Cultured Hippocampal Pyramidal Neurons Express Two Kinds of $\text{GABA}_A$ Receptors

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ABSTRACT

We combined a study of the subcellular distribution of the $\alpha_1$, $\alpha_2$, $\alpha_4$, $\beta_1$, $\beta_2/3$, $\gamma_2$, and $\delta$ subunits of the $\text{GABA}_A$ receptor with an electrophysiological analysis of $\text{GABA}_A$ receptor currents to determine the types of receptors expressed on cultured hippocampal pyramidal neurons. The immunocytochemistry study demonstrated that $\alpha_1$, $\alpha_2$, $\beta_2/3$, and $\gamma_2$ subunits formed distinct clusters of various sizes, which were colocalized with clusters of glutamate decarboxylase (GAD) immunoreactivity at rates ranging from 22 to 58%. In contrast, $\alpha_4$, $\beta_1$, and $\delta$ subunits were distributed diffusely over the cell soma and neuronal processes of cultured neurons and did not colocalize with the GAD marker. Whole-cell $\text{GABA}_A$ receptor currents were moderately sensitive to GABA and were modulated by diazepam. The whole-cell currents were also enhanced by the neurosteroid allopregnanolone (10 nM). Tonic currents, measured as changes in baseline current and noise, were sensitive to $\text{Zn}^{2+}$, furosemide, and loreclezole; they were insensitive to diazepam. These studies suggest that two kinds of $\text{GABA}_A$ receptors are expressed on cultured hippocampal neurons. One kind of receptor formed clusters, which were present at $\text{GABA}_A$ergic synapses and in the extrasynaptic membrane. The $\alpha_1$, $\alpha_2$, $\beta_2/3$, and $\gamma_2$ subunits were contained in clustered receptors. The second kind was distributed diffusely in the extrasynaptic membrane. The $\alpha_4$, $\beta_1$, and $\delta$ subunits were contained in these diffusely distributed receptors. The properties of tonic currents recorded from these neurons were similar to those from recombinant receptors containing $\alpha_4$, $\beta_1$, and $\delta$ subunits.

$\text{GABA}_A$ receptors are ligand-gated chloride ion channels with a pentameric subunit structure that mediate fast inhibitory neurotransmission in the vertebrate central nervous system. The subunits constituting these receptors are derived from several gene families that include $\alpha$, $\beta$, $\gamma$, $\delta$, $\epsilon$, $\pi$, and $\theta$; some of these gene families have several members ($\alpha_1$–$\alpha_6$, $\beta_1$–$\beta_3$, and $\gamma_1$–$\gamma_3$) (Sieg hart and Sperk, 2002). Different $\text{GABA}_A$ receptors can be assembled from these subunits conferring unique biophysical and pharmacological properties upon the receptor isoforms. There is growing evidence from electron microscopic and electrophysiological studies that distinct $\text{GABA}_A$ receptor isoforms are present at synapses and in the extrasynaptic membrane and that these isoforms mediate two different forms of inhibition: tonic inhibition and synaptic inhibition (Soltesz and Nusser, 2001).

Several previous studies have characterized $\text{GABA}_A$ receptor subunit expression in cultures of hippocampal neurons using mRNA and protein-expression studies. The mRNA for $\alpha_1$, $\alpha_2$, $\alpha_4$, $\alpha_5$, $\beta_1$, $\beta_3$, $\gamma_2$, and $\delta$ subunits is expressed in these cells (Killisch et al., 1991; Brooks-Kayal et al., 1998). The subcellular distribution of the subunit protein in these cells has also been investigated. One study described the distribution of $\text{GABA}_A$ receptors containing the $\alpha_1$, $\alpha_2$, $\alpha_3$, and $\alpha_5$ subunits in cultured neurons (Brunig et al., 2002a), suggesting that $\alpha_1$ and $\alpha_2$ subunits were clustered at synapses, whereas $\alpha_5$ clusters were extrasynaptic. Another study found both synaptic and extrasynaptic clusters of $\text{GABA}_A$ receptors containing $\alpha_1$, $\alpha_2$, $\beta_1$, $\beta_2/3$, and $\gamma_2$ subunits (Christie et al., 2002). These findings were extended to the $\alpha_5$ subunit (Christie and de Blas, 2002), which was demonstrated to form clusters at synapses and in extrasynaptic membranes.

These studies did not investigate the distribution of $\alpha_4$, $\beta_1$, and $\delta$ subunits in cultured hippocampal neurons. The mRNA

ABBREVIATIONS: HBS, HEPE$S$-buffered saline; GAD, glutamate decarboxylase; MEM, minimal essential medium; PBS, phosphate-buffered saline; sIPSC, spontaneous inhibitory postsynaptic current.
and protein for these subunits is expressed in hippocampal pyramidal neurons. In cerebellar granule cells, the δ subunit is expressed in extrasynaptic membrane, and the α6 subunit commonly associates with it. It is known that in the forebrain, the δ subunit commonly coassembles with the α4 subunit; thus, it is likely that these two subunits have similar distribution on hippocampal pyramidal neurons. However, previous studies did not compare the distribution of δ subunit-containing receptors with γ2 subunit-containing receptors. Finally, the previous immunocytochemical studies did not provide electrophysiological evidence for the expression of these subunits on cultured hippocampal neurons. Separate electrophysiological studies in cultured hippocampal neurons reveal that the biophysical and pharmacological properties of tonic currents and phasic (quantal) inhibitory synaptic currents were distinct and suggested that different types of receptors mediate two types of inhibition (Bai et al., 2001; Yeung et al., 2003). However, these electrophysiological studies did not investigate the subunits expressed on cultured hippocampal neurons. We combined a study of the subcellular distribution of α1, α2, α4, β1, β2/3, γ2, and δ subunits of the GABA<sub>A</sub> receptor with electrophysiological analysis of GABA<sub>A</sub> receptor currents to determine the types and distribution of receptors expressed on these neurons.

**Materials and Methods**

**Hippocampal Cultures.** Hippocampal neuronal cultures were prepared according to the protocol described by Banker and modified for electrophysiology (Goslin et al., 1998; Mangan and Kapur, 2004). Hippocampal neurons and glia were cultured separately and then combined to form a tissue culture “sandwich”. This approach allowed the preparation of low-density hippocampal cultures while allowing access to the neurotrophic support from glia. Glial cultures were prepared 10 days before coculturing with hippocampal neurons. Ten days after glial cell plating, hippocampal neuronal cultures were prepared using Sprague-Dawley rat fetuses (embryonic day 18) from a single litter. The hippocampi were removed from brains under a dissecting microscope and placed in a HEPES-buffered saline (HBS)–containing Petri dish. All hippocampi were transferred to a culture incubator until they were used.

Hippocampal neurons were fully suspended. After suspension, hippocampal neurons were incubated at 37°C for 15 min. After incubation, trypsin was replaced with 5 ml of HBS, and the hippocampi were rinsed with the buffer three times at 5-min intervals. Hippocampi were triturated until a single cell suspension was obtained. Because of intense nonspecific binding in the cell body, only puncta on processes were quantified for all neurons. Puncta were counted in each field after a single neuron was identified.

**Electrophysiological Recording.** Whole-cell voltage-clamp recordings were made using a technique described previously (Hamill et al., 1997). Coverslips were rinsed twice with phosphate-buffered saline (PBS) to remove any residual MEM. Neurons were fixed in 4% paraformaldehyde with 4% sucrose in PBS for 15 min. Coverslips were then washed with three quick changes of PBS followed by 5 min in PBS repeated three times. Cells were blocked in 5% normal goat serum (or 5% normal donkey serum for goat anti-α2 experiments) in PBS for 30 min and then rehydrated with two quick changes of PBS. Coverslips were incubated in the primary antibody overnight at 4°C (primary antibody concentrations: α1, 3 μg/ml; α4, β2/3, and δ, 5 μg/ml; β1, γ2, and mouse anti-GAD65, 2 μg/ml; rabbit anti-GAD65 and rabbit anti-GAD65/67, 1:1000). The next day, the first primary antibody was removed, and the coverslips were washed with three changes of PBS for 5 min each. A second primary antibody was applied overnight at 4°C.

Primary antibodies were removed, and neurons were washed three times with PBS. Cultures were incubated with secondary antibodies at room temperature in darkness for 45 min. Secondary antibodies conjugated with the fluorochromes Alexa Fluor 488 or Alexa Fluor 594 were chosen to prevent cross-reactions (Molecular Probes, Eugene, OR). Dishes were rinsed three times in PBS, and coverslips were mounted on slides, neuronal-side down, by placing a drop of Gel/Mount (Biomeda, Foster City, CA) on the slide and sealing the coverslip to the slide with clear nail polish. At least four separate experiments were performed, two dishes for each combination of antibodies in an experiment. In control experiments omitting primary antibodies, very low background staining was detected. Slides were stored at −20°C before viewing.

**Image Acquisition.** Fluorescent images of cells were captured using a Photometrics CoolSNAPcf charge-coupled device camera (Roper Scientific, Trenton, NJ) mounted on an Eclipse TE2000 fluorescence microscope (Nikon, Tokyo, Japan) with either a 40 × 1.3 or 60 × 1.4 numerical aperture lens driven by Metamorph imaging software (Universal Imaging Corporation, Downingtown, PA). Using Metamorph, the brightness and contrast of fluorescent images were adjusted to a threshold such that punctate fluorescence was two times higher than diffuse background labeling. The number of puncta was then measured. A puncta was defined as an aggregation of 0.05- to 4.4-μm<sup>2</sup> area. Because of intense nonspecific binding in the cell body, only puncta on processes were quantified for all neurons. Puncta were counted in each field after a single neuron was centered.

**Analysis of Colocalization.** Using Meta morph, a binary image was created from each threshold image. Binary images were then added together to display overlapping puncta. Puncta were counted, and percentage of colocalization was calculated as the number of colocalized puncta divided by the total number of subunit clusters. Data were analyzed using Prism 4.0 (GraphPad Software Inc., San Diego, CA). Significant differences between two groups were determined using an unpaired Student’s <i>t</i>-test.

**Photomicrograph Production.** As mentioned above, the brightness and contrast of fluorescent images were adjusted using Meta morph so that punctate fluorescence was two times higher than diffuse background labeling. Images were then saved as eight-bit tiff files and opened in Photoshop 6.0 (Adobe Systems, Mountain View, CA), in which overall brightness was increased for final production.

**Immunofluorescence Staining and Antibodies.** The antibodies indicated were used for immunocytochemistry of GABA<sub>A</sub> receptor subunits: α1 (1–16), α2 (1–19), α4 (1–14), β1 (350–404), β2/3, γ2 (319–366), and δ (1–44), and glutamate decarboxylase (GAD). Antibody against α1 was from Alomone Labs (Jerusalem, Israel); β2/3 and GAD65 were from Chemicon International (Temecula, CA); antibodies against the α2 subunit was from Santa Cruz Biochemicals (Santa Cruz, CA); and other antibodies were developed in Dr. Sieghart’s laboratory. The antibodies to the γ2, α4, δ, and β1 subunits have been characterized extensively in the past by immunoprecipitation, Western blotting, and immunocytochemistry (Sperl et al., 1997). Coverslips were rinsed twice with phosphate-buffered saline (PBS) to remove any residual MEM. Neurons were fixed in 4% paraformaldehyde with 4% sucrose in PBS for 15 min. Coverslips were then washed with three quick changes of PBS followed by 5 min in PBS repeated three times. Cells were blocked in 5% normal goat serum (or 5% normal donkey serum for goat anti-α2 experiments) in PBS for 30 min and then rehydrated with two quick changes of PBS. Coverslips were incubated in the primary antibody overnight at 4°C (primary antibody concentrations: α1, 3 μg/ml; α4, β2/3, and δ, 5 μg/ml; β1, γ2, and mouse anti-GAD65, 2 μg/ml; rabbit anti-GAD65 and rabbit anti-GAD65/67, 1:1000). The next day, the first primary antibody was removed, and the coverslips were washed with three changes of PBS for 5 min each. A second primary antibody was applied overnight at 4°C.

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**Electrophysiological Recording.** Whole-cell voltage-clamp recordings were made using a technique described previously (Hamill et al., 1981; Kapur and Macdonald, 1999). Patch pipettes (resistance of 5–8 MΩ) were pulled on a P-97 Flaming/Brown puller (Sutter Instrument Company, Novato, CA) by a three-stage pull. The extracellular recording solution consisted of 142 mM NaCl, 1.0 mM CaCl<sub>2</sub>, 8.09 mM CsCl, 6 mM MgCl<sub>2</sub>, 10 mM glucose, and 10 mM HEPES, with pH adjusted to 7.4 and osmolality of 310 to 320 mOsm unless specified otherwise. All reagents were from Sigma-Aldrich (St. Louis, MO) unless specified otherwise. Glass recording patch pipettes were filled with a solution consisting of the following: 133 mM CsCl, 1.0 mM MgCl<sub>2</sub>, 1.0 mM HEPES, and 5.0 mM EGTA, pH 7.3, osmolality of ~280 to 290 mOsm. Recording pipettes also contained an ATP
regeneration system consisting of 50 U/ml creatinine phosphokinas, 22 mM phosphocreatine, and 3 mM ATP. The ATP regeneration system maintained intracellular energy stores and reduced rundown of GABA<sub>A</sub> receptor currents as described previously (Stelzer et al., 1988). For recordings of GABA<sub>A</sub> receptor-mediated spontaneous inhibitory post synaptic currents (sIPSCs) and tonic GABAergic current, 50 μM DL-2-amino-5-phosphonopentanoic acid and 20 μM 6-cyano-7-nitroquinoline-2,3-dione (Toxic Cookson Inc., Ellisville, MO) were included in the external medium to block NMDA and α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid/kainate receptor-mediated currents, respectively.

All recordings were obtained at room temperature (24°C). Currents were recorded with an Axopatch 1-D amplifier and low-pass-filtered at 2 kHz with an eight-pole Bessel filter before digitization, storage, and display. Liquid junction potentials were corrected, and whole-cell capacitance and series resistance were compensated by 70 to 75% at a 10-μs lag. A recording was performed when series resistance after compensation was 20 MΩ or less. Access resistance was monitored with a 10 ms, ~5 mV test pulse once every 2 min. The recording was rejected when the series resistance increased by 25% during the experiment. Whole-cell currents were displayed on a chart recorder (2400S; Gould Instrument Systems Inc., Cleveland, OH), and peak whole-cell currents were measured manually from the chart paper. Currents were also digitized (20 kHz) using an Axon Instruments Digidata 1200A analog-to-digital converter and recorded to a personal computer using axotape acquisition software (Axon Instruments Inc., Union City, CA) and filtered at 5 kHz.

Drug Application. GABA, diazepam, and ZnCl<sub>2</sub> dissolved in extracellular solution were applied to neurons using a modified U-tube “multipuffer” drug application system with the tip of application pipette placed 100 to 200 μm from the cell (Greenfield and Macdonald, 1996). Diazepam was dissolved first in dimethyl sulfoxide and then diluted in extracellular buffer with the final dimethyl sulfoxide dilution being at least 1:50,000. All other drugs were dissolved in extracellular buffer.

Analysis of Whole-Cell Currents. The magnitude of the enhancement or inhibition of the current by a drug was measured by subtracting the peak amplitude of control current elicited by GABA alone from the peak amplitude of the current elicited by coapplication of GABA and a drug expressing it as a percentage fraction of the control current. Peak GABA<sub>A</sub> receptor currents (or current enhancement) at various drug concentrations were fitted to a sigmoidal function (Isom, 1975), where

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I = \frac{[\text{max}]}{1 + 10^{\text{EC}_{50} - \log \text{drug}}}
\]

where \(I\) is the GABA<sub>A</sub> receptor current at a given GABA concentration, \(I_{\text{max}}\) is the maximal current, and \(n_{H}\) is the Hill slope. Maximal current and concentration-response curves were obtained after pooling data from all neurons tested for GABA and for all drugs. Concentration-response curves were also obtained from individual neurons for GABA and diazepam. The curve-fitting algorithm minimized the sum of the squares of the actual distance of points from the curve. Convergence was reached when two consecutive iterations changed the sum of squares by less than 0.01%. The curve fit was performed on an IBM-compatible personal computer using Prism 4.0. All data are presented as mean ± S.E.M.

Analysis of Tonic and Synaptic Currents. Even in the absence of synaptic activity, a number of GABA<sub>A</sub> receptors could be opening and closing resulting in a chloride shunt (i.e., tonic current). Two methods were used to measure this current: shifts in the baseline current, and changes in baseline noise. In voltage-clamp mode, the current required to hold a neuron at a certain potential depends on resting conductance of various ion channels and receptors and on the leak conductance. The baseline noise arises from the recording apparatus (machine noise) and opening and closing of ion channels and receptors at any holding potential. When bicuculline, a selective inhibitor of the conductance associated with GABA<sub>A</sub> receptors, is applied, both the baseline current and baseline noise are diminished (Hamann et al., 2002; Stell and Mody, 2002; Yeung et al., 2003). The baseline noise was quantified by measuring the root mean square noise for a 50 ms epoch at consecutive 1000-ms intervals for 30 s using the root mean square analysis routines packaged with the Mini Analysis program (Synaptosoft, Decatur, GA). These 30 values were then averaged to obtain the mean root mean square noise. Root mean square noise was measured only in the absence of phasic synaptic currents; when such events occurred in the 50-ms interval being examined, the trace was advanced in 50-ms blocks until no phasic synaptic currents were evident. Phasic synaptic currents were detected using the Mini Analysis program with the threshold for detection set at three times the baseline root mean square noise. The accuracy of detection was confirmed visually. An event was detected when there was at least a 100-ms interval between complete decay of the previous event and the rise of the next phasic current.

Results

Immunocytochemistry

Hippocampal pyramidal neurons in culture for 14 to 18 days were examined using immunocytochemical methods to compare the distribution of α1, α2, α4, β1, β2/3, and δ subunits with GAD-containing terminals by double-labeling experiments. In initial experiments, pyramidal cells and their processes were filled with biocytin during electrophysiological recording and visualized with fluorescent dye after fixation. Large- and small-diameter dendrites emerge from the apex and base of pyramidal neurons and branch into secondary and tertiary dendrites. The dendritic arbor of pyramidal neurons extended several hundred microns from the cell soma (Fig. 1A). Nonpyramidal interneurons were morphologically distinct bipolar or multipolar neurons whose cell soma was intensely stained for GAD65. To mark synapses, cells were stained with antibody against synaptophysin (Fig. 1B), a commonly used synaptic marker (Rao et al., 2000). Synapses were present on the soma and dendrites of pyramidal neurons. To specifically determine that antibodies against GAD65 labeled GABAergic synapses, we double-labeled cultures for synaptophysin and GAD65.

GAD65-containing terminals were seen to extend from GAD-positive interneurons to the cell soma and dendrites of pyramidal neurons. The pyramidal cell soma and proximal dendrites were densely innervated, and many secondary and tertiary dendrites were also innervated, but others did not demonstrate any GAD-containing terminals. The GAD immunoreactivity was demonstrated as puncta and was distributed in a circle around the cell soma, extending in radial and branching arrays over the processes (Fig. 2B). As demonstrated in Fig. 1, C through E, GAD65 clusters colocallized with synaptophysin immunoreactivity clusters, suggesting that GAD65 clusters were present at GABAergic synapses. There are two GAD isoforms: GAD65 and GAD67; GAD65 is believed to be concentrated in GABAergic synaptic terminals, whereas GAD67 is believed to be present in the cell soma (Dupuy and Houser, 1996). We used two different antibodies against GAD, one specifically directed against GAD65, and the other directed against both GAD65 and GAD67. As shown in Fig. 1, F, G, and H, both antibodies detected the same set of terminals.

A Comparison of the Distribution of α2 and δ Subunits

The subcellular distributions of the α2 and δ subunits were compared with that of GAD65 immunoreactivity in double-
Fig. 1. GABAergic synapses on pyramidal neurons in culture for 14 to 18 days. A, a visually identified pyramidal cell filled with biocytin during electrophysiological recordings and stained with 7-amino-4-methylcoumarin-3-acetic acid-coupled streptavidin. The neuron was visualized with an ultraviolet light filter. B, a pyramidal neuron stained with antibody against synaptic marker synaptophysin to demonstrate the distribution of synapses on a pyramidal neuron. Scale bar for A and B, 20 μm. All subsequent images were visualized with a 40× objective lens. Scale bar in C, 10 μm. Dendrites of a pyramidal neuron stained for synaptophysin (C) and GAD65 (D), and the two images were superimposed (E) to demonstrate colocalization of GAD65 and synaptophysin immunoreactivity clusters. Dendrites of a pyramidal neuron stained for GAD65/67 (F) and GAD65 (G), and the two images were superimposed (H) to demonstrate colocalization of GAD65 and GAD65/67 immunoreactivity clusters.
labeled neurons to establish patterns of distribution of synaptic and extrasynaptic receptors in cultured hippocampal neurons. The γ2 subunit is present at GABAergic synapses (Nusser et al., 1998) and is required for the concentration of GABA<sub>α</sub> receptors at synapses (Essrich et al., 1998). In contrast, the δ subunit is exclusively extrasynaptic (Nusser et al., 1998). In cultured pyramidal neurons, the γ2 subunit immunoreactivity was in the form of intense round puncta distributed over the periphery of the soma and dendrites of pyramidal neurons (Fig. 2A). The γ2 immunoreactivity clusters ranged in size from 1 to 2.5 μm<sup>2</sup> (1.43 ± 0.19 μm<sup>2</sup>, 14 neurons, four cultures). The GAD65 immunoreactivity was distinctly punctate, with no staining over the cell body of the pyramidal neurons, as described above. Visual analysis of γ2 and GAD65 puncta suggested that they often localized to the same site as shown in the boxes in Fig. 2, A and B, and expanded in C and D, respectively, where colocalized clusters are marked by arrowheads. In addition to sites of colocalization, there were many γ2 clusters that did not colocalize with GAD65 immunoreactivity; typically these clusters were smaller than clusters that colocalized with GAD. To study colocalization of γ2 and GAD65 clusters on the processes of labeled neurons, 16 images of γ2 and GAD puncta from 4 culture preparations were digitally superimposed by Metamorph software. The colocalization of γ2 subunit clusters with GAD65 clusters was 31%. Thus γ2 subunit clusters were present at GABAergic synapses and in the extrasynaptic compartment. Similar synaptic and extrasynaptic clusters of GABA<sub>α</sub> receptors have been reported extensively in the past (Rao et al., 2000; Scotti and Reuter, 2001; Christie et al., 2002).

The δ subunit immunoreactivity was less intense but present on all cultured pyramidal neurons. Examination of individual pyramidal neurons revealed more intense immunoreactivity distributed over the cell soma and less intense immunoreactivity over the processes of pyramidal neurons. The immunoreactivity was diffusely distributed over most processes of pyramidal neurons, but over some processes, small-diameter (<0.05 μm<sup>2</sup>) puncta could be distinguished from the surrounding, diffuse immunoreactivity (3% of δ puncta colocalized with GAD65 clusters, 13 cells, 4 cultures). Visual analysis of small δ immunoreactivity clusters and GAD colocalization revealed that these did not tend to colocalize with each other (Fig. 2, E and F, magnified in G and H, respectively).

One possible confound with respect to diffusely distributed GABA<sub>α</sub> receptor δ subunits was their surface expression versus intracellular location. The immunostaining protocol used in this study required the permeabilization of the cell membrane before antibody application. As such, it is possible that the staining of δ subunits may have reflected unassembled subunit protein or internally localized receptors. The antibody used for staining the receptor recognizes a surface epitope (amino acids 1–44); therefore, the surface expression of this subunit could be assessed. Cultured neurons were processed for δ subunit immunoreactivity using both permeabilizing and nonpermeabilizing protocols, and the staining patterns were compared. In nonpermeabilized cells, δ immunoreactivity was diffusely but clearly expressed in the cell soma and dendrites (Fig. 3A), although the immunoreactivity was less intense than that for the cell shown in Fig. 3B, which was processed identically with an additional

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**Fig. 2.** A comparison of γ2 and δ GABA<sub>α</sub> receptor subunit immunoreactivity and their colocalization with GAD65. A, γ2 immunoreactivity was present as widespread large clusters on the cell soma and dendritic arbor, whereas in B, GAD65 immunoreactivity surrounded the cell soma and was present over processes; γ2 and GAD65 immunoreactivity were often colocalized (see arrowheads in C and D); the boxed areas in A and B are magnified in C and D, respectively. E, δ subunit immunoreactivity was diffusely and intensely distributed over the cell soma and less intensely over the processes. The same neuron showed extensive and marked GAD65 immunoreactivity (F). Even when δ subunit immunoreactivity was clustered, it did not colocalize with GAD65 immunoreactivity, see boxed areas in E and F magnified in G and H, respectively.
The α Subunits

α1 Immunoreactivity was not well developed on pyramidal neurons in culture for 14 to 18 days; it was most prominent on the soma and dendrites of interneurons, as described by other laboratories (Fritschy and Brunig, 2003). However, occasional α1 subunit staining was present on some pyramidal neurons. To quantify the rate of α1 subunit expression in these neurons, 56 pyramidal neurons from 5 separate cultures were examined; the α1 subunit was expressed in six neurons, whereas the remaining neurons showed nonspecific, low-level staining. In these six pyramidal neurons, immunoreactivity was in the form of intense round puncta (1.61 ± 0.13 µm²) distributed over the periphery of the soma and dendrites of pyramidal neurons (Fig. 4A) in a manner quite similar to that of the γ2 subunit distribution. GAD65 immunoreactivity showed a similar distribution (Fig. 4B).

The β Subunits

β1 Subunit. The β1 immunoreactivity was present over the cell soma and the processes (Fig. 5A), a pattern similar to that of the δ subunit (Fig. 2E). Clusters of β1 immunoreactivity were small (1.0 ± 0.15 µm², 29 neurons, 5 preparations), less intense, and less distinct from the surrounding immunoreactivity (Fig. 4E). The clusters of β2 immunoreactivity were present on the soma, proximal, and distal dendrites of pyramidal neurons, some of which colocalized with GAD65 immunoreactivity (Fig. 4, E–H). In 29 pyramidal cells from 5 cultures, 22% of α2 puncta colocalized with GAD65 clusters. This suggested that the α2 subunit clusters were present at GABAergic synapses.

α4 Subunit. The α4 subunit immunoreactivity was diffusely distributed over the soma and dendrites of pyramidal neurons (Fig. 4I), a pattern similar to that of the δ subunit (Fig. 2E). Clusters of α4 immunoreactivity were rare and showed little obvious colocalization with GAD65 puncta (Fig. 4, K and L). Only 2% of α4 clusters colocalized with GAD65 clusters (14 cells, 4 cultures). The antibody used for staining the receptor recognizes a surface epitope (amino acids 1–14); therefore, the surface expression of this subunit could be assessed. Cultured neurons were processed for α4 subunit immunoreactivity using both permeabilizing and nonpermeabilizing protocols, and the staining patterns were similar (Fig. 3, C and D).

β2/3 Subunit. The antibody used for this study recognizes an epitope on both β2 and β3 subunits, so the findings refer to both subunits. β2/3 subunit immunoreactivity was marked, distinct puncta (1.57 ± 0.083 µm², 25 cells, 5 preparations) on both the soma and processes (Fig. 5E) and was often colocalized with GAD65 (Fig. 5F, arrowheads in G and H). In 25 cells from 5 preparations, 58% of β2/3-positive clusters were colocalized with GAD65 immunoreactivity in nonpermeabilized neurons and neurons exposed to 0.1% Triton X-100 before primary antibody application. δ and α4 subunit immunoreactivity was diffusely expressed in both nonpermeabilized (A and C, respectively) and permeabilized (B and D, respectively) neurons.
with GAD65 clusters. The β2/3-subunit immunoreactivity clusters were also present extrasynaptically within the cell body and along the dendrites (Fig. 5, E–H).

β1 and β2/3 subunits were also compared directly by staining the cultures for both of these subunits. The diffusely distributed β1 subunit did not colocalize with β2/3 subunit clusters. This suggested that β1 and β2/3 subunits were present on the same cell but were usually found in different GABA_A receptors (Fig. 5, I–L).

GABA_A Receptor Currents Recorded from Cultured Hippocampal Pyramidal Neurons

The expression of GABA_A receptor subunits on cultured neurons was further characterized by evaluating the properties of whole-cell GABA_A receptor currents as well as tonic and phasic GABA_A receptor currents elicited from these neurons. The membrane properties, input resistance, and action-potential characteristics of pyramidal neurons in culture for 14 to 18 days were similar to those reported in the past (Mangan and Kapur, 2004). When GABA was applied to cultured pyramidal neurons voltage-clamped to −50 mV, using internal and external solutions with symmetric concentration of chloride ions, an inward current was recorded. Multiple concentrations of GABA, ranging from 0.1 to 1000 μM, with recovery intervals of at least 3 min, were applied to each cell. Low concentrations evoked a slowly activating and decaying current, and higher concentrations evoked faster rising currents with a distinct peak and slower decay. GABA concentration-response data were obtained from individual pyramidal cells by measuring peak currents evoked by GABA. GABA concentration-response data were fitted to an equation for a sigmoidal curve, and the equation for the best fit revealed an \( EC_{50} \) value of 10.7 ± 1.3 μM (\( n = 9 \)), and the maximal evoked current was 1392 ± 216 pA (\( n = 9 \)). Recombinant receptors containing the δ subunits have a high affinity for GABA (\( EC_{50} < 0.3–3 \) μM), whereas γ2 subunit-con-
taining receptors have a lower affinity for GABA (EC_{50} in 10–30 μM) (Saxena and Macdonald, 1996; Wafford et al., 1996). Thus, the EC_{50} of whole-cell GABA\_A receptor currents recorded from pyramidal neurons was closer to that of γ2 subunit-containing GABA\_A receptor than the δ subunit-containing recombinant GABA\_A receptors.

**Diazepam Sensitivity**

Benzodiazepines act on an allosteric site on GABA\_A receptors and enhance γ2 subunit-containing receptors but not δ subunit-containing receptors. In six pyramidal cells, diazepam (1–1000 nM) was coapplied with 3 μM GABA alternating with 3 μM GABA. Diazepam (3–1000 nM) uniformly enhanced GABA\_A receptor currents in a concentration-dependent fashion (Fig. 6A) in all six neurons. The concentration-response data from these cells were pooled (Fig. 6C) and fitted to an equation for sigmoidal function, which demonstrated that diazepam caused maximal enhancement of 71.5 ± 7.0% with an EC_{50} value of 46.9 nM (Fig. 6C). We further characterized cell-to-cell variability by fitting the data from individual neurons (Fig. 6B). The EC_{50} values ranged from 14.1 to 45.0 nM (median, 34.9 nM). Currents recorded from recombinant GABA\_A receptors containing any α1, -2, -3, or -5 subunit and a γ2 subunit are highly sensitive to diazepam, whereas those containing α4 and δ subunits are insensitive to micromolar concentrations of diazepam. None of the cells expressed purely diazepam-insensitive GABA\_A receptor currents. In addition, the EC_{50} of the diazepam effect (14–45 nM) was close to that of recombinant GABA\_A receptors containing the γ2 subunit (1–10 nM). These studies were consistent with the immunocytochemical finding that receptors containing α2 (or α1) and γ2 subunits were expressed on cultured pyramidal neurons.

**Allopregnanolone Sensitivity**

The immunocytochemical studies suggested that cultured pyramidal cells express α4 and δ subunit-containing recep-

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**Fig. 5.** Distribution of β1 and β2/3 GABA\_A receptor subunits immunoreactivity and their colocalization with GAD65 immunoreactivity. A, β1 immunoreactivity was diffuse with no evident puncta, whereas in B, GAD65 showed significant clustering on the soma and dendritic tree of the same cell. No colocalization between β1 and GAD65 immunoreactivity was evident, as shown in boxed areas in A and B, magnified in C and D, respectively. E, clusters of mouse anti-β2/3 immunoreactivity were evident on the soma and dendrites, which overlapped with rabbit anti-GAD65 immunoreactivity (F) as shown in boxed areas in E and F, magnified in G and H, respectively. Little colocalization was evident between β1 (I) and β2/3 (J) subunits (boxed areas in I and J, magnified in K and L, respectively).
tors; however, GABA and diazepam sensitivity of whole-cell currents did not directly suggest expression of these subunits. Because the δ subunit confers high affinity for neurosteroids on GABA<sub>6</sub> receptors, we tested the neurosteroid sensitivity of the whole-cell currents recorded from these neurons. A low concentration of allopregnanolone (10 nM) enhanced GABA<sub>6</sub> receptor currents in all eight pyramidal cells tested. The mean enhancement in eight cells was 29.9 ± 3.7% (P < 0.001, paired t test). Significant enhancement of GABA<sub>6</sub> receptor currents by a low concentration of allopregnanolone supported the immunocytochemical evidence of the expression of the δ subunit on cultured pyramidal neurons. However, the δ subunit is not essential for neurosteroid sensitivity, and receptors lacking this subunit are also modulated by neurosteroids.

**Tonic Currents in Cultured Pyramidal Neurons**

One limitation of studying the pharmacology of whole-cell currents is that all receptors expressed on the cell surface are activated by exogenous application of GABA and its modulators. In this way, whole-cell currents are dominated by low GABA-sensitive and highly diazepam-sensitive subunits, but the pharmacology does not rule out a component that is highly GABA-sensitive and diazepam-insensitive. To specifically detect δ subunit-containing receptors, we studied the properties of GABA<sub>6</sub> receptor-mediated tonic inhibition in cultured pyramidal neurons. Several studies have suggested that the δ subunit-containing receptors can mediate tonic inhibition (Hamann et al., 2002; Semyanov et al., 2004). Tonic inhibition results from persistent activation of extrasynaptic GABA<sub>6</sub> receptors by low micromolar concentrations of ambient GABA. The extrasynaptic receptors composed of α6 and δ subunits mediate tonic inhibition in cerebellar granule cells. The α6 subunit-containing receptors have a high affinity for GABA and thus can respond to low ambient concentrations of GABA. The δ subunit-containing receptors desensitize slowly, thereby remaining open for long periods of time in the presence of the neurotransmitter (Haas and Macdonald, 1999).

sIPSCs were recorded from pyramidal neurons by blocking excitatory neurotransmission with 6-cyano-7-nitroquinolin-2-3-dione and 2-amino-5-phosphonovaleric acid (Fig. 7) and the competitive GABA<sub>6</sub> receptor antagonist bicuculline (5 μM) was applied, which caused a reduction of noise and shift in baseline current (Fig. 7, top). It also abolished sIPSCs demonstrating that both tonic and synaptic inhibition were mediated by GABA<sub>6</sub> receptors.

To measure the tonic currents, the effect of bicuculline on baseline current and noise was analyzed. In the trace shown in Fig. 7, baseline noise consisted of large fluctuations before drug application; after bicuculline application, the baseline current decreased to approximately 40 pA with smaller fluctuations (Fig. 7, bottom). In recordings from five cells, the fluctuations were analyzed by determining the root mean square of the noise measured before and after bicuculline addition. The noise was measured over a 50-ms interval every 1000 ms for 30 s, and the synaptic (phasic) events were manually excluded. In each of the five cells, the root mean square noise reduction was significant (P < 0.0001, two-tailed t test). When data from these cells were pooled together, bicuculline addition decreased root mean square noise from 5.34 ± 0.07 to 4.21 ± 0.02 pA.

![Fig. 6.](https://example.com/fig6.png) Diazepam enhanced whole-cell GABA<sub>6</sub> receptor-mediated currents. A, augmentation of whole-cell currents evoked by 3 μM GABA directly coapplied with several concentrations of diazepam. B, concentration-response profiles for six cells exposed to 3 μM GABA coapplied with multiple concentrations of diazepam (1–1000 nM). EC<sub>50</sub> values for diazepam enhancement of GABAergic currents encompassed a narrow range; therefore individual responses were combined (C).

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For a full description of the figure and its content, please refer to the original publication.
Diazepam and Zn²⁺ Modulation of Tonic Currents

The pharmacology of GABA_α receptors underlying tonic GABA current was investigated initially by application of diazepam (30–50 nM, which is the approximate EC₅₀ value for enhancement of whole-cell GABA currents). Diazepam (30 nM) did not alter baseline current in any of the pyramidal cells examined (Fig. 8). The result was further confirmed using 50 nM diazepam in six pyramidal cells. The root mean square noise was also not altered significantly by diazepam (50 nM) application (5.22 ± 0.19 pA versus 5.12 ± 0.22 pA, t test). However, sIPSCs were enhanced by diazepam treatment; τ_decay increased from 36.9 ± 0.9 ms in untreated cells (111 sIPSCs with peak amplitudes from 40–60 pA) to 48.7 ± 1.2 ms after at least 2-min exposure to diazepam (91 events; P < 0.001, t test). This result suggested that GABA_α receptors mediating tonic currents contained the α4 or δ subunit or both; synaptic GABA_α receptors contained the α1, -2, -3, or -5 subunits and the γ2 subunit.

Application of 60 μM Zn²⁺ caused a significant reduction in tonic current, whereas spontaneous synaptic currents remained but with modified frequency and amplitude (Fig. 9). Baseline current (holding potential, −50 mV) declined from 134 ± 23 to 71 ± 9 pA after Zn²⁺ addition (n = 5; P < 0.001, t test). Root mean square noise reduction ranged from 7.1 to 22.0% in these five cells and was significant in each neuron (mean root mean square noise reduced from 6.21 ± 0.15 to 5.28 ± 0.2 pA; P = 0.006, paired t test). sIPSC frequency decreased from 3.4 ± 0.7 to 0.8 ± 0.3 Hz, whereas mean sIPSC amplitude decreased from 68.8 ± 9.0 to 44.9 ± 2.3 pA. Comparison of sIPSCs of similar amplitudes (50–60 pA) before and after Zn²⁺ exposure revealed no significant change in τ_decay (32.7 ± 1.2 versus 35.2 ± 1.8 ms, respectively). One explanation for Zn²⁺ inhibition of tonic currents in cultured pyramidal neurons was that δ subunit-containing receptors mediated these currents because recombinant receptors containing this subunit are highly sensitive to Zn²⁺. However, Zn²⁺ also inhibited GABA release (as demonstrated by diminished IPSC frequency), which could diminish ambient GABA levels and thus diminish tonic inhibition.

Sensitivity to Furosemide and Loreclezole

We further examined properties of tonic currents by using furosemide, an antagonist of GABA-evoked currents in α4 subunit-containing recombinant receptors (Wafford et al., 1996). Application of furosemide (100 μM) affected the baseline GABA current, reducing it from 127 ± 14 to 87 ± 9 pA (P < 0.05, t test, n = 5) (Fig. 10A). Root mean square noise reduction was significant in four of the five cells; in the fifth cell, noise reduction (from 5.52 ± 0.04 to 5.45 ± 0.05 pA) was not significant (P = 0.14). The sIPSC kinetics (τ_decay 34.3 ± 1.2 versus 37.2 ± 2.8 ms after furosemide) and frequency were unaffected by furosemide.

Loreclezole is an anticonvulsant known to enhance β2 or β3 subunit-containing receptors but not those containing β1. In addition, it can inhibit β1 subunit-containing GABA_α receptors by accelerating desensitization. We tested the loreclezole sensitivity of tonic currents to determine whether these currents were enhanced or inhibited by this drug. In six cultured neurons tested, loreclezole (30 μM) did not have a consistent action. In three cells, baseline current was decreased significantly from 124 ± 12 to 91 ± 11 pA (Fig. 10B); in three other cells, no significant change was observed. In the three cells in which loreclezole reduced baseline current, root mean square noise was reduced significantly from 5.64 ± 0.07 to 5.16 ± 0.04 pA (P < 0.001). In the three remaining cells, root mean square noise reduction was not significant (from 5.48 ± 0.13 to 5.377 ± 0.13 pA). No change was observed in sIPSC frequency or decay kinetics regardless

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**Fig. 7.** Tonic and phasic GABAergic currents recorded from cultured neurons. Currents consisted of a tonic background current on which were superimposed discrete phasic sIPSCs (left expanded trace). Application of the GABA_α receptor antagonist bicuculline decreased the tonic current and abolished phasic currents (right expanded trace). Tonic currents fluctuated 10 to 20 pA around the mean value before bicuculline addition; current fluctuations were eliminated after tonic current reduction by bicuculline (current-time plot). Each point in the current-time plot represents the mean tonic current (between sIPSCs) over a 5-ms duration (20 kHz, acquisition frequency) taken every 100 ms (first 15 s of plot) or 500 ms (remainder of plot). The higher frequency of data points expressed in the first 15 s illustrates the extent of tonic current variability.
of loreclezole's effect on the tonic inhibitory current. It should be noted that differing loreclezole effects were produced in cells from the same culture source.

Discussion

A combination of immunocytochemical and electrophysiological studies suggested that cultured hippocampal neurons expressed two types of GABA_A receptors. One type of receptors formed clusters present at GABAergic synapses and in extrasynaptic membrane. The α1, α2, β2/3, and γ2 subunits were present in these clustered receptors. The GABA and diazepam sensitivity of whole-cell currents and diazepam sensitivity of synaptic currents could be best explained by the expression of receptors containing α1 or α2 and γ2 subunits. The second type of receptor was diffusely distributed in the extrasynaptic membrane. The α4, β1, and δ subunits were contained in these receptors. The properties of tonic currents recorded from these neurons were similar to recombinant receptors containing α4, β1, and δ subunits. These studies add to the growing evidence that GABA_A receptors segregate between synaptic and extrasynaptic sites in neurons.

Diffusely Dispersed GABA_A Receptors Containing α4, β1, and δ Subunit and the Properties of Tonic Currents. The present study found that the δ subunit was diffusely distributed on the surface of cultured hippocampal neurons and that it was not concentrated at synapses. The distribution of the δ subunit has been studied extensively in brain slices previously by means of light and electron microscopy (Sperk et al., 1997; Peng et al., 2002). In adult brain, light microscopic studies revealed that this subunit was expressed in cerebellar granule cells, thalamus, hippocampal granule cells, and in CA3 and CA1 pyramidal neurons in the hippocampus. Electron microscopic studies of cerebellar granule cells suggested that the δ subunit was expressed exclusively in theextrasynaptic membrane (Nusser et al., 1998). Likewise, a recent electron microscopic study of hippocampal dentate granule cells demonstrated perisynaptic localization of the δ subunit (Peng et al., 2002).

Separate electrophysiological experiments further sup-

Fig. 8. Diazepam modulation of tonic and phasic GABAergic currents. Diazepam (30 nM) did not alter the level of tonic inhibition but did alter sIPSC kinetics (see text). The expanded traces show tonic and phasic currents before and after diazepam application.

Fig. 9. Zn²⁺ modulation of tonic and phasic GABAergic currents. Zn²⁺ (60 μM) decreased the tonic current and decreased sIPSC frequency but did not alter sIPSC kinetics (see text). The expanded traces show tonic and phasic currents before and after tonic current reduction.
ported the notion that the δ subunit was expressed on cultured hippocampal neurons. Enhancement of whole-cell GABA<sub>4</sub> receptor currents by 10 nM allopregnanolone suggested high sensitivity to neurosteroids, a property of δ subunit-containing GABA<sub>4</sub> receptors (Wohlfarth et al., 2002). Because δ subunit-containing receptors desensitize very slowly (Saxena and Macdonald, 1996; Haas and Macdonald, 1999), GABA<sub>4</sub> receptors containing this subunit are ideally suited for mediating tonic current, which could be recorded from these neurons. However, other subunits, such as the α5 subunit, may mediate tonic inhibition in pyramidal neurons (Caraiscos et al., 2004); therefore, we tested the pharmacological properties of tonic inhibition in these neurons. Tonic currents were not enhanced by 30 and 50 nM diazepam, whereas these concentrations enhanced whole-cell currents and IPSCs, further suggesting that δ subunit-containing receptors were present in extrasynaptic membrane. Moreover, Zn<sup>2+</sup> inhibited tonic currents, providing additional evidence that δ subunit-containing receptors mediated the tonic inhibition observed in these cells.

Furosemide reduction of tonic GABA currents suggested participation of α4 subunit-containing GABA<sub>4</sub> receptors in mediating tonic currents. Furosemide is known to inhibit α4 subunit-containing receptors and was demonstrated to inhibit GABA<sub>4</sub> receptor currents in dentate granule cells, which express the α4 subunit (Wafford et al., 1996; Kapur and Macdonald, 1999). Likewise, α4 subunit-containing GABA<sub>4</sub> receptors have a higher affinity for GABA than those containing the α1 subunit. It is believed that this property allows low ambient GABA levels to activate extrasynaptic receptors.

The current study did not investigate coassembly or colocalization of α4 and δ subunits and, therefore, cannot address the issue of receptor subunit composition directly. However, the distribution of these two subunits was similarly diffuse, without puncta, and the immunoreactivity did not colocalize with GAD65 clusters. Immunoprecipitation studies have demonstrated that the α4 subunit often coassembles with the δ subunit (Sur et al., 1999). In the forebrain, the distribution of α4 subunit mRNA is similar to that of the δ subunit (Wisden et al., 1992). In addition, the expression of the α4

![Fig. 10. Furosemide and loreclezole modulation of tonic currents. A, application of furosemide (100 μM) caused a decrease in tonic inhibition but did not alter the frequency or kinetics of synaptic inhibitory currents. Expanded traces show currents before and after furosemide addition. B, in this example, loreclezole (30 μM) caused a reduction in the tonic current; however, this result was obtained in only three of six cells examined. Expanded traces show currents before and after loreclezole application.](https://morphem.aspetjournals.org/doi/pdf/10.1124/mol.105.078950)
subunit is diminished in δ subunit knockout mice (Peng et al., 2002).

The distribution of β1 subunit was similar to that of α4 and δ subunits, suggesting an extrasynaptic distribution. The immunocytochemical studies were further supported by electrophysiological studies. Loreclezole is an anticonvulsant that enhances peak GABA_A receptor currents elicited by subsaturating concentrations of GABA by acting at an allosteric regulatory site on β2 and β3 subunits (Wafford et al., 1994; Fisher and Macdonald, 1997). Peak currents elicited from receptors containing the β1 subunit were not enhanced by loreclezole because the receptors lack the positive modulatory site present on the β2 and β3 subunits. In addition to the potentiation of peak currents, loreclezole inhibited steady-state GABA_A receptor currents by acting at a site distinct from the positive modulatory site on β2/3 subunits and increased the apparent desensitization. This inhibitory action of loreclezole occurred regardless of the β subunit. Thus, GABA_A receptors containing the β1 subunit may be inhibited by loreclezole or stay unaffected by it. In the current study, loreclezole inhibited tonic currents in three neurons, and in the remaining three neurons, it had no effect on these currents. These effects would be consistent with the expression of β1 subunit in receptors mediating tonic currents.

The extent of tonic current reduction with zinc and furosemide, although significant, was less than that achieved with bicuculline, suggesting that receptor isoforms containing subunits unaffected by these agents also contribute to tonic GABA currents. It is possible that α5 subunit-containing GABA_A receptors also mediate tonic inhibition in pyramidal cells, in addition to α4, δ subunit-containing receptors. Tonic currents have been reported previously in mouse embryonic cultures of pyramidal neurons, but these currents were found to be sensitive to the benzodiazepine, midazolam (Bai et al., 2001; Yeung et al., 2003). In addition, it was reported recently that α5 subunit-containing GABA_A receptors mediate tonic inhibition in CA1 pyramidal neurons in mice (Caraiscos et al., 2004). The subcellular distribution of the α5 subunit was not investigated in detail in the current study; however, in preliminary studies (data not shown), it was diffusely distributed over the membrane but also formed a few discrete clusters that colocalized with GAD65.

Clustered Receptors Containing α1, α2, β2/3, and γ2 Subunits. α2, β2/3, and γ2 subunit immunoreactivity clusters were present on all cultured pyramidal cells as demonstrated in many studies in the past (Essrich et al., 1998; Brunig et al., 2002a, b; Christie et al., 2002). Previous studies on the localization of GABA_A receptor subunits in rat brain have demonstrated that α1, α2, and α5 are present in the CA1 and CA3 subfields of the hippocampus, a finding confirmed by the immunofluorescent labeling results reported here. Consistent with previous reports, clusters of α2, α1, β2/3, and γ2 subunit clusters were found to colocalize with GAD65 and suggested that these subunits are present at synapses (Brunig et al., 2002a; Christie et al., 2002). Postembedding immunoelectron microscopic studies in the hippocampus and cerebellum further confirm that these subunits are present at synapses (Nusser et al., 1995, 1998). Electrophysiological studies demonstrating modulation of whole-cell currents and synaptic currents further supported the expression of α2 and γ2 subunits in cultured neurons. Synaptic currents and whole-cell currents were modulated by diazepam and moderately sensitive to Zn^{2+}, a combination of properties probably conferred by the α2 and γ2 subunits (Sieghart and Sperk, 2002).

Extrasynaptic GABA_A receptors containing α2, β2/3, and γ2 subunits found in this study are well described in hippocampal neuronal cultures. The relationship of extrasynaptic clusters to synaptogenesis was explored and suggested that small clusters of GABA_A receptors could be generated without any synaptic input (Scotti and Reuter, 2001) or caused by signals from glutamatergic input (Rao et al., 2000; Christie et al., 2002). In addition to the extrasynaptic membrane receptors, small extrasynaptic clusters may represent a pool of receptors endocytosed for recycling or degradation. In addition to accumulating at synapses, the surface receptors also aggregate in clathrin-coated pits, which invaginate during the process of endocytosis (Barnes, 2000; Ktittler et al., 2000). Another explanation for the lack of apposition of GABA_A receptor clusters to GAD65 terminals is that these are present at glutamatergic synapses. In cultured hippocampal neurons, clusters of receptors containing these subunits occur at glutamatergic synapses (Rao et al., 2000; Christie et al., 2002).

The physiological significance of extrasynaptic clusters of GABA_A receptors containing α2, β2/3, and γ2 subunits remains unclear. Tonic currents, which are believed to be mediated by extrasynaptic receptors, were not sensitive to diazepam but were sensitive to modulation by furosemide, Zn^{2+}, and loreclezole, properties not consistent with GABA_A receptors containing α2, β2/3, and γ2 subunits. GABA_A receptors containing these subunits are known to desensitize rapidly, and it is possible that these receptors remain desensitized. In excised extrasynaptic patches of cerebellum exposed to a desensitizing GABA concentration, three distinct conductance levels were evident, suggesting multiple GABA_A receptor isoforms (Bickley et al., 1999). It is possible that similar studies on cultured pyramidal neurons would also reveal multiple types of GABA_A receptors on extrasynaptic membrane. In summary, this study adds to the growing evidence that GABA_A receptors are segregated between synaptic and extrasynaptic sites in neurons.

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