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Tonically active $GABA_A$ receptors in hippocampal pyramidal neurons exhibit constitutive GABA-independent gating

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Running title: GABA-independent tonic currents

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ABBREVIATIONS: GABA, γ-aminobutyric acid; mIPSC, miniature inhibitory postsynaptic current; TTX, tetrodotoxin; CNQX, 6-cyano-2,3-dihydroxy-7-nitroquinoxaline; AP5, 2-amino-5-phosphonovalerate; propofol, 2,6-diisopropylphenol.

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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 mIPSCs and responses to exogenous GABA, had no effect on tonic currents. Since 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 $\alpha1\beta3\gamma2$ receptors recombinantly expressed in HEK293 cells. Propofol activated gabazine-resistant, bicuculline-sensitive currents when applied to either preparation. While 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 $\alpha1\beta1/3\gamma2$ 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, due to either anesthetic or spontaneous gating. Moreover, spontaneously active $GABA_A$ receptors mediate gabazine resistant tonic currents in pyramidal neurons.

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γ-Aminobutyric acid 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 due to 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 since $GABA_A$ receptors are pentameric containing various combinations of α1-6, β1-3, γ1-3, δ, ρ1-3, 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 α6, β2/3 and δ subunits (Nusser et al., 1998). Synaptic receptors in the same cells lack the δ subunit and contain the γ2 subunit. The δ subunit enhances the affinity of extrasynaptic $GABA_A$ receptors 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 CNS. 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 appear 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 (Stell et al., 2003; Overstreet and Westbrook, 2001). Furthermore the only known $GABA_A$ receptors that mediate gabazine-insensitive GABA evoked currents contain ρ subunits (often referred to as $GABA_C$ receptors) which are predominantly expressed in the retina (Zhang et al., 2001).

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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). Additionally, direct $GABA_A$ receptor activation by anesthetics may also contribute to the respiratory depression that compromises the therapeutic indexes of these agents. While it appears 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 GABAmimetics at the GABA binding site (Davies et al., 1997). $GABA_A$ receptors formed by the expression of the β3 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). By 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. By 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 α1β1γ2, α1β3γ2 or α1β3ε $GABA_A$ receptors.

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MATERIALS AND METHODS

Hippocampal cultures. Hippocampal tissue was dissected from 1-3 day old rats and placed in a papain solution (papain (1.4 mg/ml) and bovine serum albumen (30 mg/ml)) in Hanks Balanced Salt Solution) and incubated at 37°C for 20 mins. The papain solution was aspirated and replaced by a solution of ovomucoid (1 mg/ml) and bovine serum albumen (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-alpha (80% v/v), fetal calf serum (10% v/v), horse serum (10% v/v), glucose (5 mg/ml), 50 μg/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 X 10^5 cells/ml of medium. After 24 h the medium was replaced by an incubation medium containing Neurobasal A (98% v/v), B27 supplement (2% v/v), 50 μg/ml streptomycin and 50 U/ml penicillin. On day four the medium was replaced by incubation medium supplemented with cytosine-β-D-arabinofuranoside (5 μM) to inhibit the growth of glia. On day five the medium was replaced by incubation medium lacking cytosine-β-D-arabinofuranoside. Tissue culture reagents were supplied by Invitrogen Corp. (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 Eagles medium supplemented with 10% bovine serum, 50 μg/ml streptomycin and 50 U/ml penicillin in an atmosphere of 5% CO_2. Cells were transfected by calcium phosphate precipitation with equimolar ratios of rodent α1, β3, and γ2L $GABA_A$ subunit cDNAs in the pCDM8 vector (Davies et al., 1997). In several experiments the rat β1 or ε $GABA_A$ subunit

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cDNAs, also in the pCDM8 vector, were used instead of either the β3 or γ2 cDNAs, respectively. Cells were used 24-96 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 (in mM): NaCl 140, KCl 2.8, $MgCl_2$ 2.0, $CaCl_2$ 1.0, glucose 6.0 and HEPES-NaOH (pH 7.2). Recording electrodes contained (in mM): CsCl 140, $MgCl_2$ 2.0, $CaCl_2$ 0.1, ATP (Mg^{2+} salt) 3, EGTA 1.1 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 μM) and 2-amino-5-phosphonovalerate (AP5; 10 μM) to inhibit ionotropic glutamate receptors and tetrodotoxin (TTX; 1 μM) to block action potentials (reagents supplied by Sigma, MO, USA). Membrane currents were monitored with an Axopatch-200A amplifier (Molecular Devices Corp., Sunnyvale, CA). Currents were low-pass filtered at 2 kHz (Bessel characteristics). In experiments examining activation of $GABA_A$ receptors, GABA or propofol were applied either to the perfusate or locally by pressure ejection (Picospritzer II, General Valve Corp., Fairfield, NJ) from micropipettes positioned approximately 50 μm from the cell under investigation. Whole cell currents were digitized at a frequency of 10 kHz onto the hard drive of a Pentium PC and were analyzed with pCLAMP 8.0 software (Molecular Devices Corp.).

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 PC as described previously (Hales et al., 2006).

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GABA (1 μM) and/or gabazine (20 μM) were applied directly to patches by local pressure ejection as was picrotoxin (100 μM). Gabazine-resistant spontaneous channels were recorded prior to GABA application with gabazine (20 μM) in the recording chamber. For consistency with whole-cell recordings from hippocampal neurons, the extracellular solution was supplemented with CNQX (10 μM), AP5 (10 μM) and TTX (1 μM).

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^{o}C and diluted to their working concentrations in saline on the day of experimentation. Picrotoxin in DMSO, 2,6-diisopropylphenol (propofol) in ethanol and furosemide in ethanol were kept as stock solutions at -20^{o}C and diluted to their working concentrations in saline on the day of experimentation. All reagents were supplied by Sigma, MO, USA.

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 Corp.). 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

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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.

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RESULTS

Pharmacology of tonic $GABA_A$ receptor mediated currents recorded from cultured pyramidal neurons in the absence of exogenous GABA. Phasic miniature IPSCs (mIPSCs) and sustained tonic currents were recorded from cultured hippocampal neurons in the presence of TTX (0.5 μM) to block action potentials and CNQX (10 μM) and AP5 (10 μM) 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 μM) inhibited mIPSCs as well as the tonic currents (Fig. 1A, 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 MΩ ($n = 6$). 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 μM) also inhibited mIPSCs but was less effective than picrotoxin at reducing tonic current (Fig. 1B, F). The competitive antagonist gabazine (20 μM) had no effect on tonic currents and did not significantly alter input resistance ($n = 5$), but abolished mIPSCs (Fig. 1C, F). Zn^{2+} (100 μM), an inhibitor of $GABA_A$ receptor activity inhibited tonic currents and had relatively little effect on mIPSCs (Fig. 1D, F). In order 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. 1D, F). Nevertheless we investigated whether glycine

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receptors contribute to tonic currents of pyramidal neurons by applying strychnine (100 nM). Glycine (100 μM) activated 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). By contrast, strychnine had no effect on tonic currents recorded from pyramidal neurons (Fig. 1F).

We also examined the effect of furosemide (600 μM) 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. 2A, B). GABA (5 μM) activated large desensitizing inward currents (mean peak current amplitude = 1.2 ± 0.4 nA) that were inhibited by 94 ± 3 % (n = 6) and 97 ± 3 % (n = 4)

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by the application of either bicuculline (20 μM) or gabazine (20 μM), respectively (Fig. 2A, 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 μM).

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 μM) activated robust currents in recordings from outside-out patches (Fig. 3A). Such GABA-activated currents were prevented by application of gabazine (20 μM). However brief duration spontaneous single channel events were evident even during the application of gabazine (Fig. 3A, B). The application of picrotoxin (100 μM) abolished spontaneous channels (Fig. 3B), which had the same amplitude as channels activated by GABA (Fig. 3C and D). The chord conductance of the spontaneous events was 24.1 ± 0.5 pS (n = 9), similar to that of GABA (1 μM) activated channels (25.7 ± 0.2 pS, n = 3) subsequently recorded from several of the same patches (Fig. 3D, 3E). In these experiments it is unlikely that spontaneous channels were activated by GABA released from neurons since 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 hypothesis 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

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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 μM) caused a relatively modest inhibition (25 ± 4%, n =12) of the propofol-evoked current amplitude (Fig. 4A, B). By contrast, bicuculline (20 μM) inhibited propofol activated currents by 85 ± 5 % (n = 13). Application of gabazine (20 μM) to pyramidal neurons significantly attenuated ($p < 0.001$) the inhibition of the propofol-activated current by bicuculline (20 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 $\alpha1\beta3\gamma2$ receptors. This subunit combination was chosen in part because it is unlikely to mimic that of extrasynaptic receptors in hippocampal neurons, which likely contain the $\alpha5$ subunit (Caraiscos et al., 2004) and possibly also the $\beta1$ subunit (Mangan et al., 2005). GABA (100 μM)-evoked currents recorded from HEK293 cells expressing recombinant $\alpha1\beta3\gamma2$ receptors were abolished by bicuculline (IC_{50} = 1.9 ± 0.3 μM) and gabazine (IC_{50} = 0.4 ± 0.1 μM) (Fig. 5A). Bicuculline (IC_{50} = 3.0 ± 1.5 μM) also abolished propofol (10 μM)-activated currents mediated by $\alpha1\beta3\gamma2$ receptors (Fig. 5B). By contrast,

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gabazine (1 – 100 μM) caused a maximum inhibition of 13% (Fig. 5B). Consistent with our observations in hippocampal pyramidal neurons, gabazine (10 μM) abolished the inhibition of propofol-activated currents by bicuculline (10 μM; Fig. 5C, D). Taken together these data suggest that the failure of gabazine to block propofol-activated currents in pyramidal neurons is not due to a receptor subtype discriminative effect, but instead 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 also directly activates $GABA_A$ receptors in the absence of GABA. Loreclezole, unlike propofol, selectively affects receptors that contain either the β2 or β3 subunit, having a negligible effect on receptors containing the β1 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 applied to cultured hippocampal pyramidal neurons loreclezole (10 μM) 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 β2 and/or β3 subunits. Flunitrazepam (1 μM) 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 either flunitrazepam is enhancing tonic currents activated by low levels of ambient GABA or enhancing the gating of spontaneous currents. The $GABA_A$

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antagonist gabazine (20 μM) 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 μM) had no significant effect ($p = 0.31$) on the amplitude of flunitrazepam (1 μM)-evoked currents (-20 ± 9 pA, $n = 6$).

These data indicate that at least some of the $GABA_A$ receptors in pyramidal neurons contain β2 and/or β3 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 α1β3ε $GABA_A$ 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). Interestingly, bicuculline inhibits spontaneous currents mediated by ε subunit-containing receptors and gabazine is less efficacious in this respect (Maksay et al., 2003). Like picrotoxin, furosemide abolishes spontaneous currents mediated by human α1β3ε 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 α1β3ε 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 α1β3ε 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

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pyramidal neurons (Fig. 1F). Furthermore, furosemide (600 μM) also inhibited spontaneous current mediated by rat α1β3ε receptors (Fig. 7B). The density of current inhibited by furosemide was 1.11 ± 0.22 pA/pF (n = 9) (Fig. 7B). These data demonstrate that spontaneous currents mediated by rat α1β3ε 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 α1β1γ2 and α1β3γ2 $GABA_A$ receptors. Recombinant $GABA_A$ receptors containing the β1 subunit expressed in *Xenopus oocytes* exhibit spontaneous current (Miko et al., 2004). Furthermore, hippocampal neurons cultured from embryonic rats express the β1 subunit (Mangan et al., 2005). Therefore we examined whether α1β1γ2 $GABA_A$ receptors exhibited spontaneous gating when expressed in HEK293 cells. Since loreclezole-elicited currents in pyramidal neurons (Fig. 5A), indicating the presence of receptors containing the β2 and/or the β3 subunit, we compared spontaneous currents mediated by α1β1γ2 receptors to those mediated by α1β3γ2 receptors. Picrotoxin (100 μM) inhibited small tonic currents when bath applied to HEK293 cells expressing either α1β1γ2 or α1β3γ2 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 ± 0.11% and 0.13 ± 0.05% for α1β1γ2 and α1β3γ2 receptors, respectively. Furthermore, similar to our observations in pyramidal neurons (Fig. 1B, C, F), bicuculline (20 μM) inhibited spontaneous currents mediated by α1β1γ2 and α1β3γ2 receptors, while gabazine (20 μM) had no effect (Fig. 7D, G). However, gabazine attenuated the inhibition of tonic current

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by bicuculline when co-applied to cells expressing $\alpha1\beta1\gamma2$ or $\alpha1\beta3\gamma2$ 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 $\alpha1\beta1\gamma2$ or $\alpha1\beta3\gamma2$ receptors (Fig. 7E) having a significantly greater effect in the latter compared to 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 $\alpha1\beta3\gamma2$ receptors (-198 ± 93 pA, $n = 6$), compared to those expressing $\alpha1\beta1\gamma2$ 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 $\alpha1\beta1\gamma2$ or $\alpha1\beta3\gamma2$ 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 $\alpha1\beta1\gamma2$ and $\alpha1\beta3\gamma2$ receptors (Fig. 7G) exhibit a striking similarity to those of tonic currents recorded from pyramidal neurons (Fig. 1F).

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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. By contrast, gabazine inhibited mIPSCs and currents activated by exogenous GABA but had little effect on either tonic or propofol-activated currents. Interestingly, the pharmacological properties of tonic currents of pyramidal neurons were similar to tonic currents of HEK293 cells expressing $\alpha1\beta1\gamma2$ or $\alpha1\beta3\gamma2$ receptors, but differed from those of $\alpha1\beta3\varepsilon$ receptors.

Our data support the idea that gabazine-resistant $GABA_A$ receptor activity mediates inhibitory tonic currents (Bai et al., 2001). However, since 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 comprised 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.

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Since 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 outside-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 and persisted for prolonged time periods after patch excision, were resistant to gabazine and 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. By contrast, gabazine has negligible negative efficacy and is therefore a neutral competitive antagonist.

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In order 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 a previous study (Bieda and MacIver, 2004) gabazine was relatively ineffective as an inhibitor of propofol-activated currents. By 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 $\alpha1\beta3\gamma2$ receptors were also resistant to inhibition by gabazine and abolished by bicuculline. Furthermore, gabazine prevented the inhibition of propofol-activated $\alpha1\beta3\gamma2$ receptors by bicuculline. Again, these data support the hypothesis that gabazine has negligible negative efficacy while 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 alphaxolone and pentobarbital (Ueno et al., 1997).

Neurosteroids also directly activate $GABA_A$ receptors and, by analogy to alphaxalone, 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

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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) lead 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 Zn^{2+}-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 αxβxε and α1β3γ2ε $GABA_A$ receptors (Neelands et al., 1999; Davies et al., 2001; Maksay et al., 2003; Wagner et al., 2005). Furthermore, the β1 subunit also confers some spontaneous gating to both α2β1 and α2β1γ2 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 appear to be responsible for spontaneous gating in hippocampal pyramidal neurons. Furosemide inhibited spontaneous currents mediated by recombinant rodent α1β3ε receptors, while tonic currents in pyramidal neurons were resistant to the compound. Interestingly, 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 β1 subunit identified previously in hippocampal neurons using immunocytochemistry (Mangan et

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al., 2005). Since loreclezole activated currents when applied to pyramidal neurons, inferring the presence of β2 and/or β3 subunits, we compared the spontaneous gating of α1β1γ2 receptors to that of α1β3γ2 receptors expressed in HEK293 cells. Consistent with a previous report (Miko et al., 2004) spontaneous currents mediated by receptors containing the β1 subunit were larger than the those mediated by receptors containing the β3 subunit, when normalized to the peak GABA-evoked current. Nevertheless both α1β1γ2 and α1β3γ2 receptors mediated detectable tonic currents with similar pharmacological properties to those of pyramidal neurons. Importantly neither gabazine nor furosemide affected constitutive gating of α1β1γ2 or α1β3γ2 receptors. By 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) potentiation 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 since diazepam also enhances bicuculline-sensitive spontaneous current mediated by α1β3γ2 receptors (Bianchi and Macdonald, 2001).

Spontaneous gating of recombinant $GABA_A$ receptors can be enhanced by introducing mutations in the TM2 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. While high affinity extrasynaptic $GABA_A$ receptors activated by ambient GABA likely mediate tonic currents in some brain regions (Farrant and Nusser, 2005), our study demonstrates the need for consideration of additional mechanisms. Using bicuculline is an inverse agonist and gabazine as a neutral antagonist the prevalence of GABA-independent gating can now be quantified in different brain regions.

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FOOTNOTES:

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Legends for Figures

Figure 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 -60mV with TTX (0.5 μM), CNQX (10 μM) and AP5 (10 μM) in the extracellular solution. A, Picrotoxin (PIC; 100 μM) and B, bicuculline (BIC; 20 μM) inhibited mIPSCs and tonic currents. C, Gabazine (GBZ; 20 μM) inhibited mIPSCs but not tonic current. D, Zn^{2+} (10 μM) inhibited tonic current but had little effect on mIPSCs. Current amplitude histograms in A-D were compiled from the respective current trace as described in the Materials and Methods. Open and grey 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 μM) 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 (not shown). F, The bar graph illustrates the amplitude of currents inhibited by various antagonists including furosemide (600 μM; FUROS) expressed relative to cell capacitance (i.e. current density) and is represented as mean ± SEM 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).

Figure 2. Bicuculline and gabazine abolish GABA-evoked currents recorded from pyramidal neurons. A, The addition of GABA (5 μM) to the perfusate activated a large desensitizing current that was abolished by gabazine (GBZ; 20 μM) and bicuculline (BIC; 20 μM), applied

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either alone or in combination. B, The bar graph summarizes 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 wash out as a percentage of that measured immediately before antagonist application. Error bars, where visible, represent ± SEM.

Figure 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 (upper trace). The lower 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 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 (closed circles) and GABA-activated channels (open circles) 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.

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Figure 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).

Figure 5. Currents activated by GABA and propofol mediated by α1β3γ2 receptors have differing sensitivities to gabazine. A, GABA (100 μM)-activated current recorded from HEK293 cells expressing recombinant α1β3γ2 receptors were inhibited by gabazine (circles) and bicuculline (triangles) 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 μM and 0.4 ± 0.1 μM, respectively. Hill slopes for inhibition by bicuculline and gabazine are 0.8 ± 0.1 and 1.0 ± 0.1, respectively. B, Propofol (3 μM) activated currents mediated by recombinant α1β3γ2 $GABA_A$ receptors expressed in HEK293 cells were inhibited by bicuculline (triangles) but not by gabazine (circles). The IC_{50} value for bicuculline was 3.2 ± 0.5 μM. Hill coefficient for inhibition by bicuculline was 0.9 ± 0.1. C, Propofol (10 μM)-activated currents recorded from the same HEK293 cell expressing α1β3γ2 receptors were inhibited by bicuculline (10 μM). The inhibition by bicuculline was prevented by gabazine (10 μM). D, The bar graph illustrates the percentage inhibition of propofol-activated currents (I_{prop}) by bicuculline and

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gabazine applied either alone or in combination. In all cases data points represent the mean of at least four cells. Error bars are ± SEM.

Figure 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 following 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).

Figure 7. Inhibitors of $GABA_A$ receptor activity exhibit differing tendencies to attenuate tonic current in HEK293 cells expressing α1β3ε, α1β1γ2 and α1β3γ2 receptors. Tonic currents were recorded in the absence of GABA from HEK293 cells expressing recombinant rodent receptors. A, Picrotoxin (PIC, 100 μM) inhibited tonic current. B, Furosemide (FURO, 600 μM) applied to the same cell also inhibited tonic current. C, By contrast to α1β3ε receptors, which exhibited robust picrotoxin-sensitive tonic currents, α1β1γ2 and α1β3γ2 receptors mediated small picrotoxin-sensitive tonic currents recorded in the absence of GABA. D, Tonic currents mediated by α1β1γ2 and α1β3γ2 receptors were inhibited by bicuculline (BIC, 20 μM). The inhibitory effect of bicuculline was inhibited by co-application of the antagonist with gabazine (GBZ, 20 μM). E, Flunitrazepam (FLU, 1 μM) enhanced tonic currents recorded from α1β1γ2 and α1β3γ2 receptors, this effect was more pronounced in the latter compared to the former (see text). F,

Loreclezole (10 μM)-evoked a large current when applied to HEK293 cells expressing α1β3γ2 receptors without having an effect on cells expressing α1β2γ2 receptors. G, The bar graph illustrates the average density of currents (pA/pF) inhibited when $GABA_A$ receptor antagonists were applied to cells expressing either α1β1γ2 or α1β3γ2 receptors. Current amplitudes were measured using the amplitude histogram approach shown in Figure 1 (described in Materials and Methods). Each mean current density was determined from at least five cells, vertical lines represent ± SEM. Statistical significance compared to 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 $^{***}p < 0.001$.

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Fig. 1

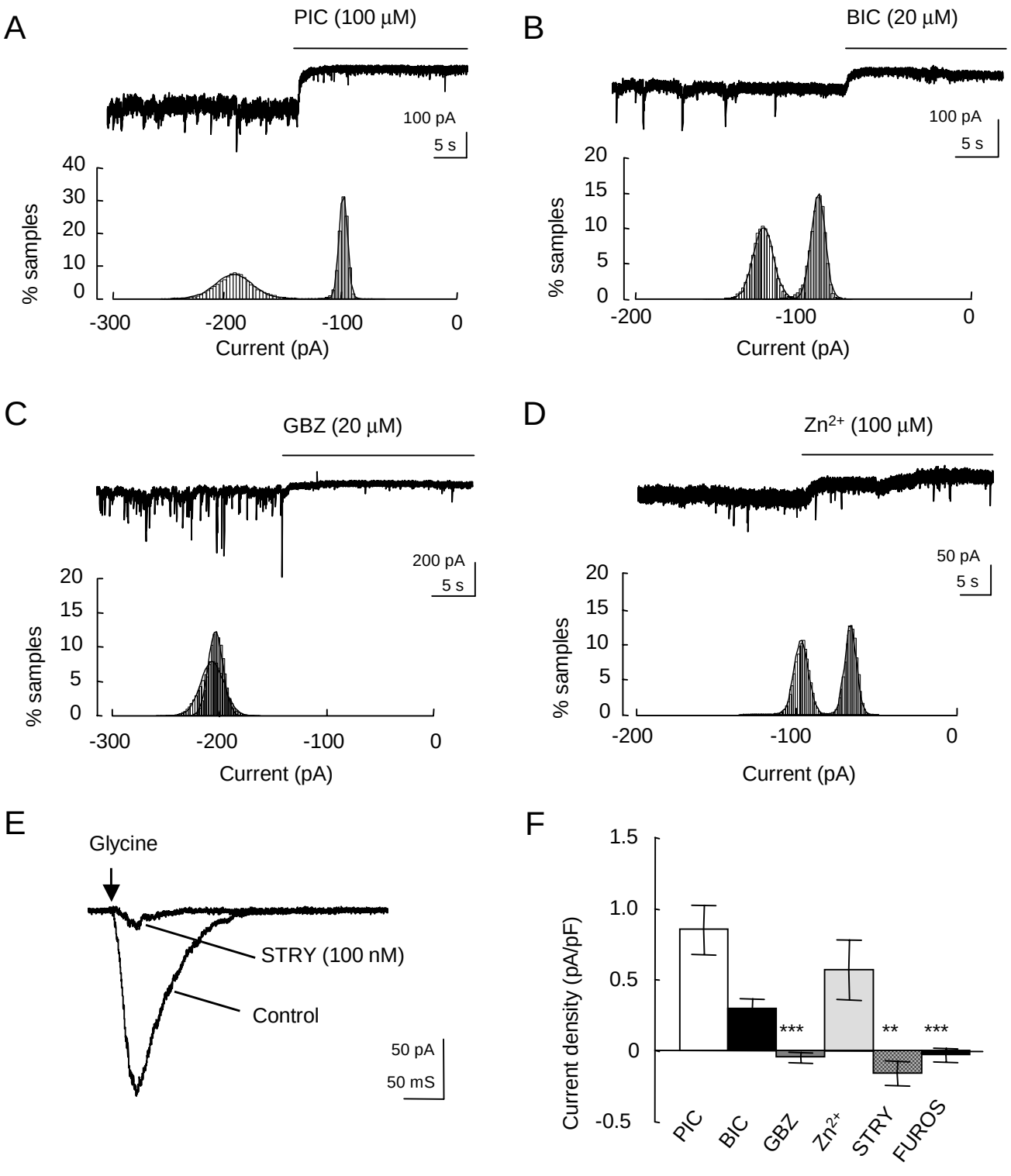

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Fig. 2

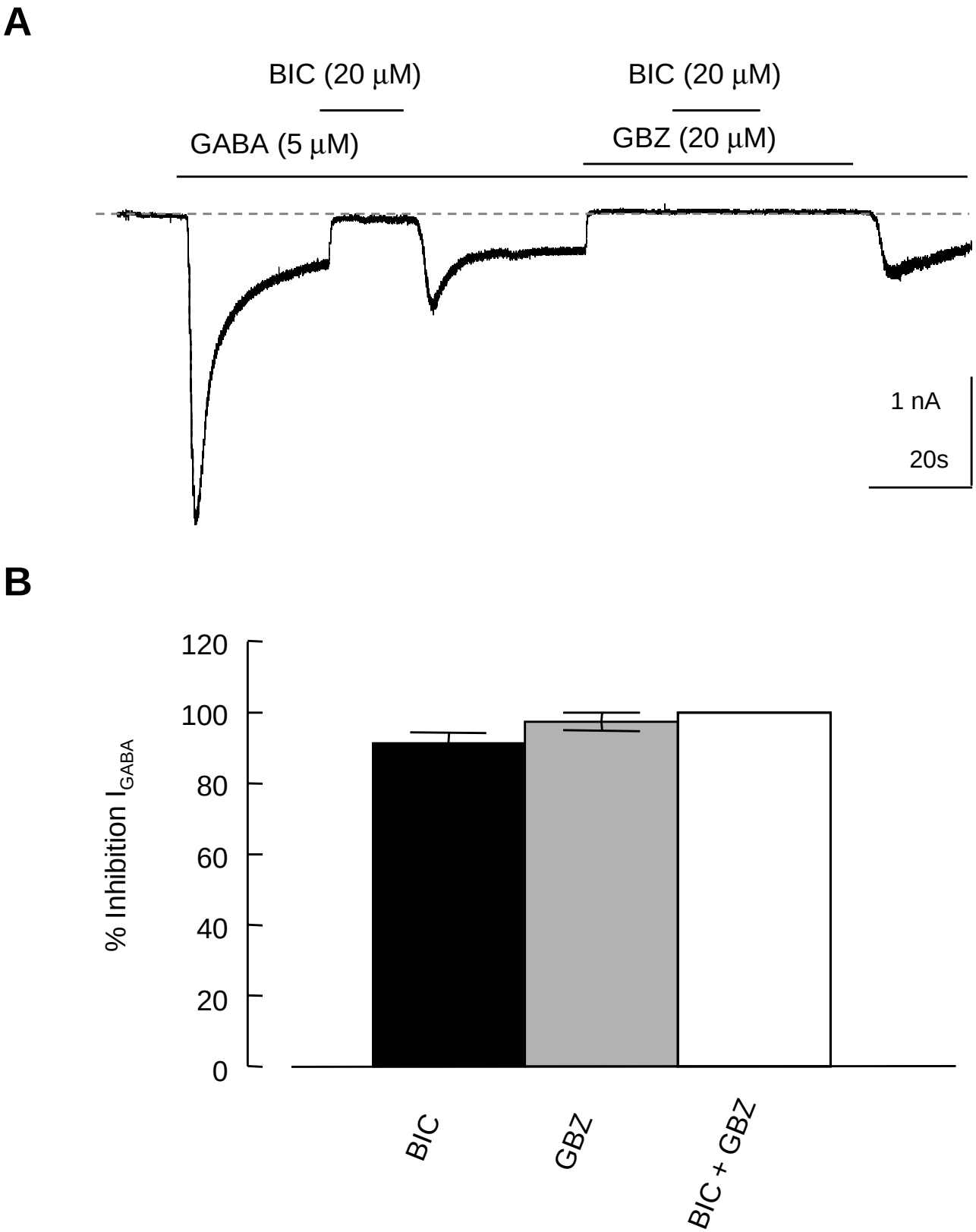

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Fig. 3

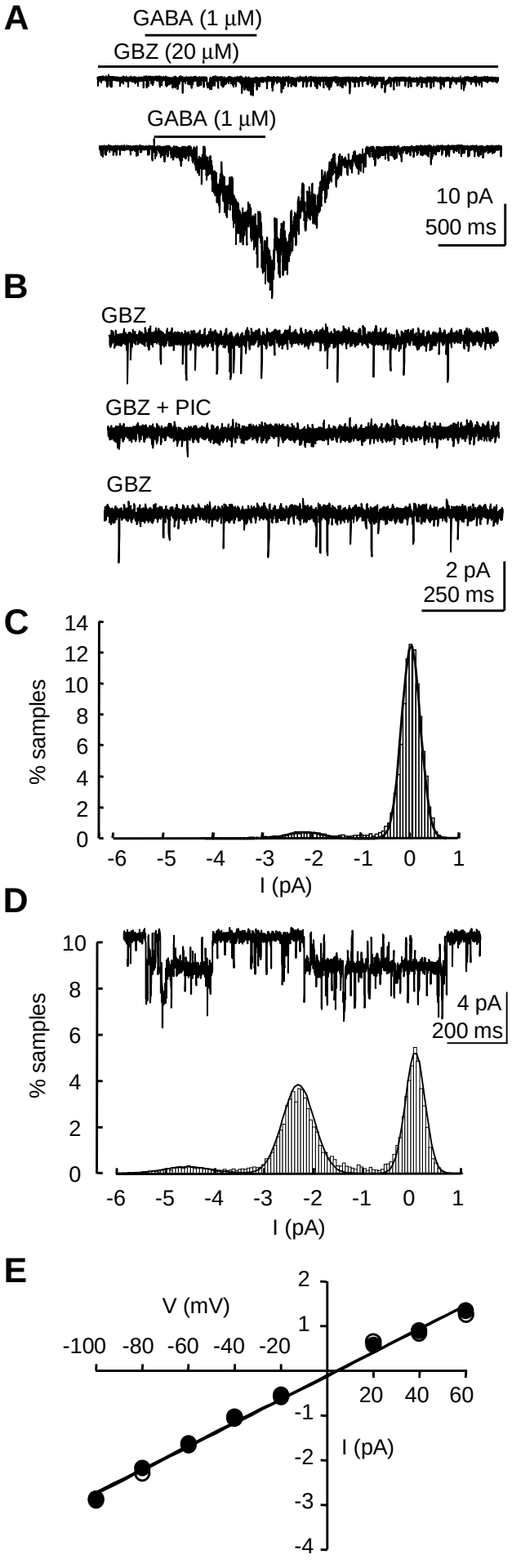

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Fig. 4

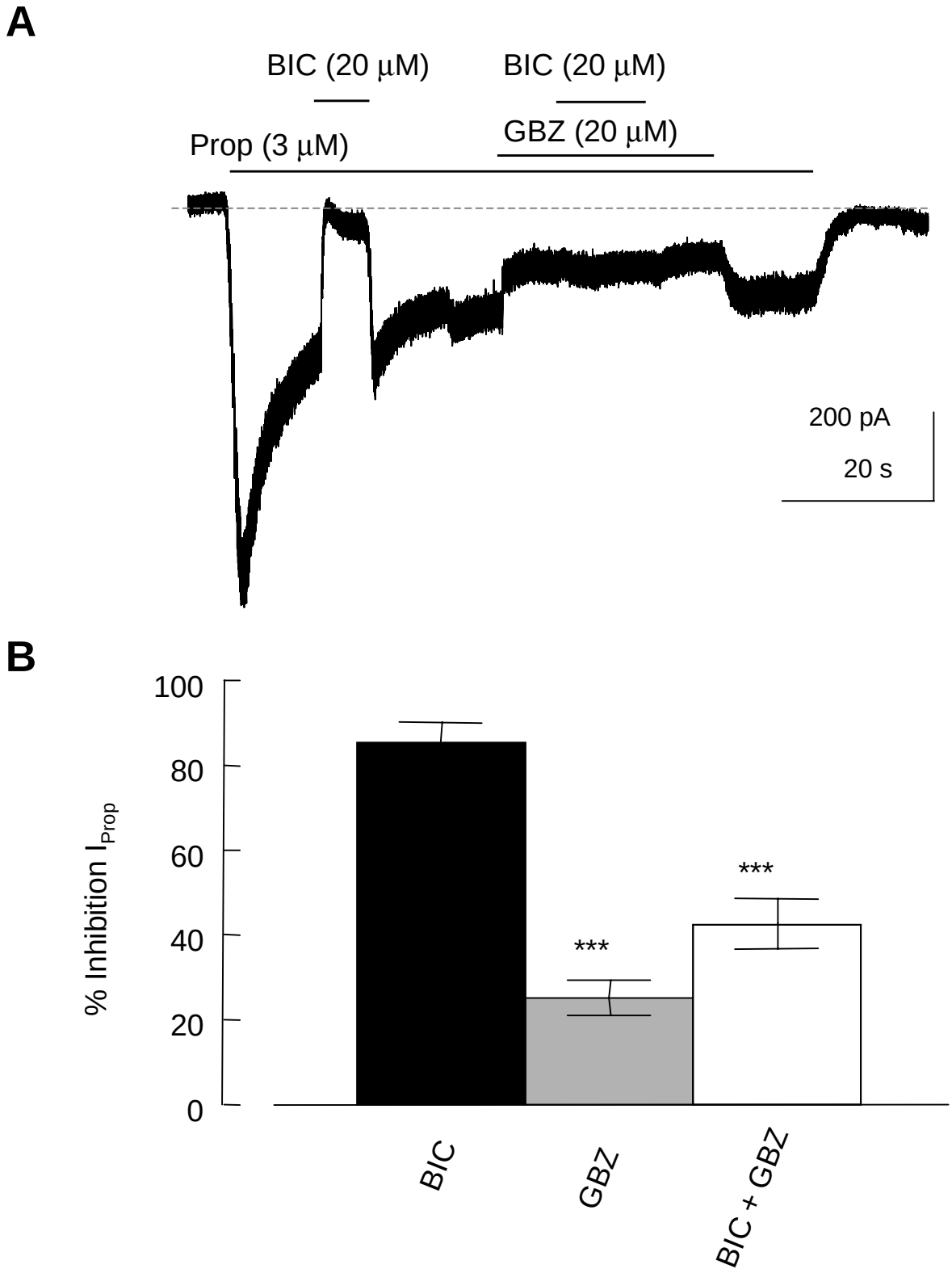

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Fig. 5

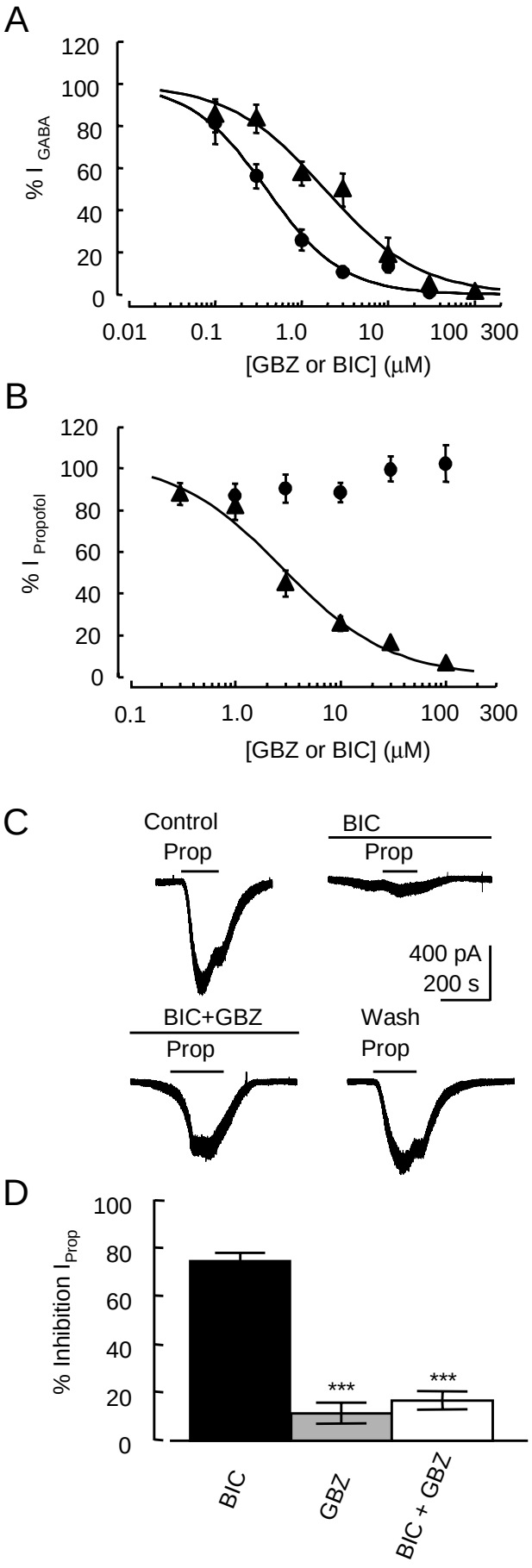

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Fig. 6

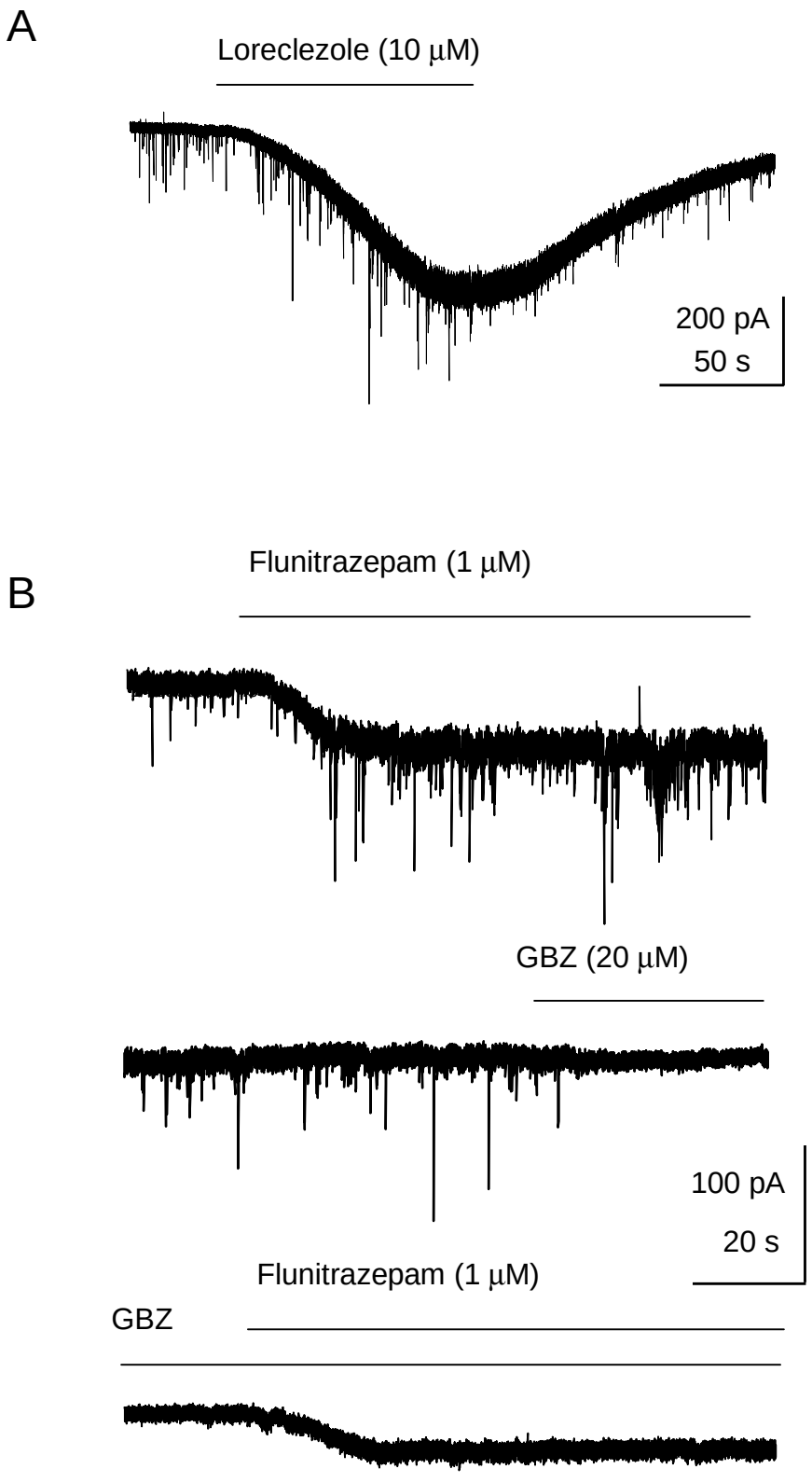

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Fig. 7