Rapid regulation of tonic GABA currents in cultured rat hippocampal neurons

By

1,2Christopher B. Ransom, 3Wucheng Tao, 4Yuanming Wu, 1,2,3William J. Spain, and 4,5George B. Richerson

1Epilepsy Center of Excellence, VA Puget Sound HealthCare System; 2Dept of Neurology and 3Dept of Physiology and Biophysics, University of Washington, Seattle, WA; 4Dept of Neurology and 5Dept. of Molecular Physiology and Biophysics, University of Iowa, Iowa City, IA

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Corresponding Author:
Christopher B. Ransom MD, PhD
Epilepsy Center of Excellence
VA Puget Sound Health Care System
Acting Assistant Professor, Dept of Neurology
University of Washington
Seattle, WA 98105
206-764-2021
cbr5@u.washington.edu

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Abstract

Subacute and chronic changes in tonic GABAergic inhibition occur in human and experimental epilepsy. Less is known about how tonic inhibition is modulated over shorter time frames (seconds). We measured endogenous tonic GABA currents from cultured rat hippocampal neurons to evaluate how they are affected by (1) transient increases in extracellular [GABA], (2) transient postsynaptic depolarization, and (3) depolarization of presynaptic cells. Transient increases in [GABA] (1 μM) reduced tonic currents; this reduction resulted from GABA-induced shifts in $E_{GABA}$. Transient depolarization of postsynaptic neurons reversed the effects of exogenous GABA and potentiated tonic currents. The voltage-dependent potentiation of tonic GABA currents was independent of $E_{GABA}$ shifts and represented postdepolarization potentiation (PDP), an intrinsic GABA$_A$ receptor property (Ransom et al., 2010). Inhibition of vesicular GABA release with concanamycin A (conA) did not affect tonic currents. In conA-treated cells, transient application of 12 mM K$^+$ to depolarize presynaptic neurons and glia produced a persistent increase in tonic current amplitude. The K$^+$-induced increase in tonic current was reversibly inhibited by SKF89976a (40 μM), indicating that this was caused by nonvesicular GABA release from GABA transporter type 1 (GAT1). Nonvesicular GABA release due to GAT1 reversal also occurred in acute hippocampal brain slices. Our results indicate that tonic GABA currents are rapidly regulated by GABA-induced changes in intracellular Cl$^-$ concentration, PDP of extrasynaptic GABA$_A$ receptors, and nonvesicular GABA release. These mechanisms may influence tonic inhibition during seizures.
when neurons are robustly depolarized and extracellular GABA and K⁺ concentrations are elevated.
Introduction

High affinity, extrasynaptic GABA_A receptors are tonically activated by ambient [GABA] in many areas of the brain, including cerebellum, thalamus, hippocampus, cortex, and striatum (Ade et al. 2008; Cope et al. 2005; Farrant and Nusser 2005). This tonic form of GABAergic inhibition occurs in vivo (Chadderton et al. 2004) and influences neuronal firing properties and network behavior (Glykys and Mody 2006; Mitchell and Silver 2003; Pavlov et al. 2009), thereby affecting many physiological and pathophysiologic processes including learning and memory, anxiety, and epilepsy (Maguire et al. 2005; Martin et al. 2010; Shen et al. 2007). In epilepsy, tonic inhibition is affected by changes in neurosteroid levels, altered expression/localization of GABA_A receptor subunits, and decreased function of GABA transporters (Maguire et al. 2005; Patrylo et al. 2001; Peng et al. 2004; Zhang et al. 2007). The development of these changes occurs over days to weeks. Relatively less is known about how tonic inhibition is affected by ongoing neural activity over shorter time periods (seconds).

Tonic inhibition can be rapidly regulated by factors that influence ambient [GABA], including vesicular release of GABA at synapses (Glykys and Mody 2007b; Tang et al. 2011), both uptake and release by GABA transporters (Scimemi et al. 2005; Wu et al. 2007; Wu et al. 2006), and GABA release via glial anion channels (Lee et al. 2010). Extrasynaptic GABA_A receptors that mediate tonic inhibition are also subject to direct voltage-dependent modulation (Pavlov et al. 2009; Ransom et al. 2010). All of these mechanisms may be influenced by ongoing neural activity. The intense neural activity that occurs during seizures is
accompanied by elevations of extracellular GABA and depolarization of pre- and post-synaptic neurons (During and Spencer 1993). How these rapid and transient changes in GABA concentration and membrane potential affect tonic inhibition is not known.

To investigate the rapid regulation of tonic inhibition, we measured tonic GABA currents in cultured hippocampal neurons and assessed how they are affected by: 1) transient increases in extracellular GABA concentration, 2) transient postsynaptic depolarization, and 3) transient depolarization of presynaptic neurons and glia. Our results indicate that tonic inhibition can be rapidly regulated by multiple mechanisms, including changes in intracellular anion concentration, voltage-dependent modulation of GABA_A receptor function, and nonvesicular GABA release mediated by GABA transporter type 1 (GAT1). These mechanisms may influence the effects of tonic inhibition during seizures.

**Materials and Methods**

*Cell culture:* Primary hippocampal cell cultures were prepared as previously described (Gaspary et al. 1998). In brief, 0-2 day old Sprague-Dawley rat pups of both sexes were decapitated and the hippocampi were dissected. The tissue was minced in sterile-filtered, HEPES-buffered solution and then treated with a digestion solution containing papain (10 U/ml), 0.5 mM EDTA, and cysteine (0.2 mg/ml) for 15 minutes. The enzyme-treated tissue was triturated in complete Minimum Essential Medium (MEM), trypsin inhibitor (1.5 mg/ml), and bovine serum albumin (1.5 mg/ml). Triturated cells were added to culture media
(MEM/10% FBS) and plated directly on poly-l-ornithine-coated round glass coverslips (Erie Scientific, Portsmouth, NH, USA) in 12 well culture dishes at a density of 2.5-5 x 10^5 cells/ml. After 1 hour, media was changed to 70% MEM/30% Neurobasal with B27 supplement (Gibco, Carlsbad, CA, USA). Cells were maintained in an incubator (Forma Scientific, model 3110, Marietta, OH) with a humidified environment containing 5% CO2 in room air at 37°C. Media was changed on day 5-6 to Neurobasal Medium with B27 supplement and 1 μM cytosine arabinoside (Ara-C), followed by half medium changes (without Ara-C) every 7 days.

**Acute brain slices:** Hippocampal brain slices were prepared from 4-6 week old Sprague-Dawley rats of both sexes. Rats were anesthetized with 4% isoflurane, decapitated, and the brain dissected free. Transverse slices were made of hippocampus with a thickness of 300 μm using a vibratome (Leica VT1200). Slices were cut and stored in a solution containing (in mM): 125 NaCl, 3 KCl, 26 NaHCO3, 1.2 NaH2PO4, 0.5 CaCl2, 4 MgCl2, 20 dextrose, and 1 kynurenic acid. Solutions were continuously gassed with 95% O2/5% CO2. Slices were allowed to recover at room temperature for 1 hour prior to recording.

**Electrophysiology:** Conventional whole-cell patch clamp techniques were used to record membrane currents from neurons aged 14-60 days in vitro. Cultured cells were visualized with an Axiovert 200 inverted microscope with DIC optics (Carl Zeiss Inc., Thornwood, NY). Dentate gyrus granule cells in brain slices were visualized with an Axioskop 2 upright microscope with fixed stage using infrared DIC optics (Carl Zeiss Inc.). Recordings were made using a Multiclamp 700B or
Axopatch 200B amplifier, a Digidata 1200 series A-D converter, and pClamp 10 software (Molecular Devices, Redwood City, CA). Data were acquired at 2-5 kHz and low-pass filtered at 1 kHz. Series resistance and whole-cell capacitance were determined by compensating the current transients produced by a –10 mV voltage step and compensated 50-70% online; whole-cell capacitance was recorded as the compensation value from the front of the amplifier or Multiclamp 700B software. Series resistance was determined at the beginning and end of experiments and compensated 50-70% online, if series resistance exceeded 20 MΩ or changed by more than 25% the experiment was discarded. Drug applications were made via a microperfusion device (SF-77B, Warner Instruments, Hamden, CT) connected to manifolds that allowed up to 5 test solutions to be applied in a single experiment. Flow through the manifolds was controlled with electronic solenoid valves (Parker Hannifin Corporation, General Valve Division, Cleveland, OH) and step application of test solutions was controlled by pClamp software. The microperfusion device produced 90% solution exchange times of 50-55 ms across open pipette tips. The recording chamber (RC-26, Warner Instruments, Hamden, CT) had a volume of ~0.3 ml and was continuously superfused at a rate of ~1-2 ml/min with bath solution that contained (in mM): 134 NaCl, 3 KCl, 1.4 NaH₂PO₄, 24 NaHCO₃, 10 Dextrose, 2 MgCl₂, 2 CaCl₂, 1 Kynurenic acid, and 0.001 CGP 55845. The pH was 7.35-7.4 when bubbled with 95% O₂/5% CO₂. Osmolarity was adjusted to 300-305 mOsm with H₂O. In acute brain slices, local application of elevated K⁺ solution was made by pressure ejection from a patch pipette. A Picospritzer II was used for
pressure ejection (General Valve Corporation). The baseline picospritzer solution contained (in mM): 150 NaCl, 3 KCl, 2 CaCl₂, 2 MgCl₂, 10 dextrose, and 10 HEPES with pH adjusted to 7.4 with NaOH. For experiments with high K⁺ (12-48 mM), KCl was substituted for an equimolar amount of NaCl.

Patch electrodes were made with borosilicate glass without filament (593400, A-M Systems, Carlsborg, WA) using a micropipette puller (P-97; Sutter Instruments, Novato, CA). Pipettes had resistances of 2.5 to 3.5 MΩ when filled with an intracellular solution containing (in mM): 125 CsCl, 10 QX-314 chloride salt, 10 HEPES, 1 EGTA (pH corrected to 7.25 with CsOH). Osmolarity was adjusted to 275-285 mOsm with H₂O as needed. All chemicals were purchased from Sigma except SKF-89976a, SNAP-5114 (Tocris-Cookson, Ellisville, MO), and QX-314 (Alomone Labs, Tel Aviv, Israel). Data acquisition was begun 3-5 minutes after establishing a whole-cell recording. Experiments were performed at room temperature, typically 23°C.

Analysis: Data analysis was performed with Clampfit (pClamp 10, Molecular Devices, Mountain View CA) and Origin (v6.1, Microcal Software, Northhampton, MA) software. Tonic current amplitudes were measured as the difference in mean holding current in the absence and presence of the GABAₐ receptor antagonist bicuculline methiodide (10-20 μM). The mean holding current values were obtained from Gaussian fits to all-point current amplitude histograms (see Fig. 4A). Histograms were constructed from 5-10 seconds of data with a bin width of 1 pA. Gaussian fits were performed using a Levenberg-Marquardt curve-fitting algorithm provided in Clampfit software, fits were constrained to exclude
the portion of the histogram skewed by synaptic currents beyond 20 pA from the peak (Glykys and Mody 2006). The slope conductance was determined by fitting I-V plots with a linear equation. Tonic conductance, g, was calculated from the following equation:

\[ g = \frac{I}{V_m - E_{GABA}} \]

where I is tonic current amplitude, \( V_m \) is membrane potential, and \( E_{GABA} \) is the reversal potential for GABA currents (a composite of the equilibrium potential for permeant anions including \( Cl^- \) and \( HCO_3^- \)). The measured reversal potential of currents evoked with exogenous GABA was used as \( E_{GABA} \) for calculations of chord conductance in Fig. 3B. We used the chord conductance equation above to calculate the theoretical shifts in \( E_{GABA} \) required to cause the tonic current changes observed in response to exogenous GABA alone or GABA with depolarization (i.e. data in Fig. 1), assuming no change in conductance. The theoretical Nernst potential for chloride was 0 mV under our experimental conditions and this value was used as the baseline value of \( E_{GABA} \) for calculations of theoretical \( E_{GABA} \) shifts presented in Fig. 3C. Charge transfer, \( Q \), was determined by integrating baseline-subtracted currents over the period of high \([K^+] \) application.

Data values are presented as mean ± standard error of mean (SEM) and all error bars represent SEM. Statistical analyses were performed using Microsoft Excel (Bellevue, WA). A two-tailed, paired or unpaired Students t-test was used with a p-value of 0.05 considered as significant. Data in Fig. 3 were analyzed
with a nonparametric test (Wilcoxon Signed Rank Test). Where appropriate the actual p-values are reported.

Results

Transient changes in extracellular GABA and postsynaptic depolarization modulate tonic GABA currents

To evaluate the effects of transient increases in extracellular GABA on tonic inhibition, tonic currents due to endogenous GABA were measured before and after application of exogenous GABA (1 \( \mu \)M, 10 s). Neurons were held at –60 mV. Transient application of exogenous GABA reduced subsequently measured endogenous tonic currents (measured 5 s after wash of GABA) (Fig. 1A-B). We next examined the effects of transient depolarization during GABA application. In contrast to effects of exogenous GABA alone, tonic currents were increased when neurons were transiently depolarized during exogenous GABA application (Fig. 1C-D). For the cell illustrated in Fig. 1A-D, exogenous GABA alone reduced tonic current from –76 pA to –41 pA while exogenous GABA paired with depolarization increased tonic current from –73 pA to –116 pA (Fig. 1E). On average, exogenous GABA alone reduced tonic currents by 25±9% (n=11 cells, p<0.05) and exogenous GABA paired with transient depolarization potentiated tonic currents by 68±13% (n=11, p<0.01) (Fig. 1F). These results indicate that transient increases in [GABA] with or without transient depolarization can rapidly (within seconds) cause a persistent modulation of endogenous tonic GABA currents.
Determination of GABA induced shifts in $E_{\text{GABA}}$

The changes in tonic current amplitudes produced by exogenous GABA and depolarization could be due to altered intracellular anion concentrations and changes in driving force (i.e. $E_{\text{GABA}}$). To evaluate shifts in $E_{\text{GABA}}$, we measured currents in response to ramp voltage commands under baseline conditions and during application of exogenous GABA (Fig. 2A). Ramp voltages were from $-30$ mV to $+10$ mV over 250 ms. A point-by-point subtraction of currents recorded in the presence and absence of GABA was performed to yield the GABA-induced ramp current. These data were used to determine reversal potential (i.e. $E_{\text{GABA}}$) and slope conductance (linear fit to ramp current data). GABA application caused a shift in $E_{\text{GABA}}$ towards more negative potentials (indicating a decrease in intracellular anion concentration) and a reduction in slope conductance (Fig. 2B). This procedure was repeated in the same neurons with a transient depolarization to $+40$ mV (5 s) during the period of GABA application (Fig. 2C). Depolarization during GABA application shifted $E_{\text{GABA}}$ towards more positive voltages (indicating intracellular anion accumulation) and increased slope conductance, in contrast to the reduction of conductance seen with GABA alone (Fig. 2D). On average, application of exogenous GABA alone reduced conductance from $23.7\pm5$ nS to $14.0\pm2$ nS ($n=8$, $p<0.01$); because our measurement of slope conductance is independent of $E_{\text{GABA}}$, this conductance change likely represents desensitization of receptors. Exogenous GABA paired with depolarization increased conductance from $22.1\pm4$ to $27\pm8$ nS ($n=8$, $p<0.05$). In the case of exogenous
GABA paired with depolarization the changes in conductance likely represent postdepolarization potentiation (PDP), an intrinsic voltage-dependent property of GABA<sub>A</sub> receptors in cultured hippocampal neurons (Ransom et al., 2010). Exogenous GABA application alone shifted $E_{\text{GABA}}$ from $-0.8 \pm 0.7$ mV at baseline to $-9.3 \pm 1.8$ mV ($p<0.01$, $n=8$), while exogenous GABA paired with depolarization shifted $E_{\text{GABA}}$ from $-1.2 \pm 0.9$ mV at baseline to $3.1 \pm 0.3$ mV ($p<0.01$, $n=8$) (Fig. 3A). We did not characterize the recovery time course for $E_{\text{GABA}}$ shifts in detail but they were fully recovered within 2 minutes (the time interval between GABA applications).

**Relationship between $E_{\text{GABA}}$ shifts and changes in tonic current**

Data presented above show that currents evoked with exogenous GABA are associated with changes in $E_{\text{GABA}}$ and changes in slope conductance. Because the receptors mediating endogenous tonic currents are already activated by ambient GABA, they may represent a distinct population of receptors from those activated by exogenous GABA. These two receptor populations are expected to have different properties (Farrant and Nusser 2005), and could be affected differently by transient changes in [GABA] and transient depolarization. For example, the change in slope conductance (a measure independent of $E_{\text{GABA}}$) seen in Fig. 2A-B is attributed to receptor desensitization while the extrasynaptic receptors mediating tonic currents are believed to desensitize very little (Bianchi et al. 2002; Glykys and Mody 2007a; Mtchedlishvili and Kapur 2006; Saxena and Macdonald 1996).
We used our measured changes in $E_{\text{GABA}}$ (Fig. 3A) to estimate changes in tonic conductance produced by exogenous GABA alone or exogenous GABA paired with transient depolarization (using data presented in Fig. 1). For GABA application alone, the calculated tonic conductance was $1.1 \pm 0.2$ nS at baseline and $0.97 \pm 0.2$ nS following the GABA application, this change of $-12 \pm 11\%$ was not significant ($p=0.22$, $n=11$) (Fig. 3B). Exogenous GABA paired with transient depolarization increased tonic conductance from $1.1 \pm 0.2$ nS at baseline to $1.6 \pm 0.2$ nS, a significant change of $57 \pm 12\%$ ($p<0.001$, $n=11$) (Fig. 3B). This analysis indicates that the reduction of tonic currents by exogenous GABA alone was primarily due to driving force changes caused by reductions in intracellular anions (i.e. changes in $E_{\text{GABA}}$). In contrast, depolarization led to absolute increases in tonic conductance independent of $E_{\text{GABA}}$ shifts. Our calculations will overestimate the influence of $E_{\text{GABA}}$ on changes of tonic current, because changes in $E_{\text{GABA}}$ will begin to recover during the 5 s interval between wash out of GABA and measurement of tonic current.

We also calculated the theoretical shift in $E_{\text{GABA}}$ (i.e. the change in driving force, delta $E_{\text{GABA}}$) required to produce the measured changes in tonic current amplitudes (assuming no change in conductance). These theoretical calculations were then compared to our observed changes in $E_{\text{GABA}}$. For GABA application alone, there was no significant difference between the observed ($-8.5 \pm 1$ mV) and theoretical delta $E_{\text{GABA}}$ ($-14.7 \pm 6$ mV; $p=0.37$, $n=8$) (Fig. 3C). With GABA plus depolarization, the theoretical $E_{\text{GABA}}$ change needed to produce the observed increases in tonic current was $+41.3 \pm 8$ mV, much larger than the experimentally
determined delta $E_{\text{GABA}}$ of $+4.3 \pm 0.3 \text{ mV}$ ($p<0.001$, $n=8$) (Fig. 3C). These considerations are consistent with our conclusions that changes in $E_{\text{GABA}}$ (and reduced driving force) were the primary cause of tonic current reduction produced by exogenous GABA alone and that depolarization produced absolute increases of tonic conductance independent of driving force changes.

Presynaptic depolarization enhanced tonic currents via nonvesicular GABA release

During seizures there is widespread neuronal depolarization and extracellular $K^+$ concentrations can rise to 12-20 mM (Hablitz and Heinemann 1987). This depolarization and increased $K^+$ concentration alters the thermodynamics of GABA transporter type 1 (GAT1) function, causing GAT1 to operate in reverse and release GABA (nonvesicular GABA release) (Richerson and Wu 2003). Because we were interested in determining whether nonvesicular GABA release can produce persistent changes in tonic currents, these experiments were performed on neurons pre-treated with the $H^+$-ATPase inhibitor concanamycin A (ConA, 1 $\mu$M for 2 h) to inhibit vesicular GABA release (Rossi et al. 2003; Wu et al. 2007). ConA by itself did not significantly affect tonic current amplitude compared to neurons on untreated coverslips; average tonic current was $-58 \pm 12$ pA under control conditions and $-39 \pm 4$ pA following ConA treatment ($p=0.24$, $n=5-7$ neurons under each condition) (Fig. 4 A-B).

As previously shown, fast application of 12 mM $K^+$ (8 s) rapidly activated inward currents that were sensitive to the GAT1 antagonist SKF89976A (SKF, 40
μM) (Fig. 4C) and bicuculline (data not shown), indicating that depolarization of presynaptic neurons and glia caused nonvesicular GABA release by GAT1 (Gaspary et al. 1998; Wu et al. 2001). Using our fast solution exchange system, we next tested whether nonvesicular GABA release triggered by high K⁺ had any persistent effects on endogenous tonic currents after the depolarizing stimulus was washed off. The effects of transient presynaptic depolarization on tonic currents was studied by voltage clamping the post-synaptic neuron to -60 mV, then measuring tonic currents at baseline and 5 s after application of high K⁺ (Fig. 4C). Depolarization of presynaptic neurons and glia with 12 mM K⁺ increased tonic current amplitude measured 5 s after wash of high K⁺ (Fig. 4C). This increase was sensitive to bath application of the GAT1 antagonist SKF89976A (SKF, 40 μM), indicating that a component of it was caused by nonvesicular GABA release from reverse transport of GAT1 (Fig. 4C, middle panel). GAT1 antagonism increased baseline tonic current amplitude but also prevented the increase in tonic currents induced by high K⁺ (Fig. 4D). The effects of transient presynaptic depolarization with high K⁺ on tonic current amplitude are summarized in Fig. 4E. The K⁺-induced increase in tonic current was significantly reduced by GAT1 antagonism with SKF and this effect was partially reversible (p<0.05, n=5). This result indicates GAT1 function is dynamic, limiting ambient [GABA] (and tonic currents) under basal conditions and increasing ambient [GABA] during periods of intense presynaptic depolarization with persistent enhancement on tonic currents.
Nonvesicular GABA release occurred in acute brain slices

We next evaluated whether GAT1 functions similarly in acute hippocampal brain slices. We made whole cell recordings from dentate gyrus granule cells and locally applied high K+ by pressure ejection from a pipette positioned 60-80 μm from the recorded cell. Vesicular GABA release was inhibited by treating slices with 1 μM conA for 2 hours. Local pressure ejection of high K+ for 10 seconds induced an inward current that was inhibited by bicuculline (10 μM), indicating that this current was due in part to GABA release and activation of GABA_A receptors (Fig. 5A). The bicuculline-insensitive current likely represents a change in leak current due to a shift in E_K. On average, bicuculline inhibited the total charge movement (Q) produced during application of high K+ by 63±7% (14±6 nC under control conditions and –5.2±2 nC in the presence of bicuculline) (p<0.05, n=5) (Fig. 5B).

Bath application of the GAT1 antagonist SKF (30 μM) had different effects on holding current (a surrogate measure of tonic currents) than it did on K+-induced currents. Holding current was increased by SKF, reflecting an increase in ambient GABA and tonic inhibition (Fig. 5C-D). However, SKF simultaneously reduced the K+-induced currents, indicating that a large component of the K+ induced GABA currents was caused by nonvesicular GABA release. On average, SKF increased holding current from -68±15 pA at baseline to -188±35 pA (p<0.01, n=8) (Fig. 5D-E). The charge movement (Q) produced by high K+ under control conditions was –10.1±2 nC (peak current of -1450±236 pA) and –4.6±1 nC (peak current of -813±172 pA) in the presence of SKF, a reduction of 55±5%.
The GAT3-selective inhibitor SNAP-5114 (SNAP, 30 μM) did not significantly affect holding currents or K⁺-induced currents. Peak K⁺-induced currents were \(-1054\pm393\) pA under control conditions and \(-1005\pm350\) pA in the presence of SNAP (p=0.18, n=3, data not shown). These results in acute brain slices are similar to those obtained in cell culture and indicate that GAT1 functions differently under basal conditions (net uptake of GABA) than during periods of robust depolarization (i.e. high K⁺), when GAT1 reverses and releases GABA.

Induction of nonvesicular GABA release in acute brain slices required use of pressure ejection solutions with higher K⁺ concentrations than was required in cell culture experiments, between 30 and 48 mM. Because of the distance of the pressure ejection pipette from recorded neurons (60-80 μM), the small volume of solution ejected, K⁺ buffering by cellular uptake mechanisms (Ransom et al. 2000), and the diffusion barriers presented by tissue (Nicholson and Sykova 1998), it is expected that the actual [K⁺] reaching the recorded neuron and adjacent cells/presynaptic terminals was considerably lower than that of the pressure ejection solution itself.

Discussion

Pathophysiological events in the brain such as ischemia and epileptic seizures are associated with significant depolarization of pre- and post-synaptic neurons and elevations of ambient [GABA] (Allen et al. 2004; During and Spencer 1993). Our results suggest that tonic GABA currents will be rapidly
(within seconds) affected by these conditions via multiple mechanisms; specifically, our data show rapid modulation of tonic GABA currents caused by GABA-induced anion shifts, intrinsic voltage-dependent modulation of GABA<sub>A</sub> receptors and PDP (Birnir et al. 1994; Gray and Johnston 1985; Pavlov et al. 2009; Ransom et al. 2010), and GABA release via reverse transport of GAT1 (Fig. 6). These mechanisms are predicted to influence the level of tonic inhibition during ongoing neural activity; other factors may also rapidly regulate tonic inhibition in addition to those described here (Glykys and Mody 2007b).

Anion shifts and tonic inhibition

It is well established that activation of GABA<sub>A</sub> receptors by synaptically released GABA or exogenous GABA can produce appreciable changes in ionic gradients (i.e. Cl<sup>-</sup> and HCO<sub>3</sub><sup>-</sup> ions) (Farrant and Kaila 2007; Foldy et al. 2010; Kaila 1994; Staley et al. 1995). This has been demonstrated electrophysiologically as a shift in the reversal potential or altered polarity of GABA currents (DeFazio and Hablitz 2001; Huguenard and Alger 1986; Staley et al. 1995; Thompson and Gahwiler 1989a) and experiments using ratiometric imaging techniques have directly measured changes in intracellular [Cl<sup>-</sup>] due to GABA<sub>A</sub> receptor activation or antagonism (Isomura et al. 2003; Kuner and Augustine 2000; Lee et al. 2010). Shifts in intracellular Cl<sup>-</sup> due to GABA<sub>A</sub> receptor activation occur in CA1 pyramidal neurons under current clamp (when membrane potential is not controlled) (Isomura et al. 2003), in cultured hippocampal neurons during gramicidin perforated patch clamp experiments (when intracellular [Cl<sup>-</sup>])
and the operation of Na- and K- coupled Cl\textsuperscript{-} cotransporters are not disturbed) (Chabwine et al. 2004), and in undisturbed cerebellar granule cells during imaging experiments (Lee et al. 2010). These results indicate that GABA\textsubscript{A} receptor activation can alter intracellular [Cl\textsuperscript{-}] (and [HCO\textsubscript{3}{-}]) at resting membrane potentials and with intact regulation of intracellular Cl\textsuperscript{-}.

Our data indicate that tonic currents are reduced by transient application of exogenous GABA (1 \textmu{M}). Exogenous GABA also produced changes in \(E_{GABA}\) and the magnitude of the measured \(E_{GABA}\) shifts were sufficient to explain the reduction of tonic currents based solely on driving force considerations, without any change in tonic conductance (Fig. 6A). \(E_{GABA}\) changes are unlikely to have recovered in the 5 s interval between wash off of exogenous GABA and measurement of tonic currents. Prior studies on our preparation showed GABA-induced changes in \(E_{GABA}\) persist for >30 s with either 15 or 135 mM intracellular Cl\textsuperscript{-} (Ransom et al. 2010). The time course of \(E_{GABA}\) changes depends on both the duration of GABA\textsubscript{A} receptor activation and the recording conditions. In cultured hippocampal neurons studied with K-based pipette solutions, brief GABA pulses (25 ms, 1 mM GABA) produce changes in intracellular Cl\textsuperscript{-} that recover over 10-15 s (Kuner and Augustine 2000). This may be a lower limit of recovery time for intracellular Cl\textsuperscript{-} shifts because in this situation Cl\textsuperscript{-} recovery will be mediated by both KCC2 extrusion and diffusion from patch pipette (DeFazio et al. 2000; Thompson and Gahwiler 1989b). In gramicidin perforated patch experiments, GABA-induced Cl\textsuperscript{-} accumulation recovers much more slowly with a time constant of 2.5 minutes (Chabwine et al. 2004). The rate of recovery was faster after
GABA-induced Cl\(^-\) depletion (i.e. after GABA application to hyperpolarized neurons), but this still recovered over minutes (\(\tau = 1.2\) min) (Chabwine et al. 2004). To the best of our knowledge, ours is the first description of endogenous tonic GABA currents being affected by anion redistribution. While this result may not be surprising, it highlights that tonic inhibition (similar to phasic inhibition) can be affected in a potentially maladaptive manner during recurrent seizures, when intracellular Cl\(^-\) accumulation could convert the inhibitory, shunting function of tonic inhibition to an excitatory, depolarizing current (Dzhala et al. 2010; Thompson and Gahwiler 1989b).

Because extrasynaptic \(\text{GABA}_A\) receptors that mediate tonic currents in the hippocampus (\(\alpha_5\)- and \(\delta\)-subunit containing receptors) are believed to desensitize very little, even in the presence of high GABA concentrations (i.e. 1 mM), exogenous GABA was not expected to reduce tonic currents by desensitizing extrasynaptic receptors (Bianchi et al. 2002; Glykys and Mody 2007a; Mtchedlishvili and Kapur 2006; Saxena and Macdonald 1996). However, recent results indicate that \(\delta\)-subunit containing receptors expressed in tsA201 cells undergo significant desensitization during prolonged exposure to low GABA concentrations (i.e. 1-3 min) (Bright et al. 2011). Our data on GABA-induced \(E_{\text{GABA}}\) shifts quantitatively explain the reduction of tonic currents caused by exogenous GABA, suggesting that desensitization of extrasynaptic receptors in our experiments was not significant.

Voltage-dependent modulation of \(\text{GABA}_A\) receptors
Tonic inhibition and phasic inhibition both exhibit voltage-dependence (Collingridge et al. 1984; Mellor and Randall 1998; Pavlov et al. 2009; Ransom et al. 2010). Extrasynaptic GABA<sub>A</sub> receptors that mediate tonic inhibition in hippocampal neurons are strongly modulated by membrane potential, with a 2-fold increase in tonic conductance when neurons are depolarized from –80 to –40 mV (Ransom et al. 2010). This voltage dependence augments tonic inhibition and offsets action potential threshold (Pavlov et al. 2009). Our results show that voltage-dependent modulation of tonic inhibition is independent of anion accumulation and outlasts the period of depolarization, a phenomena termed postdepolarization potentiation (PDP) (Fig. 6B). Although we used large depolarizations in this study, PDP occurs following smaller depolarizations (i.e. –30 mV) and in response to epileptiform patterns of depolarization (Ransom et al. 2010). The mechanism of PDP at the single channel level is not known but it may relate to the increased open probability or unitary conductance of GABA<sub>A</sub> receptors seen with depolarization (Curmi et al. 1993; Fatima-Shad and Barry 1993; Gray and Johnston 1985). Voltage dependent increases of tonic conductance are expected to affect the behavior of hippocampal neurons and networks (Glykys and Mody 2006; Pavlov et al. 2009). This would typically be an inhibitory effect but if it occurred during periods of intracellular Cl<sup>−</sup> accumulation voltage dependent potentiation could enhance an excitatory influence of GABA currents (Dellal et al. 2012; Dzhala et al. 2010).
Nonvesicular GABA release by GAT1

GAT1 is expressed by GABAergic neurons and exists at high density in presynaptic terminals, up to 800-1200 molecules per terminal (Chiu et al. 2002). It is a Na-dependent cotransporter that functions to uptake GABA (translocating 2 Na\(^+\) ions, one Cl\(^-\) ion, and one GABA molecule), thereby constraining the time course and spatial distribution of synaptically released GABA (Keros and Hablitz 2005; Thompson and Gahwiler 1992). GAT1 can also operate in the reverse direction to release GABA under appropriate thermodynamic conditions (Gaspary et al. 1998; Ransom and Richerson 2009). This type of nonvesicular GABA release has been demonstrated with physiologic and pathophysiologic depolarizations, and alterations in ion gradients (Allen et al. 2004; Pin and Bockaert 1989; Wu et al. 2006). It can also mediate direct inhibitory neurotransmission between recorded pairs of hippocampal neurons (Wu et al. 2007). The present results provide new information on the dynamic function and temporal aspects of nonvesicular release mediated by GAT1. Our rapid solution exchange allowed us for the first time to detect enhanced tonic inhibition that outlasted the duration of a depolarizing stimulus (i.e. high K\(^+\)), this enhancement lasted at least 5 s (see Fig. 4A). GAT1 antagonism with SKF prevented this increase of tonic inhibition, indicating that nonvesicular GABA release by GAT1 can rapidly and persistently modulate tonic inhibition (see Fig. 6C). GAT1 antagonism not only prevented nonvesicular GABA release, but also increased baseline tonic inhibition. These results indicate that GAT1 limits ambient GABA (and tonic inhibition) under basal conditions but GAT1 can operate in reverse to
release GABA during periods of depolarization and intracellular Na$^+$ accumulation (such as occur during seizures and ischemia), leading to transient enhancement of tonic inhibition. The temporal profile of ambient GABA elevations caused by nonvesicular release is likely determined by both diffusion and a shift in the thermodynamic operation of GAT1 back to net uptake as intracellular Na$^+$ accumulation recovers; the rapid solution exchange times make direct effects of K$^+$ on membrane potential unlikely. We also showed that similar effects occur in dentate gyrus granule cells in acute hippocampal slices, indicating that reversal of GAT1 is not an artifact of cell culture preparations (see also Allen et al. 2004).

The mechanisms described here that rapidly regulate tonic GABA currents will not work in isolation; for example, nonvesicular GABA release will increase ambient GABA causing alterations in anion concentrations and $E_{\text{GABA}}$ that will affect tonic currents. In our experiments, however, the increase in tonic conductance produced by nonvesicular GABA release overshadowed any effect of $E_{\text{GABA}}$ shifts (i.e. changes in driving force), allowing us to detect increased tonic current amplitudes. Increased ambient GABA will have other effects as well, including changes in glutamate uptake by astrocytes (Unichenko et al. 2012). GABA released by reverse transport of GAT1 is unlikely to reach the peak concentrations produced by GABA released from synaptic vesicles, but it is predicted to act in a wider spatio-temporal pattern, with the potential to affect many physiologic processes of neurons and glia.
Summary and Conclusions

Tonic inhibition, by its name, implies a static, invariant level of GABAergic signaling. This feature is likely advantageous under some conditions, allowing neurons to function on a stable background tone of inhibition. However, our results indicate that tonic inhibition is not really tonic, but instead that the level of tonic inhibition can rapidly change due to factors associated with intense neural activity, including increased ambient GABA, extracellular K⁺ accumulation, and neuronal depolarization. These factors modulate tonic inhibition via changes in ion gradients, GAT1 thermodynamics, and GABA_A receptor behavior. Activity dependent fluctuations in tonic inhibition may serve a negative feedback function for neurons, providing moment-to-moment adjustment of inhibitory tone in response to changes in the prevailing level of neural activity. In addition to the slower types of regulation of tonic inhibition due to changes in receptor expression or neurosteroid levels, our results establish that tonic inhibition can be rapidly and dynamically regulated.
Figure 1 Endogenous tonic GABA currents are decreased by transient increases in [GABA] and increased by transient depolarization. A: Membrane current recorded at –60 mV. Tonic currents were measured with bicuculline application (Bic, 10 μM) before and 5 s after exposure to exogenous GABA (1 μM). Horizontal bars indicate period of drug application in this and subsequent figures. B: Data enclosed in boxes from A displayed on expanded scale. The numbers correspond to labeled timepoints in A. The histograms (gray bars) and Gaussian fits (solid line) used to determine the mean holding current before and after bicuculline are illustrated, the histogram amplitudes were normalized to allow comparison of the distributions. Exogenous GABA reduced tonic current amplitudes. C: Tonic current measurement before and after exogenous GABA application paired with transient depolarization to +40 mV. Lower trace shows voltage clamp protocol. Same neuron as (A). D: Data enclosed in boxes from C displayed on expanded scale. The numbers correspond to timepoints in C. Transient depolarization potentiated tonic currents in contrast to the reduction seen with GABA alone. E: Tonic current amplitudes from experiment in A-D at baseline (pre-GABA) and after GABA application (post-GABA). F: Percent change in tonic current produced by exogenous GABA alone or GABA plus depolarization. Data are mean from 11 neurons. Error bars represent SEM in this and subsequent figures. *-p<0.05, **-p<0.01.
**Figure 2** Determination of GABA-induced shifts in $E_{GABA}$. A: GABA-evoked ramp currents. Ramp voltage commands were given at two time points in the absence (thin red line) and presence of exogenous GABA (thick red line). Point-by-point subtraction of these records was performed to obtain the GABA-evoked ramp currents. Note the reduction in holding current after application of GABA alone (arrow). Inset illustrates command potentials. B: GABA-evoked ramp currents from data in A. The reversal potential (i.e. $E_{GABA}$) was +1.1 mV after 2 s in GABA and shifted to −9.2 mV after 10 s in GABA, indicating a reduction of intracellular anions. The slope conductance changes are indicated, numbers correspond to labeled time points in A. C: GABA-evoked currents with transient depolarization. Experiment similar to A except a 5 s depolarization was given during GABA application between the two voltage ramps. D: GABA-evoked ramp currents before and after transient depolarization. $E_{GABA}$ shifted from −1.4 mV to +2.8 mV following transient depolarization. Slope conductances are indicated, numbers correspond to labeled time points in C.

**Figure 3** Tonic conductance was not affected by GABA alone but was increased by GABA plus depolarization. A: Average GABA- and depolarization-induced changes in $E_{GABA}$ determined from 8 neurons as in Figure 2, $E_{GABA}$ was measured after 2 s and 10 s of GABA application. B: Tonic conductance before and after GABA application. Tonic conductance was calculated using measurements of tonic current from experiments as in Fig. 1 and mean values for $E_{GABA}$ (see Methods). GABA alone did not significantly affect tonic
conductance. GABA plus depolarization produced an increase in tonic conductance. C: Comparison of observed shifts in $E_{GABA}$ to theoretical shifts that would be required to produce the observed tonic current changes, assuming no change in tonic conductance (i.e. assuming tonic current change was solely due to driving force alterations from shifts in $E_{GABA}$). With GABA application alone, there was no difference between the observed shift in $E_{GABA}$ and the theoretical value, indicating that tonic current reduction by GABA was caused by reduced driving force. With GABA plus depolarization, the theoretical $E_{GABA}$ shift was significantly larger than experimental observation, indicating that depolarization increased tonic conductance independent of driving force changes. n.s.-not significant, **-$p<0.01$, ***-$p<0.001$.

**Figure 4** Nonvesicular GABA release potentiated tonic currents. A: Endogenous tonic currents measured with Bic in a control neuron and a neuron pre-treated with concanamycin A (ConA, 1 $\mu$M for 2 hours). Right side of each panel show all-points histograms fit with a Gaussian function used to determine tonic current amplitude. B: Mean tonic current from control neurons and neurons treated with ConA. Filled circles represent individual measurements (n=5-7 cells for each condition). ConA treatment greatly reduced spontaneous synaptic currents but did not significantly affect tonic currents. C: Measurement of tonic currents before and after depolarization of presynaptic neurons with 12 mM $K^+$. Tonic currents measured 5 s after the $K^+$ application were increased and this potentiation was reversibly inhibited by the GAT1 antagonist SKF89976a (SKF, 40 $\mu$M). Shaded
area indicates timing of K\(^+\) application, solution exchange times were fast so it is expected that the wash of high K\(^+\) solution is complete prior to tonic current measurement. Dashed lines indicate baseline holding current for reference. Vesicular GABA release was inhibited by pretreatment with ConA, holding potential was \(-60\) mV. D: Tonic current amplitudes before (pre-K) and after (post-K) application of 12 mM K\(^+\) from experiment in (C). GAT1 antagonism increased baseline tonic current amplitude but also prevented the increase in tonic currents by K\(^+\)-induced nonvesicular release. E: Mean values for normalized tonic current under control conditions, with SKF, and wash (n=4). *- p<0.05.

**Figure 5** Nonvesicular GABA release occurred in acute hippocampal slices. A: Membrane currents of dentate gyrus granule cell in response to local pressure ejection of high [K\(^+\)] solution under control conditions and in the presence of Bic. B: Mean charge movement (Q) induced by K\(^+\) under control conditions and with Bic (n=5). C: Currents evoked with high K\(^+\) during bath application of SKF (40 \(\mu\)M). K\(^+\) induced currents were inhibited by SKF and this was partially reversible. D: Time course of holding current and K\(^+\)-induced peak currents for experiment in (C). GAT1 antagonism with SKF produced an increase in holding current (signifying an increase in tonic inhibition), simultaneous with a reduction in the K\(^+\)-induced current. These data indicate that in acute brain slices, similar to cultured neurons, GAT1 serves to limit ambient GABA concentration under basal conditions but also operates in reverse to release GABA during high K\(^+\) stimulation. E: Mean holding current under control conditions and with SKF (n=8
neurons). Because tonic currents were not directly measured with Bic application in these experiments, holding current is used as a surrogate measure of tonic current. F: Mean charge transfer (Q) produced by high K⁺ under control conditions and with SKF (n=8 neurons). *-p<0.05, **- p<0.01.

**Figure 6** Schematic of mechanisms rapidly regulating tonic GABA currents. A: Increased ambient [GABA] leads to GABAₐ receptor activation and anionic shifts, causing changes in E_{GABA} that will alter the polarizing influence of tonic inhibition without changing tonic conductance. B: Postsynaptic depolarization increases tonic conductance via intrinsic voltage-dependent modulation of GABAₐ receptors. This voltage-dependent increase in tonic conductance, or “post-depolarization potentiation,” outlasts the period of depolarization (Ransom et al. 2010). C: Presynaptic depolarization and intracellular Na⁺ accumulation affect the thermodynamics of GAT1, shifting the operation of GAT1 to cause nonvesicular GABA release and increase tonic conductance (Richerson and Wu 2003). In these experiments, vesicular GABA release was inhibited with ConA.
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Figure 1 Ransom et al.
Figure 2 Ransom et al.
Fig 3 Ransom et al.
Figure 4 Ransom et al.
Figure 6 Ransom et al.
A  Increased ambient GABA
presynaptic terminal

\[
\Delta [\text{Cl}^-, \text{HCO}_3^-] \\
\Delta E_{\text{GABA}}
\]

postsynaptic neuron
GAT1
GABA\textsubscript{A} receptor

B  Postsynaptic depolarization

resting V\textsubscript{m}
baseline tonic inhibition
depolarization voltage-dependent modulation of GABA\textsubscript{A} receptors
repolarization persistent increase of tonic inhibition (PDP)

C  Presynaptic depolarization

\[ \Delta \text{Vm}, [\text{Na}^+] \]

ConA

Figure 6 Ransom et al.