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Properties of Recombinant γ-Aminobutyric $Acid_A$ Receptor Isoforms Containing the $\alpha5$ Subunit Subtype

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SUMMARY

The cDNAs encoding $\alpha5$ and $\gamma2L$ subunit subtypes of the γ-aminobutyric acid (GABA) type A receptor (GABAR) were transfected into L929 cells together with cDNAs encoding either the $\beta1$, $\beta2$, or $\beta3$ subunit subtype. Properties of expressed recombinant $\alpha5\beta X\gamma2L$ (where X = 1, 2, or 3) GABARs were studied with the use of whole-cell, patch-clamp techniques. In cells voltage-clamped at −70 mV with equivalent bath and pipette chloride concentrations, the application of GABA produced a concentration-dependent inward chloride current with all three $\alpha5\beta X\gamma2L$ isoforms. Minimal or no responses were recorded from cells transfected with only two subunit cDNAs, demonstrating that all three subunits were required for functional receptor assembly in these cells. The GABA concentration producing a half-maximal current was similar for $\beta2$ and $\beta3$ subtype-containing receptors (6 μM) but higher for $\beta1$ subtype-containing receptors (26 μM). $\alpha5\beta3\gamma2L$ receptors were zinc and diazepam sensitive but zolpidem insensitive. In response to low GABA concentrations, $\beta1$ and $\beta3$ subtype-containing receptors showed outward rectification of the current-voltage relationship, whereas current-voltage responses of $\beta2$ subtype-containing receptors were relatively linear. Likewise, at high GABA concentrations, $\beta1$ and $\beta3$ subtype-containing receptors showed less desensitization at positive than at negative membrane potentials. $\beta2$ subtype-containing receptors displayed faster desensitization at depolarized potentials. These voltage-dependent properties were characteristic of $\alpha5$ but not $\alpha1$ or $\alpha6$ subtype-containing receptors and were similar to responses recorded from hippocampal CA1 pyramidal neurons. Based on the pharmacological and biophysical similarities to hippocampal GABAR responses, the $\alpha5\beta3\gamma2L$ isoform could represent a native GABAR subtype.

Theoretically, GABARs can be assembled from ≥16 subtypes of five subunit families, each a separate gene product that displays a distinct distribution pattern within the central nervous system (1, 2). At least six α ($\alpha1$–6), four β ($\beta1$–4), three γ ($\gamma1$–3), one δ, and two ρ ($\rho1$ and $\rho2$) subunit subtypes have been identified (3, 4). It is thought that native GABARs are formed through the combination of five separate subunit subtypes (5). Although the exact subunit composition of native receptors is not known, there is growing evidence that benzodiazepine-sensitive GABARs may exist in the $\alpha\beta\gamma$ conformation (6). The precise role of each subunit in regulating receptor function also is not known, but a number of studies have indicated that both α and β subunits regulate GABA binding to the receptor (7, 8). The $\gamma2$ subtype confers typical high affinity benzodiazepine binding to recombinant $\alpha\beta\gamma2$ receptors, and α subunit subtypes determine the type (I or II) of benzodiazepine receptor that is produced (6, 9). For example, expression of the $\alpha5$ subtype (10) in combination with β and $\gamma2$ subtypes produces receptors with a novel (ZOL insensitive) benzodiazepine type II pharmacology, whereas the presence of the $\alpha1$ subtype produces receptors with ZOL-sensitive benzodiazepine type I pharmacology (11). The subunit composition can therefore determine the pharmacological properties of different GABAR isoforms.

Because GABARs mediate fast synaptic inhibition within the brain, different GABAR isoforms could confer unique pharmacological properties to individual inhibitory synapses. Although a large number of GABAR isoforms are theoretically possible, distinct regional distribution patterns of specific subunit mRNAs in the brain (2) suggest that expression of structurally (and possibly functionally) distinct GABAR isoforms varies according to brain region. Specifically, in the adult rat brain, $\alpha5$ subtype mRNA seems to be primarily localized to the hippocampus. Although other α subtypes are expressed in hippocampus ($\alpha1$, $\alpha2$, and $\alpha4$), they are also expressed in a variety of brain regions. The $\alpha5$ subtype is unique in that it is preferentially expressed at high levels in

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ABBREVIATIONS: GABAR, γ-aminobutyric acid type A receptor; GABA, γ-aminobutyric acid; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; PIPES, piperazine-*N,N'*-bis(2-ethanesulfonic acid); DZP, diazepam; ZOL, zolpidem; I-V, current-voltage, V_h, membrane holding potential; β-gal, β-galactosidase; GFP, green fluorescent protein; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid.

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the hippocampus. Of the three β subtypes, β1 and β3 but not β2 mRNAs are present in high levels in the hippocampus. The γ2 subtype mRNA is also expressed in high levels in the hippocampus (2). McKernan *et al.* (12) and Mertens *et al.* (13) have shown that GABARs immunoprecipitated from hippocampus with the use of α5-specific antibodies exhibit a pharmacological profile indicative of an α5βXγ2 (X = 1, 2, or 3) configuration. Binding studies with recombinant GABARs have suggested, based on benzodiazepine binding profiles, that the α5β3γ2 isoform may be the native conformation of ZOL-insensitive GABARs in the hippocampus (14). Based on these studies, a native hippocampal GABAR isoform could be α5β3γ2.

We studied the properties of α5 subtype-containing recombinant GABARs to determine which pharmacological properties are determined by the presence of a particular β subunit (β1, β2, or β3) within the receptor. Receptors containing the α5 subtype were also compared with other α subtype-containing GABARs, as well as with hippocampal GABARs, to determine similarities or differences among recombinant and native receptor isoforms. We report that β subtypes regulate GABA binding properties as well as voltage-dependent properties of the receptor and that properties of the α5β3γ2 isoform are consistent with GABAR properties of hippocampal CA1 pyramidal cells. Preliminary results have appeared in abstract form (15).

Materials and Methods

Expression of recombinant GABARs. Mouse L929 fibroblast cells (American Type Culture Collection, Rockville, MD) were maintained in Dulbecco's modified Eagle's medium supplemented with 10% horse serum, 100 IU/ml penicillin, and 100 mg/ml streptomycin and incubated at 37° in 5% CO_2/95% air. Cells were passaged with 0.5% trypsin/0.2% EDTA as needed to prevent growth to confluency in the culture dish. Transfection of cells was performed as described previously (16, 17). Briefly, cDNAs encoding rat α1, α5, β1 (Dr. A. Tobin, Department of Biology, University of California at Los Angelos), α6, β2, γ2L (F. Tan, Department of Neurology, University of Michigan), and β3 (Dr. D. Pritchett, University of Pennsylvania) subtypes were individually subcloned into the mammalian expression vector pCMVNeo (18). Marker cDNAs encoding either *Escherichia coli* β-gal or *Aequorea victoria* GFP (Dr. M. Chalfie, Department of Biological Science, Columbia University) were also individually subcloned into pCMVNeo to allow visualization of positively transfected cells (see below). Plasmids were mixed in a 1:1:1:1 ratio (α/β/γ/marker) for three-subunit transfection or a 1.5:1.5:1 ratio for two-subunit transfection. Total transfected DNA was maintained at 16 μg. For some experiments, cDNA encoding the β2 subtype was subcloned into the vector pCDM8, which also contained the CMV promoter. Increased transfection efficiency was observed with this vector. However, responses recorded from these GABARs were qualitatively similar to those expressed with pCMVNeo. Cells were transfected according to the modified calcium phosphate coprecipitation method (19) with various combinations of constructs. After a 4–5-hr incubation in the presence of plasmid DNA, cells were exposed to a 30-sec glycerol (15% in *N,N*-bis(2-hydroxyethyl)-*N*-aminoethanesulfonic acid-buffered saline) shock. At 24 hr after transfection, cells were passaged, treated with 350 mg/ml DNase I, and replated onto gridded 35-mm dishes. Electrophysiological analysis was performed 24 hr later.

Mouse L929 cells transfect with a low efficiency (~5%) (16). To identify positively transfected cells, cDNA encoding either β-gal or GFP was cotransfected with the cDNAs encoding the GABAR subunits. Cell staining with the fluorescent β-gal substrate fluorescein di-β-D-galactopyranoside (Molecular Probes, Eugene, OR) was used to identify β-gal-positive cells. Di-β-D-galactopyranoside-stained or GFP-positive cells were identified with the use of an inverted microscope equipped with fluorescein fluorescence optics, and their positions on the gridded dish were recorded for later electrophysiological analysis.

Acute dissociation of hippocampal neurons. CA1 pyramidal cells were acutely dissociated from 28–35-day-old Sprague-Dawley rats (Harlan Sprague-Dawley, Indianapolis, IN). Rats were killed by decapitation after CO_2 narcosis, and the brains were rapidly dissected. Coronal slices (500 μm) were prepared with the use of a Vibroslice (WPI, Sarasota, FL) in ice-cold oxygenated (95% O_2/5% CO_2) PIPES saline buffer containing 120 mM NaCl, 2.5 mM KCl, 1 mM $MgCl_2$, 1.5 mM $CaCl_2$, 25 mM glucose, and 20 mM PIPES, pH 7.0. After 1 hr at room temperature, one slice was incubated for 30 min at 32° in protease Type XXIII (3 mg/ml, Sigma Chemical Co., St. Louis, MO) in PIPES buffer. After an additional 15–60-min recovery period in PIPES buffer, neurons were acutely isolated through trituration of 1-mm fragments microdissected from the CA1 region. Cells were plated onto poly-L-lysine-coated (0.1 mg/ml) 35-mm culture dishes filled with external recording medium and were allowed to adhere for 10 min before recording. Neurons used for recording were distinctly pyramidal, with an extended apical dendrite as well as smaller basal dendrites.

Solutions and drug application. For electrophysiological recording, an external recording medium was used consisting of 142 mM NaCl, 8.1 mM KCl, 6 mM $MgCl_2$, 10 mM glucose, and 10 mM HEPES, pH 7.4. Patch-clamp electrodes contained a solution consisting of 153 mM KCl, 1 mM $MgCl_2$, 5 mM EGTA, 10 mM HEPES, and 2 mM ATP, pH 7.3. The use of this combination of intracellular and extracellular recording solutions produced a calculated chloride equilibrium potential of 0 mV and a potassium equilibrium potential of −75 mV. The electrode solution used for recording from CA1 pyramidal cells also contained an ATP-regenerating system containing creatine phosphokinase (50 units/ml) and phosphocreatine (25 mM, Sigma) to prevent run-down of GABA-evoked currents during the recording period. GABAR responses recorded with or without the ATP-regenerating system were qualitatively similar. Compounds were applied with the use of a modified U-tube application system, which allowed successive applications of multiple concentrations of drugs to be applied through a micropipette (20–30-μm tip diameter) placed next to the cell. The 10–90% rise time achieved with this system was ~70 msec.

GABA (Sigma) and DZP and ZOL (RBI, Natick, MA) typically were applied for 5–10 sec, either alone or in combination. All experiments were performed at room temperature.

Electrophysiology. Whole-cell, voltage-clamp recordings according to the patch-clamp technique were obtained as described previously (20) with an EPC-7 amplifier (List Medical Instruments, Darmstadt, Germany). Patch-clamp electrodes were pulled from microhematocrit capillary tubing or thin-walled borosilicate glass (WPI, Sarasota, FL) with the use of a P-87 Flaming-Brown micropipette puller (Sutter Instrument Co., San Rafael, CA) to tip resistances of 3–10 MΩ and coated with Q-dope. In fibroblasts, input resistances were routinely >1 GΩ, and cells usually required <50 pA of negative holding current to maintain V_h = −70 mV. Signals were low-pass filtered (3 dB at 2 kHz, eight-pole Bessel filter), digitized, recorded, and analyzed with the use of Axotape software (Axon Instruments, Foster City, CA).

Data analysis. Peak current amplitudes were measured and used for I-V and concentration-response relationships. Concentration-response curves were generated using a modified Marquardt nonlinear regression method, with best fit determined through a least-squares method (PRISM, GraphPAD, San Diego, CA). To quantify whole-cell current rectification, peak amplitudes of responses to GABA (at EC_{50} concentrations) were measured at holding potentials of −50 and +50 mV. These responses exhibited no visible desensitization. An amplitude ratio (+50 mV/−50 mV) was calculated, and rectification was

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determined with respect to a linear ratio of 1.0 using the predicted chloride reversal potential = 0 mV. A larger-amplitude ratio was indicative of greater outward rectification. To quantify voltage-dependent differences in desensitization, the amount of desensitization produced by a saturating GABA concentration (100–1000 μM) was first determined at holding potentials of −50 and +50 mV. Desensitization was expressed as 1 minus (the amplitude at the end of GABA application/peak GABA amplitude). A desensitization ratio (+50 mV/−50 mV) was then calculated according to the method used to characterize rectification. A smaller desensitization ratio was indicative of less desensitization at depolarized membrane potentials. Statistical comparisons were performed with one-way analysis of variance with a post-hoc Dunnett's test for multiple comparisons. A statistical significance level of 0.05 was chosen for all analyses.

Results

Specific assembly of α5β3γ2L GABARs. Expression of functional GABARs was determined according to the ability of a high concentration of GABA (1 mM) to generate an inward chloride current in a cell voltage-clamped at −70 mV. Of cells transfected with three subunit cDNAs (α5βXγ2L, as determined through positive fluorescence staining), a high percentage responded to GABA, with currents of 100-3000 pA. Ninety percent of α5β3γ2L (40 of 44 positively stained cells), 60% of α5β2γ2L (12 of 21), and 70% of α5β1γ2L (18 of 27) cells responded to GABA. Transfections with only two-subunit cDNAs resulted in few cells expressing functional GABA receptors. Only 1 of 8 α5γ2L, 0 of 6 β3γ2L, and 3 of 8 α5β3 cells responded to GABA. Of the α5β3 cells that responded, the peak currents ranged from 20 to 50 pA, significantly less than for cells transfected with α, β, and γ subunits (Fig. 1). Specific expression of αβγ subunit-containing GABARs in L929 cells has been demonstrated previously (21) and indicates that the three subunits are necessary for proper sorting, assembly, and expression of functional GABARs in these cells.

Effects of benzodiazepine receptor ligands and Zn^{2+} on α5β3γ2L GABAR function. Allosteric regulators of GABAR function can interact with the receptor at a number of sites, including distinct binding sites for benzodiazepines and Zn^{2+}. Activities of various ligands at the benzodiazepine site have been shown to be dependent on the presence of the γ2 subtype and influenced by the α subtype present (9). The α5 subtype confers sensitivity to the benzodiazepine DZP but does not confer sensitivity to another benzodiazepine-site ligand, the imidazopyridine ZOL (11). Thus, functional expression of specific αβγ subunit-containing GABARs can also be demonstrated by benzodiazepine sensitivity. In α5β3γ2L-transfected cells, DZP enhanced current amplitudes produced by an EC_{50} (3 μM) GABA concentration (Fig. 2A). The EC_{50} for DZP enhancement was 40 nM (five experiments, Fig. 2B). This enhancement was also dependent on the presence of the γ2L subtype because currents recorded from α5β3 subtype-transfected cells were insensitive to DZP (Fig. 2C). In contrast, ZOL did not significantly potentiate responses to GABA, even at concentrations ≤10 μM (three experiments, Fig. 2, A and B). These findings confirm the benzodiazepine sensitivity and ZOL insensitivity of α5 subtype-containing GABARs and support the concept of specific assembly and expression of α5β3γ2L GABARs in L929 cells.

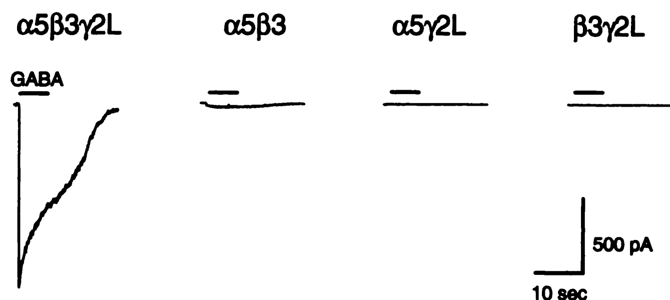

Fig. 1. Specific assembly of α5β3γ2L GABARs in L929 cells. Representative current traces recorded from cells transfected with various combinations of subunit subtype cDNAs (V_h = −70 mV). *Horizontal bar*, GABA application (1 mM for α5β3γ2L, β3γ2L, and α5γ2L; 300 μM for α5β3).

Extracellular application of the divalent cation Zn^{2+} decreased α5β3γ2L GABAR responses in a concentration-dependent manner (Fig. 3). In the presence of an EC_{50} concentration of GABA (3 μM), Zn^{2+} inhibited GABAR responses with an IC_{50} of 22 μM (three experiments). Currents were decreased to ~10% of control in the presence of 300 μM Zn^{2+}. These results are consistent with previous findings from our laboratory regarding the potency and efficacy of Zn^{2+} on other GABAR isoforms containing α subunit subtypes in combination with the γ2L subtype (22).

Incorporation of β subunit subtypes affects GABA sensitivity. Combination of α5 and γ2L subtypes with either β1, β2, or β3 subtype produced GABARs with different sensitivities to GABA. As can be seen in Fig. 4A, increasing concentrations of GABA produced an increase in peak inward current amplitudes recorded from a cell expressing α5β3γ2L GABARs. In the presence of low GABA concentrations, currents increased to a plateau with little or no evidence of acute desensitization (see also Figs. 2A and 3B). At higher GABA concentrations, currents consisted of a rapid rise to peak plus a multiphasic desensitizing component. GABA EC_{50} values for both α5β3γ2L (6 μM, 14 experiments) and α5β2γ2L (6 μM, six experiments) isoforms were similar, whereas the EC_{50} for the α5β1γ2L isoform was higher (26 μM, nine experiments, Fig. 4B). Curves for all isoforms had similar Hill coefficient values (1.1–1.3), and all reached maximum current amplitudes at GABA concentrations of ≤1 mM. These data suggest that variations in the β subtype affect GABA affinity and may indicate a role for the extracellular domain of this subunit in forming the GABA binding site on the receptor.

Voltage-dependent properties of currents from α5 subtype-containing GABARs. The peak amplitudes of currents evoked with the use of approximate EC_{50} concentrations of GABA were sensitive to changes in membrane potential. The degree of voltage sensitivity was also dependent on the subtype composition of individual GABAR isoforms. As shown in Fig. 5, outwardly rectifying currents (increased current amplitude at positive membrane potentials) were recorded from cells expressing α5β3γ2L GABARs. Outwardly rectifying I-V curves were observed in these cells under conditions of equimolar [Cl^-] across the cell membrane. In contrast, linear I-V curves were more typical of the α1β3γ2L isoform. Fig. 6 summarizes the voltage sensitivity of GABAR responses for a number of isoforms. The rectification ratio (see Materials and Methods) was dependent on expression of different α or β subtypes (larger rectification ratio denotes greater outward rectification). If the α subtype was changed (but the β3 and γ2L subtypes were unchanged), the rank order of rectification was α5β3γ2L (five experiments) >

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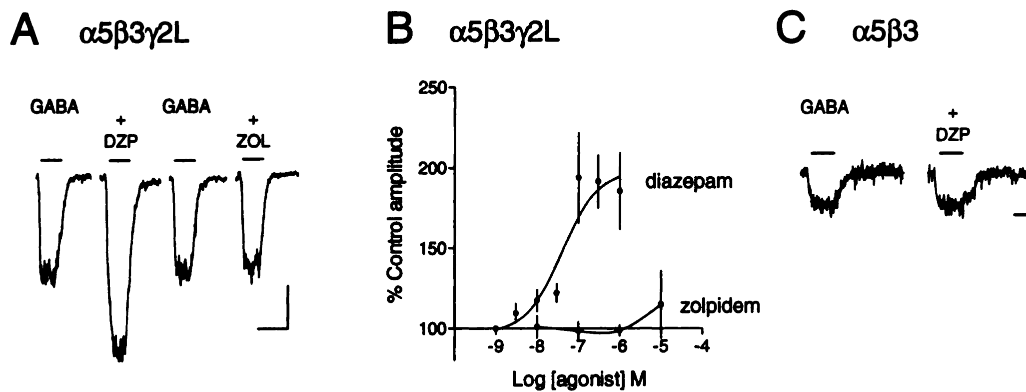

Fig. 2. Benzodiazepine and imidazopyridine sensitivity of α5β3γ2L GABARs. A, Membrane currents were recorded in response to 3 μM GABA, followed by 3 μM GABA plus 100 nM DZP, then 3 μM GABA, and finally 3 μM GABA plus 10 μM ZOL. *Horizontal bars*, drug applications made at 2-min intervals. *Calibration bar*, 100 pA, 5 sec, V_h = −70 mV. B, Concentration-response curves for enhancement of current amplitudes by both DZP (five experiments) and ZOL (three experiments). Either DZP or ZOL was coapplied with 3 μM GABA. *Ordinate*, percent of response to 3 μM GABA. Data are mean ± standard error. C, GABARs lacking the γ2L subtype were DZP insensitive. Currents were recorded in response to 1 μM GABA, followed by 1 μM GABA plus 100 nM DZP. *Horizontal bars*, drug applications made at 2-min intervals; *calibration bar*, 10 pA, 5 sec, V_h = −70 mV.

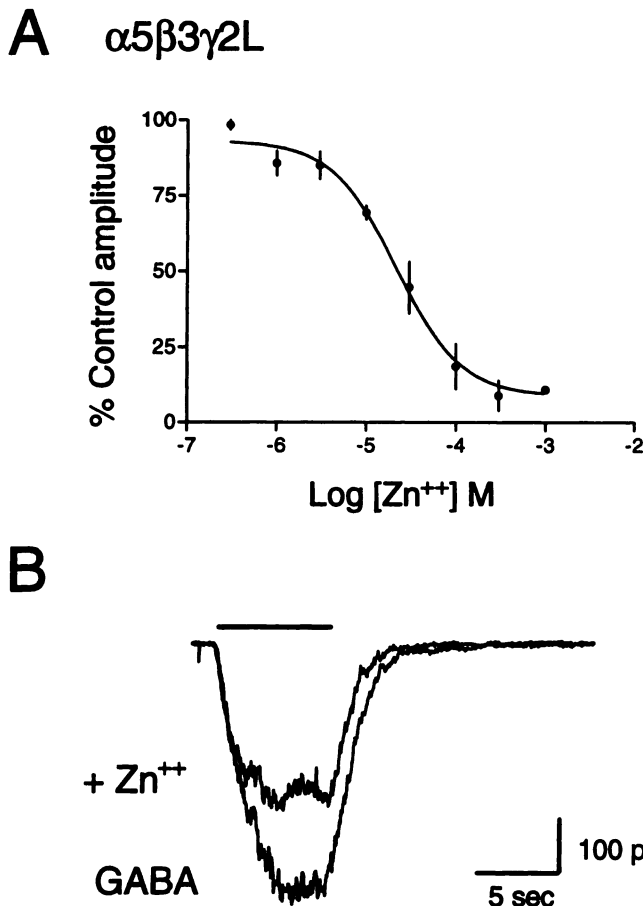

Fig. 3. Extracellular Zn^{2+} inhibited α5β3γ2L GABAR responses. A, Concentration-response curve for inhibition of current amplitudes by Zn^{2+}. *Ordinate*, percent of response to 3 μM GABA. Data are mean ± standard error (three experiments). Zn^{2+} was coapplied with 3 μM GABA. B, Superimposed currents (V_h = −70 mV) in response to successive applications of 3 μM GABA and 3 μM GABA plus 30 μM Zn^{2+}. *Horizontal bars*, drug applications made at 2-min intervals.

α6β3γ2L (six experiments) > α1β3γ2L (nine experiments (Fig. 6A). The degree of rectification in α5 subtype-containing GABARs was also dependent on the β subtype incorporated into the receptor (Fig. 6B). If the β subtype was changed (but α5 and γ2L subtypes were unchanged), the rank order of rectification was α5β3γ2L (five experiments) = α5β1γ2L (four experiments) > α5β2γ2L (four experiments). For α5 subtype-containing GABARs, the α5β2γ2L isoform produced significantly less outward rectification than α5β3γ2L. These results indicate that different GABAR isoforms can be differentially sensitive to changes in membrane potential, producing rectifying or nonrectifying whole-cell currents. Of the isoforms studied, α5β3γ2L and α5β1γ2L exhibited the most outward rectification.

In response to high GABA concentrations, desensitization of GABAR currents was evident by the decrease in amplitude of the response in the continued presence of GABA. The amount of desensitization was dependent on the membrane potential (Fig. 7). For the α5β3γ2L isoform, GABAR responses desensitized to a greater degree at negative (−50 mV) than at positive (+50 mV) membrane potentials. The opposite effect was observed for the α6β3γ2L isoform, where GABAR responses desensitized more at positive membrane potentials. A summary of voltage-dependent desensitization for a number of GABAR isoforms is shown in Fig. 8. The desensitization ratio (see Materials and Methods) was dependent on expression of different α or β subunit subtypes [a smaller desensitization ratio denotes less desensitization at positive (+50 mV) than at negative (−50 mV) membrane potentials]. If the α subtype was changed (but β3 and γ2L subtypes were unchanged), the rank order of isoforms showing more desensitization at hyperpolarized potentials was α5β3γ2L (six experiments) < α1β3γ2L (five experiments) < α6β3γ2L (six experiments, Fig. 8A). The desensitization ratio in α5 subtype-containing GABARs was also dependent on the β subtype incorporated into the receptor (Fig. 8B). If the β subtype was changed (but α5 and γ2L subtypes were unchanged), the rank order of isoforms showing more desensitization at hyperpolarized potentials was α5β3γ2L (six experiments) = α5β1γ2L (six experiments) < α5β2γ2L (seven experiments). Both α5β2γ2L and α6β3γ2L isoforms displayed significantly larger desensitization ratios than the α5β3γ2L isoform. In general, α5β3γ2L and α5β1γ2L isoforms

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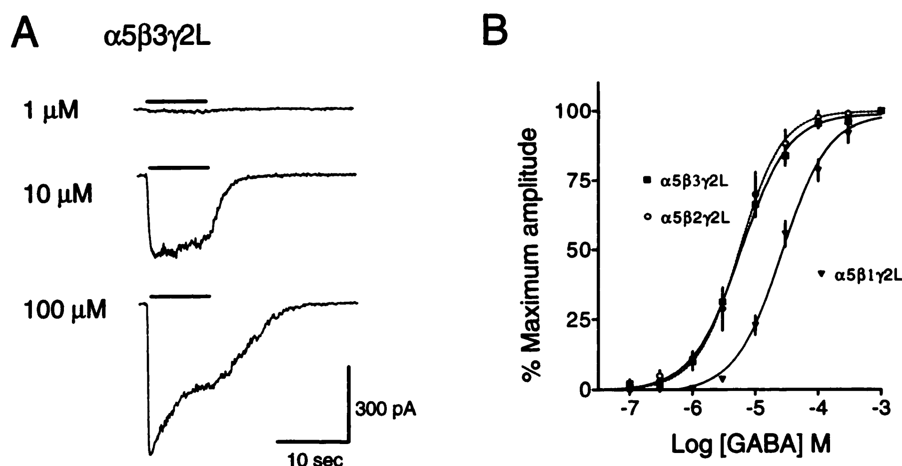

Fig. 4. GABA concentration-response characteristics for α5 subtype-containing GABARs. A, Representative traces of GABAR responses recorded from a L929 cell expressing α5β3γ2L GABARs (V_h = −70 mV). Horizontal bar, GABA application (1, 10, or 100 µM). B, GABA concentration-response curves for three α5 subtype-containing GABAR isoforms. *Ordinate*, percent maximum current amplitude measured in the presence of GABA. Data are mean ± standard error for α5β3γ2L (■, 14 experiments), α5β2γ2L (○, six experiments), and α5β1γ2L (▼, nine experiments).

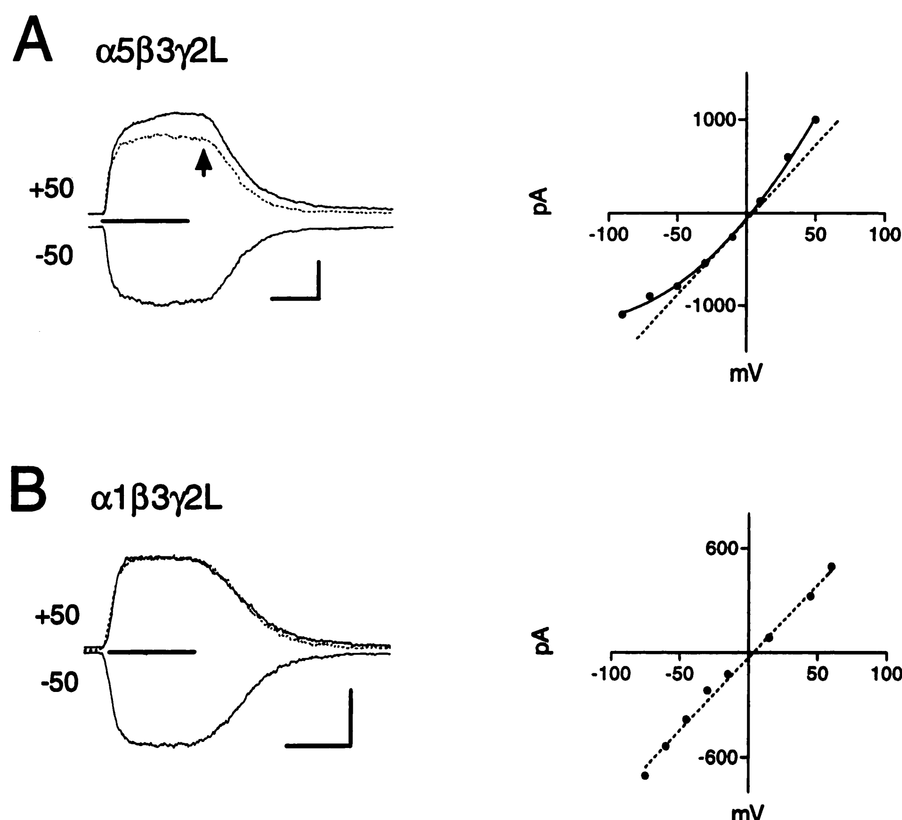

Fig. 5. Outward rectification of α5β3γ2L GABAR responses. A, Responses to 3 µM GABA recorded at holding potentials of either +50 or −50 mV. The inward current recorded at −50 mV has been inverted and superimposed (*arrowhead*) on the +50 mV response for comparison. *Calibration bar*, 400 pA, 3 sec; *right*, I-V curve from this cell. ●, Peak current amplitudes in response to individual GABA (3 µM) applications recorded at different V_h levels; *dotted line*, linear comparison. B, In a cell expressing α1β3γ2L GABARs, the responses recorded at +50 or −50 mV were identical in amplitude; *calibration bar*, 200 pA, 2 sec; *right*, I-V curve from this cell.

displayed comparably less desensitization at depolarized potentials.

GABAR responses recorded from hippocampal pyramidal neurons. Because the distribution of the α5 subtype seems to be largely hippocampal, we recorded GABAR responses from 19 acutely dissociated CA1 pyramidal neurons to determine their similarities to GABAR currents recorded from fibroblasts expressing α5 subtype-containing recombinant GABARs. GABA concentration-response curves revealed a GABA EC_{50} of ~10 µM. The voltage-dependent properties of hippocampal GABAR responses were similar to those of α5 subtype-containing GABARs (Fig. 9A). Hippocampal GABARs exhibited outward rectification in response to an EC_{50} concentration of GABA, as well as decreased desensitization at depolarized membrane potentials in the presence of a high concentration of GABA. Because pyramidal neurons undoubtedly express a number of different GABAR isoforms, we attempted to distinguish between various α subtype-containing isoforms using the benzodiazepine DZP and the benzodiazepine-site agonist ZOL. All neurons tested were DZP sensitive (EC_{50} values = 2 and 236 nM, eight experiments) (23), indicating the presence of GABARs containing both α and γ subunits (6). The two EC_{50} values represent a biphasic DZP concentration-response relationship that was characteristic of individual neurons tested. Neurons expressing primarily α5 subtype-containing GABARs should be ZOL insensitive, whereas those expressing primarily α1 or α2 subtype-containing GABARs should be relatively ZOL sensitive. We found that approximately one third of pyramidal neurons were ZOL insensitive as determined according to the inability of ZOL to cause an increase in the peak amplitude of a 10 µM GABAR response (91 ± 7%

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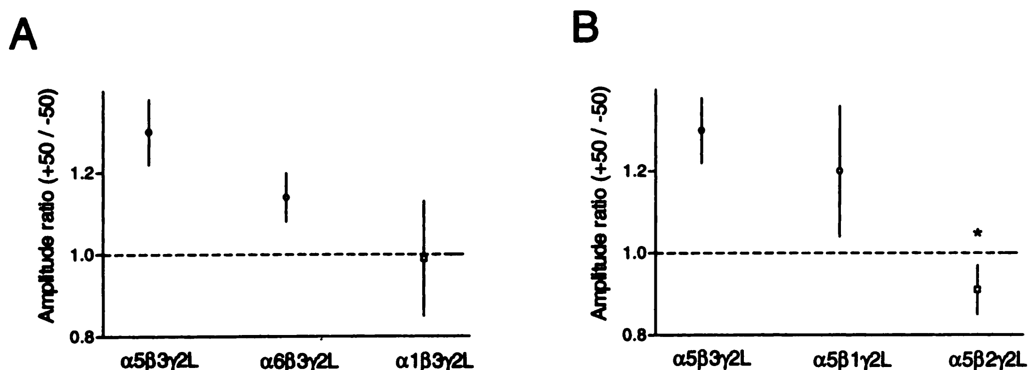

Fig. 6. Degree of rectification depends on GABAR subunit composition. A, Variations in α subtypes produced GABAR currents with different amplitude ratios (see Materials and Methods). Responses to EC_{50} concentrations of GABA were used to calculate ratios. B, Variations in β subtypes produce different amplitude ratios in α5 subtype-containing GABARs. Symbols in A and B represent mean ± standard error, and a larger ratio indicates greater outward rectification. *, Significant difference compared with the α5β3γ2L group.

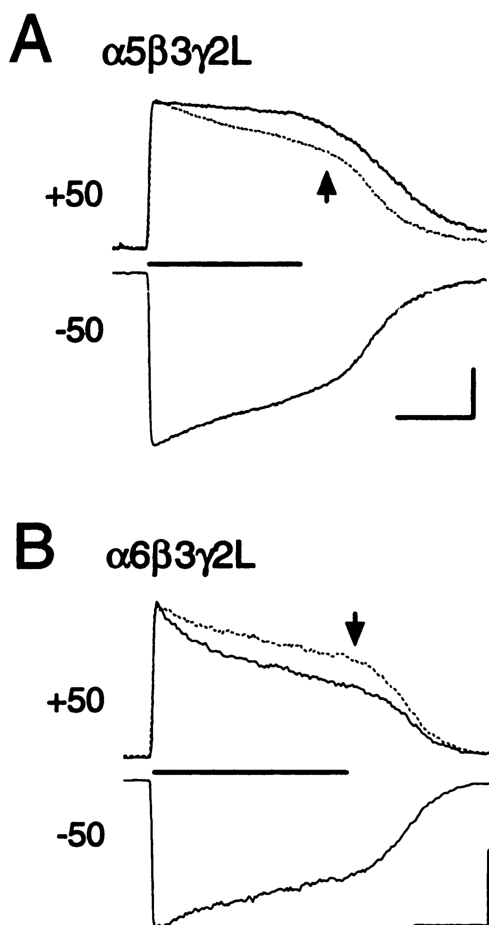

Fig. 7. Voltage-dependent desensitization of GABAR responses. A, Membrane currents recorded from α5β3γ2L GABARs in response to 300 μM GABA (*horizontal bar*). Responses were obtained at V_h levels of both +50 and −50 mV. The response recorded at −50 mV has been scaled, inverted, and superimposed (arrowhead) on the +50 mV trace for comparison. *Calibration bar*, 250 pA, 5 sec. B, Same protocol as in A, but currents were recorded from α6β3γ2L GABARs; *calibration bar*, 250 pA, 5 sec. Note less desensitization at positive membrane potentials for α5 but not α6 subtype-containing GABARs.

of control, six experiments), whereas the rest were ZOL sensitive (154 ± 9% of control, 11 experiments). No differences in DZP sensitivity were observed between ZOL-sensitive or -insensitive neurons. When neurons were grouped according to ZOL sensitivity (Fig. 9B), the ZOL-insensitive neurons showed a smaller desensitization ratio (less desensitization at depolarized membrane potentials) than ZOL-sensitive neurons. In addition, ZOL-insensitive neurons exhibited a larger amplitude ratio (greater outward rectification) than ZOL-sensitive neurons. The differences in both rectification and desensitization were not statistically significant but rather showed a trend similar to that seen in responses from α5 subunit-containing GABARs. The presence of multiple unknown GABAR isoforms in pyramidal neurons may contribute to the variability observed between cells. Although not conclusive, these results are nevertheless consistent with the presence of α5 subtype-containing GABAR isoforms α5β3γ2L or α5β1γ2L on hippocampal pyramidal neurons.

Discussion

Recombinant GABAR pharmacology. The findings of Angelotti and Macdonald (21) that preferential assembly and expression of αβγ GABARs occur in L929 cells were confirmed in the present study. Three separate subunits were required (α, β, and γ) for optimal functional expression of surface receptor proteins. Recombinant α5β3γ2L GABAR responses were blocked by Zn^{2+} and were DZP sensitive and ZOL insensitive, consistent with previous reports (11, 14), which is indicative of coassembly of all three subunits. The GABA sensitivity of α5βXγ2L GABARs was dependent on the β subtype incorporated into the receptor. Concentration-response curves for α5β3γ2L and α5β2γ2L GABARs produced a lower GABA EC_{50} value (6 μM) than that for α5β1γ2L (26 μM). This is consistent with the findings of Amin and Weiss (8), who demonstrated that the extracellular domain of the β subunit may form a portion of the GABA binding site. Other groups have also demonstrated changes in GABA affinity that coincide with changes in the β subtype (24–26). Although some discrepancy as to the absolute degree and direction of that change remains, it seems that β3 subtype-containing GABARs may have a higher affinity for GABA than β1 subtype-containing GABARs.

On hippocampal neurons, zinc interacts with an extracellular site on native GABARs, producing a noncompetitive inhibition of GABAR currents (41). Using recombinant GABARs, Draguhn *et al.* (42) and Saxena and Macdonald (22) have shown that the presence of the γ subunit results in GABARs with a lower affinity for zinc and that the subtype of

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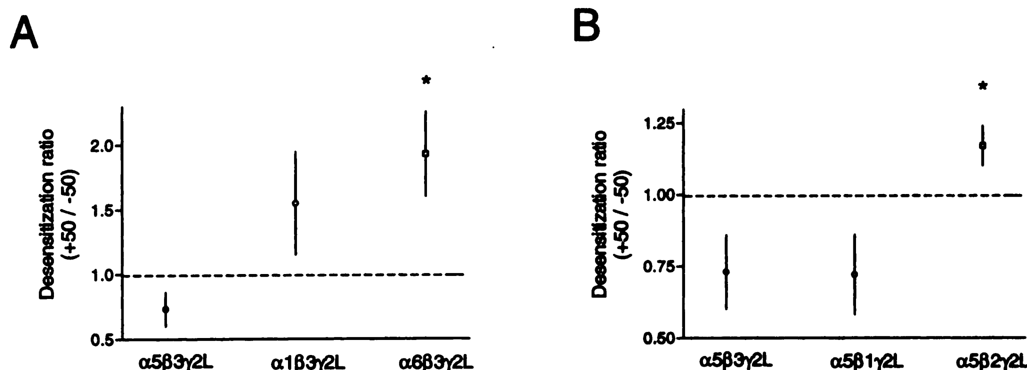

Fig. 8. Voltage-dependent desensitization depended on GABAR subunit composition. A, Variations in α subtypes produced GABARs with different desensitization ratios (see Materials and Methods). Responses to high concentrations of GABA were used to calculate ratios. B, Variations in β subtypes produced different desensitization ratios in α5 subtype-containing GABARs. Symbols in A and B represent mean ± standard error, and a smaller ratio indicates less desensitization at positive membrane potentials. *, Significant difference compared with the α5β3γ2L group.

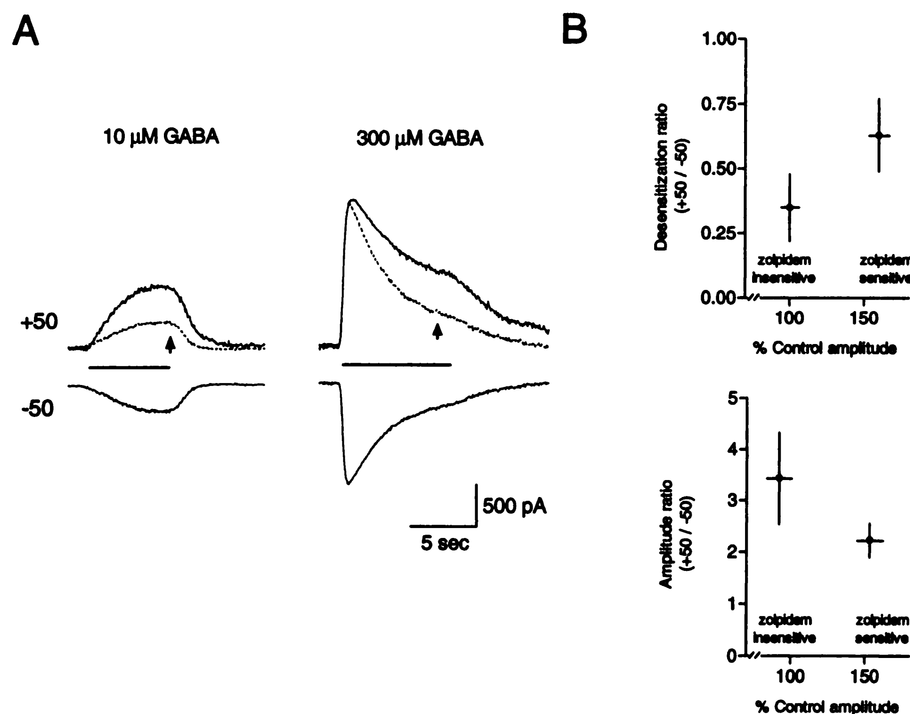

Fig. 9. Outward rectification and voltage-dependent desensitization in hippocampal CA1 pyramidal cells. A, Currents were recorded from acutely dissociated pyramidal neurons in response to 10 and 300 μM GABA (*horizontal bars*). Responses were obtained at V_h levels of both +50 and −50 mV. The responses recorded at −50 mV were inverted and superimposed (*arrowhead*) on the +50 mV traces for comparison. The responses to 300 μM GABA recorded at −50 mV (arrowhead) was also scaled to facilitate comparison of desensitization. B, Desensitization and amplitude ratios (ordinate) were calculated as described and plotted against sensitivity to ZOL. *Abscissa*, percent control amplitude of 10 μM GABAR currents recorded in the presence of 1 μM ZOL. Neurons were then classified as insensitive (desensitization ratio: four experiments; amplitude ratio, six experiments) or sensitive (desensitization ratio: eight experiments; amplitude ratio, 11 experiments). *Symbols*, mean ± standard error along both axes.

α subunit present in γ-containing GABARs also affects the zinc affinity. Our results are consistent with these reports in that α5β3γ2L GABAR currents are inhibited by a concentration of zinc (22 μM) that is greater than that required to inhibit γ-less GABARs.

Voltage-dependent GABAR responses. Outward rectification of whole-cell currents from recombinant GABARs has been demonstrated previously (27). In the current study, different GABAR isoforms were shown to exhibit varying degrees of outward rectification, with the α5β3γ2L isoform producing the most rectifying responses of the isoforms studied. Others have observed outwardly rectifying GABAR responses from a variety of neurons (28–30), yet the underlying mechanisms remain unclear. Although there is evidence of a voltage-dependent change in single-channel conductance (31, 32), there also is evidence favoring a voltage-dependent change in channel gating rather than conductance (29, 33) underlying outward rectification. It is probable that differences in subunit composition of native GABARs will determine the degree of rectification in neuronal GABARs, as shown with the use of recombinant GABARs.

Voltage-dependent changes in desensitization were evident as a decrease in the amount of desensitization at positive versus negative membrane potentials. The pattern of voltage-dependent properties of desensitization were similar to patterns of differences in rectification among isoforms. Of the isoforms studied, α5β3γ2L and α5β1γ2L produced the least desensitization at positive versus negative membrane potentials. Again, although desensitization of native neocortical (34) and hippocampal (35, 36) GABARs seemed to be voltage dependent, the underlying mechanisms remain unclear. Voltage-independent GABAR desensitization has been reported in retinal ganglion cells (37), supporting the concept of isoform-dependent differences in GABAR desensitization. However, it is still possible that desensitization may not be an intrinsic channel property because there is evidence that other cytoplasmic and/or membrane-bound constituents may contribute to desensitization (34). In addition, there seem to

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be multiple components of GABAR desensitization, with time constants ranging from 10 msec to many seconds (38). With the use of a modified U-tube drug application system, rapidly desensitizing (<100 msec) components could not be resolved. Although in the current study the focus was on a slow desensitization component, rapid GABA application to outside-out membrane patches will be required to determine the contributions of faster processes to isoform-dependent changes in desensitization.

Together, the voltage-dependent properties of outward rectification and desensitization would augment GABAR currents at depolarized membrane potentials. This could play an important role in inhibitory synaptic transmission, where postsynaptic GABARs normally function to hyperpolarize the cell after synaptic excitation-induced membrane depolarization. In a depolarized neuron, high-frequency GABAergic activation of postsynaptic $\alpha5\beta3\gamma2L$ GABARs could result in inhibitory responses of greater amplitude and duration, effectively increasing the inhibition. In addition, GABAR rectification due to Goldman constant-field effects (39) would further augment these responses.

Comparison of recombinant $\alpha5$ subtype-containing GABARs with native hippocampal GABARs. In the CA1 region of the adult rat hippocampus, relatively high expression of mRNA for $\alpha1$, $\alpha2$, $\alpha4$, $\alpha5$, $\beta1$, $\beta3$, and $\gamma2$ subtypes has been found (2), indicating the probable expression of hippocampal GABAR isoforms containing these subunit subtypes. Of these, the $\gamma2$ subtype is required for benzodiazepine modulation of GABAR currents, whereas the various α subtypes determine the overall sensitivity of the receptor to various benzodiazepine-site ligands (4). Although GABARs containing $\alpha1$, $\alpha2$, and $\alpha5$ subtypes are DZP sensitive, those containing the $\alpha4$ subtype are DZP insensitive. Similarly, GABARs containing $\alpha1$ and $\alpha2$ subtypes are modulated by the benzodiazepine-site agonist ZOL, whereas the presence of either $\alpha4$ or $\alpha5$ subtype confers ZOL insensitivity.

If the $\alpha5\beta3\gamma2$ isoform represents a native hippocampal GABAR isoform, it should have pharmacological properties similar to those of native receptors and should exhibit the basic biophysical characteristics of hippocampal GABARs. GABAR responses from CA1 pyramidal cells in the current study were DZP sensitive, indicating functional assembly of $\alpha1$, $\alpha2$, or $\alpha5$ and $\gamma2$ subtype-containing GABARs (6), with a GABA EC_{50} value of ~10 μM. Because these neurons contain mRNA for a number of α subtypes (2), it was not surprising that only a portion of them were ZOL insensitive, suggesting that a subset of pyramidal cells may express a high level of $\alpha5$ subtype-containing GABARs. Outward rectification of the GABAR I-V relationship has been previously demonstrated in both hippocampal neurons (29, 40) and membrane patches from these cells (31). Voltage-dependent differences in desensitization have also been described in hippocampal neurons (35, 36), where less desensitization of GABA-induced currents is seen at depolarized versus hyperpolarized membrane potentials. In the current study, ZOL-insensitive neurons displayed more rectification and less depolarized desensitization than ZOL-sensitive neurons, which is consistent with the presence of the $\alpha5\beta3\gamma2L$ isoform. However, these voltage-dependent properties were observed, to some degree, in almost all pyramidal neurons, regardless of the response to ZOL. In addition, both outward rectification and voltage-dependent changes in desensitization were larger in pyramidal neurons than in L929 cells expressing recombinant GABARs. This could be due to either (a) expression of other GABAR isoforms in pyramidal neurons (possibly $\alpha2$ subtype-containing) that have yet to be characterized with regard to their voltage sensitivity or (b) possible interaction of the receptor with an unknown cytoplasmic constituent that augments the voltage-dependent properties of the receptor.

Based on the present data, one might speculate that unique properties of recombinant GABAR isoforms could be used to identify native GABAR isoforms. We have shown that $\alpha5\beta3\gamma2L$ isoform responses resemble hippocampal CA1 pyramidal cell GABAR responses. These data coincide with the binding studies of Lüddens *et al.* (14), which suggest that the $\alpha5\beta3\gamma2L$ isoform may be the predominant ZOL-insensitive GABAR in the hippocampus. In contrast, cerebellar granule cells contain mRNA for $\alpha6$, $\beta3$, $\gamma2L$, and δ subunit subtypes (2). Thus, the responses of the $\alpha6\beta3\gamma2L$ isoform (less outward rectification and more desensitization at depolarized potentials) might resemble GABAR responses from cerebellar granule cells. Whether specific cerebellar neurons express the $\alpha6\beta3\delta$ instead of the $\alpha6\beta3\gamma2L$ isoform and the properties of these receptors are also of interest. Further comparisons of native versus recombinant GABAR properties will be required to resolve these issues.

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References

1. Lüddens, H., and W. Wisden. Function and pharmacology of multiple GABA-A receptor subunits. *Trends Pharmacol. Sci.* **12**:49–51 (1991).
2. Wisden, W., D. J. Laurie, H. Monyer, and P. H. Seeburg. The distribution of 13 GABAA receptor subunit mRNAs in the rat brain. I. Telencephalon, diencephalon, mesencephalon. *J. Neurosci.* **12**:1040–1062 (1992).
3. Burt D. R., and G. L. Kamatchi. GABA-A receptor subtypes: from pharmacology to molecular biology. *FASEB J.* **5**:2916–2923 (1991).
4. Macdonald, R. L., and R. W. Olsen. $GABA_A$ receptor channels. *Annu. Rev. Neurosci.* **17**:569–602 (1994).
5. Nayeem, N., T. P. Green, I. L. Martin, and E. A. Barnard. Quaternary structure of the native $GABA_A$ receptor determined by electron microscopic image analysis. *J. Neurochem.* **62**:815–818 (1994).
6. Pritchett, D. B., H. Sontheimer, B. D. Shivers, S. Ymer, H. Kettenmann, P. R. Schofield, and P. H. Seeburg. Importance of a novel GABA-A receptor subunit for benzodiazepine pharmacology. *Nature (Lond.)* **338**:582–585 (1989).
7. Levitan, E. S., P. R. Schofield, D. R. Burt, L. M. Rhee, W. Wisden, M. Kohler, N. Fujita, H. F. Rodriguez, A. Stephenson, M. G. Darlison, E. A. Barnard, and P. H. Seeburg. Structural and functional basis for GABA-A receptor heterogeneity. *Nature (Lond.)* **335**:76–79 (1988).
8. Amin, J., and D. S. Weiss. $GABA_A$ receptor needs two homologous domains of the β-subunit for activation by GABA but not by pentobarbital. *Nature (Lond.)* **366**:565–569 (1993).
9. Pritchett, D. B., H. Lüddens, and P. H. Seeburg. Type I and type II $GABA_A$-benzodiazepine receptors produced in transfected cells. *Science (Washington D. C.)* **245**:1389–1392 (1989).
10. Khrestchatisky, M., A. J. MacLennan, M.-Y. Chiang, W. Xu, M. B. Jackson, N. Brecha, C. Sternini, R. W. Olsen, and A. J. Tobin. A novel α subunit in rat brain $GABA_A$ receptors. *Neuron* **3**:745–753 (1989).
11. Pritchett, D. B., and P. H. Seeburg. $GABA_A$ receptor $\alpha5$-subunit creates novel type II benzodiazepine receptor pharmacology. *J. Neurochem.* **54**: 1802–1804 (1990).
12. McKernan, R. M., K. Quirk, R. Prince, P. A. Cox, N. P. Gillard, C. I. Ragan, and P. Whiting. $GABA_A$ receptor subtypes immunopurified from rat brain with α subunit-specific antibodies have unique pharmacological properties. *Neuron* **7**:667–676 (1991).
13. Mertens, S., D. Benke, and H. Mohler. $GABA_A$ receptor populations with novel subunit combinations and drug binding profiles identified in brain by $\alpha5$- and δ-subunit-specific immunopurification. *J. Biol. Chem.* **268**: 5965–5973 (1993).
14. Lüddens, H., P. H. Seeburg, and E. R. Korpi Impact of β and γ variants on ligand-binding properties of γ-aminobutyric acid type A receptors. *Mol. Pharmacol.* **45**:810–814 (1994).

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15. Burgard, E. C., and R. L. Macdonald. Distinct properties of α5 subunit-containing recombinant GABA$_A$ receptors produced by combinations with different β subunits. *Soc. Neurosci. Abstr.* **21**:26 (1995).
16. Angelotti, T. P., M. D. Uhler, and R. L. Macdonald. Assembly of GABA-A receptor subunits: analysis of transient single-cell expression utilizing a fluorescent substrate/marker gene technique. *J. Neurosci.* **13**:1418–1428 (1993).
17. Saxena, N. C., and R. L. Macdonald. Assembly of GABA$_A$ receptor subunits: role of the δ subunit. *J. Neurosci.* **14**:7077–7086 (1994).
18. Huggenvik, J. I., M. W. Collard, R. E. Stofko, A. F. Seasholtz, and M. D. Uhler. Regulation of the human enkephalin promoter by two isoforms of the catalytic subunit of cAMP-dependent protein kinase. *Mol. Endocrinol.* **5**:921–930 (1991).
19. Chen C., and H. Okayama. High-efficiency transformation of mammalian cells by plasmid DNA. *Mol. Cell. Biol.* **7**:2745–2752 (1987).
20. Hamill, O. P., A. Marty, E. Neher, B. Sakmann, and F. J. Sigworth. Improved patch-clamp techniques for high-resolution current recording from cells and cell-free membrane patches. *Pfluegers Arch.* **391**:85–100 (1981).
21. Angelotti, T. P., and R. L. Macdonald. Assembly of GABA-A receptor subunits: α1β1 and α1β1γ2s subunits produce unique ion channels with dissimilar single-channel properties. *J. Neurosci.* **13**:1429–1440 (1993).
22. Saxena, N. C., and R. L. Macdonald. Properties of putative cerebellar γ-aminobutyric acid$_A$ receptor isoforms. *Mol. Pharmacol.* **49**:567–579 (1996).
23. Tietz, E. I., J. Kapur, N. Esmaeil, and R. L. Macdonald. Multiphasic effects of allosteric modulators on GABA whole-cell currents in acutely dissociated hippocampal CA1 pyramidal neurons. *Soc. Neurosci. Abstr.* **21**:1346 (1995).
24. Ducic, I., H. J. Caruncho, W. J. Zhu, S. Vicini, and E. Costa. γ-Aminobutyric acid gating of Cl$^-$ channels in recombinant GABA$_A$ receptors. *J. Pharmacol. Exp. Ther.* **272**:438–445 (1995).
25. Ebert, B., K. A. Wafford, P. J. Whiting, P. Krogsgaard-Larsen, and J. A. Kemp. Molecular pharmacology of γ-aminobutyric acid type A receptor agonists and partial agonists in oocytes injected with different α, β, and γ receptor subunit combinations. *Mol. Pharmacol.* **46**:957–963 (1994).
26. Hadingham, K. L., P. B. Wingrove, K. A. Wafford, C. Bain, J. A. Kemp, K. J. Palmer, A. W. Wilson, A. S. Wilcox, J. M. Sikela, C. I. Ragan, and P. J. Whiting. Role of the β subunit in determining the pharmacology of human γ-aminobutyric acid type A receptors. *Mol. Pharmacol.* **44**:1121–1218 (1993).
27. Verdoorn, T. A., A. Draguhn, S. Ymer, P. H. Seeburg, and B. Sakmann. Functional properties of recombinant rat GABA$_A$ receptors depend on subunit composition. *Neuron* **4**:919–928 (1990).
28. Bormann, J., O. P. Hamill, and B. Sakmann. Mechanism of anion permeation through channels gated by glycine and γ-aminobutyric acid in mouse cultured spinal neurons. *J. Physiol.* **385**:243–286 (1987).
29. Segal, M., and J. L. Barker. Rat hippocampal neurons in culture: properties of GABA-activated Cl$^-$ ion conductance. *J. Neurophysiol.* **51**:500–515 (1984).
30. Weiss, D. S., E. M. Barnes, and J. J. Hablitz. Whole-cell and single-channel recordings of GABA-gated currents in cultured chick cerebral neurons. *J. Neurophysiol.* **59**:495–513 (1988).
31. Gray, R., and D. Johnston. Rectification of single GABA-gated chloride channels in adult hippocampal neurons. *J. Neurophysiol.* **54**:134–142 (1985).
32. Birnir, B., A. B. Everitt, and P. W. Gage. Characteristics of GABA$_A$ channels in rat dentate gyrus. *J. Membr. Biol.* **142**:93–102 (1994).
33. Weiss, D. S. Membrane potential modulates the activation of GABA-gated channels. *J. Neurophysiol.* **59**:514–527 (1988).
34. Frosch, M. P., S. A. Lipton, and M. A. Dichter. Desensitization of GABA-activated currents and channels in cultured cortical neurons. *J. Neurosci.* **12**:3042–3053 (1992).
35. Oh, D. J., and M. A. Dichter. Desensitization of GABA-induced currents in cultured rat hippocampal neurons. *Neuroscience* **49**:571–576 (1992).
36. Yoon, K.-W. Voltage-dependent modulation of GABA$_A$ receptor channel desensitization in rat hippocampal neurons. *J. Neurophysiol.* **71**:2151–2160 (1994).
37. Tauck, D. L., M. P. Frosch, and S. A. Lipton. Characterization of GABA- and glycine-induced currents of solitary rodent retinal ganglion cells in culture. *Neuroscience* **27**:193–203 (1988).
38. Celantano, J. J., and R. K. S. Wong. Multiphasic desensitization of the GABA$_A$ receptor in outside-out patches. *Biophys. J.* **66**:1039–1050 (1994).
39. Barker, J. L., and N. L. Harrison. Outward rectification of inhibitory postsynaptic currents in cultured rat hippocampal neurones. *J. Physiol.* **403**:41–55 (1988).
40. Ashwood, T. J., G. L. Collingridge, C. E. Herron, and H. V. Wheal. Voltage-clamp analysis of somatic γ-aminobutyric acid responses in adult rat hippocampal CA1 neurons *in vitro*. *J. Physiol.* **384**:27–37 (1987).
41. Legendre, P., and G. L. Westbrook. Noncompetitive inhibition of γ-aminobutyric acid$_A$ channels by zinc. *Mol. Pharmacol.* **39**:267–274 (1991).
42. Draguhn, A., T. A. Verdoorn, M. Ewert, P. H. Seeburg, and B. Sakmann. Functional and molecular distinction between recombinant rat GABA$_A$ receptor subtypes by Zn^{2+}. *Neuron* **5**:781–788 (1990).

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