Molecular Pharmacology Fast Forward. Published on December 21, 2004 as doi:10.1124/mol.104.007385

CULTURED HIPPOCAMPAL PYRAMIDAL NEURONS EXPRESS TWO KINDS OF $GABA_A$ RECEPTORS

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Section: cell physiology

Molecular Pharmacology Fast Forward. Published on December 21, 2004 as DOI: 10.1124/mol.104.007385
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Running Title: Synaptic and extrasynaptic $GABA_A$ receptors

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Number of text pages: 40

Number of figures: 10

Number of references: 38

Number of words in abstract: 240

Number of words in introduction: 486

Number of words in discussion: 1458

ABBREVIATIONS:

APV, 2-amino-5-phosphonovaleric acid; CCD, charge-coupled device; CNQX, 6-Cyano-7-nitroquinoxaline-2, 3-dione; EGTA, Ethylene glycol bis (beta-aminoethyl ether)-N,N,N',N'-tetraacetic acid; $GABA_A$ receptor, γ - aminobutyric acid type A receptor; GAD, glutamate decarboxylase; MEM, minimal essential medium; HBS, HEPES-buffered saline; IPSCs, inhibitory postsynaptic currents; PBS, phosphate buffered saline; sIPSC, spontaneous inhibitory postsynaptic currents

Abstract

We combined a study of the subcellular distribution of the α1, α2, α4, β1, β2/3, γ2, and δ subunits of the $GABA_A$ receptor with an electrophysiological analysis of $GABA_A$ receptor currents to determine the types of receptors expressed on cultured hippocampal pyramidal neurons. The immunocytochemistry study demonstrated that α1, α2, β2/3, and γ2 subunits formed distinct clusters of various sizes, which were colocalized with clusters of glutamate decarboxylase (GAD) immunoreactivity at rates ranging from 22-58%. In contrast, α4, β1, and δ 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 $GABA_A$ receptors 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^{2+}, furosemide, and loreclezole; and they were insensitive to diazepam. These studies suggest that two kinds of $GABA_A$ 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 α1, α2, β2/3, and γ2 subunits were contained in clustered receptors. The second kind was distributed diffusely in the extrasynaptic membrane. The α4, β1, and δ 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 α4, β1, and δ subunits.

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Introduction

γ-aminobutyric acid type A ($GABA_A$) 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 α, β, γ, δ, ε, π, and θ; some of these gene families have several members (α1-6, β1-3, γ1-3)(Sieghart and Sperk, 2002). Different $GABA_A$ 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 $GABA_A$ 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 studies in the past have characterized $GABA_A$ receptor subunit expression in cultures of hippocampal neurons using mRNA and protein expression studies. The mRNA for α1, α2, α4, α5, β1, β3, γ2, and δ 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 $GABA_A$ receptors containing the α1, α2, α3, and α5 subunits in cultured neurons (Brunig *et al.*, 2002a) suggesting that α1 and α2 subunits were clustered at synapses whereas α5 clusters were extrasynaptic. Another study found both synaptic and extrasynaptic clusters of $GABA_A$ receptors containing α1, α2, β1, β2/3, and γ2 subunits (Christie *et al.*, 2002). These findings were extended to the α5 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 α4, β1, and δ subunits in cultured hippocampal neurons. The mRNA 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 co-assembles 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 $GABA_A$ receptor with electrophysiological analysis of $GABA_A$ receptor currents to determine the types and distribution of receptors expressed on these neurons.

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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 prior to co-culturing with hippocampal neurons. Ten days after glial cell plating, hippocampal neuronal cultures were prepared using Sprague-Dawley rat fetuses (E-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 prior to incubation at 37°C for 15 minutes. After incubation, trypsin was replaced with 5 ml HBS and the hippocampi were rinsed with the buffer three times at 5 minute 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 3-4 hours, coverslips were placed neuronal-side down on sterilized longitudinally-cut Teflon O rings (3mm 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. 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 following antibodies were employed

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for immunocytochemistry of $GABA_A$ 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 (Temecula, CA); Antibody against the α2 subunit was from Santa Cruz (Santa Cruz, CA); 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 (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 minutes. Coverslips were then washed with 3 quick changes of PBS followed by 5 minutes 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 minutes then rehydrated with two quick changes of PBS. Coverslips were incubated in the primary antibody overnight at 4°C (primary antibody concentrations, α1: 3μg/ml; α4, β2/3 and δ: 5 μg/ml; α2, β1, γ2, and mouse anti-GAD65: 2 μg/ml; rabbit anti-GAD65 and rabbit anti-GAD65/67: 1:1000). The following day, the first primary antibody was removed and the coverslips were washed with 3 changes of PBS for 5 minutes each. A second primary antibody was applied overnight at 4°C.

Primary antibodies were removed and neurons were washed 3 times with PBS. Cultures were incubated with secondary antibodies at room temperature in darkness for 45 minutes. Secondary antibodies conjugated with the fluorochromes Alexa fluor 488 or Alexa fluor 594 were chosen to prevent cross-reactions. Dishes were rinsed 3 times in PBS and coverslips were

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mounted on slides, neuronal side-down, by placing a drop of Gel/Mount* (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^{o} C before viewing.

Image acquisition. Fluorescent images of cells were captured using a Roper Scientific Photometrics CoolSNAPcf^{TM} CCD camera mounted on a Nikon Eclipse TE200 fluorescent microscope (Japan) with either a 40 x 1.3 NA lens or 60 x 1.4 NA lens driven by Metamorph imaging software (Universal Imaging Corporation, Downington, PA). Using Metamorph, the brightness and contrast of fluorescent images were adjusted to a threshold such that punctuate 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 – 4.4 μm^2 area. Due to 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 thresholded image. Binary images were then added together to display overlapping puncta. Puncta were counted and percent colocalization was calculated as the number of colocalized puncta divided by the total number of subunit clusters. Data were analyzed using GraphPad Prism 4.0 (Graph Pad Software inc., San Diego, CA, USA). 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 punctuate fluorescence was two

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times higher than diffuse background labeling. Images were then saved as 8-bit tiff files and opened in Adobe Photoshop 6.0 (San Jose, CA) where overall brightness was increased for final production.

Electrophysiological recording: Whole-cell voltage clamp recordings were made using a technique described previously (Kapur and Macdonald, 1999;Hamill *et al.*, 1981). Patch pipettes (resistance of 5-8 MΩ) were pulled on a P-97 Flaming Brown puller by a three-stage pull. The extracellular recording solution consisted of (in mM): NaCl 142, $CaCl_2$ 1.0, CsCl 8.09, $MgCl_2$ 6, Glucose 10, HEPES 10, pH adjusted to 7.4 and osmolarity of 310-320 mOsm unless specified otherwise. All reagents were from Sigma (St. Louis, MO, USA) unless specified otherwise. Glass recording patch pipettes were filled with a solution consisting of the following (in mM): CsCl 153, $MgCl_2$ 1.0, HEPES 10.0, EGTA 5.0, pH 7.3, osmolarity ~280-290 mOsm. Recording pipettes also contained an ATP regeneration system consisting of creatinine phosphokinase 50 units/ml, phosphocreatine 22 mM and ATP 3 mM. The ATP regeneration system maintained intracellular energy stores and reduced rundown of $GABA_A$ receptor currents as described previously (Stelzer *et al.*, 1988). For recordings of $GABA_A$ receptor-mediated spontaneous inhibitory postsynaptic currents (sIPSCs) and tonic GABAergic current, 50 μM DL-2-Amino-5-phosphonopentanoic acid (DL-AP5) and 20 μM 6-Cyano-7-nitroquinoxaline-2,3-dione (CNQX, Tocris-Cookson Ellisville, MO) were included in the external medium to block NMDA and AMPA/Kainate receptor-mediated currents, respectively.

All recordings were obtained at room temperature (24^{o}C). Currents were recorded with an Axopatch 1-D amplifier and low-pass filtered at 2 kHz with an 8 pole Bessel filter prior to digitization, storage and display. Liquid junction potentials were corrected, and whole-cell

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capacitance and series resistance were compensated by 70 - 75% at 10 μs lag. A recording was performed when series resistance after compensation was 20 MΩ or less. Access resistance was monitored with a 10 msec, -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 Gould 2400S chart recorder, and peak whole-cell currents were measured manually from the chart paper. Currents were also digitized (20 kHz) using an Axon Instruments Digidata 1200A A-D converter and recorded to a personal computer using Axotape acquisition software (Axon Instruments, Union City, CA, USA) and filtered at 5 kHz.

Drug application: GABA, diazepam, and $ZnCl_2$ dissolved in extracellular solution were applied to neurons using a modified U-tube "multipuffer" drug application system with the tip of application pipette placed 100-200 μm from the cell (Greenfield, Jr. and Macdonald, 1996). Diazepam was dissolved first in DMSO and then diluted in extracellular buffer with the final DMSO 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 co-application of the drug and GABA; and expressing it as a % fraction of the control current. Peak $GABA_A$ 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

$$I = \frac{I_{\max}}{1+10^{(Log_{EC50}-Log_{drug})*Hill.slope}} \qquad (1)$$

where I was the $GABA_A$ receptor current at a given GABA concentration, and $I_{(max)}$ was the maximal current. 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 (Graph Pad Software inc., San Diego, CA, USA). All data are presented as mean ± standard error of the mean.

Analysis of tonic and synaptic currents: Even in the absence of synaptic activity, a number of $GABA_A$ 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 the leak conductance. The baseline noise arises from the recording apparatus (machine noise) and opening and closure of ion channels and receptors at any holding potential. When bicuculline a selective inhibitor of the conductance associated with $GABA_A$ receptors is applied, both the baseline current and baseline noise are diminished {Hamann, 2002 3022 /id}{Stell, 2002 3118 /id}{Yeung, 2003 3107 /id}. 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

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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 3 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 msec interval between complete decay of the previous event and the rise of the next phasic current.

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Results

Immunocytochemistry

Hippocampal pyramidal neurons in culture for 14-18 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). Non-pyramidal interneurons were morphologically distinct bipolar or multipolar neurons whose cell soma was intensely stained for GAD65. In order 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. In order 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, D and E,

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GAD65 clusters colocalized with synaptophysin immunoreactivity clusters suggesting 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 while 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 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 to that of GAD65 immunoreactivity in double-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 $GABA_A$ 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^2 (1.43 ± 0.19 μm^2, 14 neurons, 4 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. 2A, B, and expanded in C, D, respectively, where colocalized clusters are marked by arrowheads. In addition to sites of colocalization, there were

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many γ2 clusters that did not colocalize with GAD65 immunoreactivity, typically these clusters were smaller than clusters that colocalized with GAD. In order 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 $GABA_A$ receptors have been reported extensively in the past (Scotti and Reuter, 2001;Rao *et al.*, 2000;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^2) 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, F, magnified in G, H, respectively).

One possible confound with respect to diffusely distributed $GABA_A$ receptor δ subunits was their surface expression vs. intracellular location. The immunostaining protocol employed in this study required the permeabilization of the cell membrane prior to antibody application. As such, it is possible that the staining of δ subunits may have reflected unassembled subunit

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protein or internally localized receptors. The antibody used for staining the receptor recognizes a surface epitope (AA 1-44), therefore, the surface expression of this subunit could be assessed. Cultured neurons were processed for δ subunit immunoreactivity using both permeabilizing and non-permeabilizing protocols and the staining patterns were compared. In non-permeabilized cells, δ immunoreactivity was diffusely but clearly expressed in the cell soma and dendrites (Fig. 3A) though the immunoreactivity was less intense than that for the cell shown in Fig. 3B, which was processed identically with an additional step of a 10 minute exposure to 0.1% Triton X-100. The immunostaining pattern in these cells was similar regardless of permeabilization demonstrating that the δ subunits were expressed on the cell surface.

The α subunits

α1 immunoreactivity was not well developed on pyramidal neurons in culture for 14-18 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 6 neurons, whereas the remaining neurons showed non-specific, low level staining. In these 6 pyramidal neurons, immunoreactivity was in the form of intense round puncta ($1.61 \pm 0.13\ \mu m^2$) distributed over the periphery of the soma and dendrites of pyramidal neurons (Fig. 4A) in a manner quite similar to that of the γ2 subunit distribution. GAD65 immunoreactivity showed a similar distribution (Fig. 4B). Closer examination of processes of labeled neurons revealed that α1 and GAD65 puncta localized to the same site (arrowheads in Fig. 4 C, D; boxes in A, B,

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expanded in C, D, respectively). Quantification of double-labeled neurons showed that 32% of α1 puncta colocalized with GAD65 immunoreactivity.

α2 subunit. In contrast to the α1 subunit, all pyramidal neurons in culture for 14 days expressed the α2 subunit immunoreactivity. The clusters of α2 immunoreactivity were small ($1.0 \pm 0.15\ \mu m^2$, 29 neurons, 5 preparations), less intense and less distinct from the surrounding immunoreactivity (Fig. 4E). The clusters of α2 immunoreactivity were present on the soma, proximal and distal dendrites of pyramidal neurons, some of which colocalized with GAD65 immunoreactivity (Fig. 4E-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. 4K, 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 (AA 1-14); therefore, the surface expression of this subunit could be assessed. Cultured neurons were processed for α4 subunit immunoreactivity using both permeabilizing and non-permeabilizing protocols and the staining patterns were similar (Fig. 3C, D).

The β subunits

β1 subunit. The β1 immunoreactivity was present over the cell soma and the processes (Fig. 5A), a pattern of staining similar to that of the δ subunit. There were no distinct clusters of

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β1 immunoreactivity, though staining appeared granular in some areas in a few neurons. The β1 immunoreactivity showed no colocalization with the synaptic marker GAD65 (Fig. 5A-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 showed marked, distinct puncta (1.57 ± 0.083 μm^2, 25 cells, 5 preparations) on both the soma and processes (Fig. 5E) and was often colocalized with GAD65 (Fig. 5F, arrowheads in G, H). In 25 cells from 5 preparations, 58% of β2/3-positive clusters were colocalized with GAD65 clusters. The β2/3 subunit immunoreactivity clusters were also present extrasynaptically within the cell body and along the dendrites (Fig. 5E-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. 5I-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 14-18 days were similar to that 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

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recorded. Multiple concentrations of GABA, ranging from 0.1 to 1000 μM, with recovery intervals of at least 3 minutes, 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 fit to an equation for a sigmoidal curve and the equation for the best fit revealed an EC50 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 (EC50 < 0.3-3 μM) while γ2 subunit-containing receptors have a lower affinity for GABA (EC50 in 10 –30 μM) (Wafford *et al.*, 1996;Saxena and Macdonald, 1996). Thus the EC50 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 6 pyramidal cells, diazepam (1 to 1000 nM) was co-applied with 3 μM GABA alternating with 3 μM GABA. Diazepam (3 nM to 1000 nM) uniformly enhanced $GABA_A$ receptor currents in a concentration-dependent fashion (Fig. 6A) in all 6 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 EC50 of 46.4 nM (Fig. 6C). We further characterized cell-to-cell variability by fitting the data from individual neurons (Fig. 6B). The

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EC50 values ranged from 14.1 nM 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 μM concentrations of diazepam. None of the cells expressed purely diazepam-insensitive $GABA_A$ receptor currents. In addition, the EC50 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 receptors; 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 $GABA_A$ receptors, we tested the neurosteroid sensitivity of the whole-cell currents recorded from these neurons. A low concentration of allopregnanolone (10 nM) enhanced $GABA_A$ receptor currents in all 8 pyramidal cells tested. The mean enhancement in 8 cells was 29.9 ± 3.7% (*** $P < 0.001$, paired t test). Significant enhancement of $GABA_A$ 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.

Molecular Pharmacology Fast Forward. Published on December 21, 2004 as DOI: 10.1124/mol.104.007385
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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. In order to specifically detect δ subunit-containing receptors, we studied the properties of $GABA_A$ receptor-mediated tonic inhibition in cultured pyramidal neurons. Several studies have suggested that the δ subunit-containing receptors can mediate tonic inhibition (Semyanov *et al.*, 2004;Hamann *et al.*, 2002). Tonic inhibition results from persistent activation of extrasynaptic $GABA_A$ receptors by low μM 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;Haas and Macdonald, 1999).

Spontaneous IPSCs (sIPSCs) were recorded from pyramidal neurons by blocking excitatory neurotransmission with CNQX and APV (Fig. 7) and the competitive $GABA_A$ receptor antagonist bicuculline (5 УM) was applied, which caused a reduction of noise and shift in baseline current (Fig. 7, upper panel). It also abolished sIPSCs demonstrating that both tonic and synaptic inhibition were mediated by $GABA_A$ 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

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application; following bicuculline application, the baseline current decreased to approximately -40 pA with smaller fluctuations (Fig. 7, lower panel). In recordings from 5 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 5 cells, the root mean square noise reduction was significant (*** $p < 0.0001$, 2-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.

Diazepam and Zn^{2+} modulation of tonic currents

The pharmacology of $GABA_A$ receptors underlying tonic GABA current was investigated initially by application of diazepam (30-50 nM, which is the approximate EC50 for enhancement of whole-cell GABA currents). Diazepam (30 nM) did not alter baseline current in any of 4 pyramidal cells examined (Fig. 8). The result was further confirmed using 50 nM diazepam in 6 pyramidal cells. The root mean square noise was also not altered significantly by diazepam (50 nM) application (5.22 ± 0.19 pA vs 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 minutes exposure to diazepam (91 events; *** $p < 0.001$, t test). This result suggested that $GABA_A$ receptors mediating tonic currents contained the $\alpha 4$ or δ subunit or both; synaptic $GABA_A$ receptors contained the $\alpha 1$, 2, 3 or 5 subunits and the $\gamma 2$ subunit.

Application of 60 μM Zn^{2+} caused a significant reduction in tonic current, whereas

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spontaneous synaptic currents remained but with modified frequency and amplitude (Fig. 9). Baseline current (holding potential –50 mV) declined from 134 ± 23 pA to 71 ± 9 pA following Zn^{2+} addition (n = 5; *** $p < 0.001$, t test). Root mean square noise reduction ranged from 7.1% to 22.0% in these 5 cells and was significant in each neuron (mean root mean square noise reduced from 6.21 ± 0.15 pA to 5.28 ± 0.2 pA, ** $p = 0.006$, paired t test). sIPSC frequency decreased from 3.4 ± 0.7 Hz to 0.8 ± 0.3 Hz while mean sIPSC amplitude decreased from 68.8 ± 9.0 pA to 44.9 ± 2.3 pA. Comparison of sIPSCs of similar amplitudes (50-60 pA) before and after Zn^{2+} exposure revealed no significant change in τ_{decay} (32.7 ± 1.2 ms vs 35.2 ± 1.8 ms, respectively). One explanation for Zn^{2+} 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 Zn^{2+}. However, Zn^{2+} 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 pA to 87 ± 9 pA (* $p < 0.05$, t test n = 5; Fig. 10A). Root mean square noise reduction was significant in 4 of the 5 cells; in the fifth cell, noise reduction (5.52 ± 0.04 pA to 5.45 ± 0.05 pA) was not significant ($p = 0.14$). The sIPSC kinetics (τ_{decay} 34.3 ± 1.2 ms vs 37.2 ± 2.8 ms following furosemide) and frequency were unaffected by furosemide.

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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 $GABA_A$ 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 6 cultured neurons tested, loreclezole (30 μM) did not have a consistent action. In 3 cells, baseline current was decreased significantly from 124 ± 12 pA to 91 ± 11 pA (Fig. 10B); in 3 other cells, no significant change was observed. In the 3 cells in which loreclezole reduced baseline current, root mean square noise was reduced significantly from 5.64 ± 0.07 pA to 5.16 ± 0.04 pA (*** $p < 0.001$). In the 3 remaining cells, root mean square noise reduction was not significant (5.48 ± 0.13 pA to 5.377 ± 0.13 pA). No change was observed in sIPSC frequency or decay kinetics regardless of loreclezoles's effect on the tonic inhibitory current. It should be noted that differing loreclezole effects were produced in cells from the same culture source.

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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 current 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 in the past by means of light and electron microscopy (Peng *et al.*, 2002;Sperk *et al.*, 1997). In adult brain, light microscopic studies revealed that this subunit was expressed in cerebellar granule cells, thalamus and 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

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expressed exclusively in the extrasynaptic membrane (Nusser *et al.*, 1998). Similarly, 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 supported the notion that the δ subunit was expressed on cultured hippocampal neurons. Enhancement of whole-cell $GABA_A$ receptor currents by 10 nM allopregnanolone suggested high sensitivity to neurosteroids, a property of δ subunit-containing $GABA_A$ receptors (Wohlfarth *et al.*, 2002). Because δ subunit-containing receptors desensitize very slowly (Saxena and Macdonald, 1996;Haas and Macdonald, 1999), $GABA_A$ 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 IPSC, further suggesting that δ subunit-containing receptors were present in extrasynaptic membrane. Moreover, Zn^{2+} 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 $GABA_A$ receptors in mediating tonic currents. Furosemide is known to inhibit α4 subunit-containing receptors and was demonstrated to inhibit $GABA_A$ receptor currents in dentate granule cells, which express the α4 subunit (Wafford *et al.*, 1996;Kapur and Macdonald,

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1999). Similarly, α4 subunit-containing $GABA_A$ 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 co-assembly 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 co-assembles 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 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 (Fisher and Macdonald, 1997;Wafford *et al.*, 1994). 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 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

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loreclezole or stay unaffected by it. In the current study, loreclezole inhibited tonic currents in 3 neurons and in remaining 3 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, while 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 previously been reported in mouse embryonic cultures of pyramidal neurons but these currents were found to be sensitive to the benzodiazepine, midazolam (Yeung *et al.*, 2003;Bai *et al.*, 2001). In addition, it was recently reported 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 (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.*, 2002b;Brunig *et al.*, 2002a;Christie *et al.*, 2002). Previous studies on the localization of $GABA_A$ receptor subunits in rat brain have demonstrated that α1, α2, α4, and α5 are present in the CA1 and CA3 subfields of the hippocampus, a finding confirmed by the immunofluorescent

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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 (Christie *et al.*, 2002;Brunig *et al.*, 2002a). Postembedding immunogold electron microscopic studies in the hippocampus and cerebellum further confirm that these subunits are present at synapses (Nusser *et al.*, 1998;Nusser *et al.*, 1995). 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 likely 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 due to signals from glutamatergic input (Christie *et al.*, 2002;Rao *et al.*, 2000). 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, Jr., 2000;Kittler *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

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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 (Brickley *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.

Molecular Pharmacology Fast Forward. Published on December 21, 2004 as DOI: 10.1124/mol.104.007385
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Acknowledgment: We thank Cassie Gregory and Ashley Renick for preparing hippocampal cultures. Dedicated to the memory of Patrick S Mangan Ph.D.

Molecular Pharmacology Fast Forward. Published on December 21, 2004 as DOI: 10.1124/mol.104.007385
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Reference List

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 γ-Aminobutyric Acid A Receptors in Hippocampal Neurons. *Mol Pharmacol* **59**: 814-824.

Barnes EM, Jr. (2000) Intracellular Trafficking of $GABA_A$ Receptors. *Life Sci* **66**: 1063-1070.

Brickley SG, Cull-Candy SG and Farrant M (1999) Single-Channel Properties of Synaptic and Extrasynaptic $GABA_A$ 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 $GABA_A$ 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 γ-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 $GABA_A$ Receptors and Gephyrin. *J Neurosci* **22**: 4805-4813.

Caraiscos VB, Elliott EM, You T, Cheng VY, Belelli D, Newell JG, Jackson MF, Lambert JJ, Rosahl TW, Wafford KA, MacDonald JF and Orser BA (2004) Tonic Inhibition in Mouse Hippocampal CA1 Pyramidal Neurons Is Mediated by α5 Subunit-Containing γ-Aminobutyric Acid Type A Receptors. *Proc Natl Acad Sci U S A* **101**: 3662-3667.

Christie SB and de Blas AL (2002) α5 Subunit-Containing $GABA_A$ 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 $GABA_A$ 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 $GABA_A$ Receptor Subtypes Requires the γ2 Subunit and Gephyrin. *Nat Neurosci* **1**: 563-571.

Fisher JL and Macdonald RL (1997) Functional Properties of Recombinant $GABA_A$ Receptors Composed of Single or Multiple β Subunit Subtypes. *Neuropharmacology* **36**: 1601-1610.

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) 339-370, The MIT Press, Cambridge.

Greenfield LJ, Jr. and Macdonald RL (1996) Whole-Cell and Single-Channel α1β1γ2S $GABA_A$ Receptor Currents Elicited by a "Multipuffer" Drug Application Device. *Pflugers Arch* **432**: 1080-1090.

Haas KF and Macdonald RL (1999) $GABA_A$ Receptor Subunit γ2 and δ Subtypes Confer Unique Kinetic Properties on Recombinant $GABA_A$ 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. *Pflugers Arch* **391**: 85-100.

Kapur J and Macdonald RL (1999) Postnatal Development of Hippocampal Dentate Granule Cell γ-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 $GABA_A$ 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 $GABA_A$ 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 α1 and β2/3 Subunits of the $GABA_A$ 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 $GABA_A$ 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) $GABA_A$ Receptor Changes in δ Subunit-Deficient Mice: Altered Expression of α4 and γ2

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 $GABA_A$ Receptor Isoforms. *Mol Pharmacol* **49**: 567-579.

Scotti AL and Reuter H (2001) Synaptic and Extrasynaptic γ-Aminobutyric Acid Type A Receptor Clusters in Rat Hippocampal Cultures During Development. *Proc Natl Acad Sci U S A* **98**: 3489-3494.

Semyanov A, Walker MC, Kullmann DM and Silver RA (2004) Tonically Active $GABA_A$ Receptors: Modulating Gain and Maintaining the Tone. *Trends Neurosci* **27**: 262-269.

Sieghart W and Sperk G (2002) Subunit Composition, Distribution and Function of $GABA_A$ Receptor Subtypes. *Curr Top Med Chem* **2**: 795-816.

Soltesz I and Nusser Z (2001) Neurobiology. Background Inhibition to the Fore. *Nature* **409**: 24-5, 27.

Sperk G, Schwarzer C, Tsunashima K, Fuchs K and Sieghart W (1997) $GABA_A$ Receptor Subunits in the Rat Hippocampus I: Immunocytochemical Distribution of 13 Subunits. *Neuroscience* **80**: 987-1000.

Stelzer A, Kay AR and Wong RK (1988) $GABA_A$-Receptor Function in Hippocampal Cells Is Maintained by Phosphorylation Factors. *Science* **241**: 339-341.

Sur C, Farrar SJ, Kerby J, Whiting PJ, Atack JR and McKernan RM (1999) Preferential Coassembly of α4 and δ Subunits of the γ-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 $GABA_A$ Receptor β Subunit. *Neuron* **12**: 775-782.

Wafford KA, Thompson SA, Thomas D, Sikela J, Wilcox AS and Whiting PJ (1996) Functional Characterization of Human γ-Aminobutyric Acid A Receptors Containing the α4 Subunit. *Mol Pharmacol* **50**: 670-678.

Wisden W, Laurie DJ, Monyer H and Seeburg PH (1992) The Distribution of 13 $GABA_A$ 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 $GABA_A$ Receptors Containing the δ Subunit. *J Neurosci* **22**: 1541-1549.

Yeung JY, Canning KJ, Zhu G, Pennefather P, MacDonald JF and Orser BA (2003) Tonically Activated $GABA_A$ Receptors in Hippocampal Neurons Are High-Affinity, Low-Conductance Sensors for Extracellular GABA. *Mol Pharmacol* **63**: 2-8.

This work was supported by the NINDS grants NS 37192 to PSM and NS 02081 and NS40337 to JK, and Epilepsy Foundation grant to CS.

Molecular Pharmacology Fast Forward. Published on December 21, 2004 as DOI: 10.1124/mol.104.007385
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Figure Legends

Fig. 1 GABAergic synapses on pyramidal neurons in culture for 14-18 days. **(A)** A visually identified pyramidal cell filled with biocytin during electrophysiological recordings, and stained with AMCA-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. The scale bar shown in Fig. A, 20 μm also applies to panel B. All subsequent images were visualized with a 40X objective lens, scale bar = 10 μm was shown in Fig. C. Dendrites of a pyramidal neuron stained for synaptophysin **(C)**, 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)**, GAD65 **(G)** and the two images were superimposed **(H)** to demonstrate colocalization of GAD65 and GAD65/67 immunoreactivity clusters.

Fig. 2 A comparison of γ2 and δ $GABA_A$ 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. **(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**; boxed areas in **A** and **B** were 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

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colocalize with GAD65 immunoreactivity, see boxed areas in **E** and **F,** magnified in **G** and **H**, respectively.

Fig. 3 Comparison of δ and α4 immunoreactivity in non-permeabilized neurons and neurons exposed to 0.1% Triton X-100 prior to primary antibody application. δ and α4 subunit immunoreactivity was diffusely expressed in both non-permeabilized **(A, C,** respectively) and permeabilized **(B, D,** respectively).

Fig. 4 Distribution of α1, α2, and α4 $GABA_A$ receptor subunit immunoreactivity and colocalization with GAD65 immunoreactivity in cultured hippocampal pyramidal neurons. α1 **(A)** and α2 **(E)** immunoreactivity was in the form of clusters on the periphery of the cell soma and along dendrites. GAD65 immunoreactivity **(B, F)** showed a similar distribution. Examination of subunit/GAD65 colocalization revealed that both α1 and α2 exhibited overlap with GAD65 as indicated by arrowheads in boxed areas in **A, B,** magnified in **C, D**, respectively; boxed areas in **E, F,** magnified in **G, H**, respectively. The distribution of the α4 subunit was more diffuse with little apparent clustering of immunoreactive puncta **(I)**. Colocalization of the α4 subunit with GAD65 **(J)** was not detected as demonstrated in boxed areas in **I, J,** magnified in **K, L**, respectively.

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 GAD65 showed significant clustering on the soma and dendritic tree of the same cell **(B)**. No colocalization between β1 and GAD65 immunoreactivity was evident as shown in boxed areas in **A, B** magnified in **C, 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, F,** magnified in **G, H**, respectively. Little colocalization was evident between β1 **(I)** and β2/3 **(J)** subunits (boxed areas in **I, J,** magnified in **K, L**, respectively.

Fig. 6 Diazepam enhanced whole-cell $GABA_A$ receptor-mediated currents. **A,** augmentation of whole-cell currents evoked by 3 μM GABA directly co-applied with several concentrations of diazepam. **B,** concentration-response profiles for 6 cells exposed to 3 μM GABA co-applied with multiple concentrations of diazepam (1 - 1000 nM). EC50 values for diazepam enhancement of GABAergic currents encompassed a narrow range, therefore individual responses were combined (**C**).

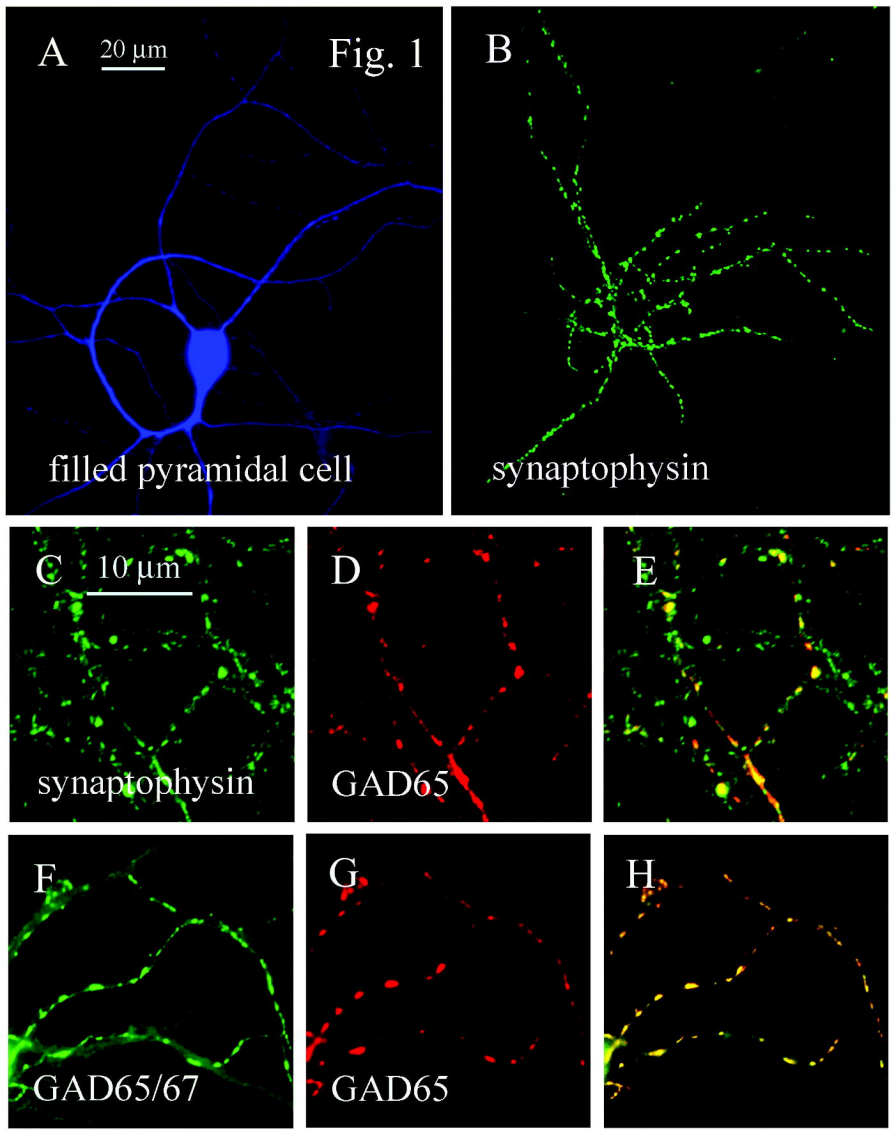
Fig. 7 Tonic and phasic GABAergic currents recorded from cultured neurons. Currents consisted of a tonic background current on which were superimposed discrete phasic spontaneous inhibitory postsynaptic currents (sIPSCs; left expanded trace). Application of the $GABA_A$ receptor antagonist bicuculline decreased the tonic current and abolished phasic currents (right expanded trace). Tonic currents fluctuated 10-20 pA around the mean value before bicuculline addition; current fluctuations were eliminated following 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.

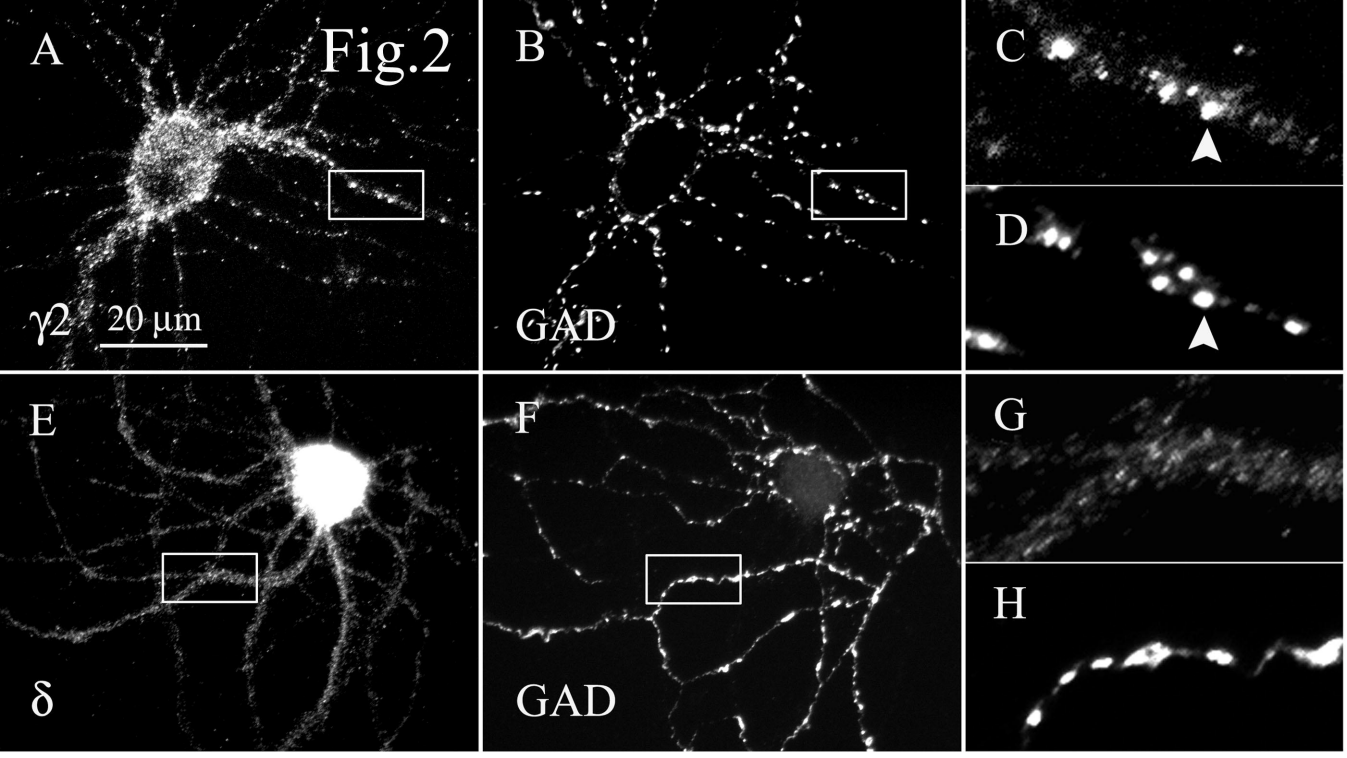
Fig. 8 Diazepam modulation of tonic and phasic GABAergic currents. Diazepam (30 nM) did not alter the level of tonic inhibition but did alter sIPSC kinetics (see text). The

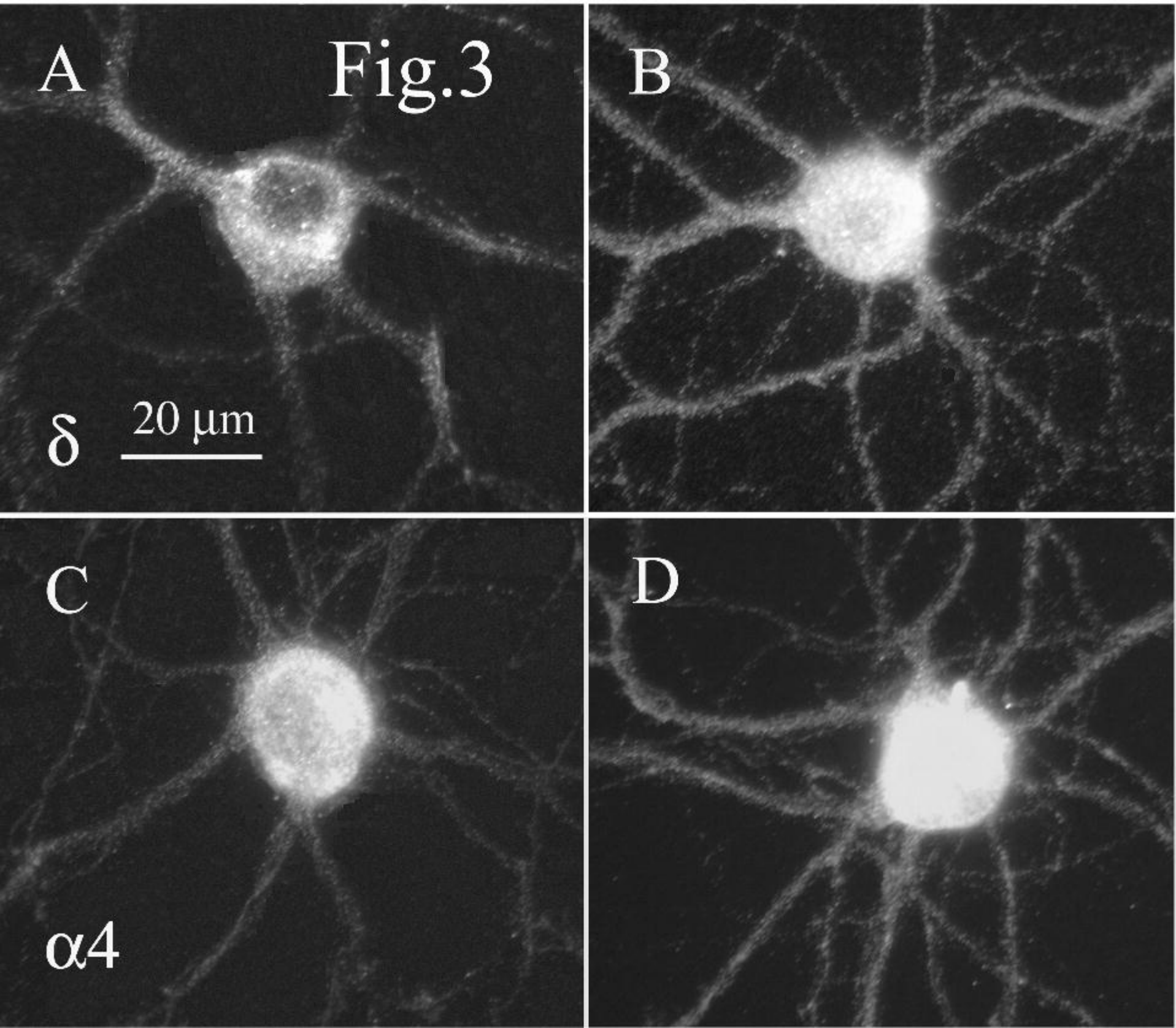
expanded traces show tonic and phasic currents before and after diazepam application.

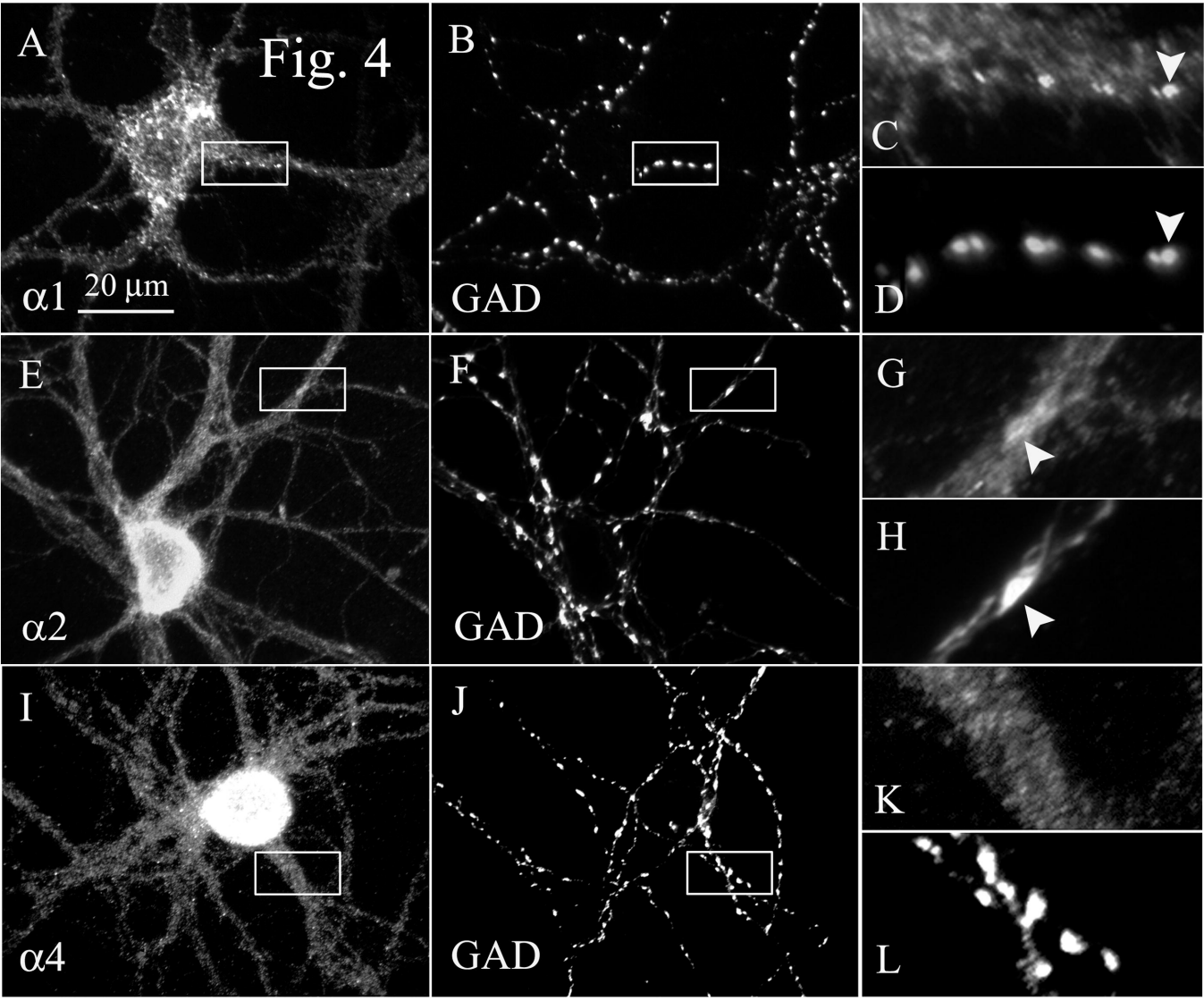
Fig. 9 Zn^{++} modulation of tonic and phasic GABAergic currents. Zn^{++} (60 μM) decreased the tonic current and decreased sIPSC frequency but did not alter sIPSC kinetics (see text). The expanded traces show tonic and phasic currents before and after tonic current reduction.

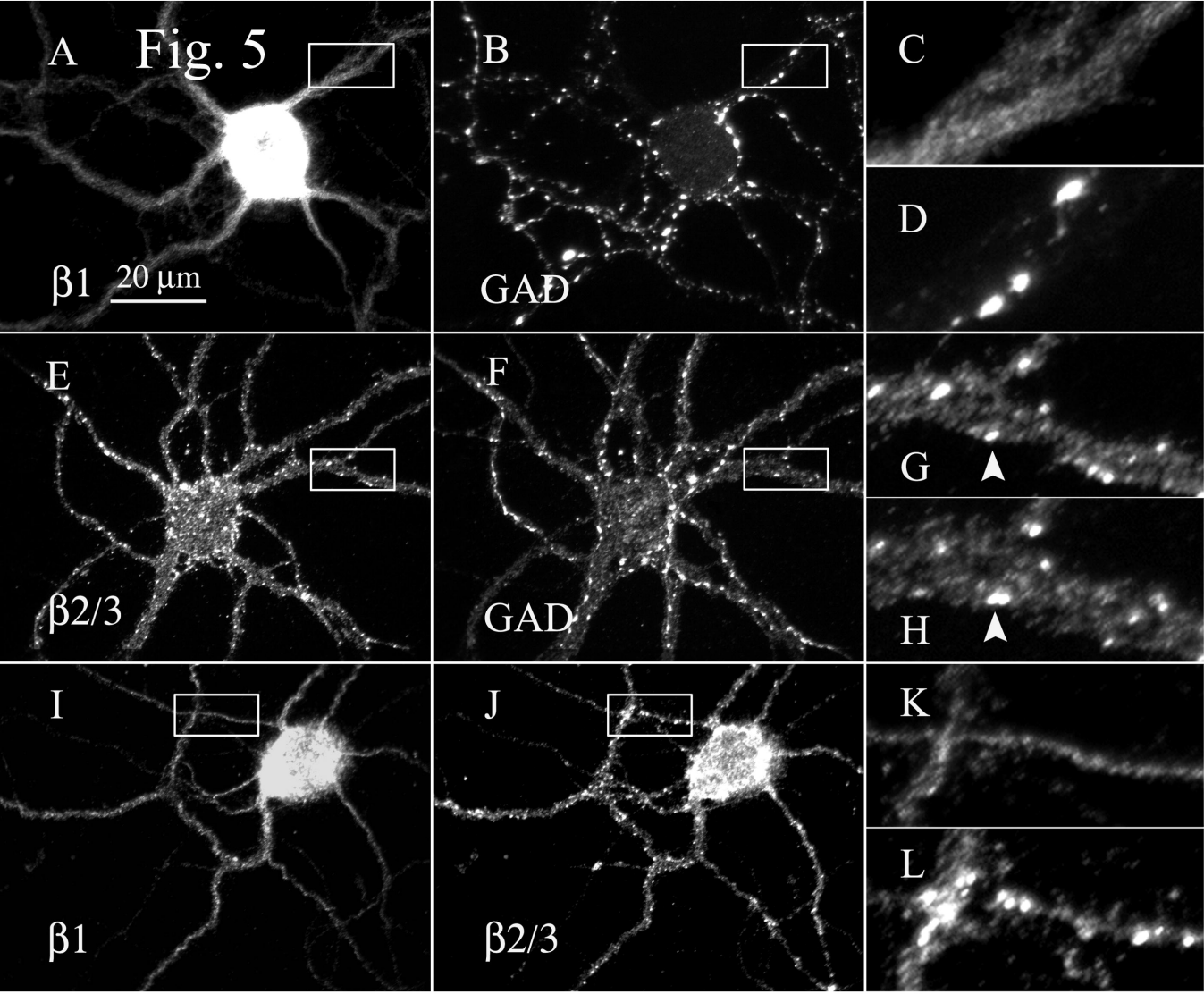
Fig. 10 Furosemide and loreclezole modulation of tonic currents. **A,** Application of furosemide (100 μM) caused a decrease in tonic inhibition but did not alter the frequency or kinetics of synaptic inhibitory currents. Expanded traces show currents before and after furosemide addition. **B,** In this example, loreclezole (30 μM) caused a reduction in the tonic current; however, this result was obtained in only 3 of 6 cells examined. Expanded traces show currents before and after loreclezole application.


A
20 µm
Fig. 1
filled pyramidal cell
B
synaptophysin
C
10 µm
synaptophysin
D
GAD65
E
F
GAD65/67
G
GAD65
H


A
Fig.2
γ2
20 μm
B
GAD
C
D
E
δ
F
GAD
G
H


A
Fig.3
B
δ
20 μm
C
α4
D


A
Fig. 4
α1
20 μm
B
GAD
C
D
E
α2
F
GAD
G
H
I
α4
J
GAD
K
L


A
Fig. 5
β1
20 μm
B
GAD
C
D
E
β2/3
F
GAD
G
H
I
β1
J
β2/3
K
L

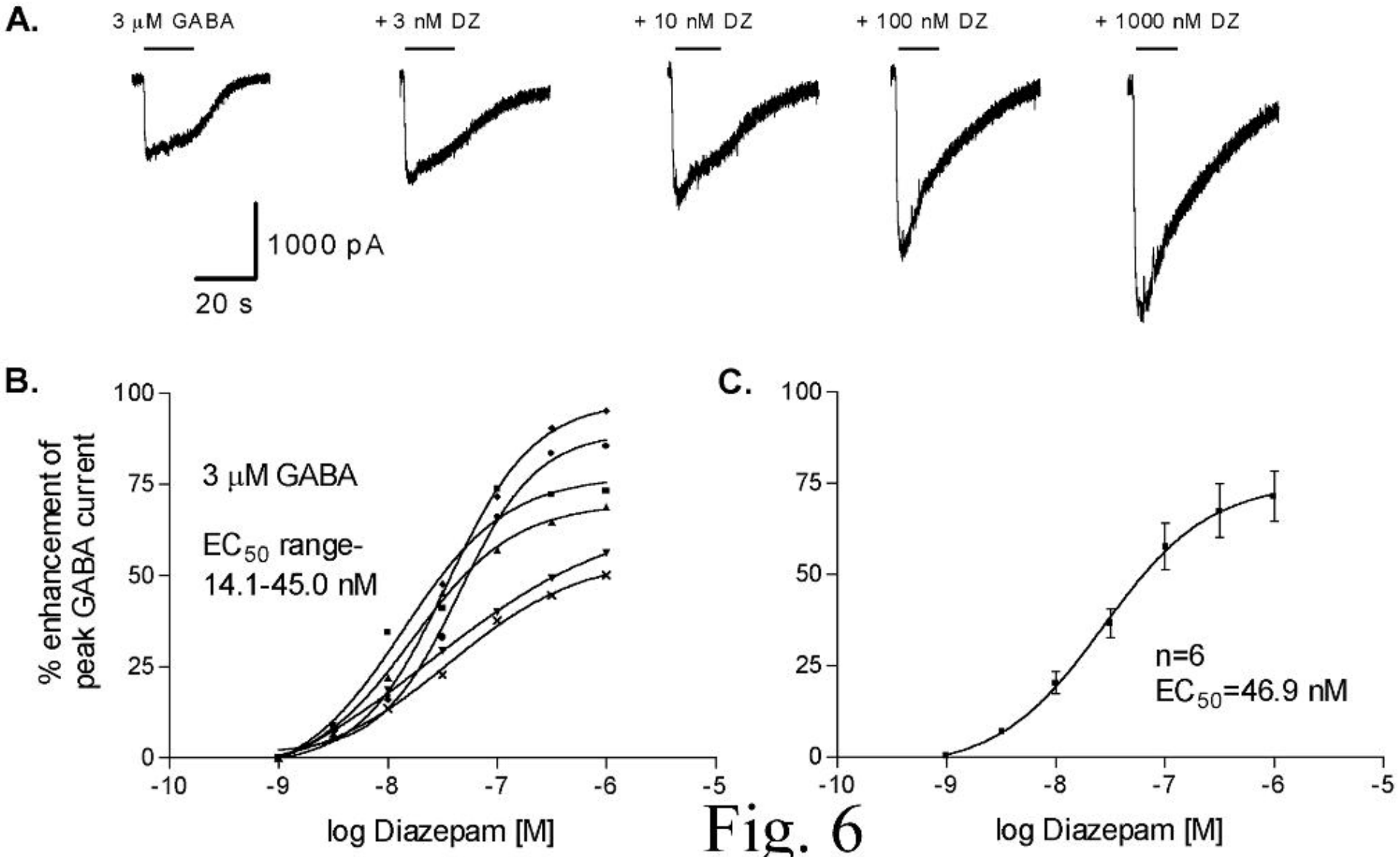

A.
3 μM GABA
+ 3 nM DZ
+ 10 nM DZ
+ 100 nM DZ
+ 1000 nM DZ
1000 pA
20 s
B.
% enhancement of peak GABA current
3 μM GABA
EC50 range-
14.1-45.0 nM
log Diazepam [M]
C.
n=6
EC50=46.9 nM
log Diazepam [M]

Fig. 6

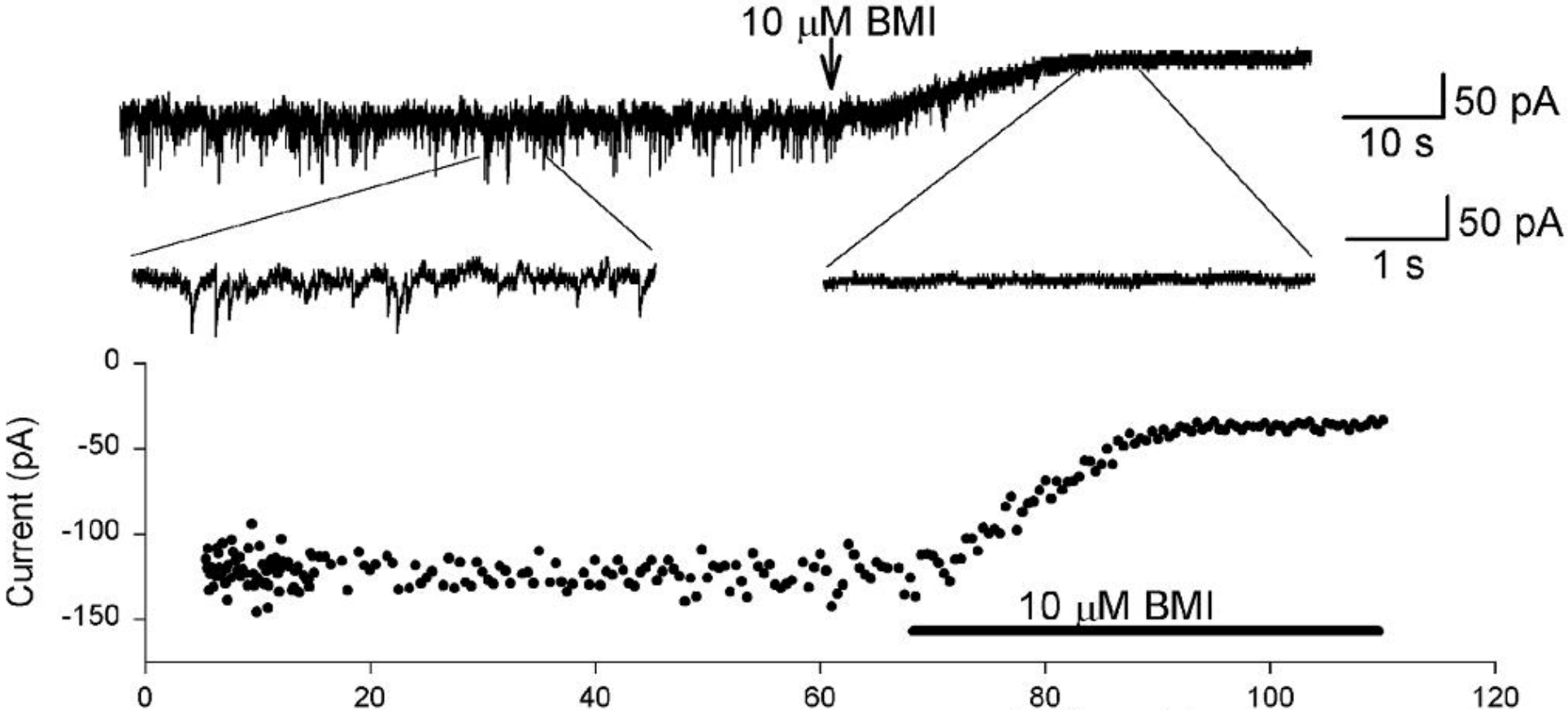

10 μM BMI
50 pA
10 s
50 pA
1 s
0
-50
-100
-150
Current (pA)
10 μM BMI
0
20
40
60
80
100
120
Time (s)

Fig. 7

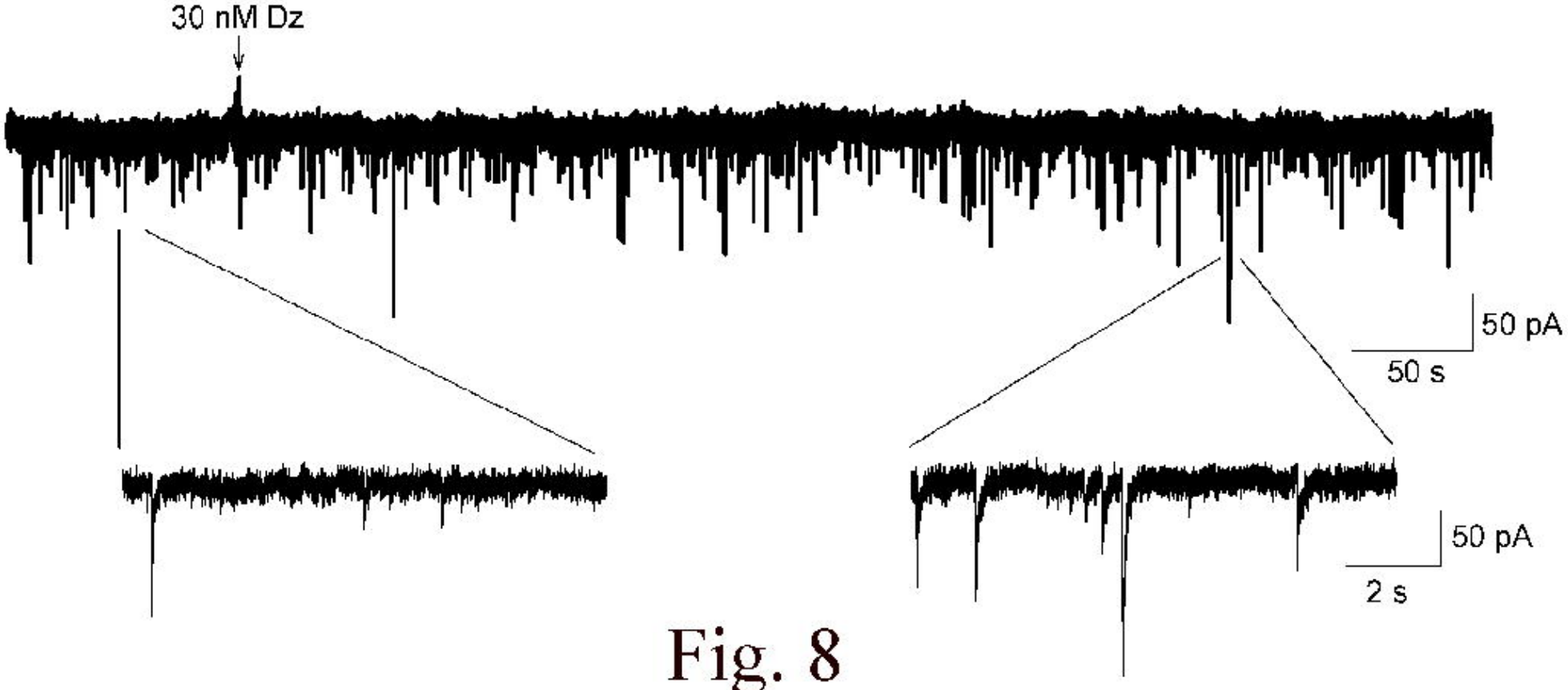


Fig. 8

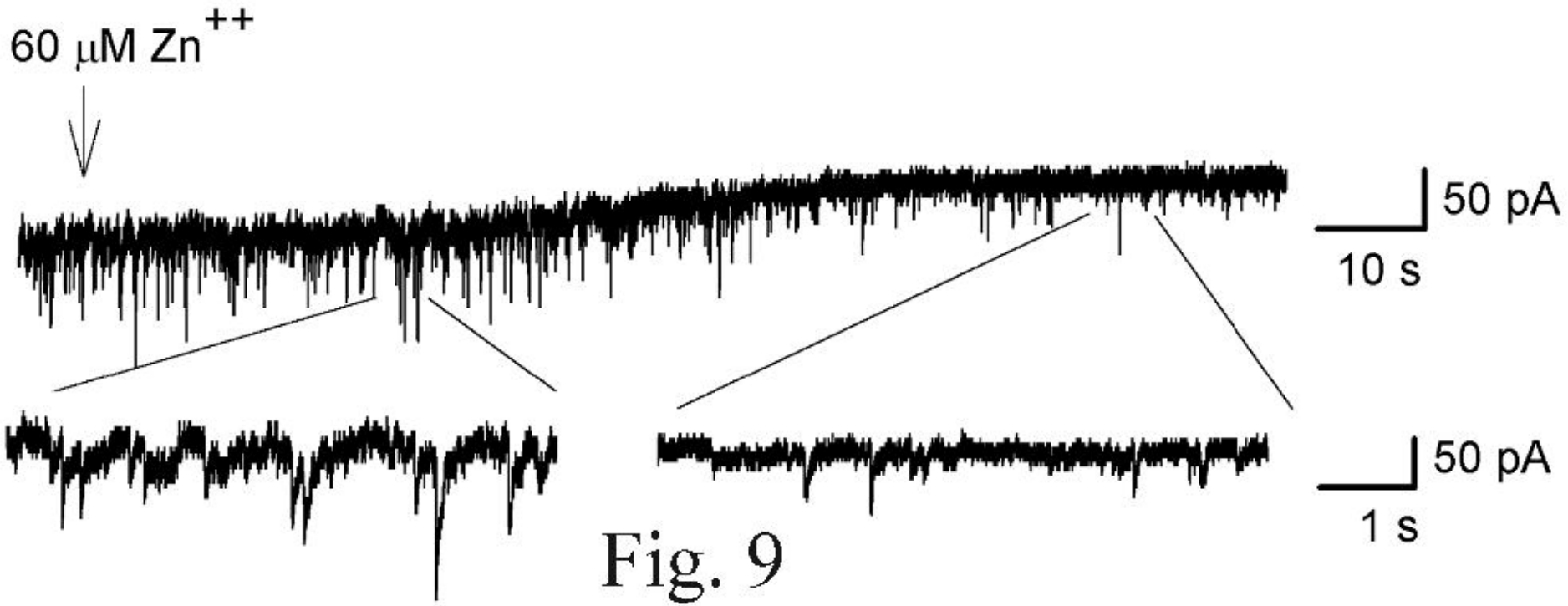
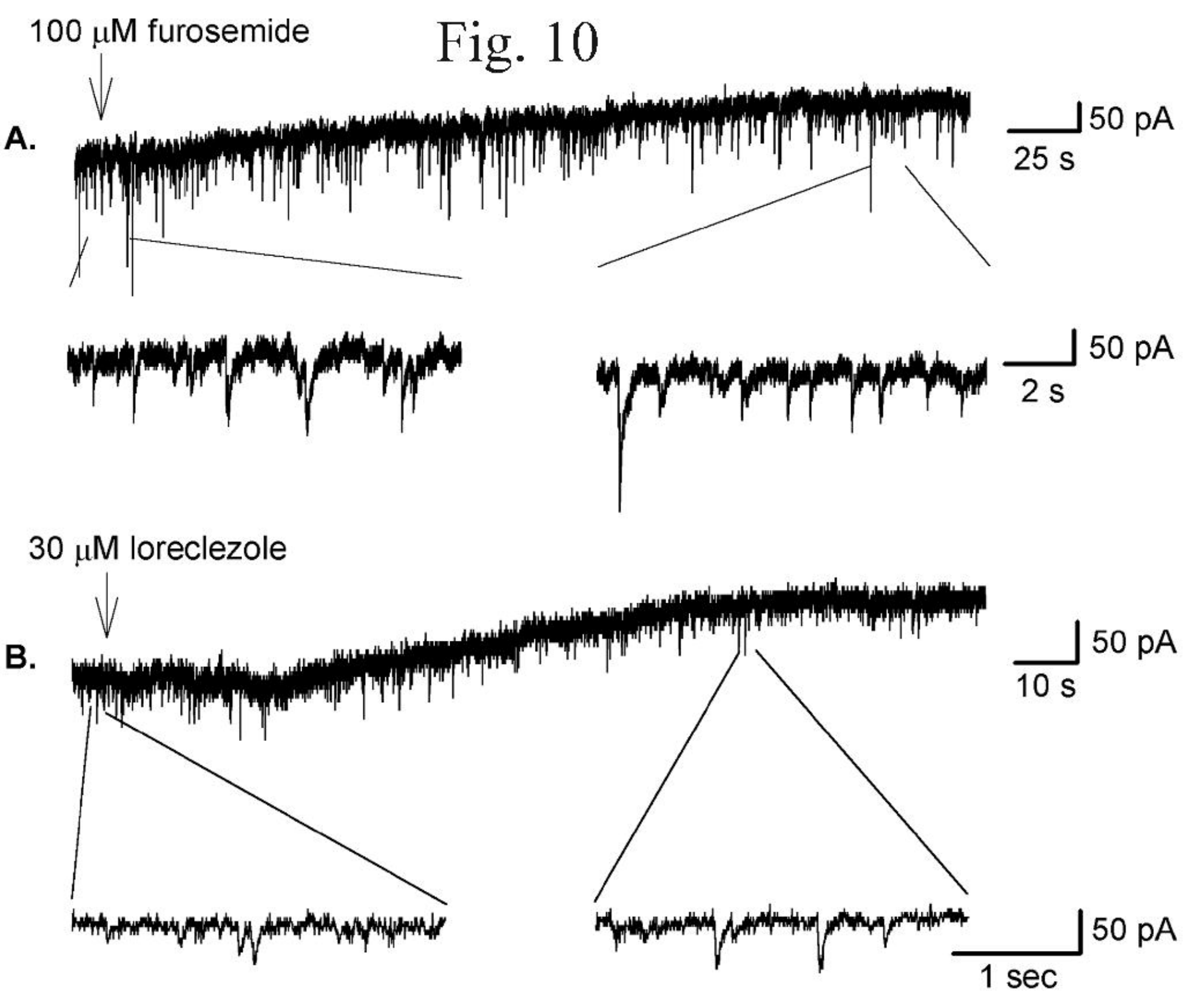


Fig. 9

Fig. 10


100 μM furosemide
A.
50 pA
25 s
50 pA
2 s
30 μM loreclezole
B.
50 pA
10 s
50 pA
1 sec