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Context-Dependent Modulation of GABA<sub>A</sub>R-Mediated Tonic Currents

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Tonic GABA currents mediated by high-affinity extrasynaptic GABA<sub>A</sub> receptors, are increasingly recognized as important regulators of cell and neuronal network excitability. Dysfunctional GABA<sub>A</sub> receptor signaling that results in modified tonic GABA currents is associated with a number of neurological disorders. Consequently, developing compounds to selectively modulate the activity of extrasynaptic GABA<sub>A</sub> receptors underlying tonic inhibition is likely to prove therapeutically useful. Here, we examine the GABA<sub>A</sub> receptor subtype selectivity of the weak partial agonist, 5-(4-piperidyl)isoxazol-3-ol (4-PIOL), as a potential mechanism for modulating extrasynaptic GABA<sub>A</sub> receptor-mediated tonic currents. By using recombinant GABA<sub>A</sub> receptors expressed in HEK293 cells, and native GABA<sub>A</sub> receptors of cerebellar granule cells, hippocampal neurons, and thalamic relay neurons, 4-PIOL evidentley displayed differential agonist and antagonist-type profiles, depending on the extrasynaptic GABA<sub>A</sub> receptor isoforms targeted. For neurons, this resulted in differential modulation of GABA tonic currents, depending on the cell type studied, their respective GABA<sub>A</sub> receptor subunit compositions, and critically, on the ambient GABA levels. Unexpectedly, 4-PIOL revealed a significant population of relatively low-affinity γ2 subunit-containing GABA<sub>A</sub> receptors in the thalamus, which can contribute to tonic inhibition under specific conditions when GABA levels are raised. Together, these data indicate that partial agonists, such as 4-PIOL, may be useful for modulating GABA<sub>A</sub> receptor-mediated tonic currents, but the direction and extent of this modulation is strongly dependent on relative expression levels of different extrasynaptic GABA<sub>A</sub> receptor subtypes, and on the ambient GABA levels.

Key words: 4-PIOL; extrasynaptic GABA receptors; GABA; partial agonist; synaptic inhibition; tonic inhibition

Significance Statement

A background level of inhibition (tonic) is important in the brain for controlling neuronal excitability. Increased levels of tonic inhibition are associated with some neurological disorders but there are no specific ligands capable of selectively reducing tonic inhibition. Here we explore the use of a GABA partial agonist as a selective chemical tool in three different brain regions. We discover that the activity of a partial agonist is heavily dependent upon the GABA<sub>A</sub> receptor subunit composition underpinning tonic inhibition, and on the ambient levels of GABA in the brain.

Introduction

GABA<sub>A</sub> receptors are the major inhibitory ligand-gated ion channels in the mammalian CNS. To date, eight classes of GABA<sub>A</sub> recep-
In particular, enhanced tonic currents, arising from elevated ambient GABA levels in the brain, are implicated in the pathology of absence seizures, cognitive disorders, and motor deficits following stroke (Cope et al., 2009; Clarkson et al., 2010; Jo et al., 2014; Wu et al., 2014). Consequently, emerging evidence indicates that antagonists and/or inverse agonists that selectively inhibit extrasynaptic α5- and/or δ-containing GABA_A receptors may prove therapeutically useful for such conditions (Navarro et al., 2002; Atack et al., 2006; Ballard et al., 2009; Cope et al., 2009; Atack, 2010; Clarkson et al., 2010; Braudeau et al., 2011; Martínez-Cué et al., 2013). Unfortunately, although several compounds selectively enhance δ- or α5-mediated tonic currents, such as THIP, DS2, AA29504, and Thio-4-PIOL (Storustov and Ebert, 2006; Wafford et al., 2009; Hoestgaard-Jensen et al., 2010, 2013; Jensen et al., 2013), only one compound (DPP-4-PIOL) appears to selectively inhibit δ-mediated tonic currents (Bodddum et al., 2014). Moreover, clinical trials involving α5-selective inverse agonists (α5IA and L-655,708) have foundered due to adverse effects (Rudolph and Mohler, 2014).

As an alternative to GABA_A receptor antagonists, we hypothesized that low-efficacy partial agonists may act as “functional antagonists”, given their ability to compete with GABA for the orthosteric binding site, and their reduced ability to activate GABA_A receptors. Moreover, low-efficacy partial agonists may be less likely to induce convulsions, or unwanted side effects (Krogsgaard-Larsen et al., 2002). Here the subtype-selective profile of the low-efficacy partial agonist, 5-(4-piperidyl)isoxazol-3-ol (4-PIOL; Kristiansen et al., 1991; Frølund et al., 1995; Mortensen et al., 2002, 2004) was assessed on recombinant and neuronal GABA_A receptors. The activity profile of 4-PIOL on tonic and phasic currents varied between three selected brain regions. This revealed a strong dependence on GABA_A receptor subunit composition, and on ambient GABA levels, and helped uncover a population of largely silent GABA_A receptors in the thalamus that can contribute to tonic inhibition under specific conditions. Overall, partial agonists may be useful as therapeutic agents, but their effectiveness will critically depend on which GABA_A receptor isoforms are present and the extent of their activation.

Materials and Methods

Transient receptor expression in HEK293 cells. Human embryonic kidney (HEK) 293 cells were cultured in DMEM, as previously described (Woolston et al., 1997). HEK cells were plated onto poly-l-lysine-coated coverslips and transfected using a calcium phosphate protocol. Briefly, GABA_A receptor pK5 cDNAs, with enhanced green fluorescent protein (eGFP) cDNA, were mixed with 340 mM CaCl_2 and an equal volume of HEPES-buffered saline (50 mM HEPES, 280 mM NaCl and 2.8 mM Na_2HPO_4, pH 7.2). One microgram of each cDNA was used, and a total of 4 μg cDNA was used for each transfection. The cdNA-calcium phosphate suspension was applied to cells, which were incubated overnight.

Culturing hippocampal neurons. Hippocampal cultures were prepared from E18 rat embryos, as previously described (Thomas et al., 2005). Cells were dissociated, as above, before plating onto glass coverslips coated with 100 μg/ml poly-o-lysine (Sigma-Aldrich) in minimum essential media (Invitrogen) supplemented with 5% v/v FCS, 5% v/v HS, 10 U/ml penicillin-G, 10 μg/ml streptomycin, 2 mM L-glutamine, and 20 mM glucose. After 2 h, the media was replaced with Neurobasal-A (Invitrogen), supplemented with 1% v/v B-27 (Invitrogen), 50 U/ml penicillin-G, 50 μg/ml streptomycin, 0.5% v/v Glutamax (Invitrogen), and 35 mM glucose. Electrophysiological recordings were performed between 11 and 21 DIV.

Acute brain slice preparation. Young rats (P14) were terminally anesthetized with isoflurane. The brain was rapidly removed and immersed in ice-cold slicing solution composed of the following (in mM): 130 K-glucionate, 15 KCl, 0.05 EGTA, 20 HEPEs, 4 Na-pyruvate, 25 glucose, and 2 kynurenic acid, pH 7.4. Coronal (thalampus and hippocampus) or parasagittal (cerebellum) slices (all 250 μm) were made using a Leica VT 1200s vibrisser, and transferred to a holding chamber incubated at 37°C. The solution was slowly exchanged for artificial CSF (aCSF) containing the following (in mM): 125 NaCl, 2.5 KCl, 1.25 Na_2HPO_4, 26 NaHCO_3, 2 CaCl_2, 1 MgCl_2, 25 glucose, and 2 kynurenic acid (pH 7.4 when bubbled with 95% O_2 and 5% CO_2). Slices were maintained in the holding chamber at room temperature until required for electrophysiology.

Drug solutions. For HEK293 cells, GABA was applied alone, or in combination with other drugs using a Y-tube application system (Mortensen and Smart, 2007). The 10–90% solution exchange times of the application system were within 20–30 ms as measured in open pipette tip recordings. GABA and bicuculline were obtained from Sigma-Aldrich, THIP and DS2 were purchased from Tocris Biosciences, and dazepamin was sourced from Roche. Drug solutions were either prepared from water or DMSO concentrated stocks, or dissolved directly into the extracellular medium, depending on the final concentration. Drug solutions were corrected for pH before use.

Data acquisition. Whole-cell currents were recorded using an Axopatch 200A amplifier. Currents were filtered at 5 kHz, digitized at 50 kHz via a Digidata 1332A (Molecular Devices), and recorded to disk (Dell Pentium Dual Core-Optiplex 960). Series resistances were monitored throughout each experiment and deviations >20% resulted in the data being excluded from further analysis.

HEK293 cell electrophysiology. HEK293 cells were continuously perfused with Krebs solution containing the following (in mM): 140 NaCl, 4.7 KCl, 1.25 MgCl_2, 2.52 CaCl_2, 11 glucose, and 5 HEPES, adjusted to pH 7.4 with 1 mM NaOH. Path pipettes were fire polished to 2–4 MΩ and filled with an intracellular solution containing the following (in mM): 125 KCl, 1 MgCl_2, 11 EGTA, 10 HEPEs, 1 CaCl_2, and 2 adenosine triphosphate, adjusted to pH 7.2 with 1 mM NaOH. HEK293 cells were voltage-clamped between −20 and −60 mV, depending on peak current size. Analysis of GABA concentration–response curves. GABA-activated currents (I_GABA) were normalized to the maximal current evoked by a saturating concentration of GABA (I_max,GABA). The normalized concentration–response curves were fitted using a modified Hill equation (Eq. (1)), using a least-squares method.

$$I_{GABA} = I_{Max_GABA} \times \frac{[GABA]^{nH}}{EC_{50}^{nH} + [GABA]}$$

EC_{50} is the concentration of GABA, [GABA], which produced 50% of the maximal response, and n_H is the Hill coefficient. I_{Min,GABA} is the minimum “plateau” response induced in the presence of 4-PIOL. For the GABA concentration–response curve constructed in the absence of 4-PIOL, I_{Min,GABA} is zero. The parameters obtained from individual curve-fittings were collated and the data were expressed as the mean ± SEM.

Neuronal whole-cell electrophysiology. Thalamic relay neurons of the dorsal lateral geniculate nucleus (dLGN), CA1 pyramidal neurons in the hippocampus, and CGCs were visualized using infrared differential interference contrast optics and a Basler scA750–60fm camera. Cells were perfused with aCSF or Krebs, supplemented with 2 mM kynurenic acid, or a mixture of 20 μM D-AP5 (Tocris Bioscience) and 10 μM CNQX.
Concentration of bicuculline (20 μM) were also present throughout the recordings. A saturating concentration of Na-ATP, and 2 QX-314 bromide. pH was adjusted using 1M CsOH. In a patch pipettes (2–4 MΩ; Abcam) to block glutamatergic currents. Recordings were made using Functional properties of recombinant α1β3γ2 GABAA receptors activated by 4-PIOL. A, Representative whole-cell current traces elicited by GABA in the presence of preapplied 4-PIOL (1 mM), for α1β3γ2-expressing HEK293 cells. In this, and subsequent figures, the horizontal bars indicate the duration of drug applications; here representing GABA (black) and 4-PIOL (green) applications. B, Mean peak GABA concentration–response curves constructed in the absence (●), or presence of 10 μM (▲), 100 μM (■) or 1000 μM (▲) 4-PIOL (n = 4–13; mean ± SEM). The peak response to each concentration of GABA was measured in relation to the holding current before 4-PIOL preapplication, and each dataset was normalized to the maximum response achieved by a saturating concentration of GABA, in the absence of 4-PIOL. The normalized concentration–response curves were fitted using a modified Hill equation (black and colored lines), to account for the elevated curve minima induced by 4-PIOL. The red hatched boxes represent concentrations of GABA proposed to exist at extrasynaptic (100 nM–1 μM) and synaptic sites (>1 mM). C, An expanded part of the GABA concentration–response curves presented in B. Note the elevated curve minima induced by 100 and 1000 μM 4-PIOL. D, Example whole-cell current traces elicited by 1 μM GABA in the absence, or presence of preapplied 10 μM 4-PIOL (blue bar).

For tonic currents, the average holding current for a 30 s epoch in each drug condition was measured using WinEDR software (v3.1; John Dempster, University of Strathclyde, Glasgow, UK). Changes in holding current were calculated by subtracting the average holding current after drug application, from the average holding current before drug application. In addition, the root mean square (rms) baseline noise was measured over a 30 s epoch, sampled every 100 ms. Because sIPSCs increase rms baseline noise, Microsoft Excel was used to calculate a threshold for eliminating contaminated 100 ms epochs. A running threshold (routinely the median) was calculated at 5 s time intervals, over a 30 s recording period, and any rms value greater than the calculated threshold, was automatically excluded from further analysis. Effective thresholding was validated by manually analyzing a small section of each recording (~10 s), and manually eliminating 100 ms epochs contaminated by synaptic currents.

Synaptic current analysis. The sIPSC frequency was determined using MiniAnalysis software (Synaptosoft). During 4-PIOL application, a significant increase in rms current noise was observed that might mask smaller sIPSCs, thereby introducing a bias toward larger events, compared with control. To limit the bias, only the largest hundred amplitude events from each condition were compared. The average decay kinetics for sIPSCs for each cell was determined by fitting uncontaminated events (>50 events for each condition) with either a mono- or bi-exponential decay function. To combine data obtained for mono- or bi-exponentially fitted events, decay times were transformed to a weighted decay time, τw, according to the following:

\[ \tau_w = \frac{(A1 \cdot \tau_1 + A1 \cdot \tau_2)}{(A1 + A2)} \]  

where τ1 and τ2 represent decay time constants, and A1 and A2 are the relative amplitude contributions of τ1 and τ2. For mono-exponential decaying events, A2 and τ2 are zero. The mean sIPSC frequency, amplitude, 10–90% rise time, and τw were calculated for each cell.

Results
Functional properties of synaptic-type GABAA receptors activated by a partial agonist
To evaluate the functional profile of 4-PIOL at synaptic-type GABAA receptors, peak whole-cell GABA currents were recorded from α1β3γ2-expressing HEK293 cells, in the absence or presence of 10, 100, or 1000 μM 4-PIOL (Fig. 1A). At these concentrations, 4-PIOL induced a rightward shift in the GABA concentration–response curve, with a discernible crossover with the control GABA curve (Fig. 1B). The GABA EC50 for α1β3γ2 receptors was increased from 4.5 ± 1.9 μM in control, to 9.4 ± 3.4, 15.4 ± 5.4, and 126.7 ± 55.6 μM in the presence of 10, 100, and 1000 μM 4-PIOL, respectively. Pre-application of 4-PIOL to
Table 1. sIPSC parameters for cultured cerebellar granule and hippocampal neurons

|                   | CGCs                  | Hippocampal neurons |
|-------------------|-----------------------|---------------------|
|                   | Control (+ 4-PIOL)    | Control (+ 4-PIOL)  |
| Frequency, Hz     | 7.2 ± 1.0             | 6.9 ± 1.7           |
| Amplitude, pA      | 409 ± 113             | 361 ± 101           |
| 10 – 90% rise time, ms | 1.1 ± 0.1           | 2.0 ± 0.3           |
| Decay tau, ms      | 17.7 ± 1.6            | 31.4 ± 3.0          |

All data are presented as mean ± SEM (n = 5–6). Control and 4-PIOL data were compared using a paired t-test.

*p < 0.05, **p < 0.01.

α1β3γ2-expressing cells revealed a small agonist response, particularly with 100 or 1000 μM 4-PIOL (Fig. 1A). This agonist activity appeared on the concentration–response curves by an elevated minimum response (Fig. 1B, C) with 100 and 1000 μM 4-PIOL inducing agonist currents that were 5.5 ± 3.2% and 6.8 ± 1.3% of the maximum GABA response (Fig. 1C). Crucially for this synaptic GABAA receptor subtype, neither 10 nor 100 μM 4-PIOL inhibited the maximum responses to higher, synaptic concentrations of GABA (percentage control: 98.8 ± 1.2% and 98.7 ± 1.3% for 10 and 100 μM 4-PIOL at 1 mM GABA; Fig. 1D), indicating that at these concentrations of 4-PIOL, the partial agonist is potentially capable of

Figure 2. Functional effects of 4-PIOL on steady-state GABA currents. Representative whole-cell currents induced by long applications of 0.1 and 1 μM GABA to α4β2δ (A), α6β2δ (B), α5β3γ2 (C), and α1β3γ2 (D) receptors. 4-PIOL (10 μM) was briefly coapplied once a steady-state GABA current was achieved. E, Quantitative analysis of data depicted in A–D. Data points for α1β3γ2 (↑), α5β3γ2 (●), α6β2δ (●), and α4β2δ (●) receptors are mean ± SEM (n = 4–5 cells). For each concentration (0.1, 0.3, and 1 μM) of preapplied GABA, the change in holding current produced by 4-PIOL is expressed as a percentage of the steady-state current to GABA alone. Negative values represent an inhibition of the steady-state GABA current.
inhibiting responses to predicted extrasynaptic concentrations of GABA, without depressing synaptic GABA currents.

**Functional properties of extrasynaptic-type GABA<sub>δ</sub> receptors activated by a partial agonist**

Given the minimal efficacy displayed by 10 μM 4-PIOL at α1βγ2 receptors, and its potential to not inhibit synaptic currents in neurons, we further characterized the effects of 10 μM 4-PIOL on extrasynaptic-type GABA<sub>δ</sub> receptors expressed in HEK293 cells. Low ambient concentrations of GABA (0.1, 0.3, and 1 μM), were preapplied to α4βδ<sub>2</sub>, α6βδ<sub>2</sub>, and α5β3γ2-expressing HEK293 cells, until a steady-state response was achieved, and subsequently, GABA was coapplied with 10 μM 4-PIOL (Fig. 2A–C).

For α4βδ<sub>2</sub> receptors, coaplication of 4-PIOL significantly inhibited the steady-state GABA current for 0.1, 0.3, and 1 μM GABA by 70.6 ± 2.7%, 56.1 ± 4.5%, and 31.5 ± 1.3%, respectively (Fig. 2A, E; p = 0.03, 0.04, and 0.02, respectively). By contrast, only a modest inhibition of steady-state currents was produced by 4-PIOL at recombinant α6βδ<sub>2</sub> receptors for the lowest concentrations of GABA (by 26.5 ± 3.9% and 20.9 ± 3.0 for 0.1 and 0.3 μM; p = 0.007 and 0.006; Fig. 2B), but not for 1 μM GABA (percentage inhibition: 9.6 ± 4.0%; p = 0.31). For recombinant α5β3γ2 receptors, the functional profile of 10 μM 4-PIOL varied, depending on the ambient GABA concentration (Fig. 2C). 4-PIOL enhanced the steady-state current elicited by 0.1 μM GABA (by 23.0 ± 1.5%; p = 0.004), was ineffective at 0.3 μM GABA (a 1.5 ± 0.8% enhancement; p = 0.2), and produced a small inhibition (6.8 ± 0.9%; p = 0.02) of steady-state GABA currents induced by 1 μM GABA.

In addition, the effects of 4-PIOL were assessed on α1β3γ2 receptors at low extrasynaptic GABA concentrations (Fig. 2D), because their presence at extrasynaptic sites has also been suggested in several neuronal cell types (Nusser et al., 1998; Mangan et al., 2005; Thomas et al., 2005; Kasai et al., 2010). 4-PIOL significantly enhanced the response to 0.1 μM GABA by 76.9 ± 21.5% (Fig. 2E; p = 0.02). However, when coapplied with 1 μM GABA, 4-PIOL produced a small inhibition of the steady-state GABA current (12.9 ± 3.3%; Fig. 2E; p = 0.05).

There is some evidence for the expression of native αβ receptors lacking either a γ or δ subunit at extrasynaptic sites in cerebellar and hippocampal neurons (Brickley et al., 1999; Mortensen and Smart, 2006). Therefore, we also assessed the effects of 4-PIOL on α1β3 receptors. Similar to the δ-containing receptors, 10 μM 4-PIOL showed no agonist behavior at these receptors, but instead inhibited steady-state currents activated by low GABA concentrations (by 69 ± 7%, 75 ± 2%, and 75 ± 2% at 0.1, 0.3, and 1 μM GABA, respectively).

Overall, these data indicate that 10 μM 4-PIOL may potentially inhibit α4βδ<sub>2</sub>-mediated tonic currents, without affecting α1β3γ2-mediated phasic currents. Moreover, 10 μM 4-PIOL is expected to produce only a modest effect on α6βδ<sub>2</sub>-mediated tonic currents (eg, in the cerebellum), whereas any inhibition of extrasynaptic γ2-containing receptors, although minimal, will be strongly dependent on the ambient GABA concentration in neuronal preparations. Although our earlier studies suggested that αβ receptors represent only a modest component of the extrasynaptic GABA<sub>δ</sub> receptor population in hippocampal pyramidal cells (~10%; Mortensen and Smart, 2006), their contribution to the tonic current will be inhibited by 10 μM 4-PIOL.

**Examining GABA<sub>δ</sub> receptor-mediated tonic and phasic inhibition with a partial agonist**

As our observations indicated that 4-PIOL may have differential effects on synaptic and extrasynaptic GABA<sub>δ</sub> receptors, we chose to explore the functional profile of 4-PIOL on tonic and phasic GABA currents in neurons. Initially, whole-cell recordings were
performed on cultured hippocampal and cerebellar granule (CGC) neurons. Under control recording conditions, a high frequency of sIPSCs were recorded from CGCs (7.2 ± 1.0 Hz) and hippocampal neurons (6.9 ± 1.7 Hz; Table 1), which displayed mean sIPSC amplitudes of 409 ± 113 and 361 ± 101 pA (Table 1), respectively. A saturating concentration of bicuculline (20 μM) was applied at the end of the recordings to confirm that all synaptic events were GABAergic, and to measure the amplitudes of GABA_A receptor-mediated tonic currents. As expected, for both cell types, bicuculline abolished the sIPSCs and induced outward shifts in the membrane holding current, indicative of GABA tonic currents (Fig. 3A, C). For CGCs and hippocampal neurons, the basal tonic current amplitudes were as follows: 22.8 ± 8.5 and 42.8 ± 6.3 pA (Fig. 3E; Table 2).

To assess the effects of 4-PIOL on the tonic and phasic currents, 10 μM 4-PIOL was applied to cells following a period of control recording (Fig. 3B, D). For CGCs, 4-PIOL showed a very small tendency to enhance the tonic current by 9.8 ± 4.0 pA (Fig. 3B, F), however, this was not statistically significant (p = 0.058). 4-PIOL exerted no significant effect on sIPSC amplitude (percentage control: 93.2 ± 14.9; p = 0.66), rise time (percentage control: 107 ± 4.1; p = 0.24), or frequency (percentage control: 64.0 ± 14.2; p = 0.057) in CGCs (Table 1).

By comparison, in hippocampal neurons, 10 μM 4-PIOL significantly enhanced the GABA-mediated tonic current by 72.0 ± 14.2 pA (p = 0.0025; Fig. 3D, F). Given that 10 μM 4-PIOL did not inhibit the synaptic-type responses of recombinant α1β2 receptors, it was unexpected that 4-PIOL also significantly inhibited both the frequency (percentage control: 49.8 ± 9.5%; p = 0.002) and the amplitude of sIPSCs (percentage control: 58.6 ± 16.8%; p = 0.047, Wilcoxon matched pairs test), but did not affect the rise time (percentage control: 110 ± 12.1; p = 0.50) or τ_w (percentage control: 100 ± 3.4; p = 0.75) of sIPSCs (Table 1).

For both types of cultured neurons, we used the δ-subunit selective agonist, THIP, to assess the presence of δ-containing GABA_A receptors. Application of 1 μM THIP evoked significant inward currents, confirming the surface expression of extrasynaptic δ-GABA_A receptors in the cultured cell preparations.
Table 4. 4-PIOL and tonic currents for cerebellar granule, CA1 hippocampal, and dLGN relay neurons in acute slices

|                        | CGCs          | CA1 pyramidal neurons | dLGN relay neurons |
|------------------------|---------------|-----------------------|--------------------|
|                        |               | Inward 4-PIOL         | Outward 4-PIOL     |                        |
| 4-PIOL current, pA     | 0.05 ± 1.44   | −24.7 ± 5.7**         | 7.8 ± 1.6**        | −57.4 ± 3.3**         |
| (pA/pF)                | (0.18 ± 0.38) | (−0.20 ± 0.06)        | (0.05 ± 0.01)      | (−0.34 ± 0.03)        |
| Tonic current, pA      | 9.8 ± 4.3     | 13.5 ± 1.7***         | 24.2 ± 4.4***      | 23.8 ± 2.1***         |
| (pA/pF)                | (2.57 ± 1.76) | (0.10 ± 0.01)         | (0.33 ± 0.03)      | (0.13 ± 0.01)         |

Data shown are the mean currents (± SEM, n = 7–42) evoked by 10 μM 4-PIOL and 20 μM bicuculline (tonic current). According to normal convention, inward and outward currents have negative and positive polarities, respectively. Currents normalized to cell capacitance (pA/pF) are shown in parentheses below the absolute values. Values for CA1 pyramidal neurons are divided according to direction of 4-PIOL response. Statistical significance was assessed using a paired t test. *p < 0.05, **p < 0.01, ***p < 0.001.

Figure 5. THIP and bicuculline modulation of 4-PIOL currents. A. Representative membrane current recorded from a dLGN relay neuron (left) in response to applied 4-PIOL (10 μM), in the absence or presence of BIC (20 μM). The bar chart (right) depicts the mean ± SEM (n = 4) for the 4-PIOL current in BIC (white) expressed as a percentage of the control 4-PIOL response (gray) recorded from the same dLGN relay neurons. B. Membrane currents recorded from a α4β2δ-expressing HEK293 cell in response to THIP (1 μM, blue bar) and 4-PIOL (10 μM, gray bar). Bar chart shows the steady-state THIP current in the absence or presence of 4-PIOL (n = 4). Significance was assessed using a paired t test, *p < 0.05. C. Membrane currents recorded from a dLGN relay neuron in response to THIP (1 μM) and 4-PIOL (10 μM). Bar chart shows the magnitude of the 4-PIOL current in the absence or presence of THIP (n = 5).

(CGCS: 22.4 ± 8.7 pA, n = 9; hippocampal neurons: 40.8 ± 5.8 pA, n = 16).

To characterize the effects of 4-PIOL under more physiological conditions, we recorded from CGCS, hippocampal CA1 pyramidal neurons and thalamic relay neurons of the dorsal lateral geniculate nucleus in acute brain slices. These three cell types were selected because their GABA<sub>A</sub> receptor-mediated tonic currents are thought to be mediated largely by α6β2δ, α5β2γ, and α4βδ GABA<sub>A</sub> receptors, respectively (Jones et al., 1997; Brickley et al., 2001; Caraiscos et al., 2004; Cope et al., 2005; Glynys et al., 2008).

Application of bicuculline (20 μM) blocked the sIPSCs (parameters shown in Table 3) and revealed GABA<sub>A</sub> receptor-mediated tonic currents in all three cell types (Fig. 4A, C, E, G). For CGCS, hippocampal neurons and dLGN relay neurons, the basal tonic current amplitudes were as follows: 9.8 ± 4.3, 19.2 ± 2.7, and 23.8 ± 2.1 pA (Fig. 4G; Table 4).

As with the cultured cells, 10 μM 4-PIOL had little impact on the tonic current recorded from CGCS in slices (change in holding current 0.05 ± 1.44 pA; Fig. 4B, H). However, in contrast to the cultured cells, 4-PIOL significantly inhibited both the frequency (percentage control: 76.7 ± 6.1%; p = 0.006; Table 3) and the amplitude of sIPSCs (percentage control: 71.2 ± 4.2%; p = 0.0002, Wilcoxon matched-pairs test), but did not affect the rise time (percentage control: 101.6 ± 3.3; p = 0.63) or decay τ<sub>w</sub> (percentage control: 105.6 ± 5.2; p = 0.32).

By comparison, in hippocampal CA1 pyramidal neurons, 10 μM 4-PIOL evoked a dichotomous response, with cells either displaying an inward current (−24.7 ± 5.7 pA, n = 7, p = 0.002) or a small, but significant outward current (7.8 ± 1.6 pA, n = 8, p = 0.001; Fig. 4D, H). Interestingly, when we divided cells according to their 4-PIOL response, we found that neurons showing an outward current had a larger basal tonic current than those cells producing an inward 4-PIOL current (outward 24.2 ± 4.4 pA, inward 13.5 ± 1.1 pA, p = 0.04, unpaired t test; Fig. 4G). Dividing cells in this way, also revealed a difference in the modulation of synaptic inhibition by 4-PIOL. For the inward current cohort, 4-PIOL caused a significant reduction in IPSC frequency (percentage control: 54.2 ± 8.1%; p = 0.04), but had no significant effect on synaptic GABA release for the outward current cohort (percentage control: 150.0 ± 43.0%; p = 0.91; Table 3).

Applying 4-PIOL to dLGN slices significantly enhanced tonic currents by 57.4 ± 3.3 pA (Fig. 4F, H), and notably reduced both the frequency (percentage control: 13.3 ± 2.2%; p = 0.001) and amplitude of sIPSCs (percentage control: 71.8 ± 2.7%; p = 0.01; Table 3), relative to synaptic events measured in control aCSF.
Because of the low frequency and amplitude of sIPSCs in the presence of 4-PIOL, and the increase in rms baseline noise induced by 4-PIOL (13.2 ± 1.6 pA), no detailed analysis of sIPSC decay or rise times was performed for dLGN relay neurons.

To further investigate the modulation of synaptic inhibition by 4-PIOL, we repeated these experiments in the presence of TTX (500 nm) to block action potential-dependent GABA release. Under these conditions, dLGN relay neurons displayed a smaller basal tonic current compared with aCSF control recordings (TTX: 13.5 ± 1.7 pA; aCSF: 23.8 ± 2.1 pA; p = 0.0005, unpaired t test) consistent with a reduced ambient GABA concentration (Bright et al., 2007). Application of 10 μM 4-PIOL induced a similar increase in tonic current as in aCSF (TTX: 43.2 ± 7.0 pA; aCSF: 57.4 ± 3.3 pA; p = 0.09, unpaired t test) but had less effect on IPSC frequency (percentage control: 76.5 ± 9.3%; p = 0.03) and amplitude (percentage control: 89.7 ± 4.7%; p = 0.04, n = 9).

Probing the identity of GABAA receptors in dLGN relay neurons
According to our recombinant expression studies, 10 μM 4-PIOL showed no discernible agonist activity at α4β5δ receptors, and was predicted to reduce GABA-mediated tonic currents at these receptors, assuming that the ambient GABA concentration in slices is ~0.1–1 μM GABA (Fig. 2E). Because tonic currents in dLGN relay neurons are thought to be mediated by α4β5δ receptors (Cope et al., 2005; Bright et al., 2007; Nani et al., 2013; Ye et al., 2013), the finding that 4-PIOL showed agonist behavior, by enhancing tonic currents in dLGN relay neurons, was unexpected. To verify the GABAergic origin of this current, we used bicuculline. Current responses to 10 μM 4-PIOL were abolished in the presence of coapplied bicuculline (percentage control 4-PIOL response: 2.2 ± 0.5; Fig. 5A), indicating that 4-PIOL was exclusively activating GABAA receptors to induce an inward current.

To investigate whether the 4-PIOL current in dLGN relay neurons was mediated by δ-containing GABAA receptors, we assessed the ability of 4-PIOL to compete with a δ subunit-selective concentration of the GABA<sub>A</sub> receptor agonist, THIP (1 μM; Brown et al., 2002; Störustov and Ebert, 2006; Mortensen et al., 2010). If THIP and 4-PIOL compete for the same orthosteric binding site, we might expect the THIP-induced currents to be reduced by 4-PIOL, given the lower efficacy and potency displayed by 4-PIOL. THIP (1 μM) was preapplied to recombinant α4β2δ receptors expressed in HEK293 cells, until a steady-state current was achieved, and subsequently 10 μM 4-PIOL was coapplied with THIP (Fig. 5B). Indeed, under these conditions, 4-PIOL reduced the steady-state THIP current, from 202.3 ± 70.7 to 68.2 ± 30.1 pA (Fig. 5B; p = 0.02), suggesting that 4-PIOL is competing with THIP for the orthosteric binding site.

If 4-PIOL is acting on δ-containing receptors in dLGN relay neurons, we might also expect 4-PIOL (10 μM) to reduce the steady-state THIP (1 μM) current in dLGN relay neurons. As expected, THIP significantly enhanced the dLGN tonic current by 96.6 ± 12.6 pA, confirming the functional expression of δ subunit-containing receptors. However, coapplication of 4-PIOL with THIP generated a further inward current (58.6 ± 6.6 pA; Fig. 5C), with a mean magnitude that was similar to the control 4-PIOL current (57.4 ± 3.3 pA; p = 0.67). These data indicate that THIP and 4-PIOL may not be competing for the same δ subunit-containing receptors in dLGN relay neurons.

To further probe whether the 4-PIOL current in dLGN relay neurons was mediated by δ-containing receptors, we investigated whether the 4-PIOL current could be modulated by the δ subunit-selective positive allosteric modulator, DS2 (Wafford et
Previously, the modulatory actions of DS2 have only been characterized on GABA-mediated currents (Wafford et al., 2009; Jensen et al., 2013), and not on responses evoked by other GABA<sub>A</sub> receptor agonists. To investigate, we monitored the effect of 10 μM DS2 on whole-cell 4-PIOL currents using recombinant α4β2δ (Fig. 6B) and α1β3γ2 (Fig. 6C) receptors, expressed separately in HEK293 cells. Under control conditions, 4-PIOL (10 μM) elicited no discernible agonist response at α4β2δ receptors (Fig. 6B), but induced a small inward current at α1β3γ2 receptors (Fig. 6C). However, coapplying DS2 with 4-PIOL, unveiled an agonist current at α4β2δ receptors, which was 8.9 ± 3.8% of the response to 1 mM GABA in the same cell (Fig. 6B). Unexpectedly, DS2 also potentiated the 4-PIOL current mediated at α1β3γ2 receptors (percentage control 4-PIOL current: 153.8 ± 24.7%; Fig. 6C; p = 0.017), albeit to a lesser extent than that observed at α4β2δ receptors. These findings complicate the interpretation of DS2-mediated potentiation of the 4-PIOL current in dLGN relay neurons, because DS2 may be modulating a population of γ2-containing GABA<sub>A</sub> receptors, or generating a δ-mediated component to the 4-PIOL current, which may not be present under control conditions.

Because 10 μM 4-PIOL activated γ2-containing receptors, but not δ-containing receptors in our recombinant expression studies, we explored the possibility that 4-PIOL was activating a population of γ2-containing receptors in dLGN relay neurons. The presence of γ2-containing receptors was examined using the benzodiazepine agonist, diazepam. Preapplication of 500 nM diazepam significantly increased the dLGN tonic current, giving rise to a bicuculline-sensitive tonic current (52.0 ± 5.6 pA) that was significantly greater than that measured in control aCSF (23.8 ± 2.1 pA; Fig. 7A; p = 0.003). Diazepam also increased the amplitude of the IPSG suggesting that GABA release at these thalamic inhibitory synapses was not saturating. Co-application of 4-PIOL with diazepam revealed a significantly larger inward current than the control 4-PIOL current (percentage control 4-PIOL response: 160.3 ± 14.3%; Fig. 7A; p = 0.003), indicating that a substantial component of the 4-PIOL current is most likely mediated by γ2-containing receptors in dLGN relay neurons.

To confirm that diazepam would only potentiate the agonist responses of γ2 subunit-containing GABA<sub>A</sub> receptors (Pritchett et al., 1989), whole-cell currents were recorded from recombinant α1β3γ2 receptors (Fig. 7B) or α4β2δ (Fig. 7C) receptors in response to brief applications of 4-PIOL (10 μM) in the absence or presence of preapplied diazepam (500 nM). At α1β3γ2 receptors, diazepam significantly potentiated 4-PIOL responses (percentage control response: 246.6 ± 50.1; Fig. 7B; p = 0.03); and, as expected, 4-PIOL (10 μM) elicited no discernible agonist response at α4β2δ receptors, either in the absence or presence of diazepam (Fig. 7C). A saturating concentration of GABA (1 mM) was applied to each α4β2δ-expressing cell, to confirm the functional expression of α4β2δ receptors (Fig. 7C).

Together, these data indicate that although δ subunit-containing receptors are expressed in dLGN relay neurons, as confirmed by THIP and DS2 modulation of basal tonic currents, the 4-PIOL current appears to be largely mediated by γ2-containing receptors, with little, or no, contribution from δ subunit-containing receptors.

Given these findings, we explored which GABA<sub>A</sub> receptor isoforms underpin tonic currents in dLGN relay neurons. Since tonic currents in these cells are thought to be mediated by δ...
subunit-containing receptors (Cope et al., 2005; Bright et al., 2007; Nani et al., 2013; Ye et al., 2013), we investigated whether there was a correlation between currents induced by a δ-selective concentration of THIP (1 μM) and by bicuculline, for individual dLGN relay neurons. 4-PIOL and bicuculline were individually applied (Fig. 8A), and to account for variation in cell size, the holding currents were normalized to whole-cell capacitance (pF). A scatter plot comparing current densities revealed a positive correlation (Fig. 8B; r = 0.61; p = 0.02). Thus, cells with a larger THIP-induced current also displayed larger GABA$_\text{A}$ receptor-mediated tonic currents. These data indicate that a higher expression of δ subunit-containing receptors may underlie the larger tonic currents, although other factors, such as the ambient GABA level, will also be important.

If, as our previous data indicates, THIP and 4-PIOL are acting at potentially different GABA$_\text{A}$ receptors (Fig. 5), no positive correlation would be expected between the THIP and 4-PIOL responses in dLGN relay neurons. Instead, a scatter-plot of THIP and 4-PIOL currents revealed no significant correlation (Pearson’s correlation coefficient, r = −0.31; p = 0.28), supporting the notion that 4-PIOL was activating a distinct receptor population from the δ-containing receptors activated by THIP (Fig. 8C).

Because 4-PIOL and THIP are likely to be acting on different GABA$_\text{A}$ receptors in dLGN relay neurons, it was intriguing to explore whether the γ2-containing receptors that mediate the 4-PIOL current, also contribute to dLGN tonic currents. A scatterplot comparing 4-PIOL and bicuculline currents recorded from individual dLGN relay neurons showed poor correlation (Fig. 8D; r = 0.32; p = 0.17), indicating that the receptors that mediate the 4-PIOL current, are unlikely to contribute substantially to basal GABA$_\text{A}$ receptor-mediated tonic currents in dLGN relay neurons.

**Modulation of tonic currents depends on ambient GABA levels**

For recombinant α1β3γ2 receptors, 4-PIOL enhanced the steady-state GABA current when the GABA concentration was low (~0.1 μM GABA), but produced a small inhibition when the ambient GABA level was raised to 1 μM (Fig. 2D). In dLGN relay neurons, the robust 4-PIOL enhancement of baseline tonic currents (mainly via γ2 subunit-containing receptors) indicates that the ambient GABA levels in this slice may be low (<1 μM).

To determine whether 4-PIOL could switch from acting as an agonist (at low ambient GABA levels), to acting as an antagonist (at higher ambient GABA levels) in the native environment of dLGN relay neurons, GABA levels were raised in slices by inhibiting GABA uptake. Because GABA uptake in the thalamus is mediated by the GABA transporters, GAT1 and GAT3 (De Biasi et al., 1998), slices were preincubated (for 30 min) and subsequently recorded in aCSF supplemented with the GAT1 inhibitor, 10 μM NNC-711 (Borden et al., 1994) and the GAT2/3 inhibitor, 20 μM SNAP-5114 (Borden, 1996). Following a period of control recording (in the presence of the GAT inhibitors), 10 μM 4-PIOL was applied to dLGN relay neurons and subsequently washed out (Fig. 9A), before application of bicuculline to measure the GABA-mediated tonic current. As expected, the tonic current was significantly larger in the presence of the GAT blockers compared with control aCSF (132 ± 19.5 and 23.8 ± 2.1 pA, respectively; Fig. 9B), even when these currents were normalized to cell capacitance (0.7 ± 0.1 pA/pF and 0.1 ± 0.01 pA/pF, respectively; p < 0.0001). These data are consistent with elevated ambient GABA levels, in GAT-blocked slices, increasing the activation of extrasynaptic GABA$_\text{A}$ receptors.

Coapplication of 10 μM 4-PIOL with GAT inhibitors enhanced the tonic current by 53.0 ± 10.1 pA (Fig. 9A,C). This 4-PIOL-induced current was similar to that observed in control aCSF (57.4 ± 3.3 pA; Fig. 9C; p = 0.47), and indicates that under control condi-
current (27.8 ± 7.9 pA) that observed in control aCSF (29.6 ± 11.5 pA; Fig. 9C). Coapplication of 10 μM 4-PIOL with 1 μM GABA also elicited an inward current (Fig. 9A), although the resultant 4-PIOL current was significantly smaller than that observed in control aCSF (29.6 ± 11.5 pA; Fig. 9C). Thus, as observed for recombinant α1β3γ2 receptors, 4-PIOL exhibited a dominant agonist profile at low GABA concentrations (≤1 μM), but produced a small inhibition of dLGN tonic currents when the ambient GABA concentration was increased.

To determine whether ambient GABA levels also influence the 4-PIOL current in cultured CGCs and hippocampal neurons, low concentrations of GABA (0.3 and 1 μM) were preapplied until steady-state currents were achieved and subsequently, 4-PIOL was coapplied (Fig. 10A,B). Similar to its effects on endogenous CGC tonic currents, coapplication of 4-PIOL produced no significant shift in the holding current, even when the preapplied GABA concentration was raised to 1 μM (Fig. 10A).

For hippocampal neurons, coapplication of 10 μM 4-PIOL with 0.3 μM GABA significantly enhanced the tonic current (Fig. 10B) by 62.8 ± 9.9 pA (p = 0.03, Wilcoxon matched-pairs test), similar to the enhancement observed under control conditions (72.0 ± 14.2 pA; p = 0.90). However, when the preapplied GABA concentration was raised to 1 μM GABA, 10 μM 4-PIOL significantly reduced the steady-state 1 μM GABA current (Fig. 10B), by 52.5 ± 7.1 pA (p = 0.0004, paired t test). This corresponded to a 73.6 ± 28.1% enhancement of the steady-state 0.3 μM GABA current, and a 12.4 ± 2.0% inhibition of the 1 μM GABA current (Fig. 10C). Thus, the modulation of tonic currents by low-efficacy partial agonists, such as 4-PIOL, appears to be highly dependent on ambient GABA levels.

### Discussion

GABA<sub>A</sub> receptor-mediated tonic inhibition is an important regulator of cell and network excitability (Mann and Mody, 2010), and its dysfunction is associated with several pathophysiological states (Belelli et al., 2009; Brickley and Mody, 2012). Selectively modulating the activity of extrasynaptic GABA<sub>A</sub> receptors may therefore be therapeutically useful for the treatment of such disorders. Given the absence of suitable subtype-selective antagonists, we took a different approach by investigating the selectivity profile of the weak partial agonist, 4-PIOL (Mortensen et al., 2002, 2004), to determine whether it could be used as a selective modulator of GABA-mediated tonic currents.

### Partial agonist modulation of GABA synaptic currents

Our receptor expression studies predicted that at high GABA (synaptic) concentrations, 4-PIOL should not affect α1β3γ2-mediated currents. However, 4-PIOL variably reduced sIPSC amplitudes and frequencies in many of our neuronal preparations. Using TTX in relay neurons revealed that 4-PIOL reduced mIPSCs to a lesser extent than sIPSCs, which is in accord with both presynaptic and postsynaptic effects. The reduced sIPSC frequency is indicative of a presynaptic action, with 4-PIOL activating extrasynaptic γ2-GABA<sub>A</sub> receptors, reducing interneuron excitability and lowering GABA release (Azmacher and Draguhn, 2004). This is supported by the bidirectional 4-PIOL responses of CA1 neurons. Cells with inward 4-PIOL currents show reduced sIPSC frequencies, consistent with inhibition of presynaptic in-
terneurons by 4-PIOL, whereas cells with outward currents show no effect on IPSC frequency, reflecting unaffected interneuron excitability. This suggests that 4-PIOL has congruent effects on extrasynaptic γ2-GABA₅ receptors on presynaptic interneurons and postsynaptic pyramidal neurons, exposed to similar GABA levels.

The residual block of mIPSCs would suggest a small inhibition of postsynaptic receptors. Although this was not resolved in recombinant α1β3γ2 receptors, native GABA₅ receptors might display a greater sensitivity to the actions of 4-PIOL, either due to endogenous factors (eg, receptor phosphorylation states), or the presence of distinct receptor subunit compositions (eg, α2 or α3βγ2 receptors).

Modulation of tonic currents depends on GABA₅ receptor subunit composition

As expected for a partial agonist, 4-PIOL exhibited both agonist- and antagonist-type behaviors at recombinant and native GABA₅ receptors. However, the direction of modulation depended on two critical factors: GABA₅ receptor composition; and critically, the ambient GABA concentration.

GABA₅ receptor-mediated tonic currents in CGCs, which are largely mediated by α6βδ receptors (Fritschy et al., 1992; Somogyi et al., 1996; Jones et al., 1997; Nußer et al., 1998; Brickley et al., 2001), were unaffected by 4-PIOL. This was unsurprising, given that 4-PIOL alone produced little if any modulation of steady-state GABA currents at recombinant α6βδ receptors. By comparison, 4-PIOL bidirectionally modulated tonic currents in hippocampal neurons, which will express an array of GABA₅ receptors, including α5βγ2, α4βδ, and αβ (Mangan et al., 2005; Mortensen and Smart, 2006; Glykys et al., 2008). This might be expected, given that α5βγ2 receptors are important for tonic currents in these cells (Caraiscos et al., 2004; Glykys et al., 2008), and 4-PIOL had a bidirectional effect on GABA currents at recombinant α5βγ2 receptors. However, other α1–3βγ2 receptor isoforms may also mediate this modulation, because tonic currents, and the 4-PIOL current itself, were previously shown to be positively modulated by benzodiazepine agonists in hippocampal neurons (Kristiansen et al., 1995; Liang et al., 2004). Interestingly, 4-PIOL generated both inward and outward currents in CA1 neurons under control conditions, suggesting that the ambient GABA concentration in this slice is close to the threshold where 4-PIOL switches from agonist to antagonist behavior.

Extrasynaptic GABA₅ receptor isoforms in thalamic relay neurons

In relay neurons, the 4-PIOL-induced enhancement of tonic currents was unexpected, because α4βδ receptors are thought to underlie tonic currents in these cells (Cope et al., 2005; Bright et al., 2007; Nani et al., 2013; Ye et al., 2013), and 4-PIOL potently inhibited the steady-state GABA currents of recombinant α4βδ receptors (by ~40–80%, when the ambient GABA concentration was varied between 0.1 and 1 mM). However, several observations suggest the 4-PIOL current in relay neurons was probably not mediated by δ-containing receptors. First, despite being able to compete with THIP for the orthosteric binding site at recombinant α4βδ receptors, 4-PIOL and THIP appeared not to compete for the same δ subunit-containing receptors in dLGN relay neurons. Next, the ability of 4-PIOL to bidirectionally modulate tonic currents, strongly depended on the ambient GABA concentration, recapitulating its actions at recombinant α1βγ2 receptors, but not α4βδ receptors. Third, the 4-PIOL current in dLGN relay neurons was potentiated by the benzodiazepine diazepam, strongly implying an action at γ2 subunit-containing receptors.

To counter this, the 4-PIOL current in dLGN relay neurons was potentiated by the δ subunit-selective modulator DS2 (Wafford et al., 2009; Jensen et al., 2013). It was notable that this modulator unveiled a previously undetected 4-PIOL current at recombinant α4βδ receptors and thus DS2 may act similarly at α4βδ receptors in dLGN, by producing a δ-mediated component to the 4-PIOL current, which is absent under control conditions. However, DS2 also potentiated the 4-PIOL current at recombinant α1β3γ2 receptors, bringing into question its isoform selectivity. DS2 modulation of α1β3γ2-GABA currents has been seen previously, and significantly, a small residual DS2-induced current was apparent in thalamic relay neurons from δ subunit knock-out mice (Jensen et al., 2013). Thus, DS2 will modulate γ2-containing receptors, albeit to a lesser extent than...
δ-containing counterparts, and so may also be potentiating the 4-PIOL current at γ2-GABA_α receptors in relay neurons.

Overall, the simplest explanation for these data are that functional effects of 4-PIOL on tonic currents in dLGN relay and hippocampal neurons, are largely dominated by its actions on γ2 subunit-containing receptors, though we cannot completely exclude a contribution from δ subunit-containing receptors. Although the significant presence of extrasynaptic γ2 subunit-containing receptors on dLGN relay neurons was unexpected, given that they are thought to accumulate at synaptic sites, immunohistochemical and functional studies indicate that a significant number of α1–α3 subunits, which associate with γ2 subunits, may also exist at extrasynaptic sites (Soltesz et al., 1990; Nuwer et al., 1998; Mangan et al., 2005; Thomas et al., 2005; Kasugai et al., 2010). Although their functional significance remains to be established in native systems, a recent study has proposed that tonically active α1β3γ2 receptors might contribute to the clinical actions of positive allosteric modulators, such as etomidate and propofol (Li and Akk, 2015). Thus, the relative expression levels of extrasynaptic δ- and γ2-containing receptors may be important in determining the functional effects of compounds, such as 4-PIOL, which can modulate both receptor isoforms under distinct conditions.

Low ambient GABA levels in neuronal preparations

Under our experimental conditions, the ambient GABA concentration in all three neuronal preparations was estimated to be significantly <1 μM. In accord with these findings, although difficult to measure precisely in the structurally tortuous environment of the brain, microdialysis studies estimate that extracellular GABA concentrations in vivo range from 30 nM to 2.9 μM (Glaeser and Hare, 1975; Lerma et al., 1986; de Groote and Linthorst, 2007; Wlodarczyk et al., 2013), whereas the activity of GABA transporters predicts that ambient GABA levels are within 0.1–0.4 μM (Attwell et al., 1993; Richerson and Wu, 2003; Wu et al., 2007).

Given the low ambient GABA levels that we estimate in slices, and the low GABA sensitivity of γ2 subunit-containing GABA_α receptors (Brown et al., 2002; Mortensen et al., 2010, 2011), it is unsurprising that the extrasynaptic population of γ2-containing receptors, detected in dLGN relay neurons, did not significantly contribute to basal tonic currents under our experimental conditions. However, this does not discount the possibility that γ2-containing receptors may contribute to tonic currents when ambient GABA levels are significantly increased, for instance, during behavioral or pathophysiological disease states. Although applying diazepam to dLGN relay neurons enhanced dLGN tonic currents, demonstrating that γ2-containing receptors can contribute to tonic currents, it is difficult to discount the possibility that diazepam may have increased the apparent affinity of these receptors for GABA (Gienel et al., 2012), thus recruiting a population of extrasynaptic γ2-containing receptors that may be inactive under control conditions.

The observation that ambient GABA levels strongly influence the functional profile of 4-PIOL is unsurprising given that both 4-PIOL and GABA act via the same binding site. Indeed, similar observations have been made for the agonist, THIP, whose enhancement of δ-mediated tonic currents in CGCs was attenuated at higher ambient GABA concentrations (Houston et al., 2012). Thus, when evaluating the potential effects of therapeutic compounds on tonic currents, an important consideration is how this modulation will be affected by variable ambient GABA concentrations.

Therapeutic potential of low-efficacy agonists

Overall, the therapeutic potential of a low-efficacy partial agonist, such as 4-PIOL, has merit, but its action will critically depend on a number of factors, including which GABA_α receptor isoforms are expressed, their expression levels on the cell surface, and ambient GABA levels. Given its enhancement of tonic currents at low ambient GABA concentrations, a weak partial agonist like 4-PIOL might be most useful for neurological conditions where an increased tonic inhibition is desirable, for example in Fragile X syndrome and sleep disorders (Brickley and Mody, 2012; Whissell et al., 2015).

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