

Tonically Active GABA_A Receptors in Hippocampal Pyramidal Neurons Exhibit Constitutive GABA-Independent Gating

Melissa R. McCartney, Tarek Z. Deeb, Tricia N. Henderson, and Tim G. Hales

Departments of Pharmacology & Physiology (M.R.M., T.Z.D., T.G.H.) and Anesthesiology & Critical Care Medicine (T.N.H., T.G.H.), The George Washington University, Washington, DC

Received July 6, 2006; accepted November 7, 2006

ABSTRACT

Phasic and tonic inhibitory currents of hippocampal pyramidal neurons exhibit distinct pharmacological properties. Picrotoxin and bicuculline methiodide inhibited both components, consistent with a role for GABA_A receptors; however, gabazine, at a concentration that abolished miniature GABAergic inhibitory postsynaptic currents and responses to exogenous GABA, had no effect on tonic currents. Because all GABA-activated GABA_A receptors in pyramidal neurons are gabazine-sensitive, it follows that tonic currents are not GABA-activated. Furthermore, picrotoxin-sensitive spontaneous single-channel events recorded from outside-out patches had the same chord conductance as GABA-activated channels and were gabazine-resistant. Therefore, we hypothesize that GABA_A receptors, constitutively active in the absence of GABA, mediate tonic current; the failure of gabazine to block tonic current reflects a lack of negative intrinsic efficacy of the antagonist. We compared the negative efficacies of bicuculline and gabazine using

the general anesthetic propofol to directly activate GABA_A receptors native to pyramidal neurons or $\alpha 1\beta 3\gamma 2$ receptors recombinantly expressed in human embryonic kidney 293 cells. Propofol activated gabazine-resistant, bicuculline-sensitive currents when applied to either preparation. Although gabazine had negligible efficacy as an inhibitor of propofol-activated currents, it prevented inhibition by bicuculline, which acts as an inverse agonist inhibiting GABA-independent gating. Recombinant $\alpha 1\beta 1/3\gamma 2$ receptors also mediated agonist-independent tonic currents that were resistant to gabazine and inhibited by bicuculline. Thus, gabazine is a competitive antagonist with negligible negative efficacy and is therefore unable to inhibit GABA_A receptors that are active in the absence of GABA because of either anesthetic or spontaneous gating. Moreover, spontaneously active GABA_A receptors mediate gabazine-resistant tonic currents in pyramidal neurons.

GABA type A (GABA_A) receptors mediate phasic and tonic inhibition of neurons in several brain regions (Farrant and Nusser, 2005). Tonic inhibition is the focus of considerable interest because of its pervasive role in governing neuronal excitability. Receptors mediating phasic and tonic inhibition in the same neurons may differ in their subunit combinations. Indeed, there is considerable scope for heterogeneity because GABA_A receptors are pentameric containing various combinations of $\alpha 1$ – $\alpha 6$, $\beta 1$ – $\beta 3$, $\gamma 1$ – $\gamma 3$, δ , $\rho 1$ – $\rho 3$, ϵ , and π subunits (Whiting et al., 1999). Most receptors contain α , β , and γ subunits. However, in cerebellar granule cells, tonically active extrasynaptic GABA_A receptors contain $\alpha 6$, $\beta 2/3$, and δ subunits (Nusser et al., 1998). Synaptic receptors in the same cells lack the δ subunit and contain the $\gamma 2$ subunit. The δ

subunit enhances the affinity of extrasynaptic GABA_A receptors by enabling them to respond to low levels of ambient GABA. By analogy to the cerebellum, the activation of extrasynaptic receptors by ambient GABA has become the model for tonic GABA_A receptor activity throughout the central nervous system. The alternative possibility that tonically active GABA_A receptors exhibit spontaneous gating has been largely ignored (Farrant and Nusser, 2005).

Unlike those of cerebellar granule cells, tonic and phasic inhibitory currents recorded from hippocampal pyramidal neurons seem to differ in their sensitivities to the antagonist gabazine (Bai et al., 2001). Indeed, several studies suggest that tonically active GABA_A receptors are gabazine-resistant. However, this is controversial because gabazine inhibits GABA_A receptors activated by exogenous GABA or when ambient GABA concentrations are increased (Overstreet and Westbrook, 2001; Stell et al., 2003). Furthermore, the only known GABA_A receptors that mediate gabazine-insensitive GABA-evoked currents contain ρ subunits (often referred to

This work was supported by grant GM058037 from the National Institutes of Health.

Article, publication date, and citation information can be found at <http://molpharm.aspetjournals.org>.
doi:10.1124/mol.106.028597.

ABBREVIATIONS: HEK, human embryonic kidney; mIPSC, miniature inhibitory postsynaptic current; TTX, tetrodotoxin; CNQX, 6-cyano-2,3-dihydroxy-7-nitroquinoxaline; AP5, 2-amino-5-phosphonovaleate; propofol, 2,6-diisopropylphenol; ANOVA, analysis of variance; SR95531, gabazine.

as GABA_C receptors), which are predominantly expressed in the retina (Zhang et al., 2001).

Additional interest in the identity of tonically active GABA_A receptors derives from the proposal that they are the preferred target of general anesthetics (Bieda and MacIver, 2004; Hemmings et al., 2005; Cheng et al., 2006). Several intravenous anesthetics and neurosteroids activate GABA_A receptors in the absence of GABA and this action may participate in a subsequent loss of consciousness (Schulz and Macdonald, 1981). In addition, direct GABA_A receptor activation by anesthetics may also contribute to the respiratory depression that compromises the therapeutic indexes of these agents. Although it seems that anesthetics activate GABA_A receptors by binding to the receptor protein, the mechanism of anesthetic-evoked activation remains elusive (Hemmings et al., 2005). However, it is clear that anesthetics do not activate GABA_A receptors by acting as GABA-mimetics at the GABA binding site (Davies et al., 1997). GABA_A receptors formed by the expression of the $\beta 3$ subunit alone are resistant to activation by GABA but can be activated by propofol. However, the activation of GABA_A receptors by anesthetics such as propofol can be inhibited by the GABA_A receptor-competitive antagonist bicuculline (Hales and Lambert, 1991). In contrast, propofol-evoked currents recorded from pyramidal neurons are resistant to gabazine, giving rise to the hypothesis that propofol selectively activates extrasynaptic receptors (Bieda and MacIver, 2004).

Here, we tested an alternative hypothesis that gabazine is effectively a neutral competitive antagonist that has negligible efficacy as an inhibitor of GABA_A receptors active in the absence of GABA. In contrast, we hypothesize that bicuculline displays significant negative intrinsic efficacy. We tested these hypotheses in cultured rodent hippocampal pyramidal neurons and in HEK293 cells expressing rodent $\alpha 1\beta 1\gamma 2$, $\alpha 1\beta 3\gamma 2$, or $\alpha 1\beta 3\epsilon$ GABA_A receptors.

Materials and Methods

Hippocampal Cultures. Hippocampal tissue was dissected from 1- to 3-day-old rats and placed in a papain solution (1.4 mg/ml papain and 30 mg/ml bovine serum albumin in Hanks' balanced salt solution) and incubated at 37°C for 20 min. The papain solution was aspirated and replaced with a solution of ovomucoid (1 mg/ml) and bovine serum albumin (30 mg/ml) in Hanks' balanced salt solution. Tissue was triturated using flame-polished Pasteur pipettes. The supernatant was aspirated after centrifugation, and dissociated cells were resuspended by trituration in a medium containing modified Eagle's medium- α [80% (v/v)], fetal calf serum [10% (v/v)], horse serum [10% (v/v)], glucose (5 mg/ml), 50 μ g/ml streptomycin, and 50 U/ml penicillin. Cells were plated on poly(L-lysine) (1 mg/ml)-coated 33-mm diameter dishes at a density of 5×10^5 cells/ml medium. After 24 h, the medium was replaced by an incubation medium containing Neurobasal A [98% (v/v)], B27 supplement [2% (v/v)], 50 μ g/ml streptomycin, and 50 U/ml penicillin. On day 4, the medium was replaced by incubation medium supplemented with cytosine- β -D-arabinofuranoside (5 μ M) to inhibit the growth of glia. On day 5, the medium was replaced by incubation medium lacking cytosine- β -D-arabinofuranoside. Tissue culture reagents were supplied by Invitrogen (Carlsbad, CA). Electrophysiological recordings were performed using hippocampal neurons that displayed pyramidal morphology maintained for 7 to 21 days in culture.

HEK293 Cell Culture and Transfection. HEK293 cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% bovine serum, 50 μ g/ml streptomycin, and 50 U/ml peni-

cillin in an atmosphere of 5% CO₂. Cells were transfected by calcium phosphate precipitation with equimolar ratios of rodent $\alpha 1$, $\beta 3$, and $\gamma 2$ L GABA_A subunit cDNAs in the pCDM8 vector (Davies et al., 1997). In several experiments, the rat $\beta 1$ or ϵ GABA_A subunit cDNAs, also in the pCDM8 vector, were used instead of either the $\beta 3$ or $\gamma 2$ cDNAs, respectively. Cells were used 24 to 96 h after transfection for electrophysiological experiments.

Electrophysiology: Whole-Cell Recording. The whole-cell configuration of the patch-clamp technique was used to record currents from single hippocampal neurons and HEK293 cells voltage-clamped at -60 mV. The recording chamber was continuously and rapidly (5 ml/min) perfused by gravity feed with an extracellular solution of the following composition: 140 mM NaCl, 2.8 mM KCl, 2.0 mM MgCl₂, 1.0 mM CaCl₂, 6.0 mM glucose, and HEPES-NaOH, pH 7.2. Recording electrodes contained 140 mM CsCl, 2.0 mM MgCl₂, 0.1 mM CaCl₂, 3 mM ATP (Mg²⁺ salt), 1.1 mM EGTA, and HEPES-CsOH, pH 7.2. Miniature GABAergic inhibitory postsynaptic currents (mIPSCs) were recorded from hippocampal neurons in the presence of 6-cyano-2,3-dihydroxy-7-nitroquinoxaline (CNQX; 10 μ M) and 2-amino-5-phosphonovaleate (AP5; 10 μ M) to inhibit ionotropic glutamate receptors and tetrodotoxin (TTX; 1 μ M) to block action potentials (reagents supplied by Sigma, St. Louis, MO). Membrane currents were monitored with an Axopatch-200A amplifier (Molecular Devices, Sunnyvale, CA). Currents were low-pass-filtered at 2 kHz (Bessel characteristics). In experiments examining activation of GABA_A receptors, GABA or propofol was applied either to the perfusate or locally by pressure ejection (Picospritzer II; General Valve Corporation, Fairfield, NJ) from micropipettes positioned approximately 50 μ m from the cell under investigation. Whole-cell currents were digitized at a frequency of 10 kHz onto the hard drive of a Pentium personal computer and were analyzed with pCLAMP 8.0 software (Molecular Devices).

Recording Single-Channel Activity. Single-channel currents recorded from outside-out patches were low-pass-filtered at 1 kHz (Bessel characteristics) and digitized at 10 kHz for acquisition onto the hard drive of a Pentium personal computer as described previously (Hales et al., 2006). GABA (1 μ M) and/or gabazine (20 μ M) was applied directly to patches by local pressure ejection, as was picrotoxin (100 μ M). Gabazine-resistant spontaneous channels were recorded before GABA application with gabazine (20 μ M) in the recording chamber. For consistency with whole-cell recordings from hippocampal neurons, the extracellular solution was supplemented with CNQX (10 μ M), AP5 (10 μ M), and TTX (1 μ M).

Drugs Used. Gabazine (SR95531), bicuculline methiodide, CNQX, AP5, TTX, strychnine, γ -aminobutyric acid, and glycine were maintained as frozen stock solutions in distilled water at -20°C and diluted to their working concentrations in saline on the day of experimentation. Picrotoxin in dimethyl sulfoxide, 2,6-diisopropylphenol (propofol) in ethanol, and furosemide in ethanol were kept as stock solutions at -20°C and diluted to their working concentrations in saline on the day of experimentation. All reagents were supplied by Sigma.

Analysis. A modified logistic equation was used to fit inhibition curves as described previously (Adodra and Hales, 1995). In preparation for the measurement of single-channel amplitudes or tonic current amplitudes, sections of digitized data were selected for the creation of all-points amplitude histograms using Fetchan (pCLAMP 8.0; Molecular Devices). In the case of tonic currents, data between synaptic events were used, first digitizing control data and then digitizing data recorded in the presence of the antagonist. In the case of single-channel records, baseline currents were leak-subtracted using Clampfit (pCLAMP 8.0). Multiple Gaussians were fitted (least-squares minimization) to amplitude histograms using the Simplex method within pSTAT (pCLAMP 8.0). The amplitude of the single-channel current recorded from each patch was determined from the difference between the mean current amplitudes determined from the Gaussians fitted to the closed- and unitary open-state currents. Single-channel conductances are reported as the chord conductances

derived from $\gamma = i/(V_m - E_{rev})$ where i is unitary current amplitude, V_m is the holding potential, and E_{rev} is the reversal potential of single-channel currents.

Results

Pharmacology of Tonic GABA_A Receptor-Mediated Currents Recorded from Cultured Pyramidal Neurons in the Absence of Exogenous GABA. Phasic mIPSCs and sustained tonic currents were recorded from cultured hippocampal neurons in the presence of TTX (0.5 μ M) to block action potentials and CNQX (10 μ M) and AP5 (10 μ M) to inhibit ionotropic glutamate receptor activity (Fig. 1). Currents were recorded in the whole-cell configuration from neurons voltage-clamped at -60 mV. Picrotoxin (100 μ M) inhibited mIPSCs and the tonic currents (Fig. 1, A and F). Picrotoxin increased the steady-state input resistance of pyramidal neurons ($p < 0.05$), measured by applying hyperpolarizing steps (15–20 mV), by 0.22 ± 0.07 M Ω ($n = 6$). All neurons that lasted the course of the experiment ($n = 25$) responded to picrotoxin, exhibiting reduced mean inward current with a range from 3.2 to 73 pA, as determined from

amplitude histogram analysis (see *Materials and Methods*). The competitive GABA_A receptor antagonist bicuculline methiodide (20 μ M) also inhibited mIPSCs but was less effective than picrotoxin at reducing tonic current (Fig. 1, B and F). The competitive antagonist gabazine (20 μ M) had no effect on tonic currents and did not significantly alter input resistance ($n = 5$) but abolished mIPSCs (Fig. 1, C and F). Zn^{2+} (100 μ M), an inhibitor of GABA_A receptor activity, inhibited tonic currents and had relatively little effect on mIPSCs (Fig. 1, D and F). To compare the antagonist sensitivities of tonic currents among neurons, we normalized the amplitude of the inhibited current to the membrane capacitance, an approach that provides a measurement of the density of antagonist-sensitive current (Fig. 1F).

Hippocampal pyramidal neurons express glycine receptors (Thio and Zhang, 2006). It is unlikely that these receptors contribute to the tonic current because their activity is potentiated by Zn^{2+} , which inhibited tonic currents (Fig. 1, D and F). Nevertheless, we investigated whether glycine receptors contribute to tonic currents of pyramidal neurons by applying strychnine (100 nM). Glycine (100 μ M) activated

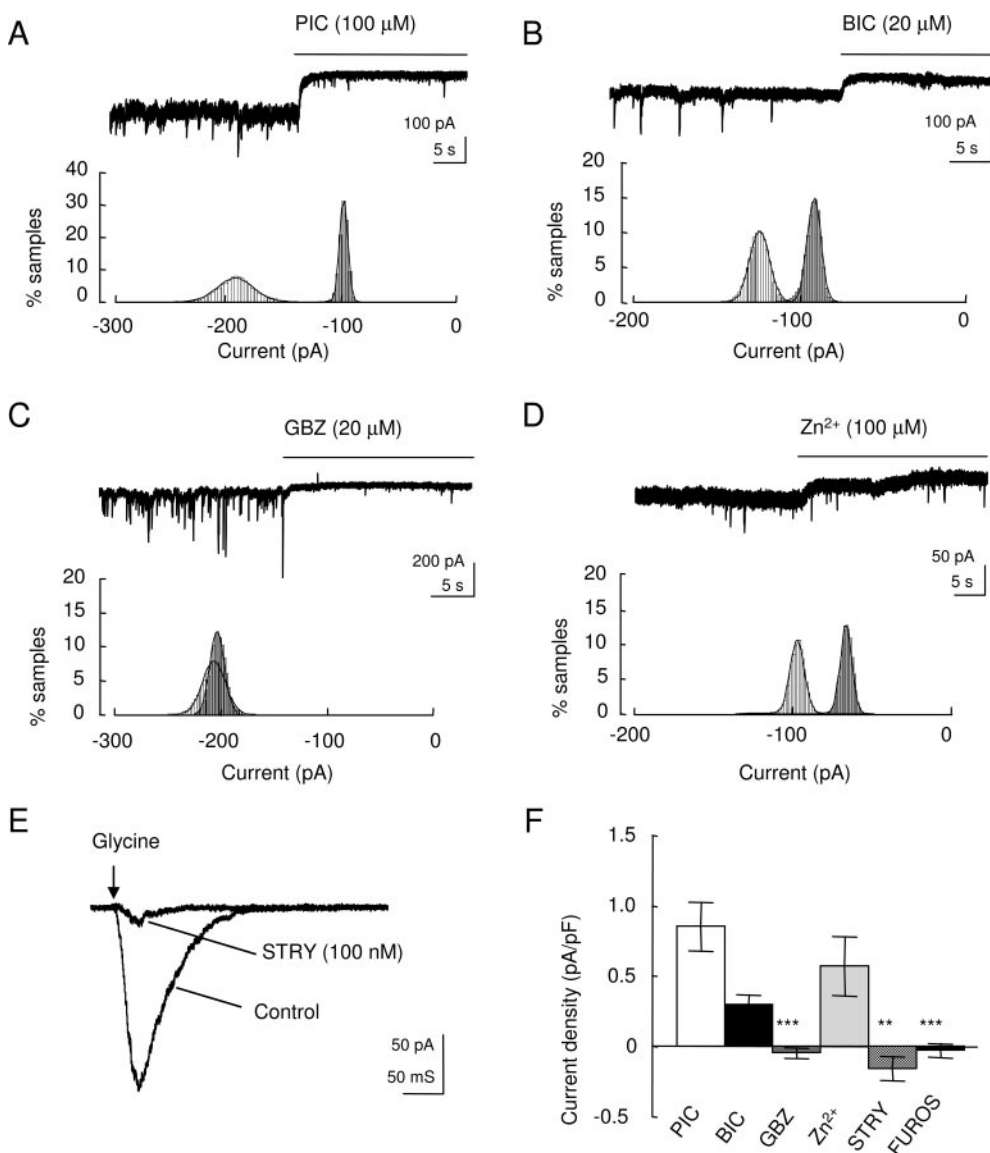


Fig. 1. Inhibitors of GABA_A receptor activity exhibit differing tendencies to attenuate tonic current in cultured hippocampal neurons. Currents were recorded from cultured hippocampal pyramidal neurons voltage-clamped at -60 mV with TTX (0.5 μ M), CNQX (10 μ M), and AP5 (10 μ M) in the extracellular solution. Picrotoxin (A, PIC; 100 μ M) and bicuculline (B, BIC; 20 μ M) inhibited mIPSCs and tonic currents. C, gabazine (GBZ; 20 μ M) inhibited mIPSCs but not tonic current. D, Zn^{2+} (100 μ M) inhibited tonic current but had little effect on mIPSCs. Current amplitude histograms in A through D were compiled from the respective current trace as described under *Materials and Methods*. Open and gray bars are the amplitudes of tonic currents, expressed as percent of the number of samples, measured in the absence and presence of drug, respectively, and were fitted with Gaussians providing a measure of mean tonic current. E, strychnine (STRY; 100 nM) inhibited currents activated by glycine (100 μ M) applied locally to a pyramidal neuron. Exemplar currents, recorded in the absence and presence of strychnine, are superimposed. The inhibition by strychnine reversed during wash (data not shown). F, the amplitude of currents inhibited by various antagonists including furosemide (600 μ M; FUROS) expressed relative to cell capacitance (i.e., current density) and is represented as mean \pm S.E.M. from at least seven different cells for each drug. Statistical significance was determined using a one-way ANOVA with post hoc Tukey test (**, $p < 0.01$ for picrotoxin versus strychnine; ***, $p < 0.001$ for picrotoxin versus both gabazine and furosemide).

small inward currents (272 ± 106 pA) when applied locally by pressure ejection to pyramidal neurons voltage-clamped at -60 mV. Glycine-activated currents were abolished by bath-applied 100 nM strychnine (Fig. 1E, $n = 5$). In contrast, strychnine had no effect on tonic currents recorded from pyramidal neurons (Fig. 1F).

We also examined the effect of furosemide (600 μ M) on tonic currents recorded from pyramidal neurons (Fig. 1F). Furosemide inhibits currents mediated by spontaneously gating recombinant GABA_A receptors containing the human ϵ subunit (Maksay et al., 2003) but had no effect on tonic currents recorded from pyramidal neurons (Fig. 1F).

These data demonstrate that tonic currents recorded from cultured hippocampal pyramidal neurons are sensitive to inhibition by picrotoxin, bicuculline methiodide (hereafter referred to as bicuculline), and Zn^{2+} but are resistant to inhibition by gabazine, strychnine, and furosemide.

Pharmacological Properties of Currents Activated by Exogenous GABA Recorded from Pyramidal Neurons. Our characterization of the antagonist sensitivities of tonic currents in pyramidal neurons is consistent with previous studies (Bai et al., 2001; Farrant and Nusser, 2005). However, there are conflicting reports of the effect of gabazine on tonic current in the literature (Stell and Mody, 2002). The addition of exogenous GABA or increasing ambient GABA levels by other means to enhance tonic GABA_A receptor activity may contribute to this discrepancy (Overstreet and Westbrook, 2001; Stell and Mody, 2002; Stell et al., 2003). To address this, we examined the pharmacology of currents activated by the addition of exogenous GABA (Fig. 2, A and B). GABA (5 μ M) activated large desensitizing inward currents (mean peak current amplitude = 1.2 ± 0.4 nA) that were inhibited by $94 \pm 3\%$ ($n = 6$) and $97 \pm 3\%$ ($n = 4$) by the application of either bicuculline (20 μ M) or gabazine (20 μ M), respectively (Fig. 2, A and B). These data demonstrate that tonic currents recorded from pyramidal neurons in the absence of added GABA (Fig. 1) are pharmacologically dissimilar to those activated by exogenous GABA (5 μ M).

Spontaneous Gabazine-Resistant Picrotoxin-Sensitive Single Channels. We investigated the properties of GABA_A receptors in outside-out membrane patches excised from pyramidal neurons. GABA (1 μ M) activated robust currents in recordings from outside-out patches (Fig. 3A). Such GABA-activated currents were prevented by application of gabazine (20 μ M). However, brief duration, spontaneous single-channel events were evident even during the application of gabazine (Fig. 3, A and B). The application of picrotoxin (100 μ M) abolished spontaneous channels (Fig. 3B), which had the same amplitude as channels activated by GABA (Fig. 3, C and D). The chord conductance of the spontaneous events was 24.1 ± 0.5 pS ($n = 9$), similar to that of GABA (1 μ M)-activated channels (25.7 ± 0.2 pS, $n = 3$) subsequently recorded from several of the same patches (Fig. 3, D and E). In these experiments, it is unlikely that spontaneous channels were activated by GABA released from neurons because patches were moved up to 1 mm from the bottom of the recording chamber without a decrease in channel activity. Furthermore, unlike GABA-evoked currents recorded from either whole pyramidal neurons (Fig. 1) or outside-out patches (Fig. 3A), spontaneous channels were resistant to gabazine. Our outside-out patch recordings support the hy-

pothesis that gabazine-resistant tonic current is mediated by GABA-independent spontaneous GABA_A receptor gating.

Antagonist Sensitivity of Propofol Activated Currents Recorded from Pyramidal Neurons. We hypothesize that the failure of gabazine to inhibit spontaneous GABA_A receptor activity stems from the antagonist's lack of negative intrinsic efficacy. The general anesthetic propofol directly activates GABA_A receptors (Hales and Lambert, 1991) through a site distinct from that of GABA (Davies et al., 1997). Based on the resistance of these currents to gabazine, it has been suggested that propofol primarily activates extrasynaptic receptors in pyramidal neurons (Bieda and MacIver, 2004). However, a lack of negative efficacy of gabazine could explain the resistance of propofol-activated currents to this antagonist. We examined the antagonist sensitivity of propofol-activated currents recorded from cultured pyramidal neurons (Fig. 4A). Gabazine (20 μ M) caused a relatively modest inhibition ($25 \pm 4\%$, $n = 12$) of the propofol-evoked current amplitude (Fig. 4, A and B). By contrast, bicuculline (20 μ M) inhibited propofol-activated currents by $85 \pm 5\%$ ($n = 13$). Application of gabazine (20 μ M) to pyramidal neurons significantly attenuated ($p < 0.001$) the inhibition of the propofol-activated current by bicuculline (20

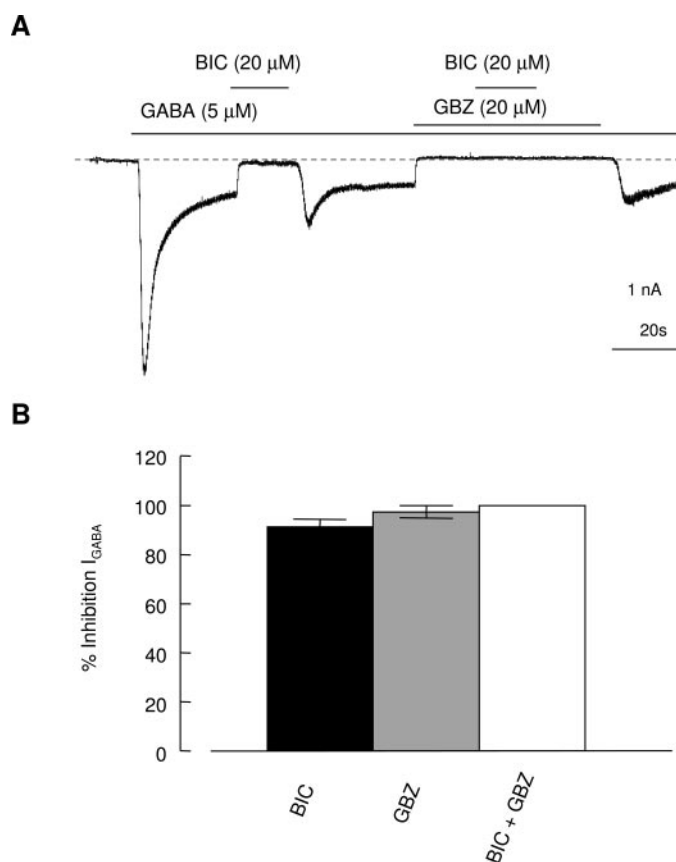


Fig. 2. Bicuculline and gabazine abolish GABA-evoked currents recorded from pyramidal neurons. A, the addition of GABA (5 μ M) to the perfusate activated a large desensitizing current that was abolished by gabazine (GBZ; 20 μ M) and bicuculline (BIC; 20 μ M), applied either alone or in combination. B, the percentage inhibition of GABA-activated current (I_{GABA}) by gabazine ($n = 4$) and bicuculline ($n = 6$) applied either alone or in combination. The inhibition was determined by expressing the amplitude of GABA-evoked current measured immediately before antagonist washout as a percentage of that measured immediately before antagonist application. Error bars, where visible, represent \pm S.E.M.

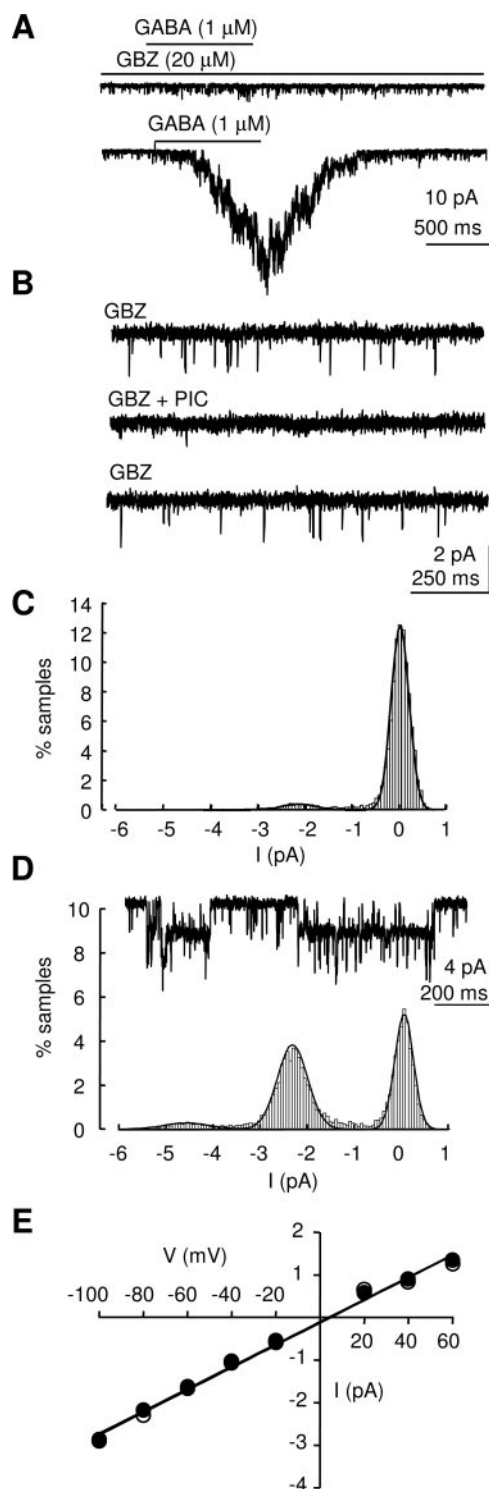


Fig. 3. Gabazine-resistant spontaneous GABA_A receptor gating. A, gabazine (GBZ; 20 μM) prevented GABA (1 μM) from activating channels in outside-out patches excised from a hippocampal pyramidal neuron, voltage-clamped at -60 mV (top trace). The bottom trace reveals numerous GABA-activated channels recorded upon washout of gabazine. B, brief duration spontaneous channel events persisted in the presence of gabazine (20 μM , top trace) but were abolished (middle trace) by the application of picrotoxin (100 μM ; PIC). Gabazine-resistant spontaneous events returned after the washout of picrotoxin (bottom trace). C, the all-points amplitude histogram for gabazine-resistant spontaneous channels recorded at -80 mV was fitted with the sum of two Gaussian distributions describing the closed and open states. These were used to establish the mean amplitude of spontaneous events, which, in this case, was -2.2 pA. D, application of GABA (1 μM) to the same patch used in C activated

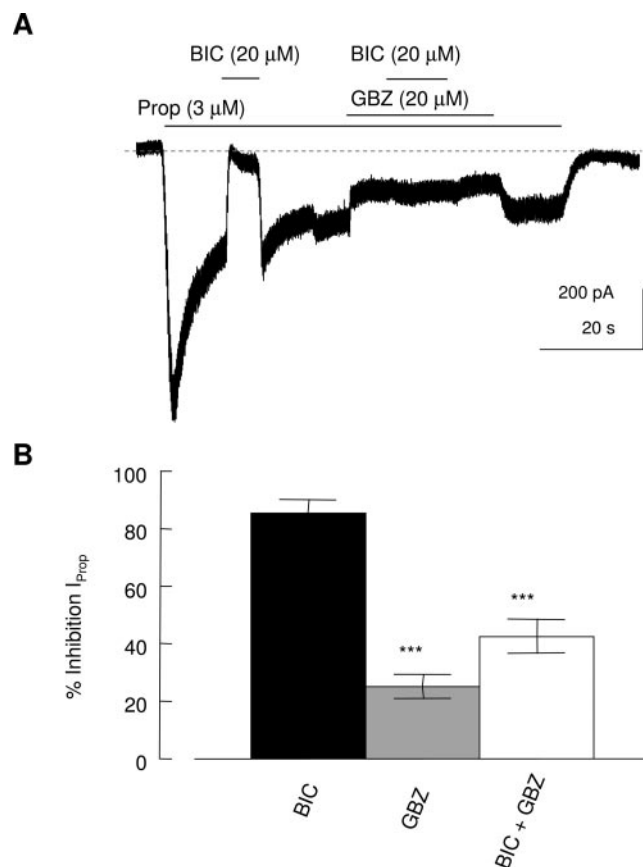


Fig. 4. Propofol-evoked currents recorded from pyramidal neurons are more effectively inhibited by bicuculline than by gabazine. A, bicuculline (BIC; 20 μM) abolished the current activated by propofol (3 μM) administered, through the perfusate, to a pyramidal neuron voltage-clamped at -60 mV. The propofol-activated current was less effectively inhibited by gabazine (GBZ; 20 μM). The inhibition by bicuculline was prevented when the antagonist was applied in combination with gabazine. B, the bar graph compares the percentage inhibition of propofol-activated currents (I_{Prop}) by bicuculline and gabazine applied alone or in combination. Statistical significance was determined using ANOVA with the post hoc Tukey test (***, $p < 0.001$ for bicuculline versus both gabazine and gabazine plus bicuculline).

μM), demonstrating that gabazine is in fact binding to propofol-activated GABA_A receptors in pyramidal neurons and yet has negligible negative efficacy (Fig. 4B).

Antagonist Sensitivity of Propofol Activated Currents Mediated by Recombinant GABA_A Receptors. We examined whether the resistance of propofol-activated currents to gabazine was unique to GABA_A receptors expressed by the hippocampus or a more general rule for propofol-activated receptors. We investigated properties of GABA and propofol-activated currents mediated by recombinant $\alpha 1\beta 3\gamma 2$ receptors. This subunit combination was chosen in part because it is unlikely to mimic that of extrasynaptic receptors in hippocampal neurons, which probably contain the $\alpha 5$ subunit (Caraiscos et al., 2004) and possibly the $\beta 1$ subunit (Mangan et al., 2005). GABA (100 μM)-evoked currents recorded from HEK293 cells expressing recombinant $\alpha 1\beta 3\gamma 2$

channels with an amplitude similar to that of spontaneous events (in this case, -2.3 pA), recorded at -80 mV. E, the current-voltage relationship reveals that spontaneous (●) and GABA-activated channels (○) have similar equilibrium potentials and chord conductances. In the exemplar data taken from the same patch in the absence and presence of GABA, chord conductances were 25.3 and 26.0 pS, respectively.

receptors were abolished by bicuculline ($IC_{50} = 1.9 \pm 0.3 \mu M$) and gabazine ($IC_{50} = 0.4 \pm 0.1 \mu M$) (Fig. 5A). Bicuculline ($IC_{50} = 3.0 \pm 1.5 \mu M$) also abolished propofol ($10 \mu M$)-activated currents mediated by $\alpha 1\beta 3\gamma 2$ receptors (Fig. 5B). In contrast, gabazine ($1\text{--}100 \mu M$) caused a maximum inhibition of 13% (Fig. 5B). Consistent with our observations in hippocampal pyramidal neurons, gabazine ($10 \mu M$) abolished the inhibition of propofol-activated currents by bicuculline ($10 \mu M$; Fig. 5, C and D). Taken together, these data suggest that the failure of gabazine to block propofol-activated currents in pyramidal neurons is due not to a receptor subtype-discriminative effect but to a lack of negative intrinsic activity of this GABA antagonist.

Flunitrazepam and Loreclezole Elicit Currents in Pyramidal Neurons in the Absence of Exogenous GABA. In addition to propofol, several other allosteric modulators of GABA_A receptor function enhance currents activated by GABA (Whiting et al., 1999). Like propofol, the sedative-hypnotic agent loreclezole potentiates GABA-evoked currents and directly activates GABA_A receptors in the absence of GABA. Loreclezole, unlike propofol, selectively affects receptors that contain either the $\beta 2$ or $\beta 3$ subunit, having a negligible effect on receptors containing the $\beta 1$ subunit. Sedative-hypnotic benzodiazepines such as flunitrazepam also enhance GABA-evoked currents but do not directly activate GABA_A receptors, and their actions are dependent on the presence of a γ subunit (Schulz and Macdonald, 1981; Whiting et al., 1999). When bath was applied to cultured hippocampal pyramidal neurons, loreclezole ($10 \mu M$) activated inward currents in all cells tested (Fig. 6A). Loreclezole-evoked currents had a mean amplitude of -265 ± 42 pA ($n = 5$). The ability of loreclezole to activate currents indicates that at least some of the GABA_A receptors in pyramidal neurons contain $\beta 2$ and/or $\beta 3$ subunits. Flunitrazepam ($1 \mu M$) also elicited inward currents, albeit smaller than those seen in response to loreclezole, in all pyramidal neurons tested ($n = 9$) with a mean amplitude of -35 ± 10 pA (Fig. 6B). These data suggest that flunitrazepam is enhancing either tonic currents activated by low levels of ambient GABA or the gating of spontaneous currents. The GABA_A antagonist gabazine ($20 \mu M$) abolished GABA-evoked currents recorded from pyramidal neurons (Fig. 2). Therefore, if flunitrazepam elicits currents in pyramidal neurons by enhancing ambient GABA, flunitrazepam-evoked currents should be abolished by gabazine. Gabazine ($20 \mu M$) had no significant effect ($p = 0.31$) on the amplitude of flunitrazepam ($1 \mu M$)-evoked currents (-20 ± 9 pA, $n = 6$).

These data indicate that at least some of the GABA_A receptors in pyramidal neurons contain $\beta 2$ and/or $\beta 3$ subunits and γ subunits. Furthermore, flunitrazepam enhances tonic currents in a manner that is resistant to gabazine.

Antagonist Sensitivity of Tonic Currents Mediated by Recombinant Rodent $\alpha 1\beta 3\epsilon$ GABA_A Receptors. We examined whether the pharmacological properties of spontaneously gating recombinant GABA_A receptors matched those of tonic currents recorded from pyramidal neurons. The human ϵ subunit enhances spontaneous gating, producing robust picrotoxin-sensitive tonic currents when incorporated into recombinant GABA_A receptors (Neelands et al., 1999; Davies et al., 2001; Maksay et al., 2003). It is interesting that bicuculline inhibits spontaneous currents mediated by ϵ subunit-containing receptors, and gabazine is less efficacious in

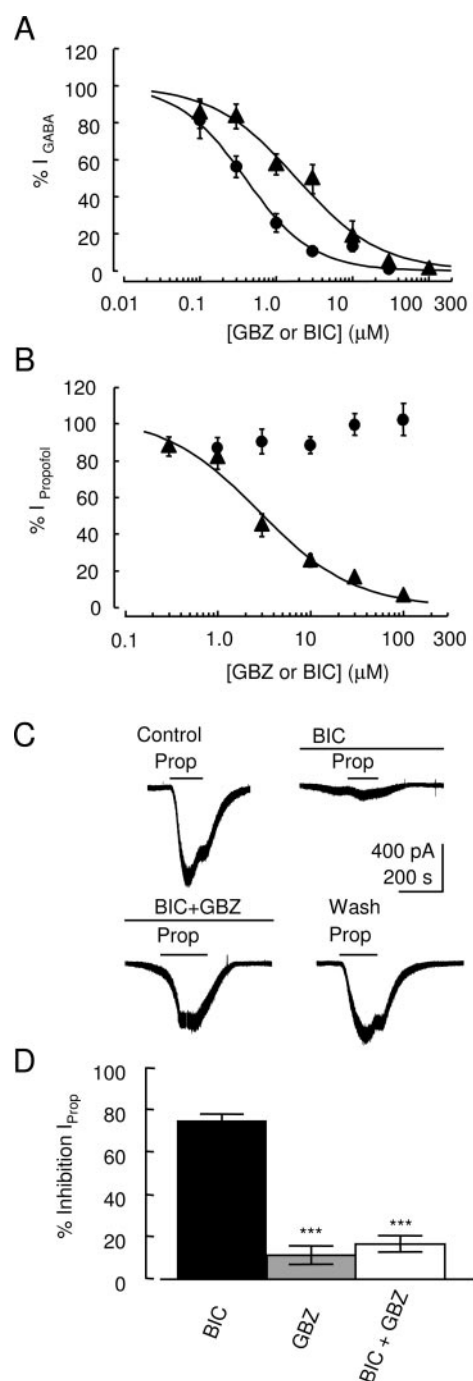


Fig. 5. Currents activated by GABA and propofol mediated by $\alpha 1\beta 3\gamma 2$ receptors have differing sensitivities to gabazine. **A**, GABA ($100 \mu M$)-activated current recorded from HEK293 cells expressing recombinant $\alpha 1\beta 3\gamma 2$ receptors were inhibited by gabazine (\bullet) and bicuculline (\blacktriangle) in a concentration-dependent manner. Data were fitted using a modified logistic equation (Adodra and Hales, 1995). The IC_{50} values for bicuculline and gabazine are 1.9 ± 0.3 and $0.4 \pm 0.1 \mu M$, respectively. **B**, propofol ($3 \mu M$)-activated currents mediated by recombinant $\alpha 1\beta 3\gamma 2$ GABA_A receptors expressed in HEK293 cells were inhibited by bicuculline (\blacktriangle) but not by gabazine (\bullet). The IC_{50} value for bicuculline was $3.2 \pm 0.5 \mu M$. Hill coefficient for inhibition by bicuculline was 0.9 ± 0.1 . **C**, propofol ($10 \mu M$)-activated currents recorded from the same HEK293 cell expressing $\alpha 1\beta 3\gamma 2$ receptors were inhibited by bicuculline ($10 \mu M$). The inhibition by bicuculline was prevented by gabazine ($10 \mu M$). **D**, the bar graph illustrates the percentage of inhibition of propofol-activated currents (I_{Prop}) by bicuculline and gabazine applied either alone or in combination. In all cases, data points represent the mean of at least four cells. Error bars are \pm S.E.M.

this respect (Maksay et al., 2003). Like picrotoxin, furosemide abolishes spontaneous currents mediated by human $\alpha 1\beta 3\epsilon$ receptors but has no effect on tonic currents recorded from either mouse (Caraiscos et al., 2004) or rat pyramidal neurons (Fig. 1F). We tested rat $\alpha 1\beta 3\epsilon$ receptors to determine whether the rodent ϵ subunit (Davies et al., 2002) also conferred furosemide-sensitive tonic currents. Picrotoxin (100 μM) applied to the bath inhibited relatively large spontaneous currents mediated by $\alpha 1\beta 3\epsilon$ receptors expressed in HEK293 cells (Fig. 7A). The density of current inhibited by picrotoxin was 1.68 ± 0.25 pA/pF ($n = 17$), somewhat larger than the picrotoxin-sensitive tonic current density of 0.85 ± 0.18 pA/pF ($n = 25$) determined in pyramidal neurons (Fig. 1F). Furthermore, furosemide (600 μM) also inhibited spontaneous current mediated by rat $\alpha 1\beta 3\epsilon$ receptors (Fig. 7B). The density of current inhibited by furosemide was $1.11 \pm$

0.22 pA/pF ($n = 9$) (Fig. 7B). These data demonstrate that spontaneous currents mediated by rat $\alpha 1\beta 3\epsilon$ receptors are sensitive to furosemide and in this way differ from tonic currents recorded from rodent pyramidal neurons (Fig. 1F).

Antagonist Sensitivities of Tonic Currents Mediated by Recombinant Rodent $\alpha 1\beta 1\gamma 2$ and $\alpha 1\beta 3\gamma 2$ GABA_A Receptors. Recombinant GABA_A receptors containing the $\beta 1$ subunit expressed in *Xenopus laevis* oocytes exhibit spontaneous current (Miko et al., 2004). Furthermore, hippocampal neurons cultured from embryonic rats express the $\beta 1$ subunit (Mangan et al., 2005). Therefore, we examined whether $\alpha 1\beta 1\gamma 2$ GABA_A receptors exhibited spontaneous gating when expressed in HEK293 cells. Because loreclezole elicited currents in pyramidal neurons (Fig. 5A), indicating the presence of receptors containing the $\beta 2$ and/or the $\beta 3$ subunit, we compared spontaneous currents mediated by $\alpha 1\beta 1\gamma 2$ receptors with those mediated by $\alpha 1\beta 3\gamma 2$ receptors. Picrotoxin (100 μM) inhibited small tonic currents when bath-applied to HEK293 cells expressing either $\alpha 1\beta 1\gamma 2$ or $\alpha 1\beta 3\gamma 2$ receptors (Fig. 7C). The picrotoxin-inhibited current densities were 0.53 ± 0.09 ($n = 6$) and 0.34 ± 0.09 pA/pF ($n = 9$), respectively, (Fig. 7G). The ratios of picrotoxin-inhibited current amplitudes to peak GABA (100 μM)-activated current amplitudes were $0.27 \pm 0.11\%$ and $0.13 \pm 0.05\%$ for $\alpha 1\beta 1\gamma 2$ and $\alpha 1\beta 3\gamma 2$ receptors, respectively. Furthermore, similar to our observations in pyramidal neurons (Fig. 1, B, C, and F), bicuculline (20 μM) inhibited spontaneous currents mediated by $\alpha 1\beta 1\gamma 2$ and $\alpha 1\beta 3\gamma 2$ receptors, whereas gabazine (20 μM) had no effect (Fig. 7, D and G). However, gabazine attenuated the inhibition of tonic current by bicuculline when coapplied to cells expressing $\alpha 1\beta 1\gamma 2$ or $\alpha 1\beta 3\gamma 2$ receptors, demonstrating that both antagonists bind to constitutively active GABA_A receptors (Fig. 7D).

Flunitrazepam (1 μM) enhanced tonic currents when bath-applied to HEK293 cells expressing either $\alpha 1\beta 1\gamma 2$ or $\alpha 1\beta 3\gamma 2$ receptors (Fig. 7E), having a significantly greater effect in the latter compared with the former ($p < 0.01$). Flunitrazepam-elicited currents were -6 ± 1 pA ($n = 6$) and -22 ± 6 pA ($n = 4$), respectively. Loreclezole (10 μM) also activated significantly larger currents ($p < 0.05$) when applied to cells expressing $\alpha 1\beta 3\gamma 2$ receptors (-198 ± 93 pA, $n = 6$) compared with those expressing $\alpha 1\beta 1\gamma 2$ receptors (-4 ± 2 pA, $n = 7$) (Fig. 7F).

We also examined the effects of Zn^{2+} (100 μM) and furosemide (600 μM) on spontaneous currents mediated by $\alpha 1\beta 1\gamma 2$ or $\alpha 1\beta 3\gamma 2$ receptors. The former but not the latter inhibited spontaneous currents recorded from cells expressing either receptor combination (Fig. 7G). The pharmacological profiles of spontaneous currents mediated by recombinant $\alpha 1\beta 1\gamma 2$ and $\alpha 1\beta 3\gamma 2$ receptors (Fig. 7G) exhibit a striking similarity to those of tonic currents recorded from pyramidal neurons (Fig. 1F).

Discussion

We characterized the antagonist sensitivities of GABA_A receptors in cultured neonatal hippocampal pyramidal neurons by recording four types of activity: 1) mIPSCs; 2) tonic inhibitory currents; 3) currents activated by exogenous GABA; and 4) currents activated by propofol. Picrotoxin and bicuculline inhibited all four types of activity. In contrast, gabazine inhibited mIPSCs and currents activated by exog-

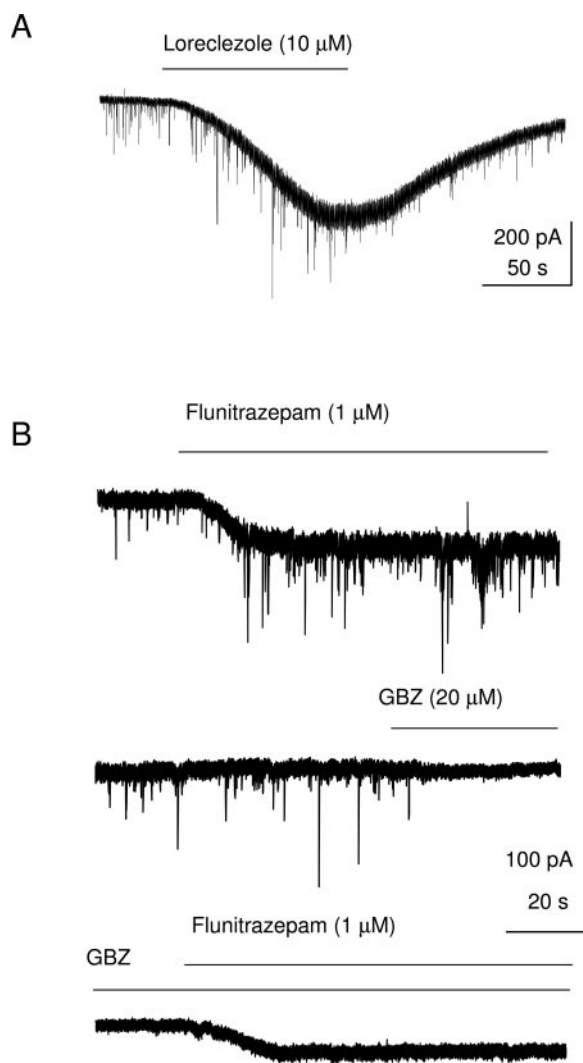


Fig. 6. Loreclezole and flunitrazepam elicit currents recorded from pyramidal neurons. **A**, the bath application of loreclezole (10 μM) to a pyramidal neuron voltage-clamped at -60 mV activated a robust inward current. **B**, top trace, flunitrazepam (1 μM) enhanced tonic current. Middle trace, in the same cell after washout of flunitrazepam, gabazine (GBZ, 20 μM) inhibited mIPSCs without affecting tonic current. Bottom trace, in the presence of gabazine, flunitrazepam caused enhanced tonic current when applied to the same pyramidal neuron. In all cases, currents were recorded in the presence of CNQX, AP5, and TTX (see *Materials and Methods*).

enous GABA but had little effect on either tonic or propofol-activated currents. It is interesting that the pharmacological properties of tonic currents of pyramidal neurons were similar to tonic currents of HEK293 cells expressing $\alpha 1\beta 1\gamma 2$ or $\alpha 1\beta 3\gamma 2$ receptors but differed from those of $\alpha 1\beta 3\epsilon$ receptors.

Our data support the idea that gabazine-resistant GABA_A receptor activity mediates inhibitory tonic currents (Bai et al., 2001). However, because picrotoxin also inhibits glycine-activated currents (Thio and Zhang, 2006), we examined whether glycine receptors mediate tonic currents. This is unlikely because Zn^{2+} , which inhibited tonic currents, enhances glycine-activated currents recorded from pyramidal neurons (Thio and Zhang, 2006). Furthermore, tonic currents were resistant to the glycine receptor antagonist strychnine. The sensitivity of tonic currents to bicuculline and picrotoxin, taken together with the similarity of the chord conductances of GABA-evoked and spontaneous channels in outside-out

patches, supports the hypothesis that GABA_A receptors mediate tonic currents. However, the only GABA receptors known to mediate GABA-evoked Cl^{-} currents that are resistant to gabazine are composed of ρ subunits (Zhang et al., 2001). These receptors are also resistant to bicuculline, and therefore, the inhibition of tonic currents by bicuculline is inconsistent with the idea that ρ subunits mediate tonic current in pyramidal neurons.

Because gabazine abolishes mIPSCs and currents activated by exogenous GABA, tonic currents are not activated by GABA. How can the apparently contradictory observations that 1) GABA_A receptors mediating tonic current are gabazine-resistant and 2) all GABA-evoked currents are inhibited by gabazine, be reconciled? We propose the following hypothesis: spontaneous GABA_A receptor gating gives rise to gabazine-resistant tonic currents. GABA_A receptor-mediated channels active in the absence of added GABA occur in out-

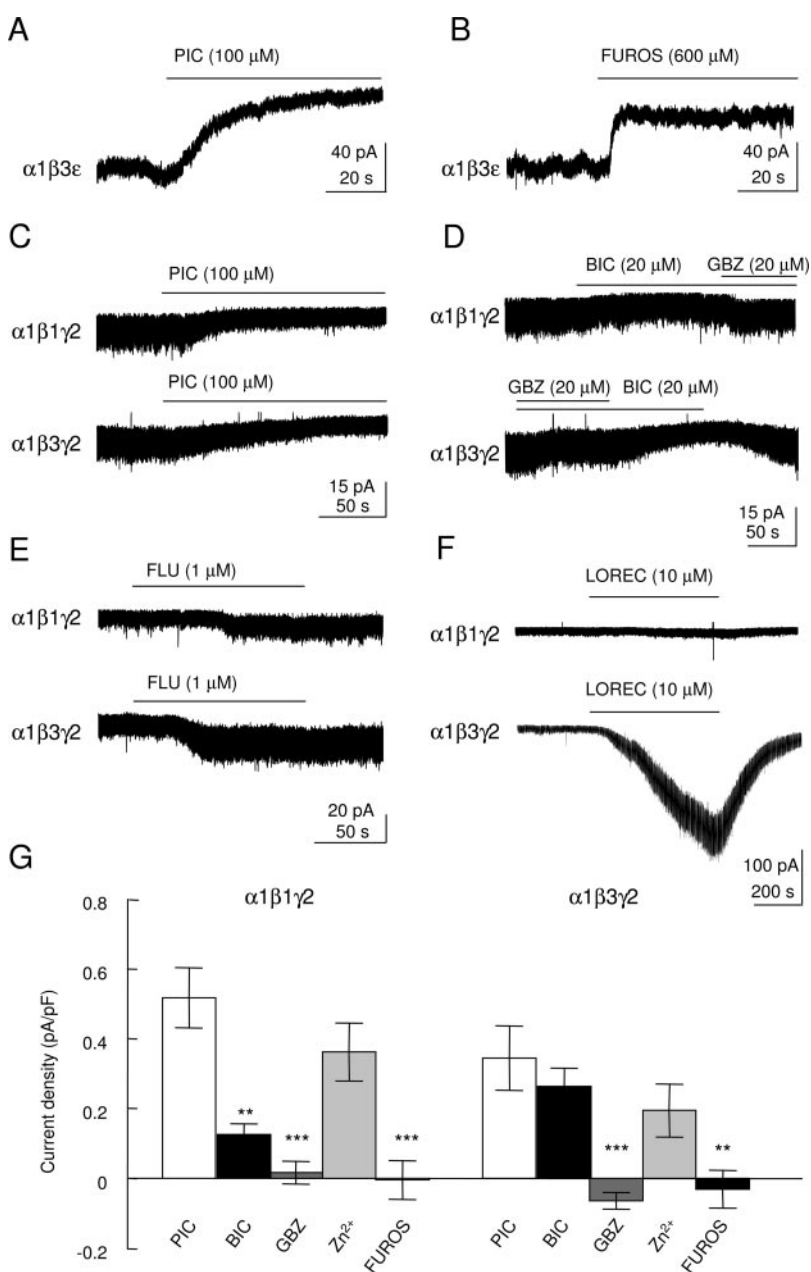


Fig. 7. Inhibitors of GABA_A receptor activity exhibit differing tendencies to attenuate tonic current in HEK293 cells expressing $\alpha 1\beta 3\epsilon$, $\alpha 1\beta 1\gamma 2$, and $\alpha 1\beta 3\gamma 2$ receptors. Tonic currents were recorded in the absence of GABA from HEK293 cells expressing recombinant rodent receptors. A, picrotoxin (PIC, 100 μ M) inhibited tonic current. B, furosemide (FURO, 600 μ M) applied to the same cell also inhibited tonic current. C, in contrast to $\alpha 1\beta 3\epsilon$ receptors, which exhibited robust picrotoxin-sensitive tonic currents, $\alpha 1\beta 1\gamma 2$ and $\alpha 1\beta 3\gamma 2$ receptors mediated small picrotoxin-sensitive tonic currents recorded in the absence of GABA. D, tonic currents mediated by $\alpha 1\beta 1\gamma 2$ and $\alpha 1\beta 3\gamma 2$ receptors were inhibited by bicuculline (BIC, 20 μ M). The inhibitory effect of bicuculline was inhibited by coapplication of the antagonist with gabazine (GBZ, 20 μ M). E, flunitrazepam (FLU, 1 μ M) enhanced tonic currents recorded from $\alpha 1\beta 1\gamma 2$ and $\alpha 1\beta 3\gamma 2$ receptors, and this effect was more pronounced in the latter compared with the former (see text). F, loreclezole (10 μ M) evoked a large current when applied to HEK293 cells expressing $\alpha 1\beta 3\gamma 2$ receptors without having an effect on cells expressing $\alpha 1\beta 1\gamma 2$ receptors. G, the average density of currents (pA/pF) inhibited when GABA_A receptor antagonists were applied to cells expressing either $\alpha 1\beta 1\gamma 2$ or $\alpha 1\beta 3\gamma 2$ receptors. Current amplitudes were measured using the amplitude histogram approach shown in Fig. 1 (described under *Materials and Methods*). Each mean current density was determined from at least five cells, and vertical lines represent \pm S.E.M. Statistical significance compared with the density of current inhibited by picrotoxin for each GABA_A receptor type was determined using a one-way ANOVA with post hoc Tukey test: **, $p < 0.01$ and ***, $p < 0.001$.

side-out patch recordings from pituitary intermediate lobe cells (Taleb et al., 1987) and in cell-attached patch recordings from hippocampal pyramidal neurons (Birnir et al., 2000) and hypothalamic neurons (Jones et al., 2006). We found that spontaneous channel activity in outside-out patches excised from pyramidal neurons were independent of GABA, persisted for prolonged time periods after patch excision, were resistant to gabazine, and were abolished by picrotoxin. GABA-activated channels recorded subsequently from the same patches had the same chord conductances as spontaneous events but were abolished by gabazine. These data support the hypothesis that spontaneously active GABA_A receptors are gabazine-resistant.

Both gabazine and bicuculline competitively antagonize GABA binding to GABA_A receptors (Straughan et al., 1971; Heaulme et al., 1986; Zhang et al., 2001). However, we propose that only bicuculline is able to significantly inhibit receptors that exhibit gating in the absence of GABA. Antagonists that are able to inhibit receptors that are active in the absence of agonist are said to have negative intrinsic efficacy and are therefore inverse agonists, terminology first developed to explain the actions of ligands that bind to the benzodiazepine site (Polc et al., 1982). We propose that bicuculline has negative intrinsic efficacy acting as an inverse agonist at the GABA binding site. In contrast, gabazine has negligible negative efficacy and is therefore a neutral competitive antagonist.

To compare the negative efficacies of bicuculline and gabazine, we activated GABA_A receptors using propofol, an anesthetic that acts at a site distinct from that of GABA (Davies et al., 1997). In agreement with results from a previous study (Bieda and MacIver, 2004), gabazine was relatively ineffective as an inhibitor of propofol-activated currents. In contrast, bicuculline abolished propofol-activated currents. The fact that gabazine prevented the abolition of propofol-activated currents by bicuculline confirms that gabazine is indeed binding to the same site with negligible negative efficacy. Consistent with this hypothesis, gabazine also attenuates the inhibition by bicuculline of tonic currents recorded from pyramidal neurons (Bai et al., 2001).

The inability of gabazine to inhibit propofol-activated currents is not indicative of a selective ability of the anesthetic to activate tonic receptors as suggested previously (Bieda and MacIver, 2004). Instead, it is a consequence of the antagonist's lack of negative efficacy. Propofol-activated currents mediated by $\alpha 1\beta 3\gamma 2$ receptors were also resistant to inhibition by gabazine and abolished by bicuculline. Furthermore, gabazine prevented the inhibition of propofol-activated $\alpha 1\beta 3\gamma 2$ receptors by bicuculline. Again, these data support the hypothesis that gabazine has negligible negative efficacy, whereas bicuculline is an inverse agonist. Furthermore, these findings are in agreement with a previous study demonstrating negligible efficacy of gabazine as an inhibitor of recombinant receptors activated by the anesthetic steroid alfaxalone and pentobarbital (Ueno et al., 1997).

Neurosteroids also directly activate GABA_A receptors, and, by analogy to alfaxalone, it is likely that this action is bicuculline-sensitive and gabazine-resistant (Ueno et al., 1997). Endogenous neurosteroids may participate in tonic GABA_A receptor activity in vivo. However, it is unlikely that in cultured neurons undergoing continuous perfusion, neurosteroids reach concentrations sufficient for GABA_A receptor

activation. A role for neurosteroids in tonic single-channel activity observed in patches excised from pyramidal neurons is even less likely.

The discovery of constitutive activity of metabotropic receptors (Costa and Herz, 1989) led to the reclassification, as inverse agonists, of several drugs found to have negative intrinsic efficacy previously considered antagonists (Kenakin, 2004; Costa and Cotecchia, 2005). The occurrence of constitutive gating in ionotropic receptors may lead to a similar reclassification of ligands such as bicuculline, previously considered antagonists. Several cysteine-loop ionotropic receptors exhibit constitutive gating, such as nicotinic acetylcholine receptors in embryonic muscle (Jackson, 1984) and recombinant Zn²⁺-activated ion channels (Davies et al., 2003).

Recombinant studies revealed that the presence of the ϵ subunit causes the appearance of robust spontaneous gating of $\alpha\beta\gamma\epsilon$ and $\alpha 1\beta 3\gamma 2\epsilon$ GABA_A receptors (Neelands et al., 1999; Davies et al., 2001; Maksay et al., 2003; Wagner et al., 2005). Furthermore, the $\beta 1$ subunit also confers some spontaneous gating to both $\alpha 2\beta 1$ and $\alpha 2\beta 1\gamma 2$ receptors (Miko et al., 2004). Spontaneously active GABA_A receptors containing the ϵ subunit may participate in tonic current in GnRH-secreting hypothalamic neurons (Jones et al., 2006). However, the ϵ subunit does not seem to be responsible for spontaneous gating in hippocampal pyramidal neurons. Furosemide inhibited spontaneous currents mediated by recombinant rodent $\alpha 1\beta 3\epsilon$ receptors, whereas tonic currents in pyramidal neurons were resistant to the compound. It is interesting that cultured embryonic hippocampal neurons exhibit furosemide-sensitive tonic currents (Mangan et al., 2005), raising the intriguing possibility that the ϵ subunit may play a developmental role in the spontaneous gating of GABA_A receptors.

We examined the possibility that spontaneous gating could be conferred by the $\beta 1$ subunit identified previously in hippocampal neurons using immunocytochemistry (Mangan et al., 2005). Because loreclezole activated currents when applied to pyramidal neurons, inferring the presence of $\beta 2$ and/or $\beta 3$ subunits, we compared the spontaneous gating of $\alpha 1\beta 1\gamma 2$ receptors to that of $\alpha 1\beta 3\gamma 2$ receptors expressed in HEK293 cells. Consistent with a previous report (Miko et al., 2004), spontaneous currents mediated by receptors containing the $\beta 1$ subunit were larger than the those mediated by receptors containing the $\beta 3$ subunit when normalized to the peak GABA-evoked current. Nevertheless both $\alpha 1\beta 1\gamma 2$ and $\alpha 1\beta 3\gamma 2$ receptors mediated detectable tonic currents with pharmacological properties similar to those of pyramidal neurons. It is significant that neither gabazine nor furosemide affected constitutive gating of $\alpha 1\beta 1\gamma 2$ or $\alpha 1\beta 3\gamma 2$ receptors. In contrast, picrotoxin and bicuculline inhibited spontaneous currents, and the actions of the latter were attenuated by gabazine. These data support our hypothesis that spontaneously active GABA_A receptors mediate tonic currents in pyramidal neurons.

Benzodiazepines enhance tonic currents recorded from pyramidal neurons (Bai et al., 2001). Unlike general anesthetics and various hypnotic compounds including loreclezole (Whiting et al., 1999), benzodiazepines do not directly activate GABA_A receptors (Schulz and Macdonald, 1981). Therefore, one of two mechanisms may account for increased tonic current: 1) enhanced affinity for ambient GABA; or 2) poten-

tiation of spontaneous gating. Gabazine had no significant effect on the enhanced tonic current induced by flunitrazepam. Furthermore, in the absence of GABA, flunitrazepam also enhanced tonic currents mediated by recombinant GABA_A receptors. Therefore, it is likely that flunitrazepam enhances spontaneous GABA_A receptor gating. This may be a property common to anxiolytic benzodiazepines because diazepam also enhances bicuculline-sensitive spontaneous current mediated by $\alpha 1\beta 3\gamma 2$ receptors (Bianchi and Macdonald, 2001).

Spontaneous gating of recombinant GABA_A receptors can be enhanced by introducing mutations in the transmembrane 2 domain (Chang et al., 1996; Thompson et al., 1999). In agreement with our findings using native and recombinant GABA_A receptors, gabazine has a negligible efficacy relative to bicuculline as an inhibitor of spontaneously active GABA_A receptors containing mutations that induce constitutive gating (Chang and Weiss, 1999; Thompson et al., 1999).

The existence of spontaneously active GABA_A receptors explains the contrasting antagonist pharmacologies of tonic currents and currents activated by exogenous GABA in hippocampal pyramidal neurons. Although high-affinity extrasynaptic GABA_A receptors activated by ambient GABA probably mediate tonic currents in some brain regions (Farrant and Nusser, 2005), our study demonstrates the need for consideration of additional mechanisms. Using bicuculline as an inverse agonist and gabazine as a neutral antagonist the presence of GABA-independent gating can now be quantified in different brain regions.

References

- Adodra S and Hales TG (1995) Potentiation, activation and blockade of GABA_A receptors of clonal murine hypothalamic GT1-7 neurones by propofol. *Br J Pharmacol* **115**:953–960.
- 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.
- Bianchi MT and Macdonald RL (2001) Agonist trapping by GABA_A receptor channels. *J Neurosci* **21**:9083–9091.
- Bieda MC and MacIver MB (2004) Major role for tonic GABA_A conductances in anesthetic suppression of intrinsic neuronal excitability. *J Neurophysiol* **92**:1658–1667.
- Birnir B, Everitt AB, Lim MS, and Gage PW (2000) Spontaneously opening GABA_A channels in CA1 pyramidal neurones of rat hippocampus. *J Membr Biol* **174**:21–29.
- Caraiscos VB, Elliott EM, You-Ten KE, Cheng VY, Belelli D, Newell JG, Jackson MF, Lambert JJ, Rosahl TW, Wafford KA, et al. (2004) Tonic inhibition in mouse hippocampal CA1 pyramidal neurons is mediated by $\alpha 5$ subunit-containing γ -aminobutyric acid type A receptors. *Proc Natl Acad Sci USA* **101**:3662–3667.
- Chang Y and Weiss DS (1999) Allosteric activation mechanism of the $\alpha 1\beta 2\gamma 2$ gamma-aminobutyric acid type A receptor revealed by mutation of the conserved M2 leucine. *Biophys J* **77**:2542–2551.
- Chang Y, Wang R, Barot S, and Weiss DS (1996) Stoichiometry of a recombinant GABA_A receptor. *J Neurosci* **16**:5415–5424.
- Cheng VY, Martin LJ, Elliott EM, Kim JH, Mount HT, Taverna FA, Roder JC, Macdonald JF, Bhambhani A, Collinson N, et al. (2006) $\alpha 5$ GABA_A receptors mediate the amnesic but not sedative-hypnotic effects of the general anesthetic etomidate. *J Neurosci* **26**:3713–3720.
- Costa T and Cotecchia S (2005) Historical review: negative efficacy and the constitutive activity of G-protein-coupled receptors. *Trends Pharmacol Sci* **26**:618–624.
- Costa T and Herz A (1989) Antagonists with negative intrinsic activity at delta opioid receptors coupled to GTP-binding proteins. *Proc Natl Acad Sci USA* **86**:7321–7325.
- Davies PA, Kirkness EF, and Hales TG (1997) Activation by general anesthetics of rat $\beta 3$ GABA_A subunit homomers expressed in human embryonic kidney 293 cells. *Br J Pharmacol* **120**:899–909.
- Davies PA, Kirkness EF, and Hales TG (2001) Evidence for the formation of functionally distinct $\alpha \beta \gamma \epsilon$ GABA_A receptors. *J Physiol* **537**:101–113.
- Davies PA, McCartney MR, Wang W, Hales TG, and Kirkness EF (2002) Alternative transcripts of the GABA_A receptor ϵ subunit in human and rat. *Neuropharmacology* **43**:467–475.
- Davies PA, Wang W, Hales TG, and Kirkness EF (2003) Novel class of ligand-gated ion channel is activated by Zn²⁺. *J Biol Chem* **278**:712–717.
- Farrant M and Nusser Z (2005) Variations on an inhibitory theme: phasic and tonic activation of GABA_A receptors. *Nat Rev Neurosci* **6**:215–229.
- Hales TG, Deeb TZ, Tang H, Bolland KA, King DP, Johnson SJ, and Connolly CN (2006) An asymmetric contribution to γ -aminobutyric type A receptor function of a conserved lysine within TM2–3 of $\alpha 1$, $\beta 2$, and $\gamma 2$ subunits. *J Biol Chem* **281**:17034–17043.
- Hales TG and Lambert JJ (1991) The actions of propofol on inhibitory amino acid receptors of bovine adrenomedullary chromaffin cells and rodent central neurones. *Br J Pharmacol* **104**:619–628.
- Heaulme M, Chambon JP, Leyris R, Molimard JC, Wermuth CG, and Biziére K (1986) Biochemical characterization of the interaction of three pyridazinyl-GABA derivatives with the GABA_A receptor site. *Brain Res* **384**:224–231.
- Hemmings HC, Akabas MH, Goldstein PA, Trudell JR, Orser BA, and Harrison NL (2005) Emerging molecular mechanisms of general anesthetic action. *Trends Pharmacol Sci* **26**:503–510.
- Jackson MB (1984) Spontaneous openings of the acetylcholine receptor channel. *Proc Natl Acad Sci USA* **81**:3901–3904.
- Jones BL, Whiting PJ, and Henderson LP (2006) Mechanisms of anabolic androgenic steroid inhibition of mammalian ϵ -subunit-containing GABA_A receptors. *J Physiol* **573**:571–593.
- Kenakin T (2004) Efficacy as a vector: the relative prevalence and paucity of inverse agonism. *Mol Pharmacol* **65**:2–11.
- Maksay G, Thompson SA, and Wafford KA (2003) The pharmacology of spontaneously open $\alpha 1\beta 3\epsilon$ GABA_A receptor-ionophores. *Neuropharmacology* **44**:994–1002.
- Mangan PS, Sun C, Carpenter M, Goodkin HP, Sieghart W, and Kapur J (2005) Cultured hippocampal pyramidal neurons express two kinds of GABA_A receptors. *Mol Pharmacol* **67**:775–788.
- Miko A, Werby E, Sun H, Healey J, and Zhang L (2004) TM2 residue in the $\beta 1$ subunit determines spontaneous opening of homomeric and heteromeric γ -aminobutyric acid-gated ion channels. *J Biol Chem* **279**:22833–22840.
- Neelands TR, Fisher JL, Bianchi M, and Macdonald RL (1999) Spontaneous and γ -aminobutyric acid (GABA)-activated GABA_A receptor channels formed by ϵ subunit-containing isoforms. *Mol Pharmacol* **55**:168–178.
- 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.
- Overstreet LS and Westbrook GL (2001) Paradoxical reduction of synaptic inhibition by vigabatrin. *J Neurophysiol* **86**:596–603.
- Pole P, Bonetti EP, Schaffner R, and Haefely W (1982) A three-state model of the benzodiazepine receptor explains the interactions between the benzodiazepine antagonist Ro 15-1788, benzodiazepine tranquilizers, β -carbolines, and phenobarbital. *Naunyn-Schmiedeberg's Arch Pharmacol* **321**:260–264.
- Schulz DW and Macdonald RL (1981) Barbiturate enhancement of GABA-mediated inhibition and activation of chloride ion conductance: correlation with anticonvulsant and anesthetic actions. *Brain Res* **209**:177–188.
- Stell BM, Brickley SG, Tang CY, Farrant M, and Mody I (2003) Neuroactive steroids reduce neuronal excitability by selectively enhancing tonic inhibition mediated by δ subunit-containing GABA_A receptors. *Proc Natl Acad Sci USA* **100**:14439–14444.
- Stell BM and Mody I (2002) Receptors with different affinities mediate phasic and tonic GABA_A conductances in hippocampal neurons. *J Neurosci* **22**:RC223.
- Straughan DW, Neal MJ, Simmonds MA, Collins GG, and Hill RG (1971) Evaluation of bicuculline as a GABA antagonist. *Nature (Lond)* **233**:352–354.
- Taleb O, Trouslard J, Demeneix BA, Feltz P, Bossu JL, Dupont JL, and Feltz A (1987) Spontaneous and GABA-evoked Cl[−] channels on pituitary intermediate lobe cells and their internal Ca²⁺ requirements. *Pfluegers Arch Eur J Physiol* **409**:620–631.
- Thio LL and Zhang HX (2006) Modulation of inhibitory glycine receptors in cultured embryonic mouse hippocampal neurons by zinc, thiol containing redox agents and carnosine. *Neuroscience* **139**:1315–1327.
- Thompson S-A, Smith MZ, Wingrove PB, Whiting PJ, and Wafford KA (1999) Mutation at the putative GABA_A ion-channel gate reveals changes in allosteric modulation. *Br J Pharmacol* **127**:1349–1358.
- Ueno S, Bracamontes J, Zorumski C, Weiss DS, and Steinbach JH (1997) Bicuculline and gabazine are allosteric inhibitors of channel opening of the GABA_A receptor. *J Neurosci* **17**:625–634.
- Wagner DA, Goldschmidt MP, Hales TG, and Jones MV (2005) Kinetics and spontaneous open probability conferred by the ϵ subunit of the GABA_A receptor. *J Neurosci* **25**:10462–10468.
- Whiting PJ, Bonner TP, McKernan RM, Farrar S, Le Bourdelles B, Heavens RP, Smith DW, Hewson L, Rigby MR, Sirinathsinghji DJ, et al. (1999) Molecular and functional diversity of the expanding GABA_A receptor gene family. *Ann NY Acad Sci* **868**:645–653.
- Zhang D, Pan ZH, Awobuluyi M, and Lipton SA (2001) Structure and function of GABA_C receptors: a comparison of native versus recombinant receptors. *Trends Pharmacol Sci* **22**:121–132.

Address correspondence to: Dr. Tim G. Hales, Department of Pharmacology and Physiology, The George Washington University, 2300 Eye Street NW, Washington, DC 20037. E-mail: phmtgh@gwumc.edu