

# Cultured Hippocampal Pyramidal Neurons Express Two Kinds of GABA<sub>A</sub> Receptors

Patrick S. Mangan, Chengsan Sun, Mackenzie Carpenter, Howard P. Goodkin, Werner Sieghart, and Jaideep Kapur

*Department of Neurology, University of Virginia, School of Medicine, Charlottesville, Virginia, (P.S.M., C.S., H.P.G., J.K.); University of Virginia, School of Medicine, Charlottesville, Virginia (M.C.); Brain Research Institute of the Medical University Vienna, Division of Biochemistry, and Molecular Biology, Vienna, Austria (W.S.)*

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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 GABA<sub>A</sub> receptor with an electrophysiological analysis of GABA<sub>A</sub> 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 synaptic marker GAD. Whole-cell GABA<sub>A</sub> 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 Zn<sup>2+</sup>, furosemide, and loreclezole; they were insensitive to diazepam. These studies suggest that two kinds of GABA<sub>A</sub> receptors are expressed on cultured hippocampal neurons. One kind of receptor formed clusters, which were present at GABAergic 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.

GABA<sub>A</sub> 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 ( $\alpha 1-6$ ,  $\beta 1-3$ , and  $\gamma 1-3$ ) (Sieghart and Sperk, 2002). Different GABA<sub>A</sub> 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 GABA<sub>A</sub> 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 GABA<sub>A</sub> 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 GABA<sub>A</sub> 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 GABA<sub>A</sub> 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

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**ABBREVIATIONS:** HBS, HEPES-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  $\delta$  subunit is expressed in extrasynaptic membrane, and the  $\alpha 6$  subunit commonly associates with it. It is known that in the forebrain, the  $\delta$  subunit commonly coassembles with the  $\alpha 4$  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  $\delta$  subunit-containing receptors with  $\gamma 2$  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  $\alpha 1$ ,  $\alpha 2$ ,  $\alpha 4$ ,  $\beta 1$ ,  $\beta 2/3$ ,  $\gamma 2$ , and  $\delta$  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 centrifuge tube, and HBS was replaced with 5 ml of 0.25% trypsin before incubation 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 hippocampal neurons were fully suspended. After suspension, neurons were counted on a hemocytometer, and 10,000 neurons were plated on each prepared coverslip in fresh MEM and horse serum.

After 3 to 4 h, coverslips were placed neuronal-side down on sterilized longitudinally cut Teflon O rings (3 mm wide, 0.5 mm thick, 22 mm i.d.; Small Parts Inc., Miami, FL), facing the prepared glial cell monolayer in a dish containing serum-free MEM with N2 supplement (Invitrogen, Carlsbad, CA). An additional 1 ml of N2 supplement was added at 10-day intervals. Cultures were kept in the incubator until they were used.

**Immunofluorescence Staining and Antibodies.** The antibodies indicated were used for immunocytochemistry of GABA<sub>A</sub> receptor subunits:  $\alpha 1$  (1–16),  $\alpha 2$  (1–19),  $\alpha 4$  (1–14),  $\beta 1$  (350–404),  $\beta 2/3$ ,  $\gamma 2$  (319–366), and  $\delta$  (1–44), and glutamate decarboxylase (GAD). Antibody against  $\alpha 1$  was from Alomone Labs (Jerusalem, Israel);  $\beta 2/3$  and GAD65 were from Chemicon International (Temecula, CA); antibody against the  $\alpha 2$  subunit was from Santa Cruz Biochemicals (Santa Cruz, CA); and other antibodies were developed in Dr. Sieghart's laboratory. The antibodies to the  $\gamma 2$ ,  $\alpha 4$ ,  $\delta$ , and  $\beta 1$  subunits have been characterized extensively in the past by immunoprecipitation, Western blotting, and immunocytochemistry (Sperk 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- $\alpha 2$  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:  $\alpha 1$ , 3  $\mu\text{g/ml}$ ;  $\alpha 4$ ,  $\beta 2/3$ , and  $\delta$ , 5  $\mu\text{g/ml}$ ;  $\alpha 2$ ,  $\beta 1$ ,  $\gamma 2$ , and mouse anti-GAD65, 2  $\mu\text{g/ml}$ ; 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 (Biomed, 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  $-20^{\circ}\text{C}$  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 TE200 fluorescent microscope (Nikon, Tokyo, Japan) with either a  $40 \times 1.3$  or  $60 \times 1.4$  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- $\mu\text{m}^2$  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 Metamorph, 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 *t* test.

**Photomicrograph Production.** As mentioned above, the brightness and contrast of fluorescent images were adjusted using Metamorph 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.

**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 M $\Omega$ ) 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 osmolarity 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: 153 mM CsCl, 1.0 mM MgCl<sub>2</sub>, 10.0 mM HEPES, and 5.0 mM EGTA, pH 7.3, osmolarity of  $\sim 280$  to 290 mOsm. Recording pipettes also contained an ATP

regeneration system consisting of 50 U/ml creatinine phosphokinase, 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 postsynaptic currents (sIPSCs) and tonic GABAergic current, 50 μM DL-2-amino-5-phosphonopentanoic acid and 20 μM 6-cyano-7-nitroquinoxaline-2,3-dione (Tocris 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 the drug and GABA and 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 using a four-parameter logistic equation (sigmoidal concentration-response) with a variable slope. The equation used to fit the concentration-response relationship was  $I = [I_{\max}/1 + 10^{(\log_{10} C_m - \log_{10} n_H) \times n_H}]$ , where  $I$  is the GABA<sub>A</sub> receptor current at a given GABA concentration,  $I_{\max}$  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 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

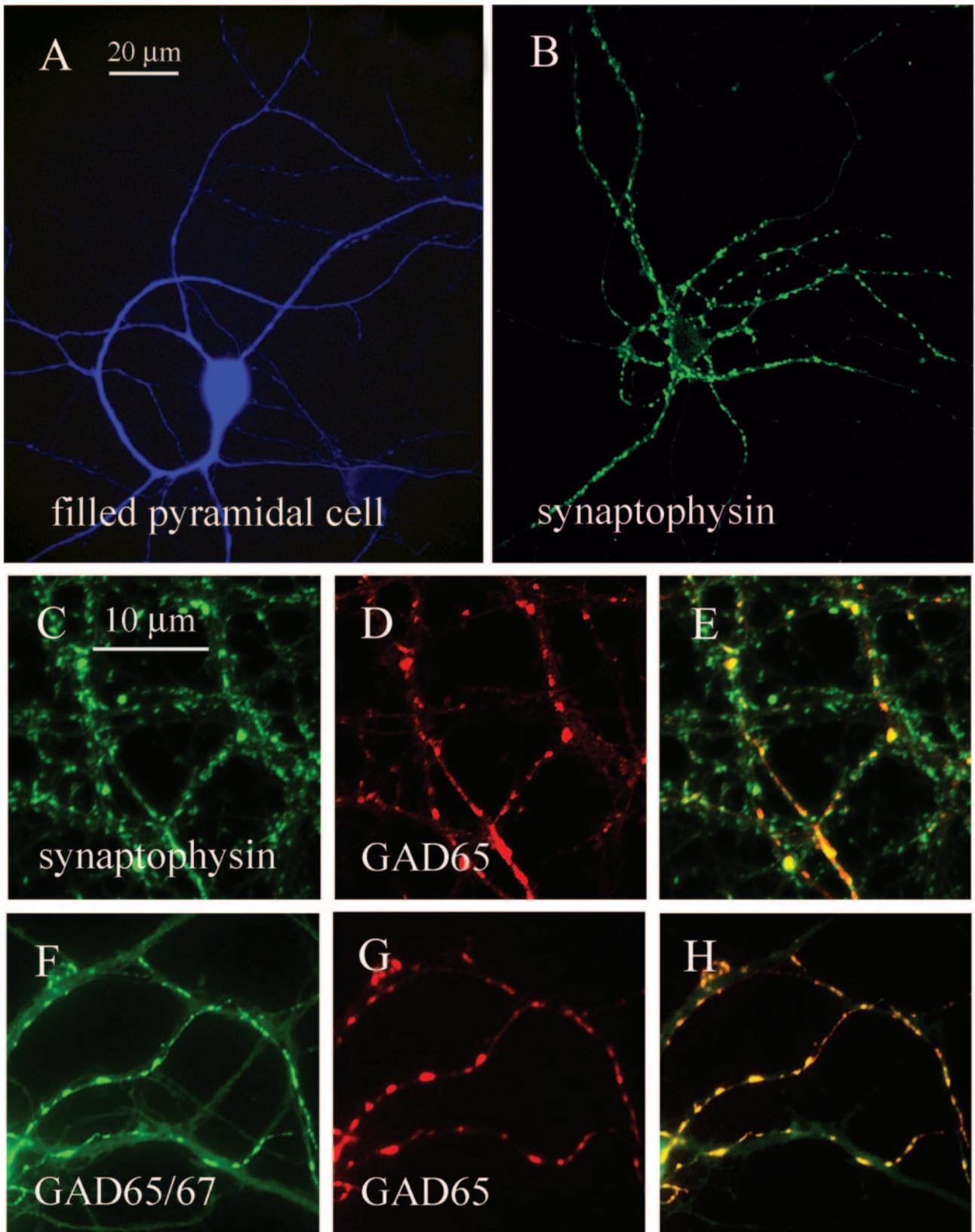
### Immunocytochemistry

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, γ2, 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 colocalized 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 γ2 and δ Subunits

The subcellular distributions of the γ2 and δ subunits were compared with that of GAD65 immunoreactivity in double-

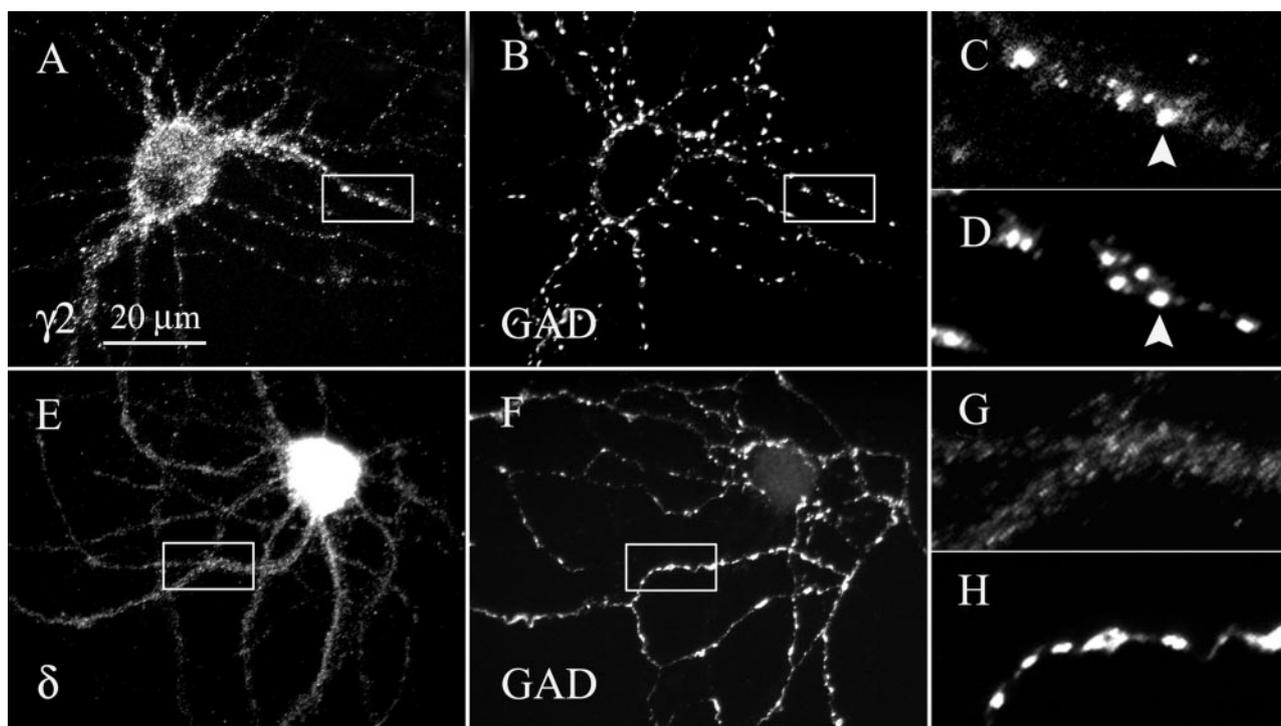


**Fig. 1.** GABAergic synapses on pyramidal neurons in culture for 14 to 18 days. A, a visually identified pyramidal cell filled with biocytin during electrophysiological recordings and stained with 7-amino-4-methylcoumarin-3-acetic acid-coupled streptavidin. The neuron was visualized with an ultraviolet 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  $\mu\text{m}$ . All subsequent images were visualized with a 40 $\times$  objective lens. Scale bar in C, 10  $\mu\text{m}$ . 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  $\gamma 2$  subunit is present at GABAergic synapses (Nusser et al., 1998) and is required for the concentration of GABA<sub>A</sub> receptors at synapses (Essrich et al., 1998). In contrast, the  $\delta$  subunit is exclusively extrasynaptic (Nusser et al., 1998). In cultured pyramidal neurons, the  $\gamma 2$  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  $\gamma 2$  immunoreactivity clusters ranged in size from 1 to 2.5  $\mu\text{m}^2$  ( $1.43 \pm 0.19 \mu\text{m}^2$ , 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  $\gamma 2$  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  $\gamma 2$  clusters that did not colocalize with GAD65 immunoreactivity; typically these clusters were smaller than clusters that colocalized with GAD. To study colocalization of  $\gamma 2$  and GAD65 clusters on the processes of labeled neurons, 16 images of  $\gamma 2$  and GAD puncta from 4 culture preparations were digitally superimposed by Metamorph software. The colocalization of  $\gamma 2$  subunit clusters with GAD65 clusters was 31%. Thus  $\gamma 2$  subunit clusters were present at GABAergic synapses and in the extrasynaptic compartment. Similar synaptic and extrasynaptic clusters of GABA<sub>A</sub> receptors have been reported extensively in the past (Rao et al., 2000; Scotti and Reuter, 2001; Christie et al., 2002).

The  $\delta$  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 ( $<0.05 \mu\text{m}^2$ ) puncta could be distinguished from the surrounding, diffuse immunoreactivity (3% of  $\delta$  puncta colocalized with GAD65 clusters, 13 cells, 4 cultures). Visual analysis of small  $\delta$  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>A</sub> receptor  $\delta$  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  $\delta$  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  $\delta$  subunit immunoreactivity using both permeabilizing and nonpermeabilizing protocols, and the staining patterns were compared. In nonpermeabilized cells,  $\delta$  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



**Fig. 2.** A comparison of  $\gamma 2$  and  $\delta$  GABA<sub>A</sub> receptor subunit immunoreactivity and their colocalization with GAD65. A,  $\gamma 2$  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;  $\gamma 2$  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,  $\delta$  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  $\delta$  subunit immunoreactivity was clustered, it did not colocalize with GAD65 immunoreactivity, see boxed areas in E and F magnified in G and H, respectively.

step of a 10-min exposure to 0.1% Triton X-100. The immunostaining pattern in these cells was similar regardless of permeabilization, demonstrating that the  $\delta$  subunits were expressed on the cell surface.

### The $\alpha$ Subunits

**$\alpha 1$  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  $\alpha 1$  subunit staining was present on some pyramidal neurons. To quantify the rate of  $\alpha 1$  subunit expression in these neurons, 56 pyramidal neurons from 5 separate cultures were examined; the  $\alpha 1$  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 ( $1.61 \pm 0.13 \mu\text{m}^2$ ) distributed over the periphery of the soma and dendrites of pyramidal neurons (Fig. 4A) in a manner quite similar to that of the  $\gamma 2$  subunit distribution. GAD65 immunoreactivity showed a similar distribution (Fig. 4B). Closer examination of processes of labeled neurons revealed that  $\alpha 1$  and GAD65 puncta localized to the same site (arrowheads in Fig. 4, C and D; boxes in A and B are expanded in C and D, respectively). Quantification of double-labeled neurons showed that 32% of  $\alpha 1$  puncta colocalized with GAD65 immunoreactivity.

**$\alpha 2$  Subunit.** In contrast to the  $\alpha 1$  subunit, all pyramidal neurons in culture for 14 days expressed the  $\alpha 2$  subunit immunoreactivity. The clusters of  $\alpha 2$  immunoreactivity were small ( $1.0 \pm 0.15 \mu\text{m}^2$ , 29 neurons, 5 preparations), less intense, and less distinct from the surrounding immunoreactivity (Fig. 4E). The clusters of  $\alpha 2$  immunoreactivity were present on the soma, proximal, and distal dendrites of pyra-

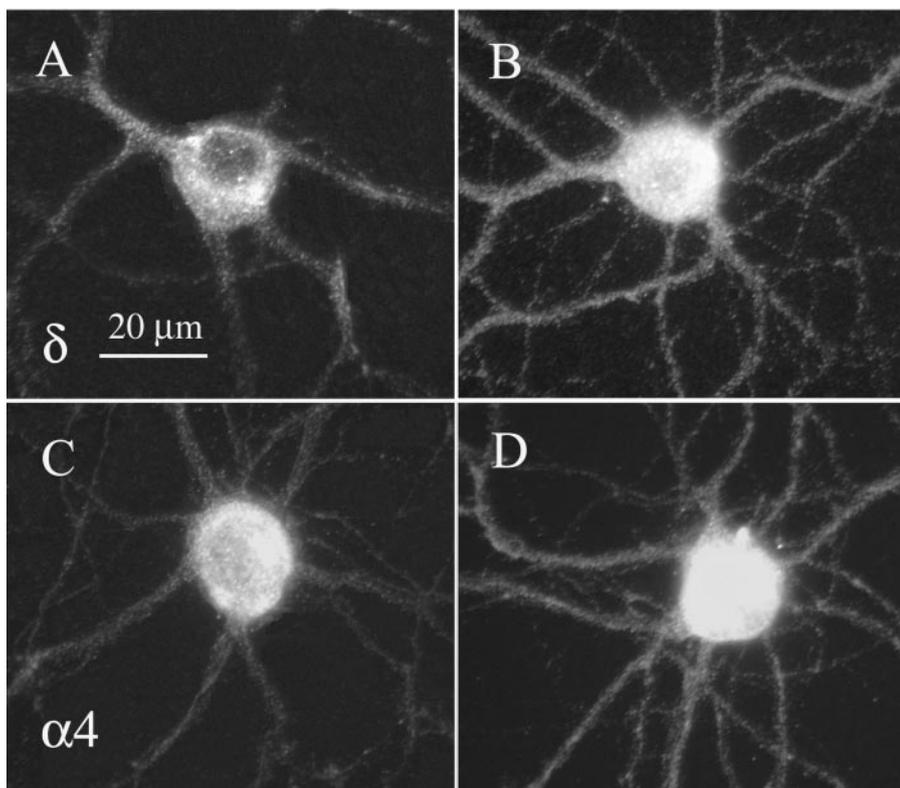
midal neurons, some of which colocalized with GAD65 immunoreactivity (Fig. 4, E–H). In 29 pyramidal cells from 5 cultures, 22% of  $\alpha 2$  puncta colocalized with GAD65 clusters. This suggested that the  $\alpha 2$  subunit clusters were present at GABAergic synapses.

**$\alpha 4$  Subunit.** The  $\alpha 4$  subunit immunoreactivity was diffusely distributed over the soma and dendrites of pyramidal neurons (Fig. 4I), a pattern similar to that of the  $\delta$  subunit (Fig. 2E). Clusters of  $\alpha 4$  immunoreactivity were rare and showed little obvious colocalization with GAD65 puncta (Fig. 4, K and L). Only 2% of  $\alpha 4$  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  $\alpha 4$  subunit immunoreactivity using both permeabilizing and nonpermeabilizing protocols, and the staining patterns were similar (Fig. 3, C and D).

### The $\beta$ Subunits

**$\beta 1$  Subunit.** The  $\beta 1$  immunoreactivity was present over the cell soma and the processes (Fig. 5A), a pattern of staining similar to that of the  $\delta$  subunit. There were no distinct clusters of  $\beta 1$  immunoreactivity, although staining seemed granular in some areas in a few neurons. The  $\beta 1$  immunoreactivity showed no colocalization with the synaptic marker GAD65 (Fig. 5, A–D).

**$\beta 2/3$  Subunit.** The antibody used for this study recognizes an epitope on both  $\beta 2$  and  $\beta 3$  subunits, so the findings refer to both subunits.  $\beta 2/3$  subunit immunoreactivity showed marked, distinct puncta ( $1.57 \pm 0.083 \mu\text{m}^2$ , 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  $\beta 2/3$ -positive clusters were colocalized



**Fig. 3.** Comparison of  $\delta$  and  $\alpha 4$  immunoreactivity in nonpermeabilized neurons and neurons exposed to 0.1% Triton X-100 before primary antibody application.  $\delta$  and  $\alpha 4$  subunit immunoreactivity was diffusely expressed in both nonpermeabilized (A and C, respectively) and permeabilized (B and D, respectively) neurons.

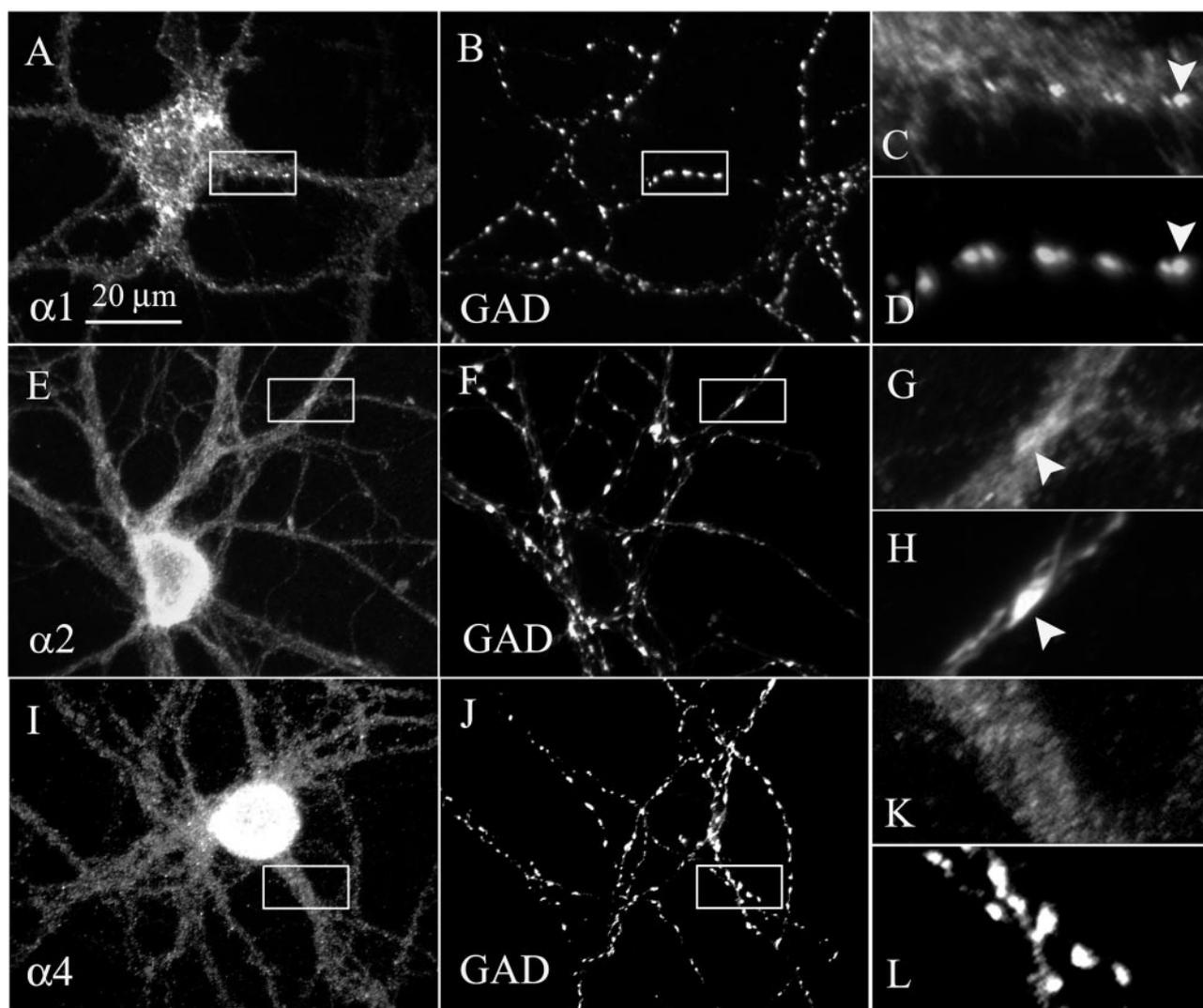
with GAD65 clusters. The  $\beta 2/3$ -subunit immunoreactivity clusters were also present extrasynaptically within the cell body and along the dendrites (Fig. 5, E–H).

$\beta 1$  and  $\beta 2/3$  subunits were also compared directly by staining the cultures for both of these subunits. The diffusely distributed  $\beta 1$  subunit did not colocalize with  $\beta 2/3$  subunit clusters. This suggested that  $\beta 1$  and  $\beta 2/3$  subunits were present on the same cell but were usually found in different GABA<sub>A</sub> receptors (Fig. 5, I–L).

#### GABA<sub>A</sub> Receptor Currents Recorded from Cultured Hippocampal Pyramidal Neurons

The expression of GABA<sub>A</sub> receptor subunits on cultured neurons was further characterized by evaluating the properties of whole-cell GABA<sub>A</sub> receptor currents as well as tonic and phasic GABA<sub>A</sub> 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  $1000$   $\mu\text{M}$ , 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  $\text{EC}_{50}$  value of  $10.7 \pm 1.3$   $\mu\text{M}$  ( $n = 9$ ), and the maximal evoked current was  $1392 \pm 216$  pA ( $n = 9$ ). Recombinant receptors containing the  $\delta$  subunits have a high affinity for GABA ( $\text{EC}_{50} < 0.3$ – $3$   $\mu\text{M}$ ), whereas  $\gamma 2$  subunit-con-



**Fig. 4.** Distribution of  $\alpha 1$ ,  $\alpha 2$ , and  $\alpha 4$  GABA<sub>A</sub> receptor subunit immunoreactivity and colocalization with GAD65 immunoreactivity in cultured hippocampal pyramidal neurons.  $\alpha 1$  (A) and  $\alpha 2$  (E) immunoreactivity was in the form of clusters on the periphery of the cell soma and along dendrites. GAD65 immunoreactivity (B and F) showed a similar distribution. Examination of subunit/GAD65 colocalization revealed that both  $\alpha 1$  and  $\alpha 2$  exhibited overlap with GAD65, as indicated by arrowheads in C and D that were magnified from boxed areas in A and B, respectively; boxed areas in E and F are magnified in G and H, respectively. The distribution of the  $\alpha 4$  subunit was more diffuse with little apparent clustering of immunoreactive puncta (I). Colocalization of the  $\alpha 4$  subunit with GAD65 (J) was not detected, as demonstrated in boxed areas in I and J, magnified in K and L, respectively.

taining receptors have a lower affinity for GABA ( $EC_{50}$  in 10–30  $\mu$ M) (Saxena and Macdonald, 1996; Wafford et al., 1996). Thus, the  $EC_{50}$  of whole-cell GABA<sub>A</sub> receptor currents recorded from pyramidal neurons was closer to that of  $\gamma$ 2 subunit-containing GABA<sub>A</sub> receptor than the  $\delta$  subunit-containing recombinant GABA<sub>A</sub> receptors.

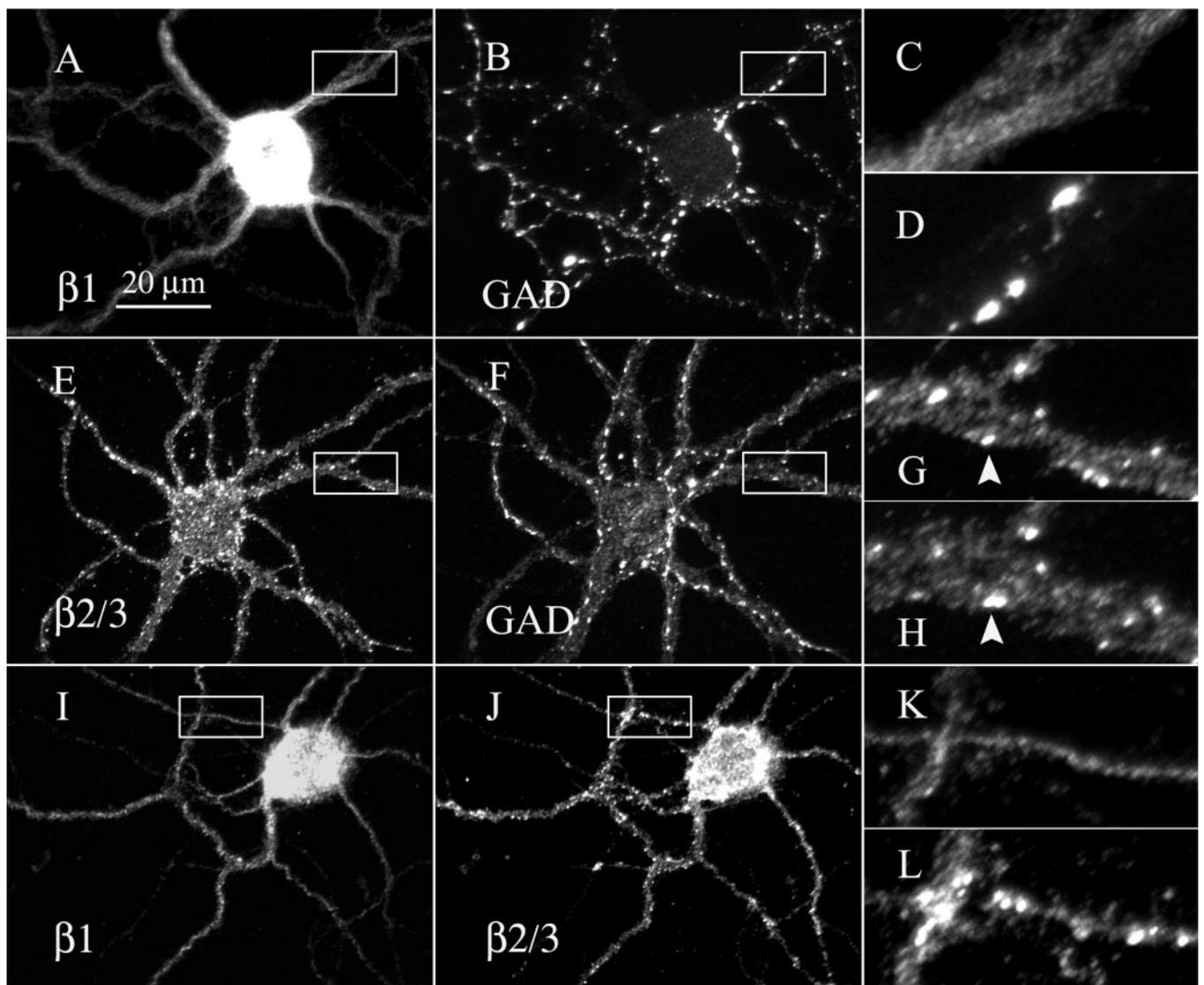
### Diazepam Sensitivity

Benzodiazepines act on an allosteric site on GABA<sub>A</sub> receptors and enhance  $\gamma$ 2 subunit-containing receptors but not  $\delta$  subunit-containing receptors. In six pyramidal cells, diazepam (1–1000 nM) was coapplied with 3  $\mu$ M GABA alternating with 3  $\mu$ M GABA. Diazepam (3–1000 nM) uniformly enhanced GABA<sub>A</sub> 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  $71.5 \pm 7.0\%$  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<sub>A</sub> receptors containing any  $\alpha$ 1, -2, -3, or -5 subunit and a  $\gamma$ 2 subunit are highly sensitive to diazepam, whereas those containing  $\alpha$ 4 and  $\delta$  subunits are insensitive to micromolar concentrations of diazepam. None of the cells expressed purely diazepam-insensitive GABA<sub>A</sub> receptor currents. In addition, the  $EC_{50}$  of the diazepam effect (14–45 nM) was close to that of recombinant GABA<sub>A</sub> receptors containing the  $\gamma$ 2 subunit (1–10 nM). These studies were consistent with the immunocytochemical finding that receptors containing  $\alpha$ 2 (or  $\alpha$ 1) and  $\gamma$ 2 subunits were expressed on cultured pyramidal neurons.

### Allopregnanolone Sensitivity

The immunocytochemical studies suggested that cultured pyramidal cells express  $\alpha$ 4 and  $\delta$  subunit-containing recep-



**Fig. 5.** Distribution of  $\beta$ 1 and  $\beta$ 2/3 GABA<sub>A</sub> receptor subunits immunoreactivity and their colocalization with GAD65 immunoreactivity. A,  $\beta$ 1 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  $\beta$ 1 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- $\beta$ 2/3 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  $\beta$ 1 (I) and  $\beta$ 2/3 (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  $\delta$  subunit confers high affinity for neurosteroids on GABA<sub>A</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>A</sub> receptor currents in all eight pyramidal cells tested. The mean enhancement in eight cells was  $29.9 \pm 3.7\%$  ( $P < 0.001$ , paired  $t$  test). Significant enhancement of GABA<sub>A</sub> receptor currents by a low concentration of allopregnanolone supported the immunocytochemical evidence of the expression of the  $\delta$  subunit on cultured pyramidal neurons. However, the  $\delta$  subunit is not essential for neurosteroid sensitivity, and receptors lacking this subunit are also modulated by neurosteroids.

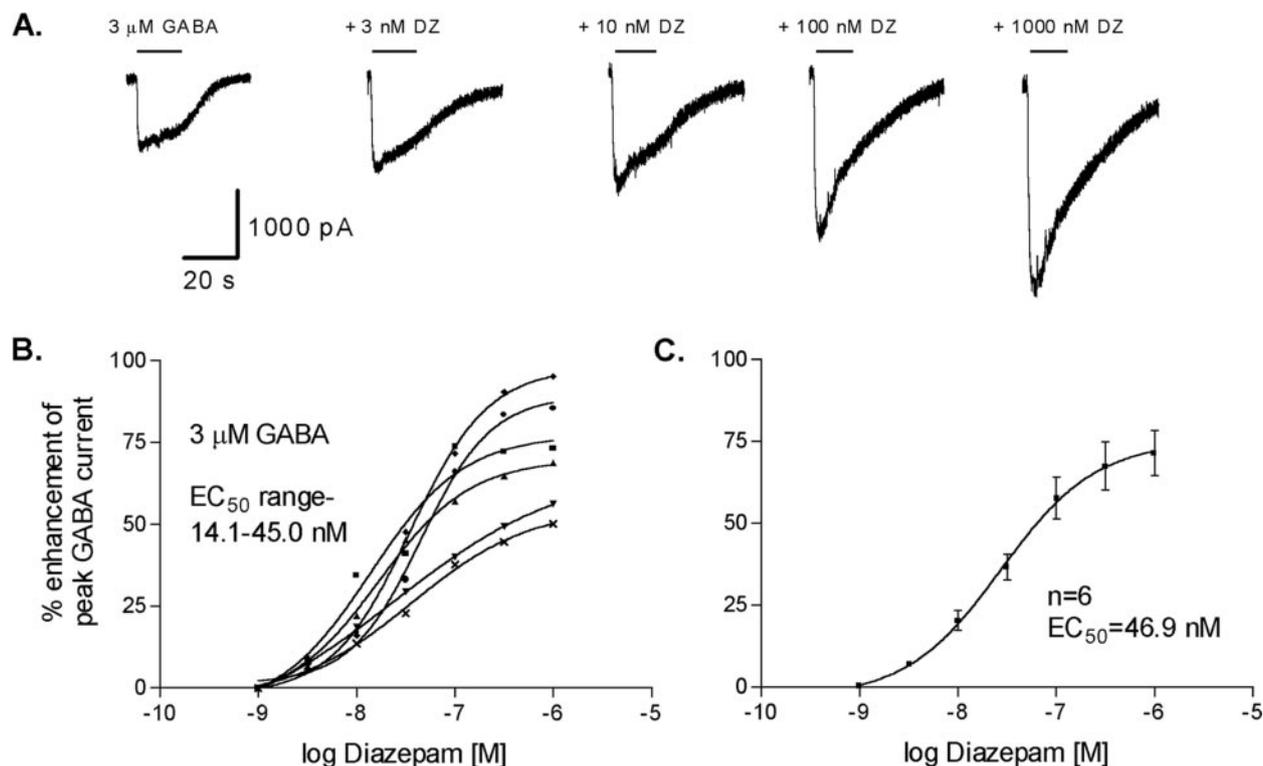
### Tonic Currents in Cultured Pyramidal Neurons

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  $\delta$  subunit-containing receptors, we studied the properties of GABA<sub>A</sub> receptor-mediated tonic inhibition in cultured pyramidal neurons. Several studies have suggested that the  $\delta$  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>A</sub> receptors by low micromolar concentrations of ambient GABA. The extrasynap-

tic receptors composed of  $\alpha 6$  and  $\delta$  subunits mediate tonic inhibition in cerebellar granule cells. The  $\alpha 6$  subunit-containing receptors have a high affinity for GABA and thus can respond to low ambient concentrations of GABA. The  $\delta$  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-nitroquinoxaline-2,3-dione and 2-amino-5-phosphonovaleric acid (Fig. 7) and the competitive GABA<sub>A</sub> receptor antagonist bicuculline (5  $\mu$ M) 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>A</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  $5.34 \pm 0.07$  to  $4.21 \pm 0.02$  pA.



**Fig. 6.** Diazepam enhanced whole-cell GABA<sub>A</sub> receptor-mediated currents. **A**, augmentation of whole-cell currents evoked by 3  $\mu$ M GABA directly coapplied with several concentrations of diazepam. **B**, concentration-response profiles for six cells exposed to 3  $\mu$ M 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**).

### Diazepam and Zn<sup>2+</sup> Modulation of Tonic Currents

The pharmacology of GABA<sub>A</sub> receptors underlying tonic GABA current was investigated initially by application of diazepam (30–50 nM, which is the approximate EC<sub>50</sub> value for enhancement of whole-cell GABA currents). Diazepam (30 nM) did not alter baseline current in any of four 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 ( $5.22 \pm 0.19$  pA versus  $5.12 \pm 0.22$  pA, *t* test). However, sIPSCs were enhanced by diazepam treatment;  $\tau_{\text{decay}}$  increased from  $36.9 \pm 0.9$  ms in untreated cells (111 sIPSCs with peak amplitudes from 40–60 pA) to  $48.7 \pm 1.2$  ms after at least 2-min exposure to diazepam (91 events;  $P < 0.001$ , *t* test). This result suggested that GABA<sub>A</sub> receptors mediating tonic currents contained the  $\alpha 4$  or  $\delta$  subunit or both; synaptic GABA<sub>A</sub> receptors contained the  $\alpha 1$ , -2, -3, or -5 subunits and the  $\gamma 2$  subunit.

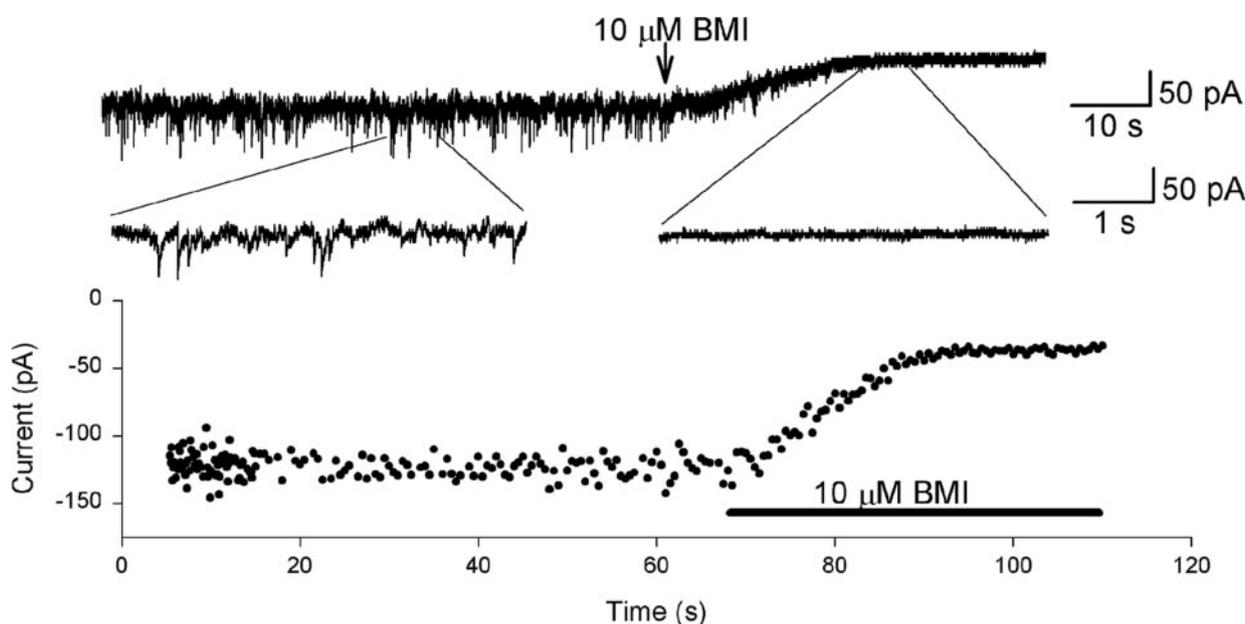
Application of  $60 \mu\text{M}$  Zn<sup>2+</sup> caused a significant reduction in tonic current, whereas spontaneous synaptic currents remained but with modified frequency and amplitude (Fig. 9). Baseline current (holding potential, -50 mV) declined from  $134 \pm 23$  to  $71 \pm 9$  pA after Zn<sup>2+</sup> 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  $6.21 \pm 0.15$  to  $5.28 \pm 0.2$  pA;  $P = 0.006$ , paired *t* test). sIPSC frequency decreased from  $3.4 \pm 0.7$  to  $0.8 \pm 0.3$  Hz, whereas mean sIPSC amplitude decreased from  $68.8 \pm 9.0$  to  $44.9 \pm 2.3$  pA. Comparison of sIPSCs of similar amplitudes (50–60 pA) before and after Zn<sup>2+</sup> exposure revealed no significant change in  $\tau_{\text{decay}}$  ( $32.7 \pm 1.2$  versus  $35.2 \pm 1.8$  ms, respectively). One explanation for Zn<sup>2+</sup> inhibition of tonic currents in cultured pyramidal neurons was that  $\delta$  subunit-containing

receptors mediated these currents because recombinant receptors containing this subunit are highly sensitive to Zn<sup>2+</sup>. However, Zn<sup>2+</sup> 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  $\alpha 4$  subunit-containing recombinant receptors (Wafford et al., 1996). Application of furosemide ( $100 \mu\text{M}$ ) affected the baseline GABA current, reducing it from  $127 \pm 14$  to  $87 \pm 9$  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  $5.52 \pm 0.04$  to  $5.45 \pm 0.05$  pA) was not significant ( $P = 0.14$ ). The sIPSC kinetics ( $\tau_{\text{decay}}$   $34.3 \pm 1.2$  versus  $37.2 \pm 2.8$  ms after furosemide) and frequency were unaffected by furosemide.

Loreclezole is an anticonvulsant known to enhance  $\beta 2$  or  $\beta 3$  subunit-containing receptors but not those containing  $\beta 1$ . In addition, it can inhibit  $\beta 1$  subunit-containing GABA<sub>A</sub> 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 ( $30 \mu\text{M}$ ) did not have a consistent action. In three cells, baseline current was decreased significantly from  $124 \pm 12$  to  $91 \pm 11$  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  $5.64 \pm 0.07$  to  $5.16 \pm 0.04$  pA ( $P < 0.001$ ). In the three remaining cells, root mean square noise reduction was not significant (from  $5.48 \pm 0.13$  to  $5.377 \pm 0.13$  pA). No change was observed in sIPSC frequency or decay kinetics regardless



**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<sub>A</sub> 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.

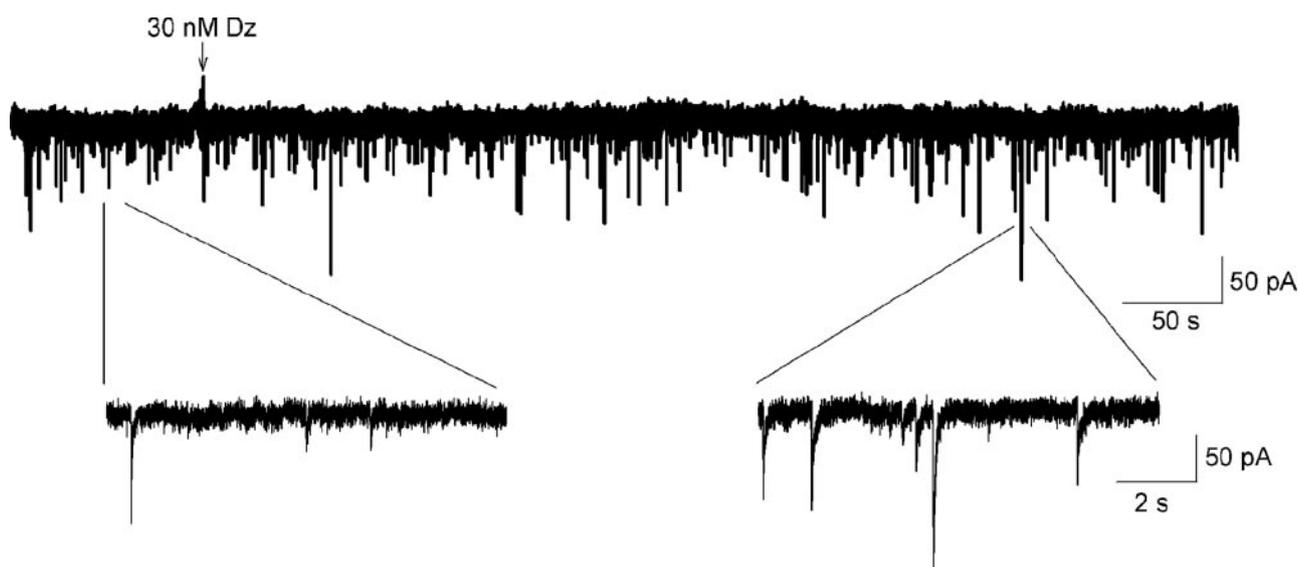
## Discussion

A combination of immunocytochemical and electrophysiological studies suggested that cultured hippocampal neurons expressed two types of GABA<sub>A</sub> receptors. One type of receptors formed clusters present at GABAergic synapses and in extrasynaptic membrane. The  $\alpha 1$ ,  $\alpha 2$ ,  $\beta 2/3$ , and  $\gamma 2$  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  $\alpha 1$  or  $\alpha 2$  and  $\gamma 2$  subunits. The second type of receptor was diffusely distributed in the extrasynaptic membrane. The  $\alpha 4$ ,  $\beta 1$ , and  $\delta$  subunits were contained in these receptors. The properties of tonic currents recorded from these neurons were similar to recombinant receptors containing  $\alpha 4$ ,  $\beta 1$ , and  $\delta$  subunits. These studies

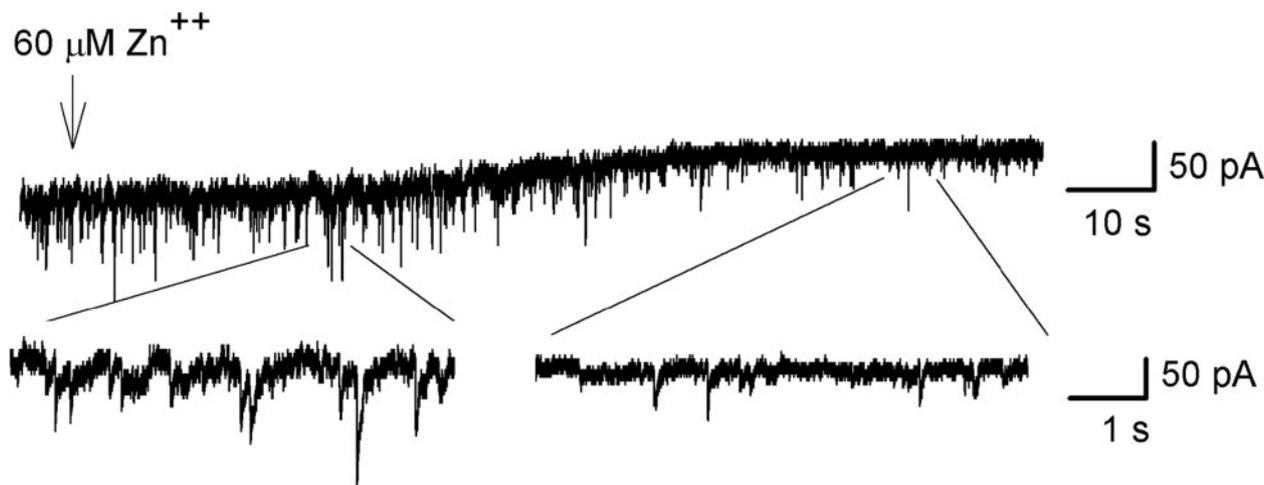
add to the growing evidence that GABA<sub>A</sub> receptors segregate between synaptic and extrasynaptic sites in neurons.

**Diffusely Dispersed GABA<sub>A</sub> Receptors Containing  $\alpha 4$ ,  $\beta 1$ , and  $\delta$  Subunit and the Properties of Tonic Currents.** The present study found that the  $\delta$  subunit was diffusely distributed on the surface of cultured hippocampal neurons and that it was not concentrated at synapses. The distribution of the  $\delta$  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  $\delta$  subunit was expressed exclusively in the extrasynaptic membrane (Nusser et al., 1998). Likewise, a recent electron microscopic study of hippocampal dentate granule cells demonstrated perisynaptic localization of the  $\delta$  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.



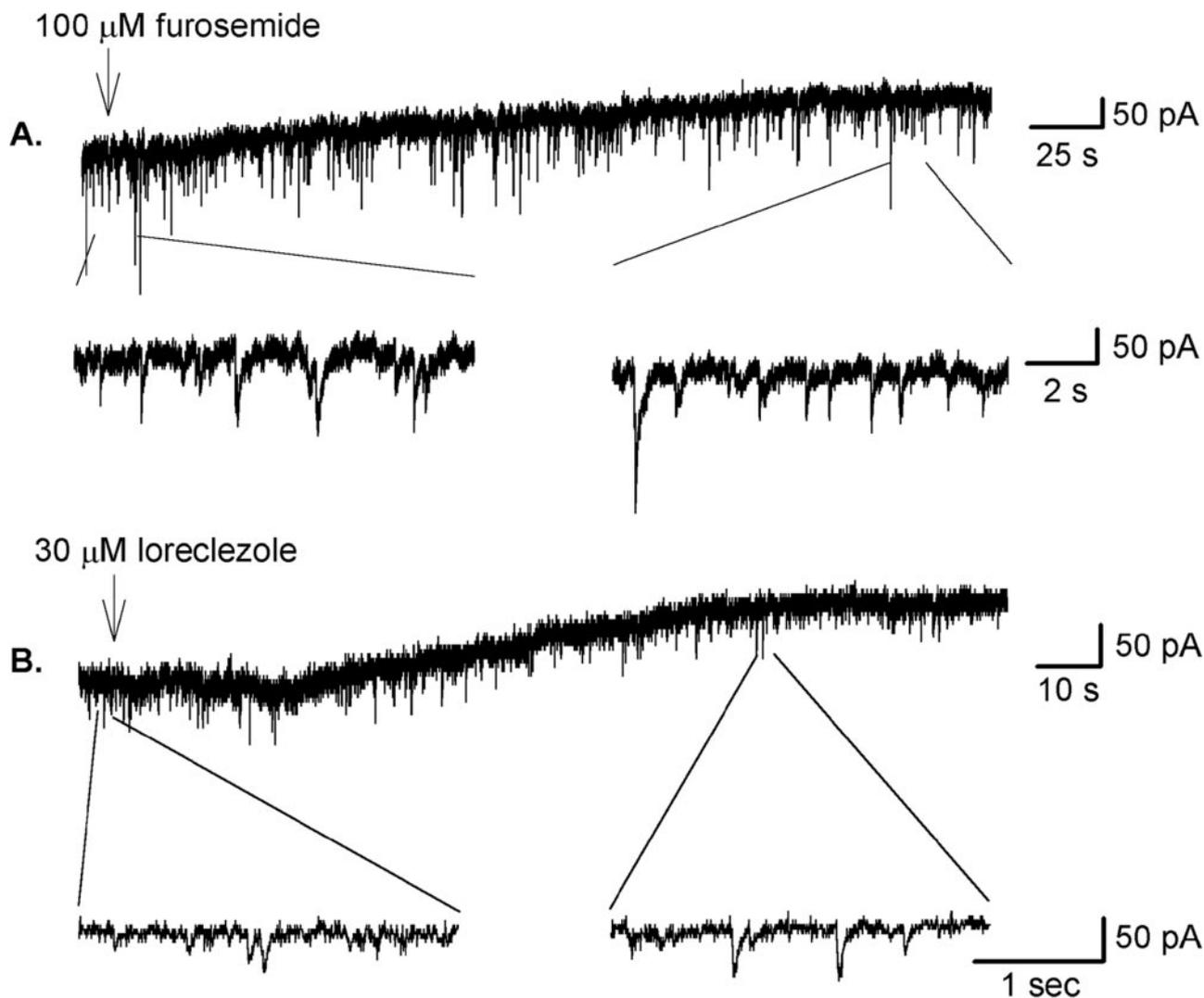
**Fig. 9.** Zn<sup>2+</sup> modulation of tonic and phasic GABAergic currents. Zn<sup>2+</sup> (60  $\mu$ M) decreased the tonic current and decreased sIPSC frequency but did not alter sIPSC kinetics (see text). The expanded traces show tonic and phasic currents before and after tonic current reduction.

ported the notion that the  $\delta$  subunit was expressed on cultured hippocampal neurons. Enhancement of whole-cell GABA<sub>A</sub> receptor currents by 10 nM allopregnanolone suggested high sensitivity to neurosteroids, a property of  $\delta$  subunit-containing GABA<sub>A</sub> receptors (Wohlfarth et al., 2002). Because  $\delta$  subunit-containing receptors desensitize very slowly (Saxena and Macdonald, 1996; Haas and Macdonald, 1999), GABA<sub>A</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  $\alpha 5$  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  $\delta$  subunit-containing receptors were present in extrasynaptic membrane. Moreover, Zn<sup>2+</sup> inhibited tonic currents, providing additional evidence that  $\delta$  subunit-containing receptors mediated the tonic inhibition observed in these cells.

Furosemide reduction of tonic GABA currents suggested

participation of  $\alpha 4$  subunit-containing GABA<sub>A</sub> receptors in mediating tonic currents. Furosemide is known to inhibit  $\alpha 4$  subunit-containing receptors and was demonstrated to inhibit GABA<sub>A</sub> receptor currents in dentate granule cells, which express the  $\alpha 4$  subunit (Wafford et al., 1996; Kapur and Macdonald, 1999). Likewise,  $\alpha 4$  subunit-containing GABA<sub>A</sub> receptors have a higher affinity for GABA than those containing the  $\alpha 1$  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  $\alpha 4$  and  $\delta$  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  $\alpha 4$  subunit often coassembles with the  $\delta$  subunit (Sur et al., 1999). In the forebrain, the distribution of  $\alpha 4$  subunit mRNA is similar to that of the  $\delta$  subunit (Wisden et al., 1992). In addition, the expression of the  $\alpha 4$



**Fig. 10.** Furosemide and loreclezole modulation of tonic currents. A, application of furosemide (100  $\mu$ M) 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  $\mu$ M) 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.

subunit is diminished in  $\delta$  subunit knockout mice (Peng et al., 2002).

The distribution of  $\beta 1$  subunit was similar to that of  $\alpha 4$  and  $\delta$  subunits, suggesting an extrasynaptic distribution. The immunocytochemical studies were further supported by electrophysiological studies. Loreclezole is an anticonvulsant that enhances peak GABA<sub>A</sub> receptor currents elicited by subsaturating concentrations of GABA by acting at an allosteric regulatory site on  $\beta 2$  and  $\beta 3$  subunits (Wafford et al., 1994; Fisher and Macdonald, 1997). Peak currents elicited from receptors containing the  $\beta 1$  subunit were not enhanced by loreclezole because the receptors lack the positive modulatory site present on the  $\beta 2$  and  $\beta 3$  subunits. In addition to the potentiation of peak currents, loreclezole inhibited steady-state GABA<sub>A</sub> receptor currents by acting at a site distinct from the positive modulatory site on  $\beta 2/3$  subunits and increased the apparent desensitization. This inhibitory action of loreclezole occurred regardless of the  $\beta$  subunit. Thus, GABA<sub>A</sub> receptors containing the  $\beta 1$  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  $\beta 1$  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  $\alpha 5$  subunit-containing GABA<sub>A</sub> receptors also mediate tonic inhibition in pyramidal cells, in addition to  $\alpha 4$ ,  $\delta$  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  $\alpha 5$  subunit-containing GABA<sub>A</sub> receptors mediate tonic inhibition in CA1 pyramidal neurons in mice (Caraiscos et al., 2004). The subcellular distribution of the  $\alpha 5$  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  $\alpha 1$ ,  $\alpha 2$ ,  $\beta 2/3$ , and  $\gamma 2$  Subunits.**  $\alpha 2$ ,  $\beta 2/3$ , and  $\gamma 2$  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<sub>A</sub> receptor subunits in rat brain have demonstrated that  $\alpha 1$ ,  $\alpha 2$ ,  $\alpha 4$ , and  $\alpha 5$  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  $\alpha 2$ ,  $\alpha 1$ ,  $\beta 2/3$ , and  $\gamma 2$  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 immunogold electron 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  $\alpha 2$  and  $\gamma 2$  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  $\alpha 2$  and  $\gamma 2$  subunits (Sieghart and Sperk, 2002).

Extrasynaptic GABA<sub>A</sub> receptors containing  $\alpha 2$ ,  $\beta 2/3$ , and  $\gamma 2$  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<sub>A</sub> 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; Kittler et al., 2000). Another explanation for the lack of apposition of GABA<sub>A</sub> 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<sub>A</sub> receptors containing  $\alpha 2$ ,  $\beta 2/3$ , and  $\gamma 2$  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<sub>A</sub> receptors containing  $\alpha 2$ ,  $\beta 2/3$ , and  $\gamma 2$  subunits. GABA<sub>A</sub> 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<sub>A</sub> receptor isoforms (Brickley et al., 1999). It is possible that similar studies on cultured pyramidal neurons would also reveal multiple types of GABA<sub>A</sub> receptors on extrasynaptic membrane. In summary, this study adds to the growing evidence that GABA<sub>A</sub> receptors are segregated between synaptic and extrasynaptic sites in neurons.

#### Acknowledgments

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#### References

- Bai D, Zhu G, Pennefather P, Jackson MF, MacDonald JF, and Orser BA (2001) Distinct functional and pharmacological properties of tonic and quantal inhibitory postsynaptic currents mediated by  $\gamma$ -aminobutyric acid A receptors in hippocampal neurons. *Mol Pharmacol* **59**:814–824.
- Barnes EM Jr (2000) Intracellular trafficking of GABA<sub>A</sub> receptors. *Life Sci* **66**:1063–1070.
- Brickley SG, Cull-Candy SG, and Farrant M (1999) Single-channel properties of synaptic and extrasynaptic GABA<sub>A</sub> receptors suggest differential targeting of receptor subtypes. *J Neurosci* **19**:2960–2973.
- Brooks-Kayal AR, Jin H, Price M, and Dichter MA (1998) Developmental expression of GABA<sub>A</sub> receptor subunit mRNAs in individual hippocampal neurons in vitro and in vivo. *J Neurochem* **70**:1017–1028.
- Brunig I, Scotti E, Sidler C, and Fritschy JM (2002a) Intact sorting, targeting and clustering of  $\gamma$ -aminobutyric acid A receptor subtypes in hippocampal neurons in vitro. *J Comp Neurol* **443**:43–55.
- Brunig I, Suter A, Knuesel I, Luscher B, and Fritschy JM (2002b) GABAergic terminals are required for postsynaptic clustering of dystrophin but not of GABA<sub>A</sub> receptors and gephyrin. *J Neurosci* **22**:4805–4813.
- Caraiscos VB, Elliott EM, You T, Cheng VY, Belevi D, Newell JG, Jackson MF, Lambert JJ, Rosahl TW, Wafford KA, et al. (2004) Tonic inhibition in mouse

- hippocampal CA1 pyramidal neurons is mediated by  $\alpha 5$  subunit-containing  $\gamma$ -aminobutyric acid type A receptors. *Proc Natl Acad Sci USA* **101**:3662–3667.
- Christie SB and de Blas AL (2002)  $\alpha 5$  Subunit-containing GABA<sub>A</sub> receptors form clusters at GABAergic synapses in hippocampal cultures. *Neuroreport* **13**:2355–2358.
- Christie SB, Miralles CP, and de Blas AL (2002) GABAergic innervation organizes synaptic and extrasynaptic GABA<sub>A</sub> receptor clustering in cultured hippocampal neurons. *J Neurosci* **22**:684–697.
- Dupuy ST and Houser CR (1996) Prominent expression of two forms of glutamate decarboxylase in the embryonic and early postnatal rat hippocampal formation. *J Neurosci* **16**:6919–6932.
- Essrich C, Lorez M, Benson JA, Fritschy JM, and Luscher B (1998) Postsynaptic clustering of major GABA<sub>A</sub> receptor subtypes requires the  $\gamma 2$  subunit and gephyrin. *Nat Neurosci* **1**:563–571.
- Fisher JL and Macdonald RL (1997) Functional properties of recombinant GABA<sub>A</sub> receptors composed of single or multiple  $\beta$  subunit subtypes. *Neuropharmacology* **36**:1601–1610.
- Fritschy JM and Brunig I (2003) Formation and plasticity of GABAergic synapses: physiological mechanisms and pathophysiological implications. *Pharmacol Ther* **98**:299–323.
- Goslin K, Asmussen H, and Banker G (1998) Rat hippocampal neurons in low density culture, in *Culturing Nerve Cells* (Banker G and Goslin K eds) pp 339–370, The MIT Press, Cambridge, MA.
- Greenfield LJ Jr and Macdonald RL (1996) Whole-cell and single-channel  $\alpha 1\beta 1\gamma 2S$  GABA<sub>A</sub> receptor currents elicited by a “multipuffer” drug application device. *Pflueg Arch Eur J Physiol* **432**:1080–1090.
- Haas KF and Macdonald RL (1999) GABA<sub>A</sub> receptor subunit  $\gamma 2$  and  $\delta$  subtypes confer unique kinetic properties on recombinant GABA<sub>A</sub> receptor currents in mouse fibroblasts. *J Physiol (Lond)* **514**:27–45.
- Hamann M, Rossi DJ, and Attwell D (2002) Tonic and spillover inhibition of granule cells control information flow through cerebellar cortex. *Neuron* **33**:625–633.
- Hamill OP, Marty A, Neher E, Sakmann B, and Sigworth FJ (1981) Improved patch-clamp techniques for high-resolution current recording from cells and cell-free membrane patches. *Pflueg Arch Eur J Physiol* **391**:85–100.
- Kapur J and Macdonald RL (1999) Postnatal development of hippocampal dentate granule cell  $\gamma$ -aminobutyric acid A receptor pharmacological properties. *Mol Pharmacol* **55**:444–452.
- Killisch I, Dotti CG, Laurie DJ, Luddens H, and Seeburg PH (1991) Expression patterns of GABA<sub>A</sub> receptor subtypes in developing hippocampal neurons. *Neuron* **7**:927–936.
- Kittler JT, Delmas P, Jovanovic JN, Brown DA, Smart TG, and Moss SJ (2000) Constitutive endocytosis of GABA<sub>A</sub> receptors by an association with the adaptin AP2 complex modulates inhibitory synaptic currents in hippocampal neurons. *J Neurosci* **20**:7972–7977.
- Mangan PS and Kapur J (2004) Factors underlying bursting behavior in a network of cultured hippocampal neurons exposed to zero magnesium. *J Neurophysiol* **91**:946–957.
- Nusser Z, Roberts JD, Baude A, Richards JG, Sieghart W, and Somogyi P (1995) Immunocytochemical localization of the  $\alpha 1$  and  $\beta 2/3$  subunits of the GABA<sub>A</sub> receptor in relation to specific GABAergic synapses in the dentate gyrus. *Eur J Neurosci* **7**:630–646.
- Nusser Z, Sieghart W, and Somogyi P (1998) Segregation of different GABA<sub>A</sub> receptors to synaptic and extrasynaptic membranes of cerebellar granule cells. *J Neurosci* **18**:1693–1703.
- Peng Z, Hauer B, Mihalek RM, Homanics GE, Sieghart W, Olsen RW, and Houser CR (2002) GABA<sub>A</sub> receptor changes in  $\delta$  subunit-deficient mice: altered expression of  $\alpha 4$  and  $\gamma 2$  subunits in the forebrain. *J Comp Neurol* **446**:179–197.
- Rao A, Cha EM, and Craig AM (2000) Mismatched appositions of presynaptic and postsynaptic components in isolated hippocampal neurons. *J Neurosci* **20**:8344–8353.
- Saxena NC and Macdonald RL (1996) Properties of putative cerebellar GABA<sub>A</sub> receptor isoforms. *Mol Pharmacol* **49**:567–579.
- Scotti AL and Reuter H (2001) Synaptic and extrasynaptic  $\gamma$ -aminobutyric acid type A receptor clusters in rat hippocampal cultures during development. *Proc Natl Acad Sci USA* **98**:3489–3494.
- Semyanov A, Walker MC, Kullmann DM, and Silver RA (2004) Tonic active GABA<sub>A</sub> receptors: modulating gain and maintaining the tone. *Trends Neurosci* **27**:262–269.
- Sieghart W and Sperk G (2002) Subunit composition, distribution and function of GABA<sub>A</sub> receptor subtypes. *Curr Top Med Chem* **2**:795–816.
- Soltesz I and Nusser Z (2001) Neurobiology. Background inhibition to the fore. *Nature (Lond)* **409**:24–25, 27.
- Sperk G, Schwarzer C, Tsunashima K, Fuchs K, and Sieghart W (1997) GABA<sub>A</sub> receptor subunits in the rat hippocampus I: immunocytochemical distribution of 13 subunits. *Neuroscience* **80**:987–1000.
- Stell BM and Mody I (2002) Receptors with different affinities mediate phasic and tonic GABA(A) conductances in hippocampal neurons *J Neurosci* **22** (RC223):1–5.
- Stelzer A, Kay AR, and Wong RK (1988) GABA<sub>A</sub>-receptor function in hippocampal cells is maintained by phosphorylation factors. *Science (Wash DC)* **241**:339–341.
- Sur C, Farrar SJ, Kerby J, Whiting PJ, Atack JR, and McKernan RM (1999) Preferential coassembly of  $\alpha 4$  and  $\delta$  subunits of the  $\gamma$ -aminobutyric acid A receptor in rat thalamus. *Mol Pharmacol* **56**:110–115.
- Wafford KA, Bain CJ, Quirk K, McKernan RM, Wingrove PB, Whiting PJ, and Kemp JA (1994) A novel allosteric modulatory site on the GABA<sub>A</sub> receptor  $\beta$  subunit. *Neuron* **12**:775–782.
- Wafford KA, Thompson SA, Thomas D, Sikela J, Wilcox AS, and Whiting PJ (1996) Functional characterization of human  $\gamma$ -aminobutyric acid A receptors containing the  $\alpha 4$  subunit. *Mol Pharmacol* **50**:670–678.
- Wisden W, Laurie DJ, Monyer H, and Seeburg PH (1992) The distribution of 13 GABA<sub>A</sub> receptor subunit mRNAs in the rat brain. I. telencephalon, diencephalon, mesencephalon. *J Neurosci* **12**:1040–1062.
- Wohlfarth KM, Bianchi MT, and Macdonald RL (2002) Enhanced neurosteroid potentiation of ternary GABA<sub>A</sub> receptors containing the  $\delta$  subunit. *J Neurosci* **22**:1541–1549.
- Yeung JY, Canning KJ, Zhu G, Pennefather P, MacDonald JF, and Orser BA (2003) Tonic activated GABA<sub>A</sub> receptors in hippocampal neurons are high-affinity, low-conductance sensors for extracellular GABA. *Mol Pharmacol* **63**:2–8.

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**Address correspondence to:** Dr. Jaideep Kapur, Department of Neurology, Box 800394, University of Virginia-HSC, Charlottesville, VA. E-mail: jk8t@virginia.edu

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