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10.1523/JNEUROSCI.4193-12.2013

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GABA-Independent GABA<sub>A</sub> Receptor Openings Maintain Tonic Currents


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Activation of GABA<sub>A</sub> receptors (GABA<sub>A</sub>Rs) produces two forms of inhibition: phasic inhibition generated by the rapid, transient activation of synaptic GABA<sub>A</sub>Rs by presynaptic GABA release, and tonic inhibition generated by the persistent activation of perisynaptic or extrasynaptic GABA<sub>A</sub>Rs, which can detect extracellular GABA. Such tonic GABA<sub>A</sub>R-mediated currents are particularly evident in dentate granule cells in which they play a major role in regulating cell excitability. Here we show that in rat dentate granule cells in ex vivo hippocampal slices, tonic currents are predominantly generated by GABA-independent GABA<sub>A</sub>R receptor openings. This tonic GABA<sub>A</sub>R conductance is resistant to the competitive GABA<sub>A</sub>R antagonist SR95531 (gabazine), which at high concentrations acts as a partial agonist, but can be blocked by an open channel blocker, picrotoxin. When slices are perfused with 200 nM GABA, a concentration that is comparable to CSF concentrations but is twice that measured by us in the hippocampus in vivo using zero-net-flux microdialysis, negligible GABA is detected by dentate granule cells. Spontaneously opening GABA<sub>A</sub>Rs, therefore, maintain dentate granule cell tonic currents in the face of low extracellular GABA concentrations.

Introduction

In addition to fast synaptic GABA<sub>A</sub> receptor (GABA<sub>A</sub>R)-mediated signaling, there is a slower form of signaling resulting from the tonic activation of GABA<sub>A</sub>Rs (Semyanov et al., 2004; Farrant and Nusser, 2005; Glykys and Mody, 2007a). Tonically active GABA<sub>A</sub>Rs can have profound effects on neuronal excitability, synaptic plasticity, network oscillations, and neurogenesis (Ge et al., 2006; Pavlov et al., 2009; Holter et al., 2010; Mann and Mody, 2010; Martin et al., 2010; Duveau et al., 2011) and have been implicated in neuronal development, information processing, cognition, and memory (Semyanov et al., 2004; Farrant and Nusser, 2005; Brickley and Mody, 2012). The conventional view is that tonic currents are mediated by high-affinity extrasynaptic GABA<sub>A</sub>Rs that can detect low concentrations of ambient GABA, and therefore the magnitude of tonic currents is regulated by the expression of these receptors and the availability of extracellular GABA (Semyanov et al., 2004; Farrant and Nusser, 2005; Glykys and Mody, 2007a).

The sources and concentration of extracellular GABA ([GABA]<sub>e</sub>) are, however, still debated. Vesicular release, “leak” through bestrophin channels, and reversal of GABA transporters have all been suggested to contribute to the extracellular GABA pool (Attwell et al., 1993; Gaspar et al., 1998; Lee et al., 2010). In vivo estimates indicate that [GABA]<sub>e</sub> is in the micromolar or submicromolar range (Lerma et al., 1986; Kuntz et al., 2004; Nyitrai et al., 2006). In vitro studies have suggested even lower concentrations of GABA, in the nanomolar range; indeed, active uptaking GABA<sub>A</sub>Rs maintain GABA at sufficiently low concentrations to prevent tonic GABA<sub>A</sub>R receptor activation (Isaacson et al., 1993). Thus, tonic currents in hippocampal neurons have often been measured in conditions that artificially raise [GABA]<sub>e</sub> by inhibiting GABA uptake or metabolism, or by adding exogenous GABA to the perfusate (Overstreet and Westbrook, 2001; Nusser and Mody, 2002; Stell and Mody, 2002; Holter et al., 2010). When GABA is not increased by these means, various magnitudes of tonic currents have been obtained using different GABA<sub>A</sub>R antagonists (Bai et al., 2001; Mtchedlishvili and Kapur, 2006; Zhan and Nadler, 2009; Mtchedlishvili et al., 2010). Here we report a novel form of tonic inhibition mediated by GABA-independent openings of GABA<sub>A</sub>Rs, which can explain these discrepancies.

We focus on dentate gyrus granule cells (DGCs), the excitability of which critically depends upon the presence of tonic GABA<sub>A</sub>R-mediated conductances (Overstreet and Westbrook, 2001; Nusser and Mody, 2002; Stell and Mody, 2002; Coulter and Carlson, 2007; Holter et al., 2010). We demonstrate that under...
baseline conditions, or when the perfusate contains the same concentration of GABA that is found in CSF, the major contributor to the tonic current in DGCs are spontaneously opening GABA\(_R\)s. This tonic GABA\(_R\) conductance is resistant to the competitive GABA\(_R\) antagonist gabazine (SR95531), but can be blocked by an open channel blocker, picrotoxin. On increasing [GABA], a SR95531-sensitive component of tonic current emerges. Together, these results indicate that the GABA-independent component of tonic current mediated by spontaneously opening GABA\(_R\)s maintains tonic inhibition in the presence of low extracellular GABA concentrations measured in vivo.

**Materials and Methods**

**Hippocampal slice preparation**

Transverse hippocampal slices (350 \(\mu\)m thick) were used for in vitro electrophysiological recordings. Slices were prepared from 3- to 4-week-old male Sprague Dawley rats and \(\delta^{+}\)/- knockouts or \(\delta^{+}\)/- littermate control mice on a C57B6 background (Mihai et al., 1999; Herd et al., 2008). Animals were killed by an overdose of isoflurane according to the United Kingdom Animals (Scientific Procedures) Act of 1986. After decapitation, brains were rapidly removed and dissected, and hippocampi were sliced with a Leica VT1200S vibratome in ice-cold sucrose-based solution containing the following (in mM): 70 sucrose, 80 NaCl, 2.5 KCl, 1.3 MgSO\(_4\), 2.5 CaCl\(_2\), 26.2 NaHCO\(_3\), 1 NaH\(_2\)PO\(_4\), 22 glucose, equilibrated with 95% O\(_2\) plus 5% CO\(_2\), pH 7.4, 315–330 mOsm. Slices were maintained in continuously oxygenated sucrose-free storage solution at 33°C for 15 min, placed to recover in a room temperature for 15 min and then placed to recover in continuously oxygenated humid interface holding chamber at room temperature for at least 1 h before recording. In experiments with concanamycin, slices were placed in exactly the same way, but before placing them in a holding chamber for the recovery they were incubated for 2 h in a submerged chamber with continuously oxygenated storage solution and 0.5 \(\mu\)M concanamycin. After that, slices were placed in an interface holding chamber and allowed to rest for about half an hour before recording. After recovering slices were transferred into recording chamber. The perfusion and storage medium contained the following (in mM): 119 NaCl, 2.5 KCl, 1.3 MgSO\(_4\), 2.5 CaCl\(_2\), 26.2 NaHCO\(_3\), 1 NaH\(_2\)PO\(_4\), 22 glucose and was gassed with 95% O\(_2\) and 5% CO\(_2\), pH 7.4; 290–298 mOsm.

In vitro electrophysiology

Whole-cell recordings. Visualized patch-clamp recordings from mature dentate granule cells (input resistance, \(R_h = 310 \pm 50\) M\(\Omega\); membrane capacitance, \(C_m = 48 \pm 6\) pF) were performed using an infrared differential interference contrast imaging system. Tonic GABA\(_R\)-mediated currents were measured in voltage-clamp mode (at holding potential \(V_{hold} = -70\) mV) in the presence of ionotropic glutamate receptor blockers DL-APV (50 \(\mu\)M) and NBQX (20 \(\mu\)M), metabotropic glutamate receptor blocker (S)-\(\alpha\)-methyl-4-carboxyphenylglycine (MCPG; 250 \(\mu\)M), and GABAB receptor blocker (2S)-3,3′-[[1S-1,3,1,3-4-dichloroethyl]amino-2-hydroxypropyl][phenethyl][phosphonic acid (CGP55845; 1 \(\mu\)M). The intracellular pipette solution contained the following (in mM): 120.5 CsCl, 2.5 KOH-HEPES, 2 EGTA, 25 NaHCO\(_3\), 2 NaCl, 2.5 CaCl\(_2\), 1.25 NaH\(_2\)PO\(_4\), 22 glucose, equilibrated with 95% O\(_2\) plus 5% CO\(_2\), pH 7.4, 315–330 mOsm. Slices were maintained in continuously oxygenated sucrose-free storage solution at 33°C for 15 min, placed to recover in a room temperature for 15 min and then placed to recover in continuously oxygenated humid interface holding chamber at room temperature for at least 1 h before recording. In experiments with concanamycin, slices were placed in exactly the same way, but before placing them in a holding chamber for the recovery they were incubated for 2 h in a submerged chamber with continuously oxygenated storage solution and 0.5 \(\mu\)M concanamycin. After that, slices were placed in an interface holding chamber and allowed to rest for about half an hour before recording. After recovering slices were transferred into recording chamber. The perfusion and storage medium contained the following (in mM): 119 NaCl, 2.5 KCl, 1.3 MgSO\(_4\), 2.5 CaCl\(_2\), 26.2 NaHCO\(_3\), 1 NaH\(_2\)PO\(_4\), 22 glucose and was gassed with 95% O\(_2\) and 5% CO\(_2\), pH 7.4; 290–298 mOsm.

### Analysis of the single-channel recordings

All analyses were performed on stretches of data that were longer than 3 min. The opening frequency of GABA\(_R\)-mediated channels was calculated as \(\Delta V_{\text{hold}} = (m_1 - m_2)/V_{\text{mem}}\), where \(m_1\) and \(m_2\) are the mode values of Gaussians, \(\sigma_1\) and \(\sigma_2\) are the SDs of corresponding modes, \(n\) is the value of electrical current, and \(p_1\) and \(p_2\) are the fitting constants. The channel conductance was calculated as \(G = (m_1 - m_2)/V_{\text{mem}}\).

The Newton–Raphson iteration method was used to obtain the value of \(n_{\text{null}}\), which is the value of \(n\) at the minimum point of the function \(F\) in the interval \(m_1 < n < m_2\). This was taken as the point of channel closure.
Since the signal was digitized at 10 kHz (i.e., at intervals of 0.1 ms), the average open time (in milliseconds) was calculated as follows (the factor of 10 in the denominator is to account for the 0.1 ms interval):

$$T = \sum_{n=n_{\text{max}}}^{n_{\text{max}} - 1} \frac{[F(n)]}{10 \times N},$$

where $n_{\text{max}} = 2m_2 - n_{\text{min}}$.

All calculations were performed using the Wolfram Mathematica 6.2 software; the general algorithm of histograms construction and interpretation was adapted from Bennett and Kears (2000). We compared results obtained using this method against those obtained by averaging all the threshold-detected openings for the data shown in Figure 3. There was no difference between these two methods (Pearson’s correlation coefficient of 0.992 for open time, and 0.979 for conductance).

**Drugs and reagents**

GABA receptor antagonists and concanamycin were purchased from Tocris Bioscience. Other reagents were from either Ascent Scientific or Sigma-Aldrich.

**In vivo microdialysis**

**Animals.** Male Sprague Dawley rats (Harlan) were housed three per cage under standard housing conditions (lights on between 5:00 A.M. and 7:00 P.M.; 21–22°C; 50–60% relative humidity) with free access to food pellets and drinking water. Animals were handled daily (~5 min/rat) starting 1 week before surgery and continuing until the day of the insertion of the microdialysis probe. At the time of surgery, rats weighed ~240 g.

Surgical procedures were performed under isoflurane anesthesia (Merial Animal Health) anesthesia. Carprofen (Rimadyl, 4 mg/kg, s.c.; Pfizer) was administered for postoperative pain relief. All procedures were conducted in accordance with the United Kingdom’s Animals (Scientific Procedures) Act 1986, and all efforts were made to minimize animal numbers and suffering.

**Surgical and microdialysis procedures.** Nine days before the start of the experiment, rats were surgically prepared for microdialysis of the hippocampus by stereotaxic implantation of a guide cannula (MAB 6.14.IC, Microbiotech/se AB) essentially as described in detail before (Droste et al., 2008). After surgery, rats were housed individually in Plexiglas cages (length × width × height = 27 × 27 × 35 cm) under similar housing conditions as described above.

Seven days after surgery, a microdialysis probe (polyethersulfone membrane, length 4 mm, 15 kDa cutoff, and outer diameter 0.6 mm; MAB 6.14.4, Microbiotech/se AB) was inserted via the guide cannula into the CA3–dentate gyrus region of the hippocampus (Linhorst et al., 1994) under short-lasting isoflurane anesthesia (see Fig. 3C, schematics). Rats were connected to a liquid swivel and a counterbalance arm (Microbiotech/se AB), allowing free movement in all directions. Fluorethylene polymer tubing with a dead volume of 1.2 µl per 100 mm length (Microbiotech) was used for all connections. Dead volumes were accounted for during the experiment. Microdialysis probes were perfused with sterile, pyrogen-free Ringer solution (Delta Pharma) at a flow rate of 2 µl/min using a micro-infusion pump (KD2820, KD Scientific).

The zero-net-flux experiment was started at 9:00 A.M. on the second day after the insertion of the microdialysis probe. Ten minute samples were collected throughout the complete experiment. After 1 h of baseline sampling, microdialysis probes were subsequently perfused with increasing concentrations of GABA (10, 20, 40, 80, 160 and 240 nM; Sigma-Aldrich) dissolved in Ringer solution (reverse microdialysis). Each GABA perfusion lasted 40 min during which four 10 min samples were collected. Microdialysis samples were collected using an automated, refrigerated fraction collector (CMA470, CMA Microdialysis AB) and were stored at ~80°C for later HPLC analysis.

**Histology.** At the end of the experiment, rats were killed using an overdose of pentobarbital (Euthatal, 200 mg/kg body weight, i.p.; Merial Animal Health), and the brains were removed and stored in a 4% buffered paraformaldehyde solution. Histological examination was performed as described previously (Linhorst et al., 1994; Droste et al., 2008). Only data from rats with correctly placed microdialysis probes were included in the analyses.

**Measurement of GABA.** HPLC with electrochemical detection was used to measure GABA in the microdialysates essentially as described previously with small modifications (de Groot and Linhorst, 2007). Briefly, GABA was separated on a TARGA C18 10 cm × 1 mm column (particle size 3 µm; Higgins Analytical) using filtered and degassed mobile phase (18% methanol, 0.1 x NaH2PO4; 0.2 mM EDTA, pH 4.72) pumped at 50 µl/min using an Atelex LC-100 pump (Antec Leyden BV).

Standards and samples were injected onto the column using a thermostatically controlled (8°C) Atelex AS-100 autosampler (Antec Leyden BV). Before injection, 13 µl of standard or sample was derivatized with 2 µl of α-phthalaldialdehyde (OPA)/sulfite solution (1.6 mM OPA, 0.5% methanol, 1.88 mM Na2SO4, 11.25 mM Na2B4O7) for 4 min to make GABA electrochemically active. Next, 10 µl of the derivatized mixture was injected onto the column and GABA was detected using a VT-03 electrochemical flow cell (Antec Leyden BV) set at +850 mV against an Ag/AgCl reference electrode. Both column and detector were housed in a Faraday-shielded oven (DECADE II, Antec Leyden BV) thermostatically controlled at 38°C. Chromatograms were recorded and analyzed using Atelex chromatography software (Antec Leyden BV). The detection limit for GABA at a signal-to-noise ratio of 3:1 was 11–15 fmol per injection on column.

**Calculations.** Perfusion of each concentration of GABA (Cout; nM) was performed for 40 min. However, to ensure that dialysis across the membrane had reached a steady state, the first 10 min sample was discarded and only the three subsequent samples were used for the calculations described below.

After measurement of the concentration of GABA in the collected samples (Cout; nM), the difference between Cout and Cin (i.e., the net loss or gain of GABA in the dialysate) was calculated (Cout − Cin) for each concentration of GABA perfused. The baseline condition represented Cin = 0. Next, for each individual animal, Cin − Cout was plotted against Cin, and, after regression analysis (GraphPad 5.0), the concentration of zero-net-flux was determined as the concentration of Cin at which Cout − Cin = 0. Next, the mean zero-net-flux concentration (±SEM; n = 5) was calculated. For graphical purposes, the mean ± SEM values for Cin − Cout were calculated for each concentration of GABA perfused and were plotted against Cin (see Fig. 3D).

**Statistics**

Statistical comparisons were made using paired and unpaired (as indicated in the text/figure legends) Student’s t test, and Wilcoxon signed ranks test (Fig. 1C, experiments). Differences were considered significant at p < 0.05. Data are presented in the text and figures as the mean ± SEM.

**Results**

**Tonic activation of GABAtpRs in DGCs does not require synaptically released GABA**

Since the accumulation of GABA in the extracellular space may result from synaptic release, we asked whether depleting vesicular GABA by incubating hippocampal slices in the vesicular H+ -ATPase inhibitor concanamycin (0.5 µM) would affect tonic currents. We confirmed that concanamycin abolished synaptic currents (Rossi et al., 2003); however, it had no significant effect on the magnitude of the tonic current revealed by application of the GABAtp antagonist picrotoxin. Application of picrotoxin produced a 11.9 ± 1.5 pA outward shift in the Ihold in control experiments, and a 13.3 ± 3.0 pA shift when slices were preincubated in concanamycin (Fig. 1A). These results indicate that in acute hippocampal slices tonic currents in DGCs are not dependent on the concentration of GABA in vesicles or on GABA release into the synapse under baseline conditions. Glycine receptors can contribute to inhibition in the hippocampus and may also be blocked by picrotoxin in a use-dependent manner (Danglot et al., 2004; Yang et al., 2007). We tested their contribution by adding the glycine receptor antagonist strychnine (1 µM) and observed that it had no effect on Ihold and did...
not occlude the effect of picrotoxin (\(\Delta I_{\text{hold}}\)) following application of strychnine: \(-0.8 \pm 0.8\) pA, \(n = 5\), \(p = 0.40\) compared with control; consecutive application of picrotoxin: \(15.6 \pm 3.3\) pA, \(n = 4\), \(p = 0.018\) compared with strychnine). We further ruled out the potential contribution of GABA\(_C\)Rs to picrotoxin-induced shift in \(I_{\text{hold}}\) by applying the GABA\(_C\) antagonist TPMPA (50 \(\mu\)M), which also did not affect the amplitude of the picrotoxin-sensitive tonic current (\(\Delta I_{\text{hold}}\)) following application of TPMPA: \(-0.14 \pm 1.78\) pA, \(p = 0.9\) compared with control; picrotoxin: \(11.7 \pm 1.7\) pA, \(p = 0.006\) compared with TPMPA; \(n = 4\)). Thus, we conclude that neither glycine receptors nor GABA\(_C\)Rs contribute to tonic conductance revealed by picrotoxin.

**Figure 1.** Pharmacology of the GABA\(_A\)-mediated tonic currents in DGCs. **A**, Concanamycin (0.5 \(\mu\)M) blocks exocytosis but does not affect \(I_{\text{hold}}\). No significant difference between the change in \(I_{\text{hold}}\) or RMS noise in control (left trace, \(n = 9\)) and concanamycin-treated slices (right trace, \(n = 6\)) upon application of picrotoxin (PTX; 100 \(\mu\)M). **B**, \(I_{\text{tonic}}\) in DGCs is insensitive to SR95531 and abolished by picrotoxin. SR95531 (0.5 \(\mu\)M) partially inhibited sIPSCs, while high concentrations (125 \(\mu\)M) completely abolished sIPSCs. Picrotoxin induced an outward shift in \(I_{\text{hold}}\) (\(p = 0.0049; n = 5\)) and decreased RMS noise (\(p = 0.0012; n = 5\)). **C**, In the presence of GABA (5 \(\mu\)M), application of SR95531 (0.5 \(\mu\)M) reduced sIPSCs, inducing an outward shift in \(I_{\text{hold}}\) (\(p = 0.016\)), and decreased RMS noise (\(p = 0.008; n = 8\)). SR95531 (25 \(\mu\)M) abolished sIPSCs, induced a further nonsignificant shift in \(I_{\text{hold}}\) (\(p = 0.44\)), and RMS noise (\(p = 0.06; n = 5\)). SR95531 (125 \(\mu\)M) affected neither \(I_{\text{hold}}\) nor RMS noise. Subsequent application of picrotoxin produced an outward shift in \(I_{\text{hold}}\) and reduced RMS noise (\(p = 0.031\) and \(p = 0.003\) respectively; \(n = 6\)). **D**, BMI (10 \(\mu\)M) blocked all synaptic activity, induced an outward shift in \(I_{\text{hold}}\) and reduced RMS noise (\(p = 0.00019\) and \(p = 0.0014\) respectively; \(n = 6\)). SR95531 (125 \(\mu\)M) resulted in inward current and an increase in RMS noise (\(p = 0.0013\) and \(p = 0.008\) respectively; \(n = 6\)), which were reversed by picrotoxin (100 \(\mu\)M) (\(p = 0.0019\) and \(p = 0.0048\) respectively; \(n = 5\)). Statistical comparisons were made using paired Student’s test in **A**, **B**, and **D**, and Wilcoxon signed-ranks test in **C**. *\(p < 0.05\); **\(p < 0.01\); ***\(p < 0.001\). \(\Delta I_{\text{hold}}\) and \(\Delta\) RMS noise values represent changes from the previous drug application.
Table 1. The effect of different GABA antagonists in control conditions with no GABA added

<table>
<thead>
<tr>
<th>Antagonist</th>
<th>GABA tonic current ($\Delta I_{\text{hold}}$, pA)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PTX (100 µM)</td>
<td>11.9 ± 1.5, n = 9, p = 0.0004 compared to control</td>
</tr>
<tr>
<td>Pentylentetrazol (1.5 mM)</td>
<td>10.6 ± 3.6, n = 5, p = 0.04 compared to control</td>
</tr>
<tr>
<td>Bicuculline (10 µM)</td>
<td>5.9 ± 0.6, n = 6, p = 0.0002 compared to control</td>
</tr>
<tr>
<td>SR95531 (125 µM)</td>
<td>−6.7 ± 1.6, n = 5, p = 0.01 compared to control</td>
</tr>
<tr>
<td>PTX (100 µM) in SR95531 (125 µM)</td>
<td>18.9 ± 3.4, n = 5, p = 0.005 compared to SR95531 (125 µM)</td>
</tr>
</tbody>
</table>

Figure 2. δ-Subunit-containing GABA$_A$ receptors are involved in generating picrotoxin-sensitive tonic currents in DGCs. The effects of SR95531 (25 µM) and picrotoxin (PTX, 100 µM) on holding currents recorded in wild-type (top trace) and knock-out mice lacking δ-subunit-containing GABA$_A$Rs (bottom trace). Picrotoxin-sensitive tonic currents are reduced in the knock-out mice compared with the wild-type littermate controls ($n = 9$ and $n = 10$ for the δ$^{+/+}$ and δ$^{-/-}$ mice, respectively; $**p < 0.01$, Student’s unpaired t test). Histogram shows mean values ± SEM.

Tonic currents in DGCs are insensitive to SR95531 under baseline conditions

These results suggest either that [GABA]$_E$ is maintained at a constant concentration despite decreased vesicular GABA release (e.g., by nonvesicular release) and/or that there is another mechanism involved in generating tonic currents that is independent of [GABA]$_E$.

To distinguish between these possibilities, we took advantage of the distinct pharmacologies of different GABA$_A$R antagonists. Picrotoxin is an open channel blocker and acts as a noncompetitive GABA$_A$R antagonist that has equivalent efficacy in blocking low-affinity synaptic and high-affinity extrasynaptic GABA$_A$Rs (Stell and Mody, 2002). In contrast, SR95531 is a competitive GABA$_A$R antagonist and displaces GABA from its binding site (Hamann et al., 1988). While low concentrations of SR95531 (0.5 µM) partially inhibited and high concentrations (125 µM) totally blocked sIPSCs, there was no significant increase in $I_{\text{hold}}$, which became more negative at high SR95531 concentrations ($\Delta I_{\text{hold}}$ 6.7 ± 1.6 pA, $n = 5$, $p = 0.014$ compared with 0.5 µM SR95531; Fig. 1B; Table 1), suggesting a partial agonist effect (this effect was not observed when SR95531 was applied after picrotoxin: $\Delta I_{\text{hold}}$ −1.2 ± 0.9 pA, $n = 3$, $p = 0.3$). Subsequent application of picrotoxin resulted in an outward shift of $I_{\text{hold}}$ by 18.9 ± 3.4 pA ($p = 0.0049$, $n = 5$), and a decrease in baseline RMS noise by 0.39 ± 0.05 pA ($p = 0.0012$, $n = 5$) in the same cells (Fig. 1B). This effect of picrotoxin was occluded by prior application of pentylentetrazol (Table 1), another noncompetitive GABA$_A$R antagonist (Huang et al., 2001). $\Delta I_{\text{hold}}$ following application of picrotoxin in the presence of pentylentetrazol was 0.4 ± 0.7 pA ($n = 5$).

As previously reported (Semyanov et al., 2003), markedly increasing GABA in the perfusate to 5 µM revealed a SR95531-sensitive component of the tonic current (Fig. 1C). Under these conditions, application of a low concentration of SR95531 (0.5 µM) caused a significant outward shift of 29.4 ± 14.4 pA in the holding current ($p = 0.016$, $n = 8$) and a significant decrease in RMS noise by 3.0 ± 0.8 pA ($p = 0.008$, $n = 8$, Fig. 1C). Even in the presence of a high concentration of SR95531 (125 µM) under these conditions, application of picrotoxin caused a further outward shift in holding current by 9.2 ± 2.1 pA ($p = 0.031$, $n = 6$) and a reduction of RMS noise by 0.51 ± 0.09 pA ($p = 0.003$, $n = 6$; Fig. 1C).

The lack of efficacy of SR95531 under baseline conditions suggests that only negligible ambient GABA can be detected by dentate granule cells. Alternatively, SR95531 may not bind to the receptors mediating tonic current. The efficacy of SR95531 in expression systems in which α48 subunit-containing receptors (the main contributor to tonic currents in dentate granule cells) (Glyks and Mody, 2007b) are expressed (Brown et al., 2002) argues against this. Nevertheless, we tested this using another competitive GABA$_A$R antagonist, bicuculline. Application of bicuculline (10 µM) blocked all synaptic activity, induced a small outward shift of holding current by 5.9 ± 0.6 pA ($p = 0.0019$, $n = 6$), and decreased baseline RMS noise by 0.72 ± 0.11 pA ($p = 0.0014$, $n = 6$; Fig. 1D). This result can be explained by the inverse agonist activity of bicuculline (Ueno et al., 1997; Bai et al., 2001; McCartney et al., 2007). If SR95531 does not bind to the same receptors as bicuculline, then it should have no effect in the presence of bicuculline. Conversely, if SR95531 does bind then it should displace the bicuculline and paradoxically induce an inward current. The effect of bicuculline was indeed reversed by application of SR95531 (25 µM), which activated an inward current of 10.7 ± 1.7 pA ($p = 0.0013$, $n = 6$), and increased baseline noise by 0.27 ± 0.06 pA ($p = 0.008$, $n = 6$). This was completely blocked by subsequent application of picrotoxin (Fig. 1D). These results suggest that all three GABA$_A$R antagonists bind to the same receptors and that these receptors are not detecting GABA under baseline conditions. Moreover, the lack of effect of SR95531 in baseline conditions indicates that other possible endogenous agonists (e.g., taurine) are also not mediating the tonic current (Jia et al., 2008).

SR95531-insensitive tonic currents in DGCs are mediated by not only δ subunit-containing GABA$_A$Rs

Tonic GABA$_A$R-mediated currents in DGCs are predominantly mediated by α4 and δ subunit-containing receptors (Stell and Mody, 2002; Stell et al., 2003; Caraiscos et al., 2004). We therefore tested whether δ-GABA$_A$R contribute to the SR95531-resistant tonic current. We took advantage of knock-out mice lacking δ subunits of GABA$_A$R, Rs. In these mice, in contrast to rats, there was a very small SR95531-sensitive tonic current. However, the majority of the tonic current was SR95531 insensitive (Fig. 2). In δ$^{-/-}$ mice in the presence of 25 µM SR95531, the effect of picrotoxin on holding current was ~60% less than that in wild-type mice (littermate controls), implying that δ-GABA$_A$R contrib-
ute to SR95531-resistant tonic currents (Fig. 2). However, there was a significant SR95531-insensitive tonic current in δ−/− mice, suggesting that other GABAAR subtypes can also contribute to the SR95531-insensitive tonic current in DGCs (Glyks et al., 2008).

Low [GABA]e is maintained both in vitro and in vivo

The fact that DGCs display SR95531-insensitive tonic currents suggests that [GABA]e in ex vivo tissue is maintained at concentrations that are not detectable by high-affinity GABAARs.

We used GABAAR openings in outside-out “sniffer” patches from dentate granule cells (Fig. 3A,B) to give a semiquantitative estimate of [GABA]e in slices. We first confirmed that channel openings could be recorded in outside-out patches in the presence of 10 μM GABA (channel openings disappeared in the absence of GABA) (Fig. 3A). These channels were blocked by picrotoxin and had an opening frequency of 20.1 ± 5.6 Hz, a conductance of 39.4 ± 7.2 pS, and an average open time of 32.1 ± 7.1 ms (n = 11). We were able to detect GABAAR openings when the patch was 5 μm above the slice (0.55 ± 0.05 Hz, n = 5), indicating that there is sufficient extrasynaptic GABA to bind to GABAARs (Fig. 3B). These openings did not occur when the patch was moved 300 μm above the slice and remained on lowering the patch again to the slice (Fig. 3B). Consequent application of 200 nM GABA to the perfusate, comparable to that found in CSF (Glaeser and Hare, 1975), almost doubled the frequency of GABAAR openings recorded above the slice surface. The effect of 200 nM GABA application was even more evident when patches were raised 300 μm above the slice. This indicates that [GABA]e is <200 nM in the slice (Fig. 3B) and is consistent with the predicted GABA transporter equilibrium [GABA]e of ~100 nM (Wu et al., 2007). We further confirmed that similarly low [GABA]e is maintained in vivo by using the zero-net-flux microdialysis method. Microdialysis probes were inserted into the CA3-dentate gyrus region via guide cannula, and in vivo [GABA]e was calculated to be 92 ± 10 nM (n = 5; Fig. 3C,D).

Since an increase in GABAAR openings occurs as the patch approaches the surface, yet there is no evidence of significant GABA-mediated tonic currents, it is likely that the activity of GABA transporters prevents extrasynaptic GABAAR receptors from being exposed to even such low concentrations under baseline conditions.
tions. When GABA in the perfusate was increased to 200 nM, comparable to that found in CSF (Glaeser and Hare, 1975), \( I_{\text{hold}} \) did not change significantly and SR95531 (25 \( \mu M \)) had no significant effect on \( I_{\text{hold}} \) (\( \Delta I_{\text{hold}} \)), following consequent application of GABA: 

\[ -1.21 \pm 2.03 \text{ pA, } p = 0.6; \Delta I_{\text{hold}} \text{ following consequent application of SR95531: } 1.7 \pm 1.4 \text{ pA, } p = 0.29; n = 5 \], indicating that there is no increase in the GABA detected by extrasynaptic GABA\(_A\)R.

**Spontaneously opening GABA\(_A\)R in DGCs**

The evidence thus far implies that tonic inhibition in DGCs *in situ* is predominantly GABA independent. Can tonic currents be mediated by spontaneously opening GABA\(_A\)R? Such receptors have been reported in excised patch and cell-attached recordings from hippocampal neurons (Birnir et al., 2000a), but not in the DGCs (Birnir et al., 1994). Failure to detect spontaneously opening GABA\(_A\)R may be due to the small area sampled using outside-
out patches and/or the dependence of such openings upon the internal neuronal environment. Indeed, we noted that in very few excised patches (<10%) could we detect infrequent channel openings (less than one every 20 s). We therefore performed recordings from nucleated patches (i.e., whole-cell excisions containing intact nuclei). In this preparation, spontaneous GABAAR openings were recorded in the majority of patches (Fig. 4). These openings had a conductance (37.4 ± 6.1 pS) similar to that observed in excised outside-out patches with applied GABA (39 ± 7.2 pS; Fig. 3A), and comparable to that determined in other studies (Bright et al., 2011). As expected, spontaneous openings were not affected by SR95531 (25 μM) but were fully blocked by picrotoxin (Fig. 4A–D). Since a high concentration of SR 95521 (125 μM) had acted as a partial agonist (Fig. 1), we tested the effect of SR 95521 (125 μM) on spontaneous openings in nucleated patches and, as predicted, found that it increased the frequency of openings (Fig. 4E). Addition of 10 μM GABA to the nucleated patches revealed SR95531-sensitive channels with the same conductance (Fig. 5).

Discussion

Our results indicate that GABA-independent openings of GABAARs are the major contributor to tonic currents in dentate granule cells in vivo, even when GABA concentrations in the perfusate are increased to levels comparable to those measured in vivo.

We took advantage and confirmed the different pharmacologies of GABAAR antagonists (Table 1), and have shown that in situ DGC tonic currents are resistant to SR95531, indicating that they are not being generated by GABA binding to the receptors. This is consistent with similar observations in cultured neurons, other brain areas and cell types (Birnir et al., 2000b; McCartney et al., 2007). Importantly, SR95531 has a paradoxical effect of inhibiting the action of the inverse agonist bicuculline. This result suggests that SR95531 is displacing bicuculline from the receptors and consequently that the lack of effect of SR95531 is not due to a failure to bind to the receptors that are mediating the tonic current. At high concentrations (125 μM), we also found that SR95531 can act as a partial agonist. A partial agonist effect of SR95531 (100 μM) and bicuculline (1 mM) has been described previously at GABAARs in which there is a point mutation in the γ subunit (Ueno et al., 1997). We did not test bicuculline at this concentration and so cannot exclude a partial agonist effect of very high concentrations of bicuculline at extrasynaptic receptors.

This lack of GABA binding explains why inhibiting vesicular GABA release in our studies has no effect on the tonic current. The low concentration of GABA despite vesicular release is al-

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**Figure 5.** Application of GABA to nucleated patches from dentate granule cells induces SR95531-sensitive channel openings. A, Left, Representative traces illustrating single-channel openings at consecutive stages of the experiment performed in the presence of 10 μM GABA. From top to bottom: control, SR95531 (25 μM), washout of SR95531. Horizontal dashed lines indicate mean amplitude values of an open (O) and closed (C) state. Right, Normalized all-points amplitude histograms. B–D, Normalized average opening frequency (B), conductance (C), and open time (D) of channel openings induced by GABA application (n = 5 nucleated patches). **p < 0.01, ***p < 0.001, paired Student’s t test. W/O, Washout.
most certainly due to efficient GABA uptake, because, when GABA transporters are blocked, tonic currents become dependent upon vesicular GABA release (Glykys and Mody, 2007b). The effect of decreasing vesicular release has variable results depending upon the nature of the tissue studied, suggesting that GABA concentrations and the consequent detection of extracellular GABA by GABA, Rs vary between brain regions. For example, in thalamocortical neurons, 50 μM SR95531 has been demonstrated to reveal robust tonic GABA, R-mediated currents (Cope et al., 2005). There may even be local inhomogeneities of GABA concentrations due to regional differences in the distribution of GABA transporters (Semyanov et al., 2003). Indirect evidence of this in the hippocampus is supplied by the observation that tonic currents are reduced in CA1 interneurons, but not in pyramidal cells of GAD65-deficient mice (Song et al., 2011). However, whether the heterogeneity of extracellular GABA concentrations observed in vitro occurs in vivo is unclear.

Spontaneous openings of GABA, Rs have been previously described in some preparations (e.g., in isolated hippocampal pyramidal cells and in outside-out patches from pyramidal neurons, but not in DGCs). However, the role for these spontaneous openings, and whether they can contribute to GABA, R-mediated signaling in situ, has remained unclear (Macdonald et al., 1989; Birnir et al., 2000b). Here, we recorded from nucleated patches to show directly that spontaneously opening receptors are present in dentate granule cells. As predicted from other studies (McCartney et al., 2007), they are not affected by the competitive GABA, R antagonist SR95531 and are completely blocked by the open channel blocker picrotoxin. Lack of evidence of such openings in outside-out patches from dentate granule cells in previous studies may be related both to the small area of membrane and also to the effects of the internal cellular milieu on GABA, R channel gating.

A vexed question is how GABA in a slice relates to the in vivo situation. Direct measurements of GABA in CSF have revealed concentrations on the order of 200 nM (Glaeser and Hare, 1975). Using the zero-net-flux microdialysis method, we have estimated the concentration in the extracellular fluid to be even lower. This is somewhat at odds with an earlier study that estimated the GABA concentration in the extracellular fluid of the hippocampus to be 800 nM (Lerma et al., 1986). This measurement, however, was confounded by the complex method used to retrieve absolute concentrations from dialysate concentrations and reliance on estimates of diffusion across the dialysis membrane. It should also be noted that these previous estimates were performed in rats under urethane anesthesia, and sampling was performed 1 h after probe implantation, when the blood–brain barrier could still be disrupted. The microdialysis method averages over a large area, and so we cannot exclude the possibility that the regional GABA concentrations may differ and, in particular, that the concentration detected by neurons may be greater than this depending upon the balance of local release and uptake; however, this was not observed by us (and others) using the nsniffer patch technique. Indeed, 100 nM is close to the EC20 for extrasynaptic δ-subunit-containing GABA, Rs (Bright et al., 2011), and so the absence of a detectable GABA-mediated current under baseline conditions or when GABA in the perfusate is increased to 200 nM indicates that the GABA detected by neurons is less than that present in the extracellular space, perhaps due to efficient local GABA uptake. These results strongly argue against the widespread addition of high GABA concentrations (usually 5 μM) to the perfusate during ex vivo experiments. Indeed, these concentrations of GABA are 50-fold greater than those we detect in vivo.

Our findings have several important implications. First, our results indicate that studies that use SR95531 to measure tonic currents may significantly underestimate them. The results may also explain discrepancies in the measurement of tonic currents in DGCs, which in one study (and in contrast to others) was proposed not to be present in control tissue because of the lack of effect of SR95531 (Zhan and Nadler, 2009). Furthermore, the widespread use of SR95531 and on occasion high concentrations of SR95531, which could have a partial agonist effect, may significantly underestimate the magnitude of tonic currents present. Even when GABA is gating the receptor, displacement by SR99531 would not prevent spontaneous openings, and may therefore not accurately measure the tonic current. Moreover, our results indicate that there is always a tonic current present in dentate granule cells even when extracellular GABA concentrations are low (as they are most of the time). The importance of maintaining such an inhibitory tone in neurons is further underscored by the observation that knocking out receptors generating tonic current in cerebellar granule cells leads to an upregulation of a two-pore potassium channel to maintain the conductance (Brickley et al., 2001).

References


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