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Synaptic-type α1β2γ2L $GABA_A$ receptors produce large persistent currents in the presence of ambient GABA and anesthetic drugs

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Running title: persistent currents from synaptic-type $GABA_A$ receptors

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ABSTRACT

Synaptic $GABA_A$ receptors respond to synaptically-released GABA and are considered to be unaffected by the low levels of ambient transmitter in the brain. We show that synaptic-type α1β2γ2L $GABA_A$ receptors expressed in HEK 293 cells respond with large steady-state currents to combinations of a low concentration (0.5 μM) of GABA and clinically used GABAergic modulators propofol, etomidate, pentobarbital, or the steroid alphaxalone. At a maximally effective concentration of modulator, the current levels at the end of 2 min applications of drug combinations were over 10% of the peak response to saturating GABA. In the absence of modulators, 0.5 μM GABA generated a steady-state response of 1% of the peak response to saturating GABA. The concentration-response curves for enhancement of steady-state currents by propofol, etomidate, pentobarbital, or alphaxalone were at lower drug concentrations compared to concentration-response relationships for enhancement of peak responses. We propose that modulation of tonically-activated synaptic-type $GABA_A$ receptors contributes to the clinical actions of sedative drugs.

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INTRODUCTION

$GABA_A$ receptors in the brain are exposed to phasic high (near mM) concentrations of GABA during synaptic transmission and low ambient (<1 μM) concentrations of GABA that is continuously present in the cerebrospinal fluid (reviewed in Farrant and Nusser, 2005). Ambient GABA activates extrasynaptic $GABA_A$ receptors whose major identifying characteristics are high affinity to the transmitter and weak desensitization in the persistent presence of an agonist. The target of phasic GABA are synaptic $GABA_A$ receptors that are characterized by relatively low affinity to GABA and high maximal peak open probability. The conventional view is that synaptic-type receptors are minimally affected by the presence of low, ambient GABA. Indeed the concentration of extracellular GABA (Houston et al., 2012) is several tens of folds lower than the apparent affinity to GABA of synaptic subtypes of $GABA_A$ receptors (Li et al., 2006; Picton and Fisher, 2007).

Under physiological conditions, $GABA_A$ receptors, including synaptic receptors, are exposed to endogenous modulators such as neuroactive steroids (Carver and Reddy, 2013). Under clinical conditions, $GABA_A$ receptors can be exposed to GABAergic sedative drugs such as propofol, etomidate, or barbiturates. While these drugs are ineffective at modulating peak responses of synaptic events due to saturation of GABA binding and a high maximal open probability of GABA-activated receptors, there remains a possibility that GABAergic modulators potentiate the low-level activity from synaptic-type receptors exposed to ambient GABA.

To test this hypothesis, we exposed heterologously-expressed rat α1β2γ2L receptors to a low concentration (0.5 μM) of GABA, intended to mimic steady-state GABA, in the absence and presence of several common modulators of the $GABA_A$ receptor. The data show that a combination of a low concentration of GABA with clinical concentrations of propofol, etomidate, pentobarbital, or the steroid alphaxalone enhances the level of quasi steady-state current by approximately 10-fold. Concentration-response measurements indicate that the EC_{50}s for

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potentiation of steady-state currents are at similar or lower concentrations than those measured for potentiation of peak responses. Our data raise the possibility of a role for tonically-activated synaptic-type $GABA_A$ receptors in the clinical actions of some anesthetic drugs.

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MATERIALS AND METHODS

Experiments were carried out on rat wild-type α1β2γ2L $GABA_A$ receptors. Receptors were transiently expressed in HEK 293 cells, using a modified calcium phosphate precipitation-based transient transfection technique (Akk, 2002). The amino terminus of the α1 subunit was tagged with the FLAG epitope (Ueno et al., 1996), and bead-binding with immunobeads against the FLAG epitope was used to identify cells expressing high levels of $GABA_A$ receptors.

Electrophysiological recordings were done on lifted cells using the standard whole-cell voltage clamp approach. Cells were clamped at -60 mV. Recordings were conducted at room temperature. The bath solution contained (in mM): 140 NaCl, 5 KCl, 1 $MgCl_2$, 2 $CaCl_2$, 10 D-glucose and 10 HEPES; pH 7.4. The pipette solution contained (in mM): 140 CsCl, 4 NaCl, 4 $MgCl_2$, 0.5 $CaCl_2$, 5 EGTA, 10 HEPES, pH 7.4. Agonist (GABA) and modulators (propofol, etomidate, pentobarbital, or alphaxalone) were applied onto cells through bath using an SF-77B fast perfusion stepper system (Warner Instruments, Hamden, CT). We have previously found that the solution exchange time with this system is 10-20 ms with a typical size HEK cell (Li and Akk, 2008). Durations of drug applications were 2 min in most recordings. Consecutive drug applications were separated by 2-4 min washouts in bath solution. The currents were recorded using an Axopatch 200B amplifier (Molecular Devices, Sunnyvale, CA), low-pass filtered at 2 kHz and digitized with a Digidata 1320 series interface (Molecular Devices) at 10 kHz. The analysis of current traces was conducted using pClamp 9.0 software (Molecular Devices). Current traces were analyzed with respect to the amplitudes of peak and residual currents at the end of a 2 min drug application. No visually noticable current decay was observed at the end of 2 min applications when 0.5 μM GABA was coapplied with modulators. Each cell was also exposed to 50 μM GABA to obtain a reference point for maximal peak response. This concentration, at approximately 5 times the macroscopic EC_{50} value (Li et al., 2006), produces a near-maximal response. Amplitudes of responses to low GABA and combinations of low GABA and modulators

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are expressed as % of the peak response to 50 μM GABA from the same cell. The data are reported as mean ± S.E.M.

Concentration-response curves were fitted, individually for each cell, with the following equation: $Y=Y_{min}+(Y_{max}-Y_{min})*([drug]/([drug]+EC_{50}))^{n}$, where EC_{50} is the concentration of drug producing a half-maximal effect, n describes the slope of relationship, and Y_{min} and Y_{max} are the low and high concentration asymptotes, respectively. Fitting was conducted using the NFIT software (The University of Texas, Medical Branch at Galveston, Galveston, TX). Parameters of the fit are reported as mean ± S.E.M.

The potentiating effect of alphaxalone on steady-state current elicited by GABA is given as response ratio, calculated as response in the presence of steroid / response in the absence of steroid. Statistical analysis was performed using Excel (Microsoft, Redmond, WA). Statistical significance was determined by comparing the response ratio to 1, i.e., no effect, using a 2-tailed paired t-test. This test is equivalent to a one-sample t-test with a hypothetical value of 1, and is designed to verify whether the effect of the drug is statistically significant.

GABA, inorganic salts used in buffers, pentobarbital, and alphaxalone were bought from Sigma-Aldrich (St. Louis, MO). Propofol was from MP Biomedicals (Solon, OH). Etomidate was purchased from R&D Systems (Minneapolis, MN).

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RESULTS

Rat α1β2γ2L $GABA_A$ receptors expressed in HEK 293 cells have a whole-cell GABA EC_{50} of approximately 10 μM (Li et al., 2006). Activation of α1β2γ2L receptors by 50 μM GABA resulted in fast-rising large inward currents that desensitized to 14 ± 1 % (mean ± S.E.M.; 14 cells) of the peak response by the end of a 2 min drug application. Exposure of same set of cells to 0.5 μM GABA elicited a peak response that was 3 ± 1 % and a steady-state current that was 1 ± 1 % of the peak response to 50 μM GABA. Sample currents are shown in Figure 1A-B.

The anesthetic compound propofol potentiates activity from α1β2γ2L receptors elicited by submaximal concentrations of GABA (Ruesch et al., 2012). We found that coapplication of 1 μM propofol with 0.5 μM GABA potentiated the peak response by 7 ± 2 fold (14 cells). Propofol alone at this concentration elicited a negligible response (0.3 ± 0.1 % of the peak response to 50 μM GABA). The current at the end of a 2 min drug application was enhanced by 14 ± 5 fold when 1 μM propofol was coapplied with 0.5 μM GABA. Relative to the peak response to 50 μM GABA, the steady-state current at the end of a 2 min application of 0.5 μM GABA + 1 μM propofol was 9 ± 2 % (14 cells).

To investigate the concentration-response relationship of propofol-elicited enhancement of steady-state currents, we tested, in a separate set of 5 cells, the effect of 0.1-3 μM propofol on steady-state currents elicited by low GABA. Compared to the peak response to 50 μM GABA in the same cell, the mean current level at the end of a 2 min drug application was 2 ± 1%, 5 ± 1%, 8 ± 2%, or 8 ± 1%, in the presence of 0.5 μM GABA and 0.1, 0.3, 1, and 3 μM propofol, respectively. Curve-fitting the data from each cell individually yielded an EC_{50} of 0.44 ± 0.06 μM and a Hill coefficient of 2.4 ± 0.6 (mean ± S.E.M.; Figure 2B). The fit maximum was 9 ± 1% (5 cells).

To determine whether other common $GABA_A$ receptor potentiators modulate steady-state currents from α1β2γ2L receptors, we examined the effects of etomidate, and pentobarbital on

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currents elicited by 0.5 μM GABA. Coapplication of 0.1-10 μM etomidate with 0.5 μM GABA enhanced steady-state current. The relative (compared to peak in the presence of 50 μM GABA) steady-state current levels were 1.3 ± 0.4%, 3 ± 1%, 6 ± 1%, 11 ± 1%, or 11 ± 1% when receptors were activated by 0.5 μM GABA in the presence of 0.1, 0.3, 1, 3, or 10 μM etomidate respectively (a set of 7 cells). Curve-fitting yielded an EC_{50} of 0.9 ± 0.4 μM, a Hill coefficent of 2.2 ± 0.4, and a fitted maximal response of 11 ± 1%. Sample traces and the concentration-response plot are shown in Figure 3A.

Steady-state currents elicited by 0.5 μM GABA were also enhanced in the presence of pentobarbital. Coapplication of 3, 10, 30, or 100 μM pentobarbital with GABA resulted in relative current levels of 2 ± 1%, 6 ± 1%, 13 ± 1%, or 12 ± 2% (5 cells at each condition), respectively. Curve-fitting yielded an EC_{50} of 12 ± 2 μM, a Hill coefficient of 2.6 ± 0.4, and a maximal current level of 13 ± 2% (Figure 3B).

We previously showed that the application of a neuroactive steroid enhances peak responses from α1β2γ2L $GABA_A$ receptors activated by allosteric activators or combinations of low GABA and an allosteric activator (Li et al., 2014). To test whether a steroid can enhance steady-state currents from receptors activated by GABA and an allosteric drug, we coapplied 10-1000 nM alphaxalone with 0.5 μM GABA + 1 μM propofol. Due to numerous controls and comparisons in this experimental protocol, the effects of increasing concentrations of steroid were tested on separate cells. The steady-state current level at the end of a 2 min application was 8 ± 1% (10 nM alphaxalone; 3 cells), 15 ± 2% (30 nM; 3 cells), 11 ± 2% (100 nM; 3 cells), 10 ± 2% (300 nM; 4 cells), and 16 ± 6% (1000 nM; 3 cells) of the peak response to 50 μM GABA. These values were not significantly different (two-sample t-test, Excel, Microsoft) from the mean steady-state response observed for 0.5 μM GABA + 1 μM propofol in the absence of alphaxalone (9 ± 2% of the peak response to 50 μM GABA; above). Sample traces are shown in Figure 3C.

To verify that alphaxalone is capable of potentiating steady-state responses to GABA alone, we evaluated data obtained as part of the experimental protocol in the preceding experiment

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where each cell had additionally been challenged with 0.5 μM GABA + alphaxalone. In six cells exposed to 0.5 μM GABA + 1000 nM alphaxalone, the steady-state response at the end of a 2 min drug application was 9 ± 2% of the peak response to 50 μM GABA. This represented an 11 ± 2 fold increase in the current level compared to steady-state response to 0.5 μM GABA alone in the same set of cells. Note that the number of cells here is greater than in the preceding experiment because not all cells yielded data for all drug conditions. Analogous data evaluation for other steroid concentrations showed that steady-state currents from receptors activated by 0.5 μM GABA were potentiated by 2.4 ± 0.3 (11 cells) and 4.1 ± 0.4 (4 cells) fold in the presence of 100 and 300 nM alphaxalone, respectively. Coapplication of 10 or 30 nM alphaxalone with 0.5 μM GABA had a statistically insignificant effect on steady-state response. We infer that alphaxalone concentration-dependently enhances steady-state response to low GABA. Sample current traces and the concentration-response relationship are given in Figure 3C.

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DISCUSSION

The potentiating effects of $GABA_A$ receptor modulators are typically studied with regard to effect on the peak response. Here, we demonstrate that some common, clinically used $GABA_A$ receptor potentiators (propofol, etomidate, pentobarbital and the steroid alphaxalone) potentiate quasi steady-state currents from α1β2γ2L receptors activated by 0.5 μM GABA. The steady-state response under control conditions was 1% of the peak response to saturating GABA. At a maximally effective concentration of modulator, the amplitude of steady-state current was potentiated by approximately 10-fold.

$GABA_A$ receptors containing α1β2γ2L receptors have a high (>0.8) maximal open probability in the presence of GABA (Steinbach and Akk, 2001). This predicts that the steady-state open probability of α1β2γ2L receptors in the presence of 0.5 μM GABA and propofol, etomidate, pentobarbital or alphaxalone reaches ~0.1. Although the latter value may be overestimated if rapid desensitization reduces the maximal peak estimate we believe that the error is insignificant, for the following reasons. First, a more rapid solution exchange when 1 mM, instead of 50 μM GABA, is used to activate receptors has only minor effect on the peak response. Second, the peak response to saturating GABA is minimally affected by potentiators, indicative of high open probability during peak current. Finally, our relative steady-state responses to saturating GABA are generally similar to those obtained from rapid drug applications to excised patches (e.g., Haas and Macdonald, 1999).

The EC_{50}s for potentiation of steady-state currents from α1β2γ2L were lower, by a factor of 2 or more, than the EC_{50}s from available data for potentiation of peak responses. For example, we estimate that the half-maximal concentration of etomidate producing an increase of steady-state currents is 0.9 μM (Figure 3A) whereas our recent study on the effect of etomidate on peak currents from α1β2γ2L receptors revealed an EC_{50} of 2 μM (Li et al., 2014). Similarly, while the EC_{50} for potentiation of steady-state currents by propofol was 0.4 μM (Figure 2B), estimates for

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EC_{50} for potentiation of peak currents from α1β2γ2 receptors range from 2 μM (Krasowski et al., 2001) to 25 μM (Lam and Reynolds, 1998). The differences in EC_{50} estimates may be due to state-dependence of potentiation or simply reflect greater occupancy of the transmitter site in steady-state recordings.

The concentration of free propofol in plasma of surgical patients is ~1.3 μM (Rehberg and Duch, 1999). Thus, potentiation of steady-state currents from α1β2γ2L receptors may contribute to the sedative effect of propofol. The concentration of steady-state GABA to which the receptors are exposed under physiological conditions is estimated at or below 0.5 μM (multiple references in Houston et al., 2012). While exposure of α1β2γ2L to 0.5 μM GABA elicited an almost negligible response (1% of the response to saturating GABA), the combination of 0.5 μM GABA and 1 μM propofol is expected to generate a steady level of ~10% of maximal activity from these receptors.

Maximal steady-state current levels were similar for other potentiators. Coapplication of 100 μM pentobarbital with 0.5 μM GABA produced steady-state activity that was 12% of the peak response to 50 μM GABA. The mean steady-state current was 11% of the maximal peak response in the presence of 0.5 μM GABA and 10 μM etomidate, and 9% in the presence of 0.5 μM GABA and 1000 nM alphaxalone. Interestingly, the addition of 10-1000 nM alphaxalone to the combination of 0.5 μM GABA + 1 μM propofol did not lead to further enhancement of steady-state current. This is in contrast to our recent finding that neuroactive steroids can potentiate peak currents elicited by a combination of GABA and an allosteric activator (Li et al., 2014).

The ambient GABA concentration is likely dynamic, showing greater fluctuations at or near the synapse where the high mM concentration of GABA during a synaptic event rapidly drops due to uptake and diffusion. A recent study found that the ability of $GABA_A$ receptor potentiators to enhance tonic currents depends on ambient GABA concentration (Houston et al., 2012). This introduces yet another variable capable of affecting the final level of tonic inhibition in the presence of sedative drugs.

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Two major questions are raised by these observations. The first is the mechanism by which the steady-state response is enhanced. One possibility is that desensitization is reduced. This notion is supported by prior work that showed slower desensitization or potentiation of steady-state currents elicited by saturating GABA in the presence of some anesthetic drugs (Feng and Macdonald, 2004; Feng et al., 2004; Hall et al., 2004). Potentiation of steady-state currents from receptors activated by low concentrations of GABA additionally includes an effect on channel gating. Our data obtained with 0.5 μM GABA demonstrate major contribution from gating given that the peak response, for which desensitization-based effect is minimal, was enhanced by 7-fold, whereas the steady-state response, sensitive to changes in both desensitization and gating, was enhanced by 14-fold during coapplication with 1 μM propofol. In future single-channel work, it will be interesting to determine the precise kinetic makeup of potentiation of currents elicited by ambient GABA, taking into consideration.

The second question relates to the physiological consequences of the additional, tonic current. Receptors containing the α1 and γ2 subunits constitute the majority of $GABA_A$ receptors in the brain (Whiting et al., 1999). If it is assumed that these receptors are present at about 10-fold higher numbers than the classic tonic receptors (largely α4/δ or α5/γ2 subtypes) then the steady-state contribution we have demonstrated would be a considerable additional tonic inhibitory influence on the cells. This rough calculation will require much more refinement as information on the relative numbers of different receptor types is gathered, as well as further studies on the abilities of these modulators to enhance responses to endogenous levels of GABA. There is evidence that α1/γ2 receptors can be located outside the immediate postsynaptic membrane (Thomas et al., 2005; Mortensen and Smart, 2006; Kasugai et al., 2010). This pool of extrasynaptically-located α1/γ2 $GABA_A$ receptors is expected to be minimally active in the presence of steady-state GABA but respond with a ~10-fold increase in current amplitude to exposure to anesthetics. Unlike synaptic α1/γ2 receptors that undergo periodic activation and

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desensitization by high mM concentrations of GABA, extrasynaptic α1/γ2 receptors would be ideally suited to provide steady inhibitory current until exposure to the anesthetic is terminated.

To recapitulate, the data show that synaptic-type α1β2γ2L $GABA_A$ receptors exposed to a low, steady-state concentration of GABA and clinical concentrations of several anesthetic drugs produce persistent currents that have an amplitude of ~10% of the maximal peak response. This type of potentiation of inhibitory tone is distinct from the anesthetic-mediated prolongation of synaptic currents or enhancement of tonic inhibition mediated by classic α4/6 or α5-containing extrasynaptic receptors. We propose that these persistent, non-desensitizing currents from α1βγ2 $GABA_A$ receptors, in addition to previously identified anesthetic targets or modes of potentiation, contribute to the clinical effects of anesthetic drugs.

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AUTHORSHIP CONTRIBUTIONS

Participated in research design: Akk.

Conducted experiments: Li.

Contributed new reagents or analytical tools: N/A

Performed data analysis: Li and Akk.

Wrote or contributed to the writing of the manuscript: Li and Akk.

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FOOTNOTES

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FIGURE LEGENDS

Figure 1. Activation of rat α1β2γ2L $GABA_A$ receptors by GABA. Currents were elicited by 2 min applications of 50 μM GABA (**A**) or 0.5 μM GABA (**B**). In B, the framed inset shows the same current trace at a higher current resolution. Traces in A and B are from the same cell.

Figure 2. Exposure to propofol enhances steady-state currents from α1β2γ2L $GABA_A$ receptors. (**A**) Sample current responses from receptors activated by 50 μM GABA (red line), 0.5 μM GABA (blue line), or 0.5 μM GABA + 0.1, 0.3, 1, or 3 μM propofol (black lines). For clarity, only the first 10 sec of the 50 μM GABA trace are shown. The inset shows the final 30 sec of the traces. All traces are from the same cell. (**B**) A concentration-response relationship for propofol-induced enhancement of steady-state currents. The steady-state currents were normalized to the peak response to 50 μM GABA from the same cells. Data show mean ± S.E.M. from a set of 5 cells. The curve shows the prediction of the equation $Y=Y_{min}+(Y_{max}-Y_{min})*([propofol]/([propofol]+EC_{50}))^n$, where EC_{50} is the concentration of propofol producing a half-maximal effect, n the slope of the curve, and Y_{min} and Y_{max} are the low and high concentration asymptotes, respectively. The curve was generated with the overall mean parameters from the five cells. The EC_{50} was 0.44 ± 0.06 μM (mean ± S.E.M.), n was 2.4 ± 0.6, and Y_{max} was 9 ± 1%. The value for Y_{min} was held at the value obtained from the relative steady-state current from the same 5 cells in the presence of 0.5 μM GABA alone (1 ± 0.3%).

Figure 3. Exposure to etomidate, pentobarbital or alphaxalone enhances steady-state currents from α1β2γ2L $GABA_A$ receptors. (**A**) Sample current responses from receptors activated by 50 μM GABA (red line), 0.5 μM GABA (blue line), or 0.5 μM GABA + 10 μM etomidate (ETO, black line). For clarity, only the first 10 sec of the 50 μM GABA trace are shown. All traces are from the same cell. The lower portion of A shows the concentration-response relationship for etomidate-

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induced enhancement of steady-state current levels. The steady-state currents were normalized to the peak response to 50 μM GABA from the same cells. Data show mean ± S.E.M. from 7 cells exposed to 0.5 μM GABA and 0.1-10 μM etomidate. The curve was generated as in Figure 1 with the following overall parameters: EC_{50} = 0.9 ± 0.4 μM, n = 2.2 ± 0.4, Y_{min} = 0.6 ± 0.2 %, Y_{max} = 11 ± 1%. (**B**) Sample current traces from receptors activated by 50 μM GABA (red line), 0.5 μM GABA (blue line), or 0.5 μM GABA + 100 μM pentobarbital (PEB, black line). The lower portion of B shows the concentration-response relationship for pentobarbital-induced enhancement of steady-state current levels. Data show mean ± S.E.M. from 5 cells exposed to 0.5 μM GABA and 3-100 μM pentobarbital. The curve was generated as in Figure 1 with the following overall parameters: EC_{50} = 12 ± 2 μM, n = 2.6 ± 0.4, Y_{min} = 0.7 ± 0.1 %, Y_{max} = 13 ± 2%. (**C**) Sample current traces from receptors activated by 50 μM GABA (red line), 0.5 μM GABA (blue line), 0.5 μM GABA + 1 μM alphaxalone (ALF, black line), or 0.5 μM GABA + 1 μM alphaxalone + 1 μM propofol (PRO, grey line). The lower portion of C shows the effect of 10, 30, 100, 300, or 1000 nM alphaxalone on steady-state responses to 0.5 μM GABA. The data give mean ± S.E.M. for the response ratio (1 = no effect) from 4-11 cells at each steroid concentration. All datapoints were obtained from individual cells. Statistical analysis was conducted by comparing the response ratio to 1 using 2-tailed paired t-test (Excel, Microsoft, Redmond, WA). †, P > 0.05; *, P < 0.05; **, P < 0.01.

Figure 1

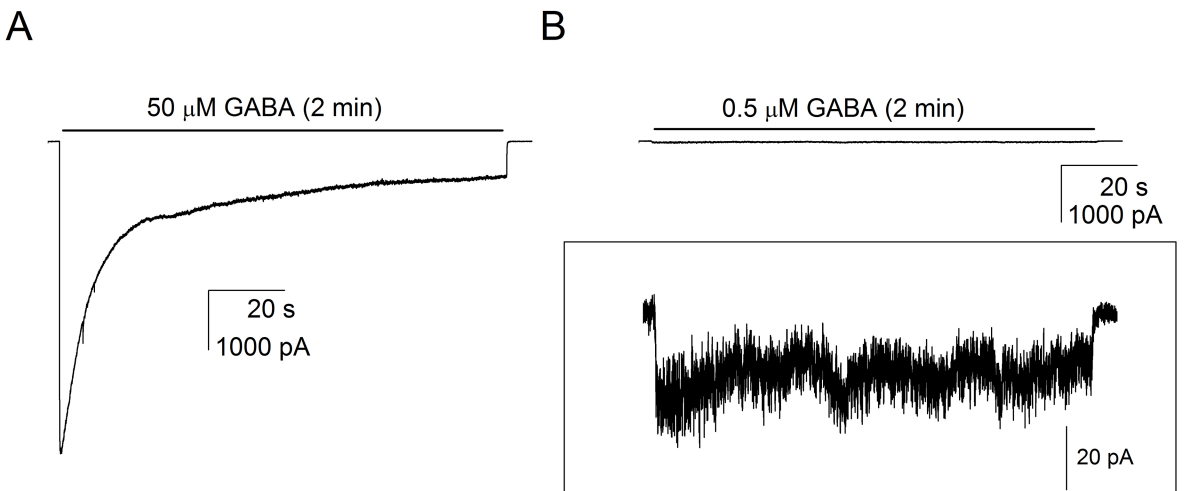


Figure 2

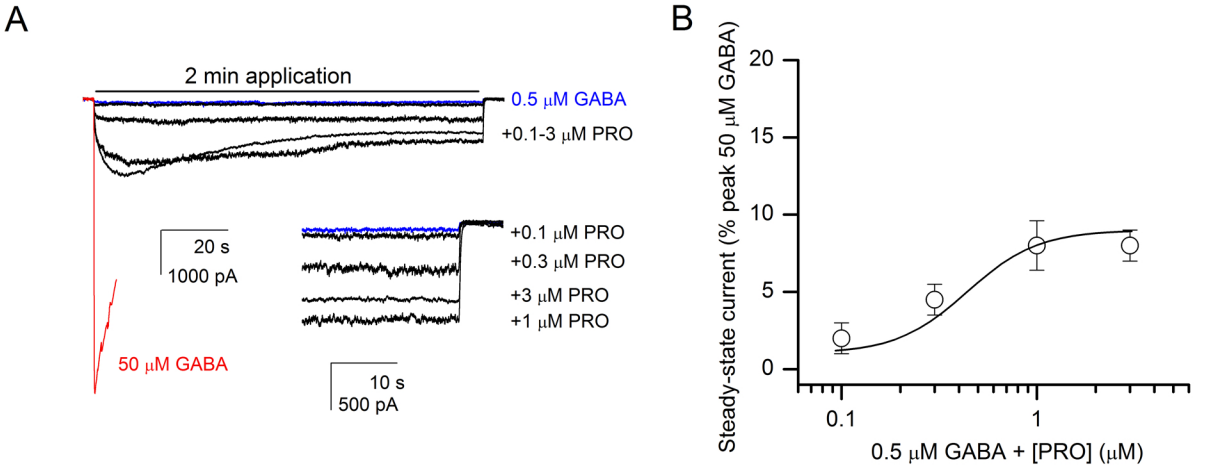
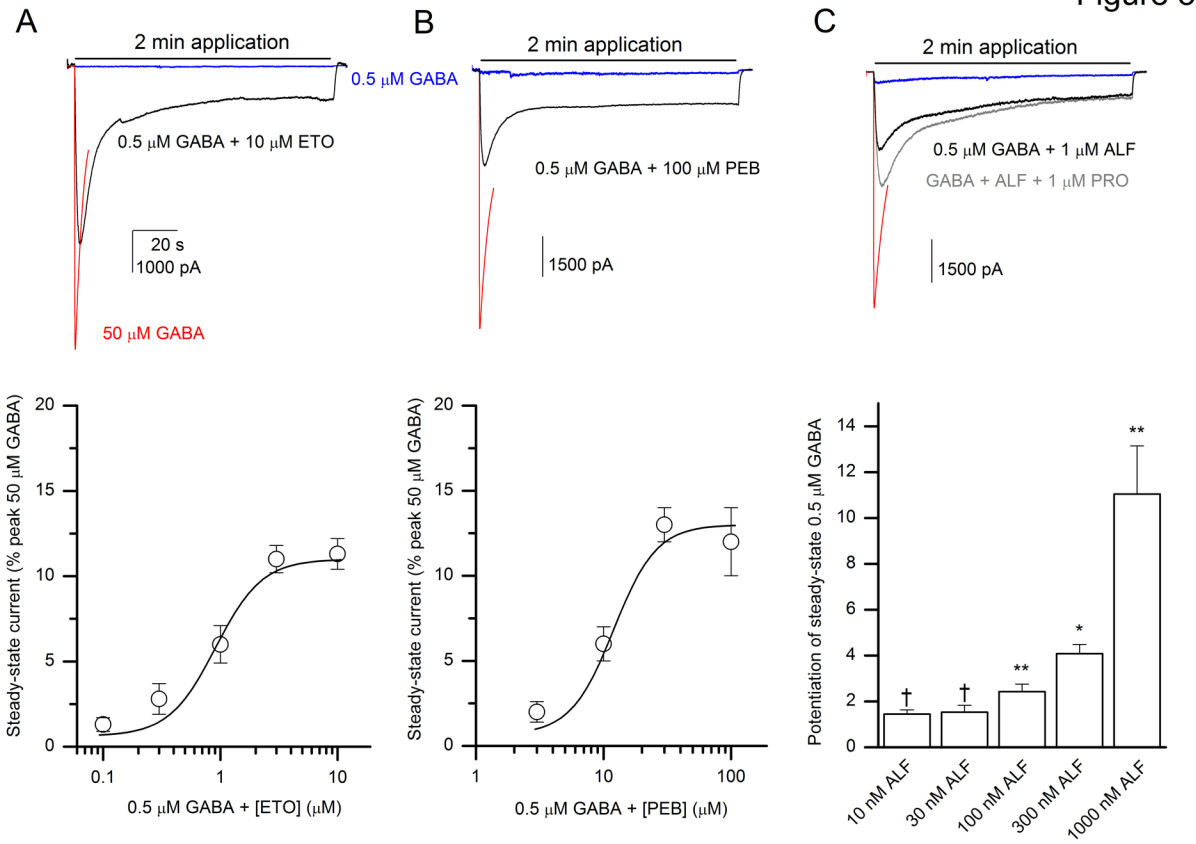

A
2 min application
0.5 μM GABA
+0.1-3 μM PRO
20 s
1000 pA
+0.1 μM PRO
+0.3 μM PRO
+3 μM PRO
+1 μM PRO
50 μM GABA
10 s
500 pA
B
Steady-state current (% peak 50 μM GABA)
20
15
10
5
0
0.1
1
0.5 μM GABA + [PRO] (μM)

Figure 3


A
2 min application
0.5 μM GABA
0.5 μM GABA + 10 μM ETO
20 s
1000 pA
50 μM GABA
B
2 min application
0.5 μM GABA + 100 μM PEB
1500 pA
C
2 min application
0.5 μM GABA + 1 μM ALF
GABA + ALF + 1 μM PRO
1500 pA
Steady-state current (% peak 50 μM GABA)
0.5 μM GABA + [ETO] (μM)
Steady-state current (% peak 50 μM GABA)
0.5 μM GABA + [PEB] (μM)
Potentiation of steady-state 0.5 μM GABA
10 nM ALF
30 nM ALF
100 nM ALF
300 nM ALF
1000 nM ALF