures (ie. Giustetto et al. 1998; Kneussel et al. 1999; Christie et al. 2002b; Christie et al. 2006; Christie and De Blas 2003). Specificity tests included immunostaining in host-transfected cell lines, light microscopy, EM immunocytochemistry, displacement of immunoreactivity in these assays by gephyrin and the use of a gephyrin knockout mouse (Feng et al. 1998). The mouse mAb to the GluR2 subunit of the AMPA receptor, (to amino acids 175-430, catalog number MAB397; lot number 19090047) was from Chemicon (Temecula, CA). This antibody recognizes a 102 kDa protein in immunoblots of rat brain and has been used in light microscopy and EM immunocytochemistry of rat brain (manufacturer's technical information and Rubio 2006). Specificity tests included the use of a GluR2 mouse knockout (Sans et al. 2003). The GP anti-GABA (to GABA coupled to KLH by glutaraldehyde, catalog number AB175; lot number 23071453) was from Chemicon. This antibody has been used to identify GABAergic neurons by light microscopy immunocytochemistry (manufacturer's technical information) and GABAergic terminals in the rat brain by EM immunocytochemistry. Specificity tests included the absence of immunoreactivity of brain tissue with antibody adsorbed with GABA coupled with glutaraldehyde to bovine serum albumin (BSA) while immunoreactivity was not affected by adsorption with glutamate or glycine coupled with glutaraldehyde to BSA (Rubio and Juiz 2004). The mouse mAb anti-PSD-95 (to amino acids 77-299) was from Upstate 6 Biotechnology (Lake Placid, NY; clone K28/43; catalog number 05-494; lot number 28879). This antibody recognizes a 95 kDa protein in immunoblots of rat brain (manufacturer's technical information). Immunoreactivity is blocked by PSD-95 (amino acids 77-299). At the light microscopy level specifically labels glutamatergic synapses and at the EM level specifically labels Gray's type 1 postsynaptic densities (Rameau et al. 2004; Becamel et al. 2004). The sheep anti-GAD (lot number 1440) was a gift of Dr. Irwin J. Kopin (NINDS, Bethesda). This antibody to purified rat GAD recognizes a 65 kDa protein on rat brain immunoblots and it has been widely used in the literature, since it was first generated by Oertel et al. 1981a, for the localization of GABAergic terminals in the rat brain and neuronal cultures by light microscopy immunocytochemistry including adsorption controls (Oertel et al. 1981a,b). We have previously shown that there is co-localization in GABAergic presynaptic terminals of the anti-GAD immunoreactivity with that of GABAergic synaptic vesicle marker vGAT (vesicular GABA transporter, Li et al. 2005b) and this immunoreactivity is apposed to that of postsynaptic GABA_ARs (Christie et al, 2002a,b, Christie and De Blas 2002, 2003). Fluorophore-labeled (FITC, Texas Red or AMCA) and colloidal gold-labeled secondary antibodies (species-specific anti-IgG), made in donkey, were from Jackson ImmunoResearch Laboratories (West Grove, PA). Colloidal gold-labeled (10 nm) goat anti-mouse secondary antibody was from ICN (Irvine, CA). All other colloidal gold-labeled secondary antibodies,

goat anti-Rb IgG (18 nm), donkey anti-GP IgG (18 nm) and donkey anti-Rb IgG (12 nm), were from Jackson ImmunoResearch Laboratories.

Brain tissue fractions and immunoblots

The crude synaptosomal (P2) and a purified synaptosomal fractions (after centrifugation on a discontinuous sucrose density gradient) from 3-month old Sprague-Dawley male rats were prepared from homogenates of rat hippocampi by the method of Carlin et al. (1980). A fraction enriched in postsynaptic densities (PSDs) was prepared by treating the purified synaptosomes with 0.5% Triton X-100, 0.32M sucrose, 6mM Tris-HCl pH 8.1 for 15 min at 4°C followed by centrifugation at 100,000 × g for 1 hr at 4°C. The pellet containing PSDs (Cho et al., 1992) was resuspended in 50 mM Tris-HCl, pH 7.4. Samples were subjected to SDS-PAGE and immunoblotted with specific antibodies as described elsewhere (De Blas and Cherwinski, 1983).

Light microscopy immunocytochemistry

This procedure has been described elsewhere (De Blas 1984; De Blas et al, 1988, Moreno et al, 1994). Briefly, 60 day old Sprague-Dawley rats were perfused through the ascending aorta under anesthesia (80 mg/kg ketamine-HCl, 8 mg/kg xylazine, 2 mg/kg acepromazine maleate via IP) with fixative consisting of 4% paraformaldehyde, 1.37% lysine and 0.21% sodium periodate in 0.1 M phosphate buffer (PB), pH 7.4. Brains were frozen and sliced in parasagittal sections with a freezing microtome. Free floating tissue sections were incubated for 24 hours at 4 °C with the affinity-purified Rb anti- $\alpha 5$ antibody in 0.3% Triton X-100, 0.1 M PB, pH 7.4. The washed tissue sections were incubated with a biotin-labeled anti-rabbit IgG and avidin-biotin-horseradish peroxidase complex (Vectastain Elite ABC Kit, Vector Laboratories; Burlingame, CA). The reaction product was visualized by incubation with 3-3' diaminobenzidine tetrahydrochloride in the presence of cobalt chloride and nickel ammonium sulfate. Sections were washed and mounted on gelatin-coated glass slides. No tissue immunolabeling was detected when the Rb anti- $\alpha 5$ antibody was incubated with 20 μ g/ml of the $\alpha 5$ antigenic peptide or when the primary antibody was omitted.

Low density hippocampal cultures

Hippocampal cultures were prepared by the method of Banker et al. (1998) as described elsewhere (Christie et al., 2002a). Briefly, dissociated neurons from embryonic day 18 Sprague Dawley rat pup hippocampi were plated at low density (3,000-8,000 cells per 18mm diameter circular coverslip) and maintained in glial cell conditioned medium for 19-22 days. These cultures contained 90-95% pyramidal cells and 5-10% interneurons.

Immunofluorescence of hippocampal cultures

Double or triple label immunofluorescence detection of various antigens with specific antibodies raised in various species was done as described elsewhere (Christie et al., 2002a,b), with modification for labeling of surface $\alpha 5$ -containing GABA_ARs in living neurons, prior to fixation with paraformaldehyde (Christie and De Blas 2002, Christie et al. 2006). Living hippocampal neurons were surface labeled with rabbit anti- $\alpha 5$ antibody by incubation at 37 °C for 30 min in DMEM containing N2 supplement (Gibco), 1% D-glucose, 0.1% ovalbumin, 1mM sodium pyruvate and 26 mM NaHCO₃ in a 5% CO₂ atmosphere. Then the neurons cultured on coverslips were washed and fixed by immersion in 4% paraformaldehyde, 4% sucrose in phosphate buffered saline (PBS) for 12 min at room temperature (RT) followed by cell permeabilization with 0.25% Triton X-100 in PBS for 5 minutes and by treatment with 5% donkey serum in PBS for 30 min at RT. The cultures were incubated with a mixture of the other primary antibodies, raised in various species, in 0.25% Triton X-100 PBS for 2 hrs at room temperature, washed and incubated for 1 hr at room temperature with a mixture of

species-specific secondary anti-IgG antibodies all raised in donkey and conjugated to either Texas Red, FITC, or AMCA fluorophores (1:200 dilution in 0.25% Triton X-100 PBS). The coverslips were washed with PBS, and mounted using Prolong anti-fade mounting solution (Molecular Probes; Eugene, Oregon). Alternatively the same procedure was used except that living cells were surface-labeled with the anti- $\alpha 5$ antibody by incubation for 3 h at 4 °C (Christie and De Blas 2002). Alternatively, labeling with the anti- $\alpha 5$ and other secondary antibodies was also done after cell fixation in methanol at -20 °C for 10 min (instead of fixation with paraformaldehyde) as explained elsewhere (Christie and De Blas 2002). Specificity of the immunolabeling was demonstrated by blocking the binding of the primary anti- $\alpha 5$ or anti- $\gamma 2$ antibody with 20 $\mu\text{g}/\text{ml}$ of the corresponding antigenic peptide. Moreover, no immunolabeling was obtained when the primary antibody was omitted. Images were collected using a 60X pan-fluor objective on a Nikon Eclipse T300 microscope with a Sensys KAF 1401E CCD camera, driven by IPLab 3.0 (Scanalytics, Fairfax, VA) acquisition software. Image files were then processed and merged for color colocalization using PhotoShop 7.0 (Adobe).

Post-embedding electron microscopy immunocytochemistry

The tissue preparation, freeze substitution, and post-embedding immunogold labeling were done as reported previously (Riquelme et al., 2002; Charych et al. 2004a,b; Li et al. 2005a). Briefly, 35-70 day old Sprague Dawley rats were anesthetized as described above and perfused with 800 ml 4% paraformaldehyde and 0.5% glutaraldehyde in 0.1 M PB, pH 7.4 fixative. Vibratome sections, 300-500 μm -thick, were cryoprotected with 2 M sucrose and plunge-frozen in liquid propane cooled by liquid nitrogen (-186 °C). Samples were immersed in 1.5% uranyl acetate in anhydrous methanol at -90 °C for 30 hours, infiltrated with Lowicryl HM20 resin (Polysciences, Warrington, PA) and polymerized with UV light (-45 to 0 °C) during 72 hours in a Leica (Vienna, Austria) AFS freeze substitution instrument.

Sections, 70-80 nm-thick, were collected from the embedded tissue blocks on 400-mesh gold-gilded nickel grids coated with Coat-Quick "G" grid coating pen (Daido Sangyo, Japan) and the immunoreaction procedure was done as described elsewhere (Riquelme et al., 2002). After incubations first with the affinity-purified primary antibody (or the mixture of two antibodies raised in different species for double-label experiments) and second with colloidal gold-labeled anti-IgG secondary antibody (or a mixture of two gold-labeled species-specific anti-IgG secondary antibodies raised in the same species), the tissue sections were counterstained with uranyl acetate and lead citrate. No immunolabeling was observed when the primary antibody was omitted. We used the following antibody combinations in double-label experiments: I) Rb anti- $\alpha 5$ with mouse mAb to $\beta 2/3$, or PSD-95 or GluR2 or gephyrin (with 18 nm colloidal gold goat anti-Rb IgG and 10 nm colloidal gold goat anti-mouse IgG as secondaries) or II) Rb anti- $\alpha 5$ with GP anti-GABA or anti- $\gamma 2$ (with 12 nm colloidal gold donkey anti-Rb IgG and 18 nm colloidal gold donkey anti-GP IgG secondary antibodies). EM images were stored in PhotoShop 7.0 (Adobe) and contrast/brightness was adjusted with this program.

For quantitative analysis of the distribution of the $\alpha 5$ gold particles at the synapse, we used the method of Valtschanoff and Weinberg (2001). A line was drawn along the midline of the synaptic cleft (the zero position), equidistant from both the pre- and postsynaptic membrane. The axodendritic distance was measured as the length of a perpendicular line between the center of the gold particle and the midline of the synaptic cleft. To plot the particle density, the positions of the particles were sorted into 5 nm bins. A negative or positive number indicates that the particle was located on the presynaptic or postsynaptic side of the midline, respectively. Binned data were smoothed with a five-point weighted running average using SigmaPlot (Rockware, Inc., Golden, CO). The normalized lateral position of each gold particle inside the synapse was calculated as the absolute value of $(a-b)/(a+b)$, where a and b are the distances of the center of each gold particle to each of the two parallel lines drawn at the edge of the PSD

perpendicular to the synaptic cleft. The gold particles were distributed in bins corresponding to 0.1 of the total length of the synapse (a+b, for particles inside the synapse). These values vary between 0 and 1. For gold particles located outside the synapse, the normalized lateral position of a gold particle with respect to the nearest synapse was calculated as the absolute value of (a+b)/(a-b). These values were distributed in bins corresponding to 0.1 of the total length of the synapse (a-b, for particles outside the synapse). The normalized equivalent synaptic length of extrasynaptic membrane that is adjacent to the synapse includes the gold particles with absolute (a+b)/(a-b) values between 1 and 2.

RESULTS

Immunoblots of a hippocampal fraction enriched in postsynaptic densities show the presence of the 55 kDa $\alpha 5$ GABA_AR subunit in that fraction

A rabbit antiserum was raised to the N-terminus (amino acids 1-13) of the rat $\alpha 5$ GABA_AR. The antibodies were affinity-purified on immobilized peptide (Khan et al. 1994). In immunoblots of a P2 fraction from the rat hippocampus, the anti- $\alpha 5$ antibody recognized a 55 kDa peptide (Fig 1, lane 1, arrow). A protein band of the same mobility was found in a rat hippocampal fraction enriched in PSDs (Fig1, lane 3). The immunoreactivity of the antibody with the 55kDa peptide of the P2 and PSD fractions was blocked by the antigenic peptide (Fig1, lanes 2 and 4 respectively). Others have shown that the $\alpha 5$ GABA_AR subunit is 55,000 Mr (reviewed by Macdonald and Olsen 1994; De Blas 1996). These results and the results of Fig. 2 described below, show that the anti- $\alpha 5$ antibody is specific for this subunit. The immunoblots also show that the hippocampal fraction enriched in PSDs also contain markers of GABAergic synapses such as the 51 kDa $\alpha 1$ GABA_AR subunit (Fig.1, lane 5, asterisk) and the 93 kDa gephyrin doublet (Fig1, lane 6, double asterisk), supporting the notion that $\alpha 5$ -GABA_ARs are also present in hippocampal GABAergic synapses.

Light microscopy immunocytochemistry

Fig. 2 A shows the distribution of the $\alpha 5$ subunit expression in the rat brain as revealed by light microscopy immunocytochemistry with the anti- $\alpha 5$ antibody. Immunoreactivity is highest in the hippocampus, particularly in CA1 (Fig. 2A, C), in the strata oriens and radiatum (Fig. 2E and F). Much less immunoreactivity is found in CA2 and CA3 (Fig. 2 C and D). In the dentate gyrus, the immunoreactivity was relatively high in the molecular layer (Fig. 2A and G), particularly in the boundary between the molecular layer and the granule cell layer (Fig. 2 G). High immunoreactivity was also found in the olfactory bulb particularly in the external plexiform layer and the granule cell layer (Fig. 2 A and B). The olfactory glomeruli had low levels of immunoreactivity (Fig. 2A and B). A comparatively lower level of immunoreactivity to that observed in the hippocampus and olfactory bulb was detected in the cerebral cortex, where it was highest in layers V and VI (Fig. 2A) of the anterior part of the cortex (ventral orbital cortex and secondary motor cortex). Some immunoreactivity was also detected in the superior colliculus. No immunoreactivity was detected in the cerebellum. Very little or no immunoreactivity was detected in other brain areas. Our results are in agreement with the *in situ* hybridization studies on the distribution of the $\alpha 5$ mRNA in the brain (Wisden et al. 1992; Persohn et al. 1992). The *in situ* hybridization studies showed that the $\alpha 5$ mRNA is expressed mainly in the pyramidal cells of the hippocampus, granule cells in the dentate gyrus, granule cells of the olfactory bulb, various layers of the cerebral cortex (particularly layers V and VI) and the superior colliculus.

An anti- $\alpha 5$ antibody made in another laboratory shows an immunocytochemical distribution profile in the rat brain identical to the one obtained with our anti- $\alpha 5$ antibody (compare Fig. 2 of this manuscript with Fig. 1 of Fritschy and Mohler 1995). This similarity also includes the higher expression of $\alpha 5$ in the CA1 region of the hippocampus over CA3. Thus, our

immunocytochemistry and immunoblotting results indicate that our anti- $\alpha 5$ antibody is both specific and valuable for immunolabeling $\alpha 5$ -GABA_ARs in the brain.

The light microscopy immunofluorescence results obtained with another antibody have been interpreted by the authors as $\alpha 5$ -GABA_ARs being extrasynaptic (Brunig et al. 2002; Crestani et al. 2002). Nevertheless, no subcellular localization studies of $\alpha 5$ -GABA_ARs at the EM level have been reported using this antibody. A light microscopy immunocytochemical study using a third anti- $\alpha 5$ antibody shows a similar immunocytochemical distribution profile of the $\alpha 5$ subunit in the various regions of the rat brain (Pirker et al. 2000). This third antibody has not been used to determine the synaptic and/or extrasynaptic localization of $\alpha 5$ -GABA_ARs at the light microscopy or EM levels.

In hippocampal cultures, the $\alpha 5$ subunit often forms clusters at GABAergic synapses

The live cell incubation of cultured hippocampal neurons with our Rb anti- $\alpha 5$ antibody (Fig. 3A-F) was followed by fixation, permeabilization and incubation with a mixture of the sheep anti-GAD and either the mouse anti-gephyrin mAb or guinea pig anti- $\gamma 2$ and followed by incubation with the fluorophore-labeled secondary species-specific anti-IgG antibodies. The results showed that, in cultured pyramidal neurons, the $\alpha 5$ -GABA_ARs form three classes of clusters: The first class were the larger $\alpha 5$ -GABA_AR clusters (arrows, Fig. 3A and D). They always colocalized with postsynaptic gephyrin (arrows, Fig. 3B) or $\gamma 2$ -GABA_ARs (arrows, Fig. 3E) and always were apposed to presynaptic GAD⁺ terminals (arrows, Fig. 3C and F, respectively). There were also small $\alpha 5$ -GABA_AR clusters that were not apposed to GABAergic terminals. Some of the small $\alpha 5$ -GABA_AR clusters (filled arrowheads, Fig. 3A and D) colocalized with gephyrin (filled arrowheads, Fig. 3B) or $\gamma 2$ -GABA_AR clusters (filled arrowhead, Fig. 3E) but were not apposed to GAD⁺ terminals (filled arrowheads, Fig. 3C and F, respectively). Many of the small $\alpha 5$ -GABA_AR clusters (empty arrowheads, Fig. 3A and D), neither co-localized with gephyrin (empty arrowheads, Fig. 3B) or $\gamma 2$ -GABA_ARs (empty arrowheads, Fig. 3E) nor were apposed to GAD⁺ terminals (empty arrowheads, Fig. 3C and F, respectively).

In hippocampal cultures, paraformaldehyde fixation prior to incubation with the primary antibody, interfered with the immunolabeling of neurons by the anti- $\alpha 5$ antibody. That is why we did the immunolabeling in living cells, before fixation with paraformaldehyde. We also detected localization of $\alpha 5$ -GABA_ARs at GABAergic synapses with the anti- $\alpha 5$ antibody I) when the hippocampal cultures were fixed in methanol and permeabilized with Triton X-100 prior to the incubation with the anti- $\alpha 5$ antibody or II) when live cell incubation with anti- $\alpha 5$ was done at 4 °C (not shown), indicating that the localization of $\alpha 5$ at GABAergic synapses was not an artifact induced by antibody capping or internalization. A similar live cell incubation protocol has also been used by others to visualize extrasynaptic $\alpha 5$ -GABA_ARs in hippocampal cultures (Brunig et al. 2002). Although aldehyde fixation of cultures interfered with the immunolabeling of the cells with this antibody, slow perfusion of the intact brain with aldehydes allowed significant immunolabeling of the hippocampus and other structures, as shown above by light microscopy immunocytochemistry (Fig. 2) and postembedding EM immunogold (see below).

In the intact hippocampus and cerebral cortex there is both anti- $\alpha 5$ -GABA_AR immunoreactivity that is not associated with synapses and anti- $\alpha 5$ -GABA_AR immunoreactivity that is associated with synapses

We have done single-label and double-label postembedding EM immunogold to study the subcellular localization of $\alpha 5$ -GABA_ARs and evaluate their presence at GABAergic synapses in the CA1 region of the intact hippocampus. We have also done a few studies on the synaptic localization of $\alpha 5$ -GABA_ARs on the cerebral cortex. We have previously used this technique

to study the localization of various GABA_AR subunits and other antigens in the rat brain at the EM level (Riquelme et al. 2002, Christie et al. 2002; Charych et al. 2004a,b; Li et al. 2005a).

Postembedding EM immunogold with the anti- $\alpha 5$ antibody (single-label) showed that, in hippocampal pyramidal cells of the CA1 region, the $\alpha 5$ immunogold particles (arrows, Fig. 4A) are found in the rough endoplasmic reticulum or rough ER (arrowheads, Fig. 4A). Sometimes the gold particles are arranged in paracircular structures (arrows, Fig. 4A), probably resulting from decorated vesicles or transversely sectioned tubular structures. Anti- $\alpha 5$ immunogold particles (arrows, Fig. 4B and C) are also found on or near microtubules (arrowheads, Fig. 4B and C), which suggests that $\alpha 5$ -GABA_ARs are actively transported in vesicles along microtubules. These experiments show that there is a pool of intracellular $\alpha 5$ GABA_AR subunits, revealed by the antibody, corresponding to the synthesis of this subunit and the trafficking of $\alpha 5$ -GABA_ARs.

Single-label experiments also show that the anti- $\alpha 5$ immunogold particles (arrows, Fig. 4 D-H) are frequently associated with dendritic plasma membranes (arrowheads, Fig. 4D-F and H), and sometimes with submembranous vesicular structures (arrowhead, Fig. 4G), in areas devoided of morphologically identifiable synapses. Note the intravesicular localization of the gold particles in Fig. 4G, which is expected for this anti- $\alpha 5$ antibody that recognizes the extracellular N-terminus of $\alpha 5$. Single-label experiments also show anti- $\alpha 5$ immunogold particles that are associated with synapses (arrows, Fig. 4 I-K), frequently having symmetric, or Gray's type 2, morphology (Fig. 4 I and J).

The postembedding immunogold technique is mainly designed to preserve the immunoreactivity of the tissue. In single-label experiments, sometimes it is difficult to determine whether a synapse that is decorated with gold particles is GABAergic or glutamatergic, based just on morphological criteria. We have done double-label immunogold experiments to immunochemically identify GABAergic and glutamatergic synapses, in addition to the morphological identification. The double-label immunogold experiments show that $\alpha 5$ -immunogold particles (arrows, large particles, Fig. 4L) can be localized on membranes distant from symmetric Gray's type 2 GABAergic synapses (arrowhead, Fig. 4L). These synapses were identified as GABAergic by the immunoreactivity with an anti- $\beta 2/3$ GABA_AR antibody (arrowhead, small particles, Fig. 4L). On occasions, some $\alpha 5$ -GABA_ARs (arrows, large particles, Fig. 4M and N) were also found in dendritic spines (Sp) receiving glutamatergic synapses, identified as such by anti-PSD95 or anti-GluR2 AMPA receptor subunit immunoreactivity (arrowheads, small particles, Fig. 4M and N respectively). These synapses also had Gray's type 1 asymmetric morphology (Fig. 4M and N) with a prominent postsynaptic density, as expected for glutamatergic synapses. These results are consistent with the existence of a pool of membrane $\alpha 5$ -GABA_ARs located outside GABAergic synapses, including dendritic spines, and another pool of $\alpha 5$ -GABA_ARs located at GABAergic synapses.

In the intact hippocampus there is anti- $\alpha 5$ GABA_AR immunoreactivity that is localized at GABAergic synapses

To determine whether $\alpha 5$ -GABA_ARs are present at GABAergic synapses, we did double-label postembedding immunogold combining the anti- $\alpha 5$ antibody with antibodies to GABA (presynaptic GABAergic marker) or to GABA_ARs or gephyrin (postsynaptic GABAergic markers). The $\alpha 5$ immunogold particles (small particles, arrows, crossed arrows and double crossed arrows, Fig. 5 A-E) were present mainly at plasma membranes, likely postsynaptic membranes, associated with presynaptic terminals that were heavily labeled with anti-GABA immunogold particles (large particles, arrowheads, Fig. 5 A-E). The $\alpha 5$ immunogold particles were frequently associated with the membranes (arrows, Fig. 5A-E) and sometimes with the submembranous cytoplasm (double crossed arrow, Fig. 5 E). Occasionally, some $\alpha 5$ gold particles were found in the presynaptic terminal (crossed arrows, Fig. 5 A and C). Although

the membranes decorated with the $\alpha 5$ gold particles (arrows, Fig. 5 A, B and D), could correspond to symmetric Gray's type 2 synapses, this could not be entirely ascertained with these experiments. For this purpose we used GABAergic postsynaptic markers, as shown below.

Double-labeling experiments with the postsynaptic GABAergic markers $\beta 2/3$ or $\gamma 2$ GABA_AR subunits or gephyrin (Fig. 5 F-Q), showed that the $\alpha 5$ immunoreactivity (arrows, Fig. 5 F-N), was associated with synaptic membranes at GABAergic synapses. These synapses had symmetric Gray's type 2 morphology and were also labeled with gold particles corresponding to $\beta 2/3$ GABA_ARs (arrowheads, Fig. 5 F-J), $\gamma 2$ GABA_ARs (arrowheads, Fig. 5 K) or gephyrin (arrowheads, Fig. 5 L-N) immunoreactivity.

Double-label experiments also showed sometimes the presence of $\alpha 5$ gold particles in the postsynaptic cytoplasm (double crossed arrows, Fig. 5 H, O-Q) of GABAergic synapses, as shown by $\beta 2/3$ GABA_AR immunoreactivity (arrowheads, Fig. 5H, O-Q). The $\alpha 5$ -GABA_ARs located in the postsynaptic cytoplasm might represent exocytosis or internalization of these receptors at GABAergic synapses. Sometimes, the gold particles in the postsynaptic cytoplasm showed a paracircular arrangement suggesting that they are associated with subsynaptic vesicles, whose structures presumably has not been preserved (double crossed arrows, Fig 5 P and Q). Sometimes microtubules were associated with these paracircular structures (not shown), suggesting the existence of a pool of subsynaptic transport vesicles that contain $\alpha 5$ -GABA_ARs.

Note the higher concentration of gold particles corresponding to $\beta 2/3$ and $\gamma 2$ than to $\alpha 5$ in the labeled synapses. This was expected given that $\beta 2/3$ and $\gamma 2$ subunits also co-assemble in the hippocampus with α subunits other than $\alpha 5$ (i.e. $\alpha 1$ and $\alpha 2$) forming GABA_ARs. Moreover, as indicated above, the $\alpha 5$ -immunoreactivity is more sensitive to glutaraldehyde fixation than that of $\beta 2/3$ or $\gamma 2$.

The localization of an individual gold particle might not correspond to the exact localization of the antigen due to the size of the primary and secondary antibodies and the size of the gold particle. Thus, for 12 and 18 nm gold particles, the antigen could be located up to 29 and 32 nm, respectively, from the center of the gold particle (Kellenberger and Hayat, 1991). Therefore, quantitative analysis of the distribution of gold particles is necessary to ascertain the postsynaptic localization of $\alpha 5$. The axodendritic distribution of $\alpha 5$ immunogold particles (n=110) in GABAergic synapses showed that particle density was highest in the postsynaptic membrane and subsynaptic cytoplasm (Fig. 6A). Within 30nm on each side of the synaptic cleft midline of GABAergic synapses, 65% of the $\alpha 5$ gold particles were postsynaptic and 35% were presynaptic (n=65). Moreover, 75% of all gold particles located within 50nm on each side of the synaptic cleft midline (n=75) were within the range expected for an antigen localized at the postsynaptic membrane (± 30 nm from the postsynaptic membrane). Some of the $\alpha 5$ immunogold particles in the postsynaptic cytoplasm could also represent exo-or endo-cytosis of synaptic $\alpha 5$ -GABA_ARs.

Quantitative analysis of the normalized lateral distribution of the $\alpha 5$ gold particles located ± 30 nm from the postsynaptic membrane in GABAergic synapses (n=47 gold particles from 38 synapses) showed that the gold particles were distributed along the GABAergic synapse (Fig. 6 B). There was a sharp decline in $\alpha 5$ -GABA_AR immunoreactivity beyond the edge of GABAergic synapses (Fig. 6B). The ratio of the total number of gold particles associated to the synaptic membranes inside the GABAergic synapses versus the number of gold particles associated to the normalized equivalent synaptic length of adjacent extrasynaptic membrane was 8 to 1. At further distance from the synapse, the particle ratio between synaptic and non-synaptic membranes was 24 to 1. These results indicated that the concentration of $\alpha 5$ -

GABA_AR in these GABAergic synapses was considerably higher than the perisynaptic and extrasynaptic concentration of these receptors respectively.

DISCUSSION

It is thought that activation of synaptic and extrasynaptic neuronal GABA_ARs leads to phasic and tonic GABAergic currents respectively (recently reviewed by Farrant and Nusser 2005; Mody 2005). Nevertheless, the possibility that synaptic receptors are also involved in tonic GABAergic currents cannot be excluded (Kullmann et al. 2005).

The GABA_ARs that contain the δ subunit (δ -GABA_ARs) are non-synaptic, as shown by immunoelectron microscopy in the granule cells of the cerebellum (Nusser et al., 1998) and dentate gyrus where they concentrate perisynaptically (Wei et al., 2003). These δ -GABA_ARs are responsible for the tonic GABAergic inhibition in the granule cells of the cerebellum and dentate gyrus. They sense the ambient levels and/or spillover of GABA released from synapses. In these and other cell types, the γ_2 and δ subunits do not co-assemble within the same GABA_AR (Quirk et al., 1994, 1995; Jechlinger et al., 1998). Thus, it is widely accepted that the synaptic GABA_ARs contain the γ_2 subunit while the δ -GABA_ARs are extrasynaptic or perisynaptic (Essrich et al 1998; Nusser et al 1998; Wei et al 2003; Li et al 2005b; Christie et al. 2006; Mangan et al. 2005).

Tonic GABAergic currents have been recorded in hippocampal pyramidal cells (Bai et al. 2001; Yeung et al. 2003; Caraiscos et al. 2004; Semyanov et al. 2004; Mangan et al. 2005; Glykys and Mody 2006). These tonic currents are robust during early development (Demarque et al. 2002), but they become very small in adult hippocampal pyramidal cells (Wisden et al. 2002; Semyanov et al. 2004). The tonic GABA_AR currents in hippocampal pyramidal cells are due to extrasynaptic GABA_ARs (Banks and Pearce 2000; Petrini et al. 2004; Mody 2001). It has been proposed that the tonic GABAergic inhibition observed in hippocampal pyramidal cells, is largely mediated by extrasynaptic $\alpha 5$ -GABA_ARs (Caraiscos et al. 2004). Since the $\alpha 5$ -GABA_ARs are involved in hippocampal-dependent learning and memory (Collinson et al. 2002; Kelly et al. 2002; Crestani et al. 2002; Chambers et al. 2003; Maubach 2003), it has also been proposed that these extrasynaptic $\alpha 5$ -GABA_ARs are involved in hippocampal learning and memory (Caraiscos et al. 2004). However, the experiments that led to these conclusions were done at elevated GABA concentration, after treatment with the GABA transaminase inhibitor vigabatrin, addition of GABA uptake inhibitors or addition of GABA itself. In contrast, under baseline conditions of GABA, tonic currents in hippocampal pyramidal neurons are both very small and not mediated by $\alpha 5$ -GABA_ARs (Wisden et al. 2002; Semyanov et al. 2003; Scimemi et al. 2005). Only at high GABA concentration are the tonic currents partially mediated by $\alpha 5$ -GABA_ARs (Caraiscos et al. 2004; Scimemi et al. 2005; Glykys and Mody 2006). Thus, additional research is needed to clarify the functional role of the extrasynaptic $\alpha 5$ -GABA_ARs under normal physiological conditions.

By using immunofluorescence of hippocampal cultures and/or brain slices we and others had shown that, in hippocampal pyramidal cells, many $\alpha 5$ -GABA_ARs are extrasynaptically localized (Brunig et al. 2002; Christie and De Blas 2002; Crestani et al. 2002). By using immunofluorescence and EM immunogold we have also revealed the existence of a pool of $\alpha 5$ -GABA_ARs that is localized at GABAergic synapses in hippocampus and cerebral cortex. To the best of our knowledge, this is the first time that the subcellular localization of the $\alpha 5$ -GABA_ARs subunit has been reported at the EM level. Moreover, this is the first demonstration at the EM level of the presence of $\alpha 5$ -GABA_ARs in GABAergic synapses of the hippocampus and cerebral cortex. Additional support for the presence of $\alpha 5$ -GABA_ARs at GABAergic synapses comes from our finding that the $\alpha 5$ subunit was present in a subcellular brain fraction enriched in hippocampal postsynaptic densities. In agreement with the notion that $\alpha 5$ -

GABA_ARs are present in GABAergic synapses, mIPSCs resulting from the activation of synaptic α 5-GABA_ARs, have been recorded in neurons of the mouse cerebral cortex (Dunning et al. 1999). These experiments have also shown that the decay time of the synaptic α 5-GABA_ARs is slower than that of the α 1-GABA_ARs (Dunning et al. 1999). Moreover, after inducing capping with an anti- α 5 antibody, Brunig et al. (2002) reported that in their cultured hippocampal neurons, 24% of α 5 positive clusters were apposed to synapsin I positive boutons. Although the authors emphasized the non-synaptic localization of α 5-GABA_ARs, they also stated that they did not exclude the possibility that some α 5-GABA_ARs could be targeted to postsynaptic sites.

In the α 5^{-/-} mouse mutant, the hippocampal pyramidal cells show reduced peak amplitude and slower decay time of the evoked or action potential-dependent IPSCs (Collinson et al 2002) than the control wt mouse. Moreover, the α 5^{-/-} mouse mutant has reduced tonic currents while they show no change in the mIPSCs when compared with the control non-mutant mouse (Caraiscos et al. 2004). These experiments have been interpreted as I) evidence for the absence of α 5-GABA_ARs at these GABAergic synapses and II) evidence for the extrasynaptic localization of the α 5-GABA_ARs, whose activation requires the induced multivesicular release of GABA to the synaptic cleft, the spillover of GABA outside the synaptic cleft and the activation of the extrasynaptic α 5-GABA_ARs.

How do we explain the apparent discrepancy between the morphological presence of α 5-GABA_ARs at the GABAergic synapses in hippocampal pyramidal cell dendrites on one hand, and the lack of mIPSCs reduction in the α 5^{-/-} mutant mouse with respect to the wild type, on the other hand (Caraiscos et al. 2004)? A possible explanation is that the α 5-GABA_ARs at GABAergic synapses are at significantly lower concentration than the combination of synaptic α 1-GABA_ARs, α 2-GABA_ARs and α 3-GABA_ARs, which are frequently co-expressed by the same pyramidal cell. GABA_ARs with different α subunit composition frequently colocalize at the same GABAergic synapse in both hippocampal cultures (Brunig et al. 2002; Christie et al 2002a,b; Christie and De Blas 2002, 2003) and in the intact hippocampus (Nusser et al 1996; Nyiri et al 2001), even though in the latter, the α 2 subunit preferentially concentrate at some GABAergic synapses (Nusser et al 1996; Nyiri et al 2001). In the hippocampus, 20% of all GABA_ARs have the α 5 subunit (Fritschy et al. 1997; Sur et al. 1998) plus a large proportion of these receptors are extrasynaptic, as indicated above. Thus, at individual GABAergic synapses in the wild type mouse, the α 5-GABA_ARs could represent less than 10% of all the synaptic GABA_ARs. Therefore, in whole cell recording or whole cell patch clamp recording, which do not differentiate between GABA_ARs localized in the same or in different synapses, the effect of pharmacologically blocking or knocking down α 5-GABA_ARs on the synaptic GABAergic currents, is predicted to be very small. Moreover, in the α 5^{-/-} mutant mouse, compensatory mechanisms might bring other α subunit-containing GABA_ARs to occupy the GABAergic synaptic sites otherwise occupied by the α 5-GABA_ARs, regardless of whether there is significant compensatory upregulation or not of the other α subunit isoforms in the α 5^{-/-} mutant. If that were the case, there would be no apparent difference in the mIPSCs between the α 5^{-/-} mutant and the wild type.

The hippocampal α 5-GABA_ARs also contain the β 3 and γ 2 subunits (Sur et al. 1998). The presence of the γ 2 subunit makes the α 5-GABA_ARs sensitive to benzodiazepines. Therefore, even though the γ 2 subunit is necessary for the clustering of GABA_ARs (Essrich et al. 1998; Li et al. 2005b) and a pool of the α 5-GABA_ARs is synaptic, as we are reporting in this communication, another large pool of hippocampal α 5 β 3 γ 2 receptors is extrasynaptic as we and others have shown (Brunig et al. 2002; Crestani et al. 2002; Christie and De Blas 2002). Moreover, we have recently shown that in cultured hippocampal pyramidal neurons there is a relatively large extrasynaptic pool of γ 2-GABA_ARs whose clustering can be induced by anti- γ 2 antibody-induced capping (Christie et al. 2005). In the intact cerebellum, γ 2-GABA_ARs

have been found outside GABAergic synapses (Nusser et al., 1998). Therefore, although necessary (Essrich et al. 1998; Li et al. 2005b), the presence of a $\gamma 2$ subunit is not sufficient for the synaptic clustering of GABA_ARs (Wisden et al. 2002).

The EM postembedding immunogold experiments have also shown that the synaptic $\alpha 5$ -GABA_ARs can be present in hippocampal GABAergic synapses that contain gephyrin. Moreover, we have also shown that in cultured hippocampal pyramidal cells, gephyrin colocalizes with the large synaptic $\alpha 5$ -GABA_AR clusters and with some of the small $\alpha 5$ -GABA_AR clusters. However, gephyrin is not present in many of the small $\alpha 5$ -GABA_AR clusters. The presence of gephyrin in the GABAergic synapses containing $\alpha 5$ -GABA_ARs (plus GABA_ARs with other α subunits) does not mean that gephyrin is involved in the clustering or stabilization of synaptic $\alpha 5$ -GABA_ARs. In the spinal cord of the gephyrin knockout mouse mutant the number of both $\alpha 5$ -containing and $\alpha 1$ -containing GABA_ARs clusters, observed at the light microscopy level, is unaffected, while the number of $\alpha 2$, $\alpha 3$, $\beta 2/3$ and $\gamma 2$ clusters is significantly reduced (Kneussel et al. 2001) suggesting that gephyrin is not required for the clustering or stabilization of the $\alpha 5$ -containing or $\alpha 1$ -containing GABA_ARs. Levi et al. 2004 also reported that there was no reduction in $\alpha 1$ -containing GABA_AR clusters in hippocampal cultures of the gephyrin knockout mouse. Nevertheless, gephyrin seems to be involved in the stabilization of other GABA_ARs at GABAergic synapses (Levi et al. 2004; Luscher and Keller 2004; Studler et al. 2005; Jacob et al. 2005), even though it is thought that gephyrin does not directly interact with GABA_ARs.

Our results suggest that hippocampal pyramidal cells have both synaptic and extrasynaptic $\alpha 5$ -GABA_ARs. The $\alpha 5$ -GABA_ARs are also present in GABAergic synapses in the cerebral cortex. The results are consistent with the notion that the synaptic and the extrasynaptic $\alpha 5$ -GABA_ARs are involved in phasic and tonic GABAergic inhibition respectively.

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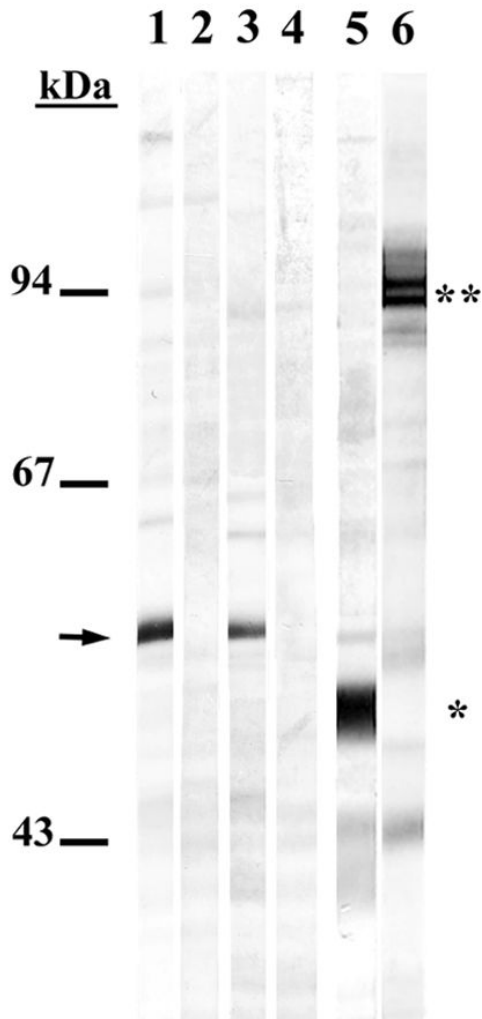


Fig. 1. Immunoblots of a hippocampal subcellular fraction enriched in postsynaptic densities show the presence of the 55 kDa $\alpha 5$ GABA_AR subunit in this fraction

Lanes 1 and 2 contain the crude synaptosomal P2 fraction. Lanes 3-6 contain a Triton X-100 insoluble fraction enriched in PSDs. The anti- $\alpha 5$ antibody recognizes a 55 kDa polypeptide (arrow, lanes 1 and 3). The immunoreactivity of the anti- $\alpha 5$ antibody with the 55 kDa GABA_AR subunit was blocked by incubating the antibody with 20 $\mu\text{g}/\text{ml}$ of the synthetic antigenic peptide (lanes 2 and 4). The hippocampal fraction enriched in PSDs also contained the 51 kDa polypeptide (asterisk, lane 5) corresponding to $\alpha 1$ GABA_AR subunit, as shown with an anti- $\alpha 1$ antibody, and the 93 kDa gephyrin polypeptide doublet (double asterisk, lane 6) as shown with an anti-gephyrin antibody. The same amount of protein (10 μg) was transferred to each strip.

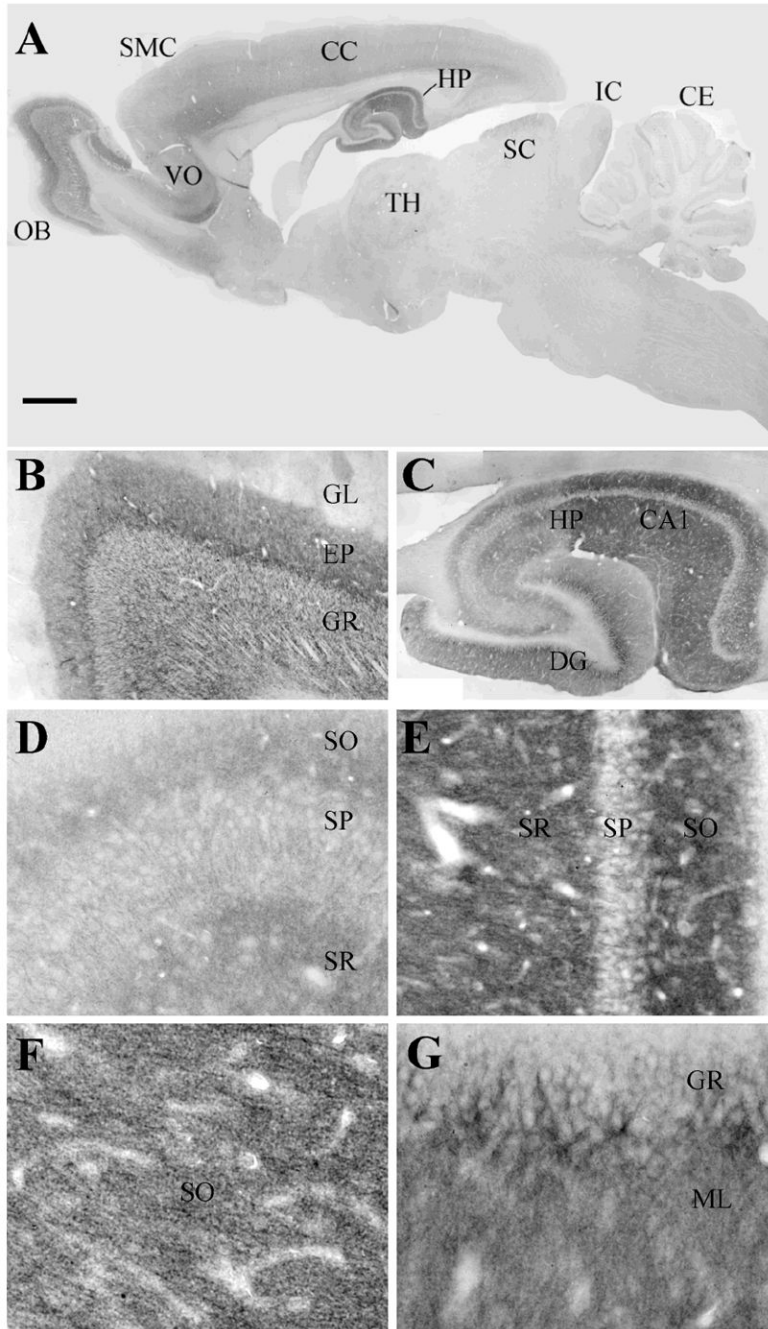


Fig. 2. Light microscopy immunocytochemistry of the rat brain with anti- $\alpha 5$ antibody

A: Whole brain; B: Olfactory bulb; C: Hippocampus; D: CA3 region of the hippocampus; E: CA1 region of the hippocampus; F: Stratum oriens of the CA1 region of the hippocampus; G: dentate gyrus. Note the strong immunoreaction in the CA1 region of the hippocampus, dentate gyrus and olfactory bulb and the absence of immunoreactivity in the cerebellum. Abbreviations are CC cerebral cortex; CE cerebellum; EP external plexiform layer; GL glomerular layer; GR granule cell layer; IC inferior colliculus; ML molecular layer; OB olfactory bulb; SC superior colliculus; SMC secondary motor cortex; SO stratum oriens; SP stratum pyramidale; SR stratum radiatum; TH thalamus; VO ventral orbital cortex. Scale bar = 1.5 mm (A); 250 μ m (B and C); 50 μ m (D and E); 25 μ m (F and G).

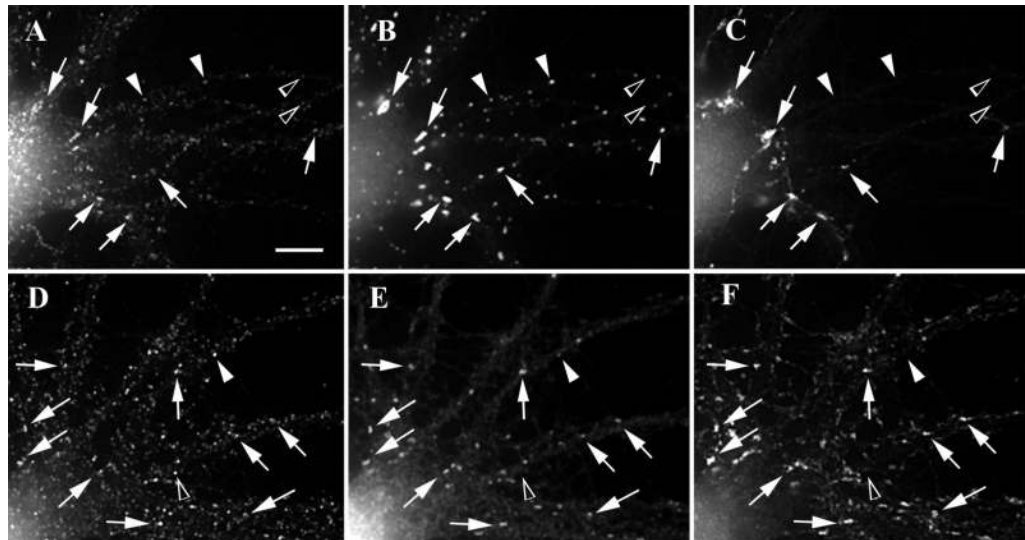


Fig. 3. In hippocampal cultures, some $\alpha 5$ -GABA_AR clusters co-localize with gephyrin and the $\gamma 2$ -GABA_ARs at GABAergic synapses

Triple-label immunofluorescence using combinations of the Rb anti- $\alpha 5$ antibody (A and D), the mouse mAb to gephyrin (B) or the GP anti- $\gamma 2$ (E) and the sheep anti-GAD (C and F). Arrows show $\alpha 5$ clusters that colocalize with postsynaptic gephyrin or $\gamma 2$ -containing GABA_ARs and that are apposed to presynaptic GAD⁺ terminals. Filled arrowheads show $\alpha 5$ clusters that colocalize with gephyrin or $\gamma 2$ -containing GABA_ARs in the absence of GAD⁺ terminals. Empty arrowheads show $\alpha 5$ clusters that do not colocalize with gephyrin, $\gamma 2$ or GAD⁺ terminals. Note the larger size and brighter fluorescence of the $\alpha 5$ clusters at GABAergic synapses (arrows) compared to the non-synaptic clusters (filled and empty arrowheads). Scale bar 5 μ m.

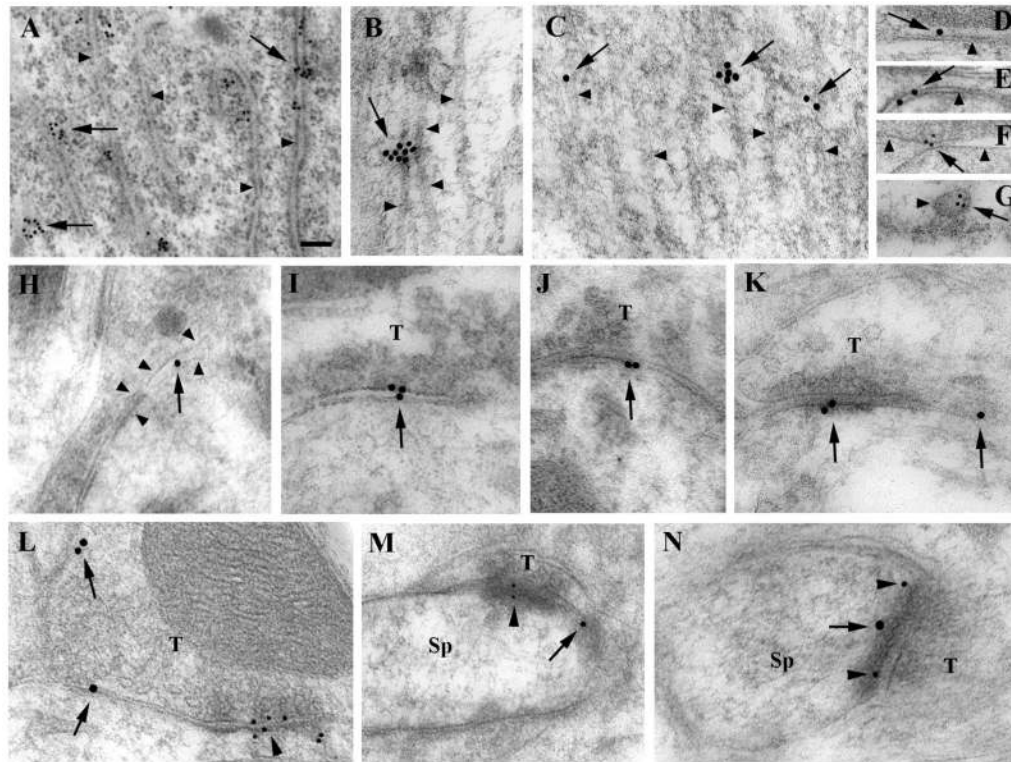


Fig. 4. In the intact rat hippocampus and cerebral cortex there is both $\alpha 5$ immunoreactivity that is not associated with synapses and $\alpha 5$ immunoreactivity that is associated with synapses
 Postembedding EM immunogold of the CA1 region of the hippocampus (A-H, K, M and N) and cerebral cortex (I, J and L) with the Rb anti- $\alpha 5$ GABA_AR subunit antibody in single-label (A-K) or double-label (L-N) experiments with a mouse anti- $\beta 2/3$ (L), or mouse anti-PSD 95 (M), or mouse anti-GluR2 AMPA receptor subunit (N) monoclonal antibody. Gold particles corresponding to $\alpha 5$ GABA_AR subunit (arrows, A-N) are present in the rough ER (arrowheads, A), on or near microtubules (arrowheads, B and C), on plasma membranes (arrowheads, D-F, H), in cytoplasmic vesicles (arrowhead, G), in synapses (I-K), or distant from GABAergic synapses labeled with anti- $\beta 2/3$ (arrowhead, L), or on dendritic spines (Sp, M and N) receiving glutamatergic synapses, as shown by immunogold particles corresponding to mouse anti-PSD 95 (arrowhead, M) or mouse anti-GluR2 (arrowheads, N). The presynaptic terminals in I-N are labeled with a T. The secondary goat anti-rabbit IgG antibody was conjugated to 18 nm diameter and the goat anti-mouse IgG antibody to 10 nm diameter colloidal gold particles respectively. Scale bar = 190 nm for A; 120 nm for F and G; 110 nm for M; 90 nm for B-E and H-K; 75 nm for L and 70 nm for N.

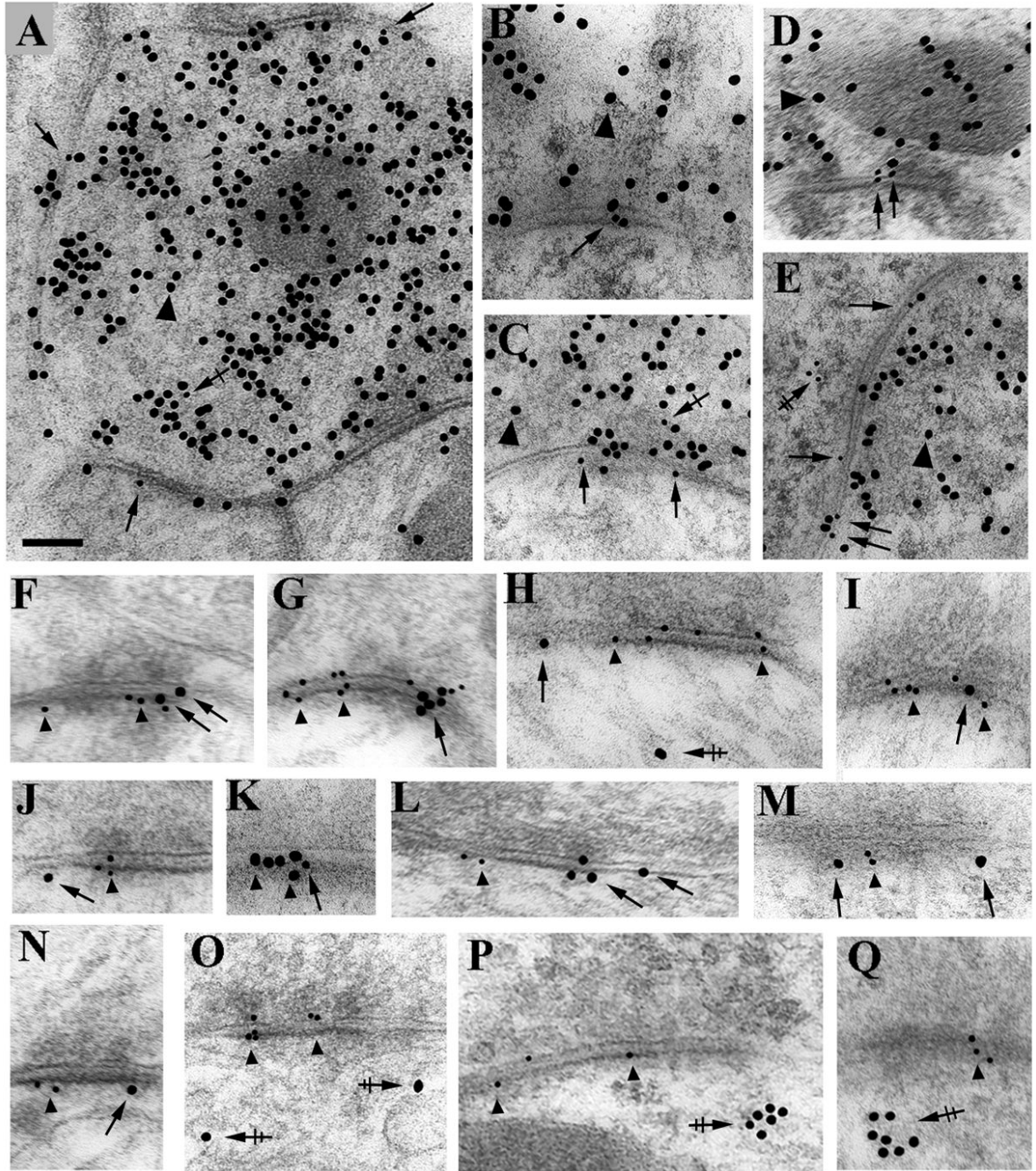


Fig. 5. In the intact rat hippocampus, $\alpha 5$ immunoreactivity is also associated with GABAergic synapses

Double-label postembedding EM immunogold of the CA1 region of the hippocampus (A-Q) with the Rb anti- $\alpha 5$ GABA_AR subunit in combination with GP anti-GABA (A-E), or mouse mAb to $\beta 2/3$ GABA_AR subunit (F-J, O-Q), or GP anti- $\gamma 2$ GABA_AR subunit (K) or mouse mAb to gephyrin (L-N). In all synapses the presynaptic terminal is on top and the postsynaptic element is at the bottom except in panel E where the presynaptic terminal is at the right side of the figure. Gold particles corresponding to $\alpha 5$ immunoreactivity are frequently localized on membranes (arrows, A-E), likely postsynaptic, that are associated with presynaptic GABAergic terminals containing the large gold particles corresponding to GABA

immunoreactivity (arrowheads, A-E). Sometimes the $\alpha 5$ gold particles are located postsynaptically in the subsynaptic cytoplasm (double crossed arrow, E). Less frequently, $\alpha 5$ gold particles are found in the presynaptic terminal (crossed arrow, A and C). Gold particles corresponding to $\alpha 5$ -GABA_AR immunoreactivity (arrows, F-Q) are found at GABAergic synapses as determined by their symmetric type 2 morphology and the presence of colocalizing GABAergic synaptic markers $\beta 2/3$ GABA_AR subunit (arrowheads, F-J, O-Q), $\gamma 2$ GABA_AR subunit (arrowheads, K) or gephyrin (arrowheads L-N). Gold particles corresponding to $\alpha 5$ immunoreactivity frequently localize on or near the synaptic membranes (arrows, F-N). Sometimes, the $\alpha 5$ gold particles localize postsynaptically in the subsynaptic cytoplasm (double crossed arrows, H, O-Q), sometimes forming paracircular clusters (double crossed arrows, P and Q). In A-E and K the secondary donkey anti-guinea pig IgG was conjugated to 18 nm diameter and the donkey anti-rabbit IgG to 12 nm diameter colloidal gold particles. In F-J, L-Q the secondary goat anti-rabbit IgG antibody was conjugated to 18 nm diameter and the goat anti-mouse IgG antibody to 10 nm diameter colloidal gold particles. Scale bar = 160 nm for A, C and E and 120 nm for B, D, F-Q.

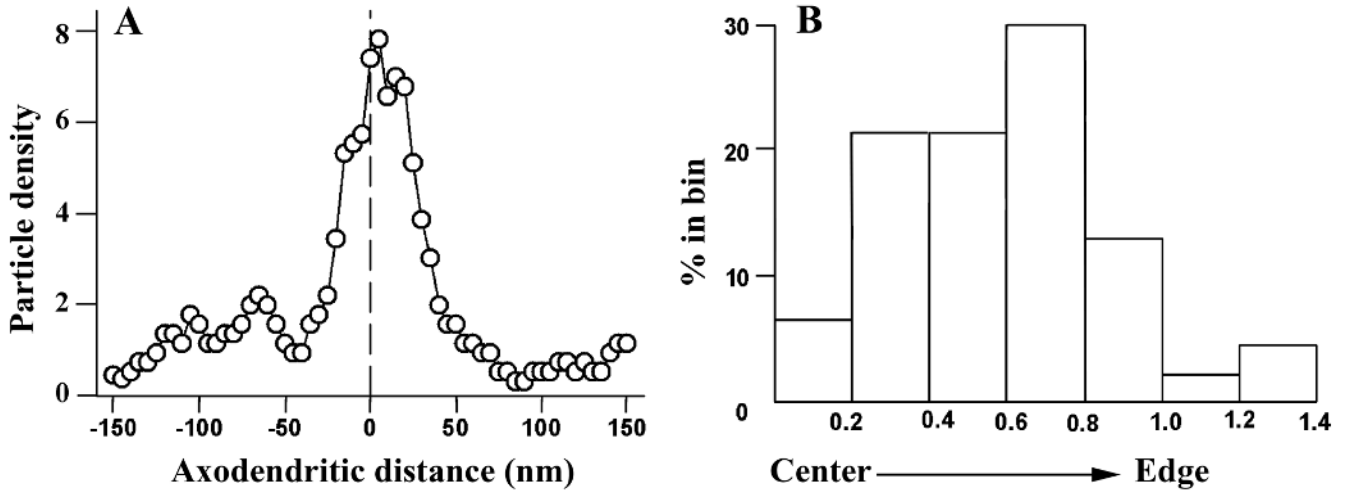


Fig. 6. Gold particle distribution of $\alpha 5$ -GABA_AR immunoreactivity at GABAergic synapses
 Axo-dendritic (A) and lateral distribution (B) of gold particles corresponding to $\alpha 5$ -GABA_AR immunoreactivity in GABAergic synapses. The gold particles in A (n=110) were distributed in 5 nm bins according to the distance from the center of each gold particle to the midline of the synaptic cleft, defined as the zero point. Double label experiments with the Rb anti- $\alpha 5$ and an antibody to a GABAergic synaptic marker (mouse mAb to $\beta 2/3$ GABA_AR subunit or GP anti- $\gamma 2$ GABA_AR subunit or mouse mAb to gephyrin) were used for identifying $\alpha 5$ in GABAergic synapses. Negative and positive values represent presynaptic and postsynaptic localization respectively. Particle density is the number of particles in a 5 nm bin. The graph was smoothed using a five point weighted running average. In B, a histogram was generated by plotting the distribution of gold particles localized ± 30 nm from the postsynaptic membrane (n=47) along the axis parallel to the synaptic membranes. The lateral position values were normalized as described in the Methods section.