An Extrasynaptic GABA$_A$ Receptor Mediates Tonic Inhibition in Thalamic VB Neurons

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Jia, Fan, Leonardo Pignataro, Claude M. Schofield, Minerva Yue, Neil L. Harrison, and Peter A. Goldstein. An extrasynaptic GABA$_A$ receptor mediates tonic inhibition in thalamic VB neurons. J Neurophysiol 94: 4491–4501, 2005. First published September 14, 2005; doi:10.1152/jn.00421.2005. Whole cell patch-clamp recordings were obtained from thalamic ventrobasal (VB) and reticular (RTN) neurons in mouse brain slices. A bicuculline-sensitive tonic current was observed in VB, but not in RTN, neurons; this current was increased by the GABA$_A$ receptor agonist 4,5,6,7-tetrahydroisothiazolo-[5,4-c]pyridine-3-ol (THIP; 0.1 μM) and decreased by Zn$^{2+}$ (50 μM) but was unaffected by zolpidem (0.3 μM) or midazolam (0.2 μM). The pharmacological profile of the tonic current is consistent with its generation by activation of GABA$_A$ receptors that do not contain the α$_1$ or γ$_2$ subunits. GABA$_A$ receptors expressed in HEK 293 cells that contained α$_1$β$_2$δ subunits showed higher sensitivity to THIP (gaboxadol) and GABA than did receptors made up from α$_1$β$_2$δ, α$_1$β$_2$γ$_2$, or α$_1$β$_2$γ$_2$ subunits. Western blot analysis revealed that there is little, if any, α$_1$ or α$_3$ subunit protein in VB. In addition, co-immunoprecipitation studies showed that antibodies to the δ subunit could precipitate α$_{4}$ but not α$_1$ subunit protein. Confocal microscopy of thalamic neurons grown in culture confirmed that α$_1$ and δ subunits are extensively co-localized with one another and are found predominantly, but not exclusively, at extrasynaptic sites. We conclude that thalamic VB neurons express extrasynaptic GABA$_A$ receptors that are highly sensitive to GABA and THIP and that these receptors are most likely made up of α$_1$β$_2$δ subunits. In view of the critical role of thalamic neurons in the generation of oscillatory activity associated with sleep, these receptors may represent a principal site of action for the novel hypnotic agent gaboxadol.

INTRODUCTION

The activation of GABA$_A$ receptors inhibits neurons in two ways—via a fast or transient inhibition after GABA binding to synaptically localized receptors and by a sustained inhibition due to GABA binding to extrasynaptic receptors (Brickley et al. 1996; Farrant and Nusser 2005; Kanaeda et al. 1995; Mody 2001). Extrasynaptic receptors are excellent sensors for extracellular GABA due to their high affinity for GABA and slow rates of desensitization (Bai et al. 2001; Brickley et al. 1999; Yeung et al. 2003). The activation of these receptors regulates neuronal input resistance and, hence, excitability (Brickley et al. 2001; Semyanov et al. 2003). The δ subunit appears to be present in many extrasynaptic GABA$_A$ receptors. In cerebellar granule cells, these receptors likely contain α$_2$, β, and δ subunits (Brickley et al. 2001; Nusser et al. 1998; Pirker et al. 2000), whereas granule cells in the dentate gyrus likely contain α$_{4}$, β, and δ subunits (Nusser and Mody 2002; Sperk et al. 1997; Sun et al. 2004; Wei et al. 2003). Extrasynaptic GABA$_A$ receptors in CA1 pyramidal neurons in the hippocampus, however, likely contain α$_3$, β$_{2/3}$, and γ$_2$ subunits (Carasico et al. 2004).

Several GABA$_A$ receptor subunits are expressed in the thalamus. α$_1$, α$_4$β$_2$, γ$_2$, and δ subunits are found in thalamocortical neurons in the ventrobasal (VB) complex, whereas α$_3$, β$_3$, and γ$_2$ subunits are heavily expressed in the GABAergic neurons of the reticular nucleus (RTN) (Fritschy and Möhler 1995; Pirker et al. 2000). In particular, the thalamus shows a high level of expression of the δ subunit, suggesting that extrasynaptic GABA$_A$ receptors might be found in this region.

Whole cell patch-clamp recordings were obtained from thalamic neurons in acutely prepared mouse brain slices. A bicuculline-sensitive tonic current was recorded in neurons in the VB but not in the RTN. We have characterized the pharmacology of the tonic current and compared this with the profile of a variety of recombinant GABA$_A$ receptors expressed in HEK 293 cells, including the subtypes likely to be present in the thalamus. We also studied the nature of these receptors using Western blotting, immunohistochemistry, and co-immunoprecipitation and investigated their localization using confocal microscopy. Our results suggest that the tonic current in VB neurons is generated by GABA$_A$ receptors containing α$_{4}$, β, and δ subunits, and that these are found primarily at extrasynaptic locations.

METHODS

Brain slice preparation

Brain slices were acutely prepared as described (Ying and Goldstein 2005) from P12-20 C57Bl/6 mice (Charles River, Wilmington, MA) in accordance with institutional and federal guidelines. Briefly, mice were anesthetized with halothane and decapitated, and their brain were submerged in ice-cold carbogenated (95% O$_2$, 5% CO$_2$) slicing solution. The slicing solution contained (in mM): 217 sucrose, 2.5 KCl, 10 MgCl$_2$, 0.5 CaCl$_2$, 26 NaHCO$_3$, 1.25 Na$_2$HPO$_4$ and 11 glucose. Thick horizontal slices (300–400 μm) containing the thalamus were obtained using a vibrating microslicer (DTK, Kyoto, Japan). Slices were incubated in carbogenated artificial cerebrospinal fluid (ACSF) at 35°C for 30–60 min prior to use and then maintained at room temperature (20–22°C). ACSF contained (in mM): 124 NaCl, 2.5 KCl, 2 MgSO$_4$, 2 CaCl$_2$, 26 NaHCO$_3$, 1.25 Na$_2$HPO$_4$, and 10 glucose.

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Electrophysiological recordings in slices

Once transferred to the recording chamber, a brain slice was held in place by nylon threads attached to a platinum frame and continuously superfused with carbogenate ACSF. Thalamic neurons in the VB and RTN were visually identified using a Zeiss Axioskop FS microscope (Jena, Germany) equipped with DIC-IR optics. Whole cell patch-clamp recordings were performed under voltage-clamp using an Axopatch 200A (Axon Instruments, Union City, CA) amplifier. Cells were voltage clamped at −65 mV after correcting for liquid junction potential. Recording electrodes were made of borosilicate glass and had a resistance of 3–5 MΩ when filled with intracellular solution, which contained (in mM): 140 CsCl, 4 NaCl, 1 MgCl₂, 10 HEPES, 0.05 EGTA, 2 ATP-Mg₂⁺, and 0.4 GTP-Mg₂⁺; pH was 7.25 and osmolality was adjusted to 280–290 mOsm with sucrose.

During recordings, GABA₆ receptor-mediated spontaneous inhibitory postsynaptic currents (iSPSCs) were pharmacologically isolated by bath application of the ionotropic glutamate receptor blocker, kynurenic acid (3–5 mM). Access resistance was monitored using a 5-mV test pulse throughout the recording period; cells were included for analysis only if the series resistance was <25 MΩ and the change of resistance was <25% over the course of the experiment. Data were acquired at 5–10 kHz using pClamp 8 (Axon) and filtered at 2 kHz. Drugs were dissolved in ACSF solution and applied by bath superfusion. All compounds were obtained from Sigma (St. Louis, MO).

Data analysis

Off-line analysis was performed using MiniAnalysis 5.5 (Synaptosoft, Decatur, GA), SigmaPlot 6.0 (SPSS, Chicago, IL) and Excel 2000 (Microsoft, Redmond, WA). The holding current shift was measured as the difference in the holding current before and during drug application. All-point histograms were generated from 60-s segments before and during drug application and fitted by single Gaussian distribution (Nusser and Mody 2002). Unless otherwise indicated, averaged data are expressed as mean ± SE. Statistical significance was assessed using Student’s t-test and P < 0.05 was considered statistically significant.

Immunohistochemistry and co-immunoprecipitation

Mouse thalami from adult C57Bl/6 mice were dissected and homogenized in a Teflon-glass homogenizer with 4 vol/wt ice-cold buffer [50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1 mM EDTA] and a protease inhibitor cocktail that contained (in µM): 20 4-(2-aminoethyl)benzenesulfonyl fluoride (AEBSF), 10 EDTA, 1.3 bestatin, 0.14 E-64, 10 leupeptin, and 3 aprotinin (reagents from Sigma). The crude homogenate was centrifuged at 2,900 g for 20 min at 4°C to separate the nuclei, and the supernatant containing the membrane and cytosolic fractions was centrifuged again at 29,000 g for 1 h. The pellet containing membranes was re-suspended in complete buffer [buffer with 1% vol/vol ethylphenyl-polyyethylene glycol (Nonidet P40) and 0.25% wt/vol sodium deoxycholate] with the protease inhibitor cocktail. Protein concentration was determined with the Bradford reagent (OD value using the function: OD = Log10 255/(255 – gray value)]. The OD values of the α subunit proteins were normalized to the eIF4E internal standard to compensate for loading and transference variations. All compounds were obtained from Sigma (St. Louis, MO).

Co-immunoprecipitation experiments were carried out using the ProFound mammalian co-immunoprecipitation kit from Pierce (Rockford, IL). Affinity-purified rabbit polyclonal antibodies (50 µg), raised against GABA₆ receptor γ, δ subunits (Alpha Diagnostics), were coupled to AminoLink Plus gel as specified by the manufacturer. Thalami were re-suspended in M-PER (mammalian protein extraction reagent) with protease inhibitors at ~0.12 mg tissue/µl and homogenates were centrifuged at 27,000 g for 15 min to remove cellular debris. Supernatants were incubated with the antibody-coupled gel overnight at 4°C with gentle rocking. Gels were washed four times with buffer, and complexes were recovered with 130 µl of the elution buffer provided with the kit. Controls were performed by quenching the gel before coupling antibodies and by using a control gel provided with the kit to test for proteins that may bind nonspecifically to the gel. Approximately 40 µl of the eluates were analyzed by immunoblotting as described above with affinity purified rabbit polyclonal anti-GABA₆ receptor α₁, α₂, or α₃ subunit antibodies.

To determine the specificity of δ antibody, immunoblotting was performed by incubating the antibody overnight at 4°C with a 10-fold mass excess (20 µg/ml) of δ unrelated control peptide in TBS. The specificity of the α₄ antibody has been previously described (Bencsits et al. 1999).

Immunohistochemistry

Young adult mice were killed with an overdose of halothane and then perfused with 4% ice-cold paraformaldehyde in PBS. After dissection, brains were postfixed for 2 h in the same solution, washed extensively with PBS and then immersed in sucrose solutions of increasing concentration (10–30% wt/vol) for cryoprotection. Brains were frozen in Optimal Cutting Temperature (OCT) compound. Cryostat sections (20 µm) were collected on poly-L-lysine coated slides and stored at −80°C until use. Sections from at least five mice were examined for each immunohistochemistry (IHC) experiment. The figures shown in RESULTS are representative of at least three different IHC experiments on tissue from two mice that yielded essentially identical results. Sections were first processed for antigen retrieval by microwaving them at ~700 W for 90 s in 0.1 M sodium citrate buffer (pH 7.5); sections were then washed in PBS, permeabilized with 0.1% vol/vol Triton X-100, washed again and finally blocked for 1 h in 10% wt/vol normal donkey serum, 3% wt/vol IgG-free bovine serum albumin in PBS. Sections were incubated overnight at 4°C with primary antibody diluted in the blocking solution. The next day, sections were washed with PBS and incubated for 1 h at room temperature with the corresponding anti-IgG affinity purified conju-
gated secondary antibody (1:200 dilution) when primary antibodies were unlabeled. After three PBS washes, sections were mounted with ProLong Gold antifade reagent (Molecular Probes, Eugene, OR).

Optical sections (0.8–10 µm) were acquired with an inverted Zeiss Axiovert 200 confocal microscope (LSM 510 META; Carl Zeiss Meditec, Thornwood, NY) equipped with diode (405 nm), argon (458, 477, 488, 514 nm), HeNe1 (543 nm), and HeNe2 (633 nm) lasers and an X-Y motorized stage for stitching images (in 2 dimensions) of large objects. To prevent bleed through of signals to neighboring detecting channels, especially when investigating the co-localization of the GABA<sub>A</sub> receptor subunits, images were acquired using the multi-track configuration of the microscope detection system. Antibodies used for immunohistochemistry were: affinity-purified rabbit polyclonal anti-GABA<sub>A</sub> receptor α<sub>4</sub> subunit antibody (10 µg/ml, Novus Biologicals) and affinity-purified rabbit polyclonal anti-GABA<sub>A</sub> receptor δ subunit antibody (10 µg/ml; Alpha Diagnostics). In the co-localization experiments, no secondary antibodies were used; instead, the anti-α<sub>4</sub> antibody was labeled with fluorescein-EX dye and the anti-δ antibody with Texas red-X using Molecular Probes labeling kits.

Immunocytochemistry and electrophysiology using primary cultures of thalamic neurons

To investigate α<sub>4</sub> and δ subunit localization to synaptic and/or extrasynaptic sites, primary cultures of thalamic neurons were established. Dissection of thalami from E18 mouse pups obtained from time-mated females was performed according to published prenatal anatomical landmarks (Schambra et al. 1992). Cultures were established and maintained following the technique used for low-density hippocampal neurons (Goslin et al. 1998). Cells were maintained in culture and used for immunocytochemistry or electrophysiological experiments no earlier than 11 days after plating.

Immunostaining to detect surface GABA<sub>A</sub> receptors on live neurons was performed with fluorescent dye-labeled anti-α<sub>4</sub> and anti-δ antibodies (20 µg/ml), which were added to the culture medium and incubated for 15 min at 37°C. After one wash with warm PBS, cells were fixed with warm 4% paraformaldehyde in PBS containing 0.12 M sucrose for 15 min at 37°C. Cells were then washed with PBS containing 0.2 M glycine, permeabilized with 0.1% vol/vol Triton X-100, washed again, and blocked with 10% vol/vol normal donkey serum and 3% wt/vol IgG-free bovine serum albumin in PBS for 1 h. The rest of the procedure was performed as described for the immunohistochemistry of tissue sections. Cells were mounted with ProLong Gold antifade reagent containing DAPI (Molecular Probes). The same considerations explained for tissue immunohistochemistry were observed for confocal microscopy. Optical sections were 0.8–1.2 µm thick. To determine the presence of synaptic or extrasynaptic GABA<sub>A</sub> receptors, cells were stained with affinity-purified mouse monoclonal anti-synaptophysin 1 antibody (10 µg/µl, Synaptic Systems, Göttingen, Germany) and Cy5-conjugated affinity-purified donkey anti-mouse IgG (minimal cross-reactivity) from Jackson Immunoresearch (West Grove, PA).

The degree of co-localization of α<sub>4</sub>, δ, and synaptophysin immunoreactivity was estimated using the ImarisColoc module of Imaris program V 4.2.0 (Bitplane AG, Zurich, Switzerland). Confocal images were restored by subtracting background and by thresholding to separate image voxels (volume elements or 3-dimensional pixels) from background voxels. Considering how the images were generated (use of labeled primary antibodies and use of confocal microscope in the multi-track configuration), no further attempts were made to reduce cross-reactivity of fluorescent probes or optical cross-talk. Co-localization was determined using the algorithm of Costes and colleagues (2004), in which voxels are considered co-localized when the intensity of the two channels is above threshold in a two-dimensional (2-D) histogram (each voxel represents a point in the 2-D histogram where the x coordinate is the intensity of the voxel in one channel and the y coordinate is the intensity for the same voxel in the other channel). The percentage of co-localization was obtained by dividing the volume of co-localized voxels by the entire volume of the image above threshold. The data presented here are expressed in terms of the percentage of co-localized weighted voxels (essentially as explained in the preceding text but with weight assigned according to the brightness of each voxel). The data are expressed as means ± SE from ≥10 cells in experiments performed in duplicate using two independent cultures.

Recordings from HEK 293 cells expressing recombinant GABA<sub>A</sub> receptors

The cDNAs encoding the α<sub>4</sub>, α<sub>2</sub>, β<sub>2</sub>, γ<sub>2</sub>, and δ subunits were sub-cloned into the pcDNA3.1 expression vector and transiently expressed in human embryonic kidney (HEK) 293 cells (American Type Culture Collection, Rockville, MD). For electrophysiological recordings, cells were plated onto glass coverslips coated with poly-L-lysine (Sigma) and transfected with 2.5 µg of each cDNA plasmid (5 µg for δ) using the calcium phosphate precipitation method. Cells were washed after 24 h of contact with cDNA precipitate and used for patch-clamp recording 48–72 h post transfection. GABA and 4.5,6,7-trihydrodiospirothiazolo-[5,4-c]pyridine-3-ol (THIP)–gated currents were recorded at room temperature using the whole cell patch-clamp method (voltage clamped at −60 mV) using an Axopatch 1C amplifier (Axon). The extracellular solution contained (in mM): 145 NaCl, 3 KCl, 1.5 CaCl<sub>2</sub>, 1 MgCl<sub>2</sub>, 6 d-glucose, and 10 HEPES, pH adjusted to 7.4 with NaOH. Patch pipettes had a resistance of 5 MΩ when filled with the intracellular solution, which contained (in mM): 145 N-methyl-D-glucamine hydrochloride, 0.1 CaCl<sub>2</sub>, 5 ATP-K<sup>+</sup>, 1.1 EGTA, 2 MgCl<sub>2</sub>, and 5 HEPES, pH adjusted to 7.2 with KOH. GABA or THIP was rapidly applied (∼50-ms exchange time) to the cell via a multichannel infusion pump and motor-driven solution exchange device (Rapid Solution Changer RSC-100; Molecular Kinetics, Pullman, WA).

The peak current amplitude of each agonist response was measured for each cell, and the agonist concentration-response amplitude data were fitted using a sum of least squares method to a Hill equation of the form: \[ I = I_{\text{Max}} \times \frac{[\text{agonist}]^{n_I}}{[\text{agonist}]^{n_I} + EC_{50}^{n_I}} \], where I is the peak current, \( I_{\text{Max}} \) is the maximum whole cell current amplitude, \( [\text{agonist}] \) is the agonist concentration, \( EC_{50} \) is the agonist concentration eliciting a half-maximal current response, and \( n_I \) is the Hill coefficient.

RESULTS

In recordings from chloride-loaded VB neurons under voltage-clamp at −70 mV, we observed sIPSCs (Fig. 1A, left). These sIPSCs were abolished by bicuculline (20 µM), which also produced a shift in the holding current of ∼20 pA, consistent with the block of a tonic GABA<sub>A</sub> receptor-mediated inward current. This is conveniently illustrated by the all-points histogram, which shows that bicuculline produced a shift in mean current from 0 to 25.0 ± 3.0 pA in the example illustrated (Fig. 1A, right).

Voltage-clamp recordings from RTN neurons also revealed the presence of bicuculline-sensitive sIPSCs (Fig. 1, B and C). In contrast to its effect on VB neurons, bicuculline did not change the holding current in RTN neurons (Fig. 1, B and C). The all-points histogram from the recording of a typical RTN neuron shows that the mean current was unchanged in the presence of bicuculline (Fig. 1B, right). Analysis of the spontaneous events showed that the sIPSCs recorded from RTN neurons were less frequent, of smaller amplitude, and decayed more slowly than those seen in VB neurons (Fig. 1C). The characteristics of the sIPSCs are provided in Table 1 and are
Pharmacological characterization of the tonic current in VB neurons

To investigate the subtype(s) of the GABAA receptor contributing to the tonic current, we characterized the pharmacology of the tonic current recorded in VB neurons. The agonist THIP (0.1 μM), a structural analogue of muscimol, induced an inward current in VB neurons that was typically around –50 pA in amplitude (Fig. 2A), whereas this concentration of THIP elicited ±2–3 pA of inward current in RTN neurons (data not shown).

Micromolar concentrations of Zn2+ have little effect on α1β2γ2 GABAA receptors but inhibit current in receptors containing δ subunits (Saxena and Macdonald 1994, 1996). Addition of 50 μM Zn2+ decreased the tonic current by +5 to +10 pA in VB neurons (Fig. 2B). Unlike classic benzodiazepines, the imidazobenzodiazepine inverse agonist Ro 15–4513 potentiates GABA-evoked currents in cells expressing α3β2γ2 receptors (Brown et al. 2002; Knoflach et al. 1996) but not α1β2δ receptors (Brown et al. 2002). We found that 3 μM Ro15-4513 had no effect on the holding current in VB neurons (Fig. 2C).

The α1 subunit is highly expressed in VB neurons (Fritschy and Möhler 1995; Pirker et al. 2000), where it undoubtedly contributes to synaptically activated GABAA receptors (Huntsman and Huguenard 2000; Zhang et al. 1997). We tested whether α1β2γ2 subunit-containing receptors also contribute to the tonic current using the α1 subunit-selective imidazopyridine, zolpidem (Goldstein et al. 2002; Pritchett and Seeburg 1990; Vicini et al. 2001; Wafford et al. 1993). Zolpidem (0.3 μM) had no effect on the holding current in VB neurons (Fig. 2D), although it clearly prolonged the decay time of sIPSCs at this concentration (Table 1). Similarly, the nonsynaptic benzodiazepine midazolam (0.2 μM) had no effect on the holding current in VB neurons, although it did prolong the decay time of sIPSCs (Table 1).

Localization of αδ and δ subunits in thalamic neurons

These electrophysiological data strongly suggested that the tonic current in VB neurons was not mediated by GABAA receptors consisting of the typical “synaptic” receptor subunit combinations, for example α1β2γ2. Several combinations of subunits have been implicated in extrasynaptic GABAA receptor function, notably the α2, α5, α6, and δ subunits (Farrant and Nusser 2005). Of these, the α2 subunit is expressed only in the cerebellum (Jones et al. 1997; Pirker et al. 2000). We first examined the expression of the α2 and δ subunits in homogenates of mouse thalamus by immunoblotting. Using specific antisera, the imidazobenzodiazepine inverse agonist Ro 15–4513 induced an inward current in VB neurons (Fig. 2C).

TABLE 1. Pharmacological profile of tonic and phasic GABAAergic currents in ventrobasal neurons

<table>
<thead>
<tr>
<th>Drug</th>
<th>Cells (n)</th>
<th>Current, pA</th>
<th>Phosphorylation</th>
<th>Decay Time, ms</th>
<th>Amplitude, pA</th>
<th>Frequency, Hz</th>
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</thead>
<tbody>
<tr>
<td>Control</td>
<td>21</td>
<td>+16.5 ± 2.0*</td>
<td>(by BIC)</td>
<td>14.0 ± 1.2</td>
<td>58.3 ± 6.5</td>
<td>7.6 ± 0.7</td>
</tr>
<tr>
<td>THIP</td>
<td>10</td>
<td>–47.7 ± 8.7*</td>
<td>–1.1</td>
<td>13.1 ± 1.5</td>
<td>59.8 ± 5.0</td>
<td>7.9 ± 1.2</td>
</tr>
<tr>
<td>ZnCl2</td>
<td>11</td>
<td>+6.9 ± 1.1*</td>
<td>+1.2</td>
<td>11.7 ± 1.4</td>
<td>49.4 ± 8.4</td>
<td>6.7 ± 1.1</td>
</tr>
<tr>
<td>Ro 15-4513</td>
<td>10</td>
<td>0.7 ± 0.8</td>
<td>+3.1</td>
<td>14.1 ± 1.7</td>
<td>59.5 ± 12.4</td>
<td>7.1 ± 1.3</td>
</tr>
<tr>
<td>Zolpidem</td>
<td>10</td>
<td>–0.5 ± 1.1</td>
<td>+1.2</td>
<td>19.9 ± 2.1*</td>
<td>67.2 ± 10.1</td>
<td>6.9 ± 1.4</td>
</tr>
<tr>
<td>Midazolam</td>
<td>8</td>
<td>–0.1 ± 1.1</td>
<td>+1.2</td>
<td>17.9 ± 0.9*</td>
<td>56.5 ± 8.8</td>
<td>7.7 ± 1.7</td>
</tr>
</tbody>
</table>

Values are means ± SE. sIPSC, spontaneous inhibitory postsynaptic current; BIC, bicuculline; THIP, 4,5,6,7-tetrahydroisothiazolo-[5,4-c]pyridine-3-ol. *, P < 0.05 compared to control, Student’s t-test.
antibodies directed against α4 and δ subunits, we observed immunoreactive bands with apparent mass of ~60–65 kDa in both total homogenates and membrane fraction obtained from the thalamus (Fig. 3A). We also determined the relative expression of a variety of other α subunits in total homogenates of the mouse thalamus. Our results confirmed that α1 and α4 are the most abundant α subunits in the thalamus as determined by semi-quantitative immunoblot analysis (Fig. 3B, C).

Immunohistochemistry on mouse brain sections demonstrated that α4 and δ subunits were both present in VB (Fig. 4, A–C). High-magnification images demonstrated that α4 punctate staining overlapped with δ staining at the cellular level (Fig. 4, A–C, insets). Co-localization of α4-δ immunostaining as demonstrated, however, is not proof of physical interaction between the two proteins. To determine if α4 and δ subunits interact in vivo, co-immunoprecipitation experiments were performed. Thalamic homogenates were immunoprecipitated with anti-δ antibody, and the precipitate was immunoblotted using anti-α4 antibody. As seen in Fig. 3D, the immunoprecipitate contained an α4-immunoreactive band similar to that observed in the thalamic homogenates. We also investigated the proportion of protein complexes formed between δ subunit and other α subunits and found that neither α1 nor α5 immunoreactive protein co-immunoprecipitated with the δ subunit (Fig. 3, E and F). We did observe, however, some α4 subunit protein in association with γ2 (Fig. 3G). Those data strongly suggest that GABAA receptors containing α4 in the thalamus are likely to include α4β2γ2δ and α4β2γ2γ complexes.

To establish the specificity of the immunoblots, the δ antibody was preabsorbed with a 10-fold mass excess of δ control peptide. The peptide completely blocked the immunostaining of the 65-kDa band in the thalamic membrane fraction (Fig. 3H). In contrast, preincubation of δ antibody with an unrelated peptide did not affect the staining of the δ-reactive band (Fig. 3H). The specificity of the α4 antibody used in this work has been documented previously (Bencsits et al. 1999).

To determine the sub-cellular localization of the α4-δ-containing GABAA receptors, immunocytochemistry was performed on primary cultures of thalamic neurons. As shown in Fig. 4, D–G, the punctate pattern of α4 immunoreactivity co-localizes extensively with that for the δ subunit. The extent of co-localization was carefully analyzed and was ~70% for α4 and δ subunits (Table 2). We also studied the distribution of the synaptic protein synaptophysin in the same neurons. Visual inspection of the confocal images suggested that the α4-δ immunoreactive puncta did not co-localize with presynaptic elements, consistent with the idea that α4-δ subunits are co-localized primarily at extrasynaptic sites. The extent of co-localization with synaptophysin was then rigorously quantified (Table 2) and was on the order of 10% for δ subunits, confirming the idea that δ subunits are located largely at nonsynaptic sites. This was significantly higher (~22%) for α4 subunits. Consistent with these data, we recorded a bicuculline-sensitive tonic current in these cultured mouse thalamic neurons (Fig. 4, H and I).

**Pharmacological analysis of α4β2δ, α4β2δ, α4β2γ22s, and α4β2γ2γ2 receptors in HEK 293 cells**

We next evaluated the sensitivity to GABA and THIP of a variety of recombinant receptor subtypes expressed in HEK 293 cells (Fig. 5). As reported previously (Brown et al. 2002), THIP was clearly a partial agonist at α4β2γ2γ2 receptors but was more efficacious than GABA at α4β2δ receptors (Fig. 5, A and D). The receptors containing α4β2δ subunits were less sensitive to THIP and GABA than those containing α4β2γ2γ2 receptors (Fig. 5, A, C, and D). The threshold concentration of THIP for the activation of the α4β2δ receptors was 300 nM, whereas micromolar concentrations were required for THIP activation of α4β2δ, α4β2γ2γ2, and α4β2γ2γ2 receptors (Table 3; Fig. 5E).

**Discussion**

**Subunit composition of GABAA receptors responsible for the tonic current in VB neurons**

Bicuculline-sensitive tonic currents were recorded in VB neurons in acutely prepared mouse brain slices (Fig. 1), con-
sistent with previous reports of a tonic GABA\(\Lambda\) receptor-mediated conductance in VB neurons (Porcello et al. 2003). The \(\alpha_1\) subunit-selective imidazopyridine zolpidem (Pritchett and Seeburg 1990; Wafford et al. 1993) increased the decay time of sIPSCs recorded in VB neurons, in agreement with previous reports (Browne et al. 2001; Huntsman and Huguenard 2000; Okada et al. 2000; Zhang et al. 1997), but had no effect on the tonic current (Fig. 2D; Table 1); similar effects were observed with midazolam. These results with zolpidem and midazolam are consistent with the incorporation of the \(\alpha_1\) and \(\gamma_2\) subunits into synaptic, but not extrasynaptic, GABA\(\Lambda\) receptors in VB neurons. The inverse agonist Ro 15–4513, which potentiates GABA-evoked currents in receptors in VB neurons (Brown et al. 2002; Knoflach et al. 1996), had no effect on the tonic current (Fig. 2D). Previous reports (Browne et al. 2001; Huntsman and Huguenard 1996) indicated that the extrasynaptic GABA\(\Lambda\) receptors are unlikely to contain combinations of \(\alpha_4\) and \(\gamma_2\) subunits.

The electrophysiological data from our brain slice recordings in VB neurons appeared to eliminate many potential GABA\(\Lambda\) receptor subtypes as generators of the tonic current. Our recordings from a variety of recombinant receptor subtypes expressed in HEK 293 cells confirmed the higher efficacy of THIP, relative to GABA, in \(\alpha_1\beta_2\gamma_2\) and \(\alpha_2\beta_2\delta\) GABA\(\Lambda\) receptors (Fig. 5C) and the higher potency of GABA in these receptors relative to \(\alpha_1\beta_2\gamma_2\) (Fig. 5A). Of special interest to us was the fact that \(\alpha_1\beta_2\delta\) receptors responded to very low concentrations of THIP (300 nM; Fig. 5D) with small but highly reproducible currents that were \(\sim 3\%\) of the maximal current amplitude. In contrast, those low concentrations of THIP produced no significant current in \(\alpha_1\beta_2\delta, \alpha_2\beta_2\gamma_2, or \alpha_1\beta_2\gamma_2\) GABA\(\Lambda\) receptors. It is worth noting, however, that normalizing the THIP-evoked current (\(I_{\text{THIP}}\)) against the maximum GABA-evoked current (\(I_{\text{GABA-MAX}}\)) in Fig. 5D may overestimate the efficacy of THIP as the efficacy of GABA is not constant across the subunit combinations.

Of the GABA\(\Lambda\) receptor \(\alpha\) subunits, \(\alpha_1\) and \(\alpha_4\) are the most heavily expressed in the thalamic nuclei that comprise the VB because \(\alpha_1\) is restricted to the cerebellum, \(\alpha_4\) is only weakly detected in VB (Fig. 3, B and C), and \(\alpha_2\) and \(\alpha_3\) are apparently undetectable (Jones et al. 1997; Pirker et al. 2000). Immunoprecipitation data indicates that \(\alpha_1\)-subunit containing receptors represent 20–30% of the total GABA\(\Lambda\) receptor population in the thalamus (Khan et al. 1996; Sur et al. 1999). Of the \(\alpha_4\)-subunit containing population, 60–70% of the \(\alpha_4\) subunit co-precipitates with the \(\delta\) subunit, while essentially all of the \(\delta\) subunit is thought to be associated with \(\alpha_4\)-containing receptors (Sur et al. 1999). GABA\(\Lambda\) receptors containing the \(\delta\) subunit are likely to consist of combinations of \(\alpha_1\) and \(\gamma_2\) subunits. Receptors containing the \(\alpha_4\) subunit also show a high degree of sensitivity to Zn\(^{2+}\) (Brown et al. 2002), but this is independent of whether \(\delta\) or \(\gamma_2\) subunits are present. GABA\(\Lambda\) receptors containing both \(\alpha_4\) and \(\gamma_2\) subunits are potentiating by Ro 15–4513 (Brown et al. 2002; Knoflach et al. 1996). This was not observed in VB neurons, indicating that the extrasynaptic GABA\(\Lambda\) receptors are unlikely to contain combinations of \(\alpha_4\) and \(\gamma_2\) subunits.
subunit are thought to be exclusively extrasynaptic (Brickley et al. 2001; Nusser and Mody 2002; Nusser et al. 1998; Sassoe-Pognetto et al. 2000) and contribute to a tonic GABA-mediated conductance in granule neurons of the cerebellum and dentate gyrus (Brickley et al. 2001; Nusser and Mody 2002). The presence of a tonic current in VB neurons is, therefore, consistent with the expression of δ subunit-containing receptors.

We examined whether the α4 and δ subunits, in fact, co-localized to extrasynaptic sites on the cell membrane using immunohistochemistry. The α4 and δ subunits were clearly detected in the membrane fraction of thalamic homogenates (Fig. 3A) and, in agreement with previous observations (Sur et al. 1999), were found to co-immunoprecipitate from these homogenates (Fig. 3E). The failure to observe either α1-δ or α5-δ complexes using co-immunoprecipitation from these homogenates suggests that neither α1β2δ nor α5β2δ receptors are found in the thalamus.

Immunofluorescent labeling of cultured thalamic neurons with antibodies directed against the α4 and δ subunits demonstrated that the subunits were co-localized (Fig. 4, A–G) and also showed that the punctate pattern of fluorescence did not overlap with that of the synaptic marker, synaptophysin (Tixier-Vidal et al. 1988; Wiedenmann and Franke 1985). These data confirm that α4 and δ subunits are found in the same receptor complexes and that they are present at predominantly extrasynaptic sites.

**TABLE 2. Quantification of the co-localization of α4, δ, and synaptophysin staining in thalamic cultures**

<table>
<thead>
<tr>
<th>Co-localization percent of:</th>
<th>α4</th>
<th>δ</th>
<th>synaptophysin</th>
</tr>
</thead>
<tbody>
<tr>
<td>with:</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>α4</td>
<td>79.5 ± 6.9</td>
<td>69.4 ± 6.9</td>
<td>synaptophysin</td>
</tr>
<tr>
<td>δ</td>
<td>10.7 ± 2.8* †</td>
<td>9.3 ± 0.6* †</td>
<td>synaptophysin</td>
</tr>
<tr>
<td>synaptophysin</td>
<td>α4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>α4</td>
<td>22.8 ± 7.1* †</td>
<td>21.2 ± 4.9* †</td>
<td>synaptophysin</td>
</tr>
</tbody>
</table>

Values are expressed as mean ± SE of >10 cells from duplicate experiments using two independent cultures. Data are the percentage of co-localized weighted voxels (volume of co-localized voxels over the entire volume of the image above threshold). *Significantly different from α4-δ; †, significantly different from δ-α4 by one-way ANOVA with Tukey’s post test (in all cases \( P < 0.001 \)).
The identity of the β subunit in the extrasynaptic GABA_A receptors is unknown, although one can make some intelligent inferences. VB neurons do not appear to express the β3 subunit (Pirker et al. 2000), but it is found in neurons of the RTN. The genetic deletion of the β3 subunit had no significant effect on sIPSCs recorded in VB neurons (Huntsman et al. 1999). In rats older than P12, no mRNA coding for the β1 subunit could be detected in the region of the VB (Laurie et al. 1992; Wisden et al. 1992), and this is consistent with the minimal detection of β1 subunit protein in that region (Pirker et al. 2000). In contrast, β2 mRNA is strongly detected in this region beginning at age P12 (Laurie et al. 1992; Wisden et al. 1992); this correlates with the dense expression of β2 protein in adult animals (Pirker et al. 2000). It is likely, therefore that the β2 subunit is present in both the extrasynaptic and synaptic receptors expressed by VB neurons. Our data would appear consistent with the proposition that extrasynaptic receptors in VB neurons have an α4β2δ configuration.

**TABLE 3. Activation of recombinant GABA_A receptors by GABA and THIP**

<table>
<thead>
<tr>
<th>Subunit Combination</th>
<th>n</th>
<th>Threshold</th>
<th>EC_{50}</th>
<th>n_{hit}</th>
<th>( \frac{I_{GABA_{max}}}{PA} )</th>
</tr>
</thead>
<tbody>
<tr>
<td>α4β2δ</td>
<td>11</td>
<td>100 nM</td>
<td>3.0 ± 0.3</td>
<td>1.22 ± 0.11</td>
<td>−236 ± 25</td>
</tr>
<tr>
<td>α1β2γ2δ</td>
<td>16</td>
<td>3 μM</td>
<td>46.4 ± 3.9</td>
<td>0.96 ± 0.03</td>
<td>−327 ± 37</td>
</tr>
<tr>
<td>α1β2γ2μ</td>
<td>14</td>
<td>1 μM</td>
<td>4.9 ± 0.4</td>
<td>1.32 ± 0.04</td>
<td>−1033 ± 121</td>
</tr>
<tr>
<td>α1β2γ2μ</td>
<td>30</td>
<td>3 μM</td>
<td>21.1 ± 3.0</td>
<td>1.27 ± 0.08</td>
<td>−2058 ± 182</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Subunit Combination</th>
<th>n</th>
<th>Threshold</th>
<th>EC_{50}</th>
<th>n_{hit}</th>
</tr>
</thead>
<tbody>
<tr>
<td>α4β2δ</td>
<td>16</td>
<td>300 nM</td>
<td>51 ± 4</td>
<td>1.38 ± 0.03</td>
</tr>
<tr>
<td>α1β2γ2δ</td>
<td>23</td>
<td>3 μM</td>
<td>183 ± 10</td>
<td>1.06 ± 0.06</td>
</tr>
<tr>
<td>α1β2γ2μ</td>
<td>10</td>
<td>3 μM</td>
<td>86 ± 4</td>
<td>0.85 ± 0.02</td>
</tr>
<tr>
<td>α1β2γ2μ</td>
<td>9</td>
<td>3 μM</td>
<td>166 ± 5</td>
<td>0.59 ± 0.02</td>
</tr>
</tbody>
</table>

Threshold is defined as the concentration of GABA or THIP required to produce a current that is significantly different (\( P < 0.05 \), one-tailed unpaired t-test) from a baseline holding current of 0 ± 2.6 pA.
Physiological significance of extrasynaptic GABA<sub>A</sub> receptors in VB neurons

The driving force for chloride ions is an important factor that regulates the net effect(s) of GABA<sub>A</sub> receptor activation. Perforated patch-clamp recordings have shown that GABA currents reverse at a more negative membrane potential in VB (~81 mV) than in RTN neurons (~71 mV) (Ulrich and Huguenard 1997), consistent with a difference in the chloride equilibrium potential ($E_{Cl}$) between these two regions. The difference in $E_{Cl}$ likely reflects the differential distribution of the KCl co-transporter, KCC2, which is found in thalamic VB relay neurons but not in RTN neurons (Bartho et al. 2004). Given that the resting potential of thalamic relay neurons, at approximately ~70 mV, is more positive than the GABA reversal potential, the tonic activation of extrasynaptic GABA<sub>A</sub> receptors would be expected to hyperpolarize the membrane potential and to produce shunting inhibition, thereby altering the input-output relationship in neurons (Brickley et al. 2001; Mitchell and Silver 2003). Alterations in the amount of tonic inhibition may be associated with significant behavioral consequences. For example, in GABA transporter subtype 1 (GAT-1) knockout mice, there is an increase in the amplitude of the GABA-induced tonic conductance in granule and Purkinje cells in the cerebellum, and the transporter deficiency is associated with tremor, ataxia, and nervousness in these animals (Chiu et al. 2005).

Therapeutic implications

Recurrent interactions between thalamic relay neurons and reticular nucleus interneurons are responsible for the generation of synchronized oscillations in neuronal populations, such as sleep spindles, and the slow wave activity associated with non-REM sleep and absence seizures (McCormick 2002); higher frequency γ oscillations in thalamocortical circuits are thought to be important in establishing different levels of consciousness and attention (Jones 2002; McCormick and Bal 1997; Steriade 2000). A wide range of psychoactive compounds with hypnotic effects, including thiopental, propofol, and isoflurane, disrupt gamma frequency oscillations both in vitro (Antkowiak and Hentschke 1997; Faulkner et al. 1998; Whittington et al. 1996) and in vivo (Munglani et al. 1993). It has been argued that anesthetic disruption of such fast oscillatory activity within thalamocortical circuits is the basis for anesthetic-induced unconsciousness (Alkire et al. 2000; John and Prichep 2005). Modeling of neuronal networks has shown that fast oscillations can be desynchronized by an increase in the tonic GABA<sub>A</sub> receptor conductance (Maex and Schutter 1998). Indeed, several general anesthetics are known to increase a tonic current in CA1 hippocampal neurons (Bai et al. 2004; Yeung et al. 2003), while ethanol enhances the GABA<sub>A</sub> receptor-mediated tonic current in dentate (Wei et al. 2004) and cerebellar granule cells (Hanchar et al. 2005). It will be of interest, therefore, to investigate further the sensitivity of the tonic current in VB neurons to both anesthetics and alcohol.

The GABA agonist, THIP, was first synthesized in 1977. THIP was designed to be a lipid-soluble GABA agonist that would cross the blood-brain barrier and, hence, be orally active (Krogsgaard-Larsen et al. 1977). THIP has been described as a “super” agonist at GABA<sub>A</sub> receptors containing the α<sub>4</sub> and δ subunits because it has a higher efficacy at these receptors than GABA, whereas it acts as a partial agonist at conventional GABA<sub>A</sub> receptors containing α<sub>1</sub>β<sub>2</sub>γ<sub>2</sub> subunits (Brown et al. 2002; Ebert et al. 1994, 1997). Behaviorally, THIP (“gaboxadol”) increases non-REM sleep in rats (Lancel 1997; Lancel and Faulhaber 1996; Lancel and Langebartels 2000) and humans (Faulhaber et al. 1997; Lancel et al. 2001; Mathias et al. 2001), and a low incidence of reported side-effects suggests that the drug may be superior to currently available agents (Iversen 2004; Krogsgaard-Larsen et al. 2004).

We have shown that THIP significantly increases the amplitude of the tonic current in thalamic VB neurons, presumably by mimicking the actions of endogenous GABA. These data hint at the important role of tonic inhibition in the regulation of sleep and provide an anatomical locus for the hypnotic action of the drug gaboxadol. Further progress in our understanding of the identity of the GABA<sub>A</sub> receptors responsible for mediating the tonic conductance in different brain regions should facilitate the development of subtype-selective therapeutic agents that can produce hypnosis, while minimizing undesirable side effects (respiratory depression, ataxia) that may occur due to nonselective interactions with GABA<sub>A</sub> receptors (Krogsgaard-Larsen et al. 2004; Lambert and Belelli 2002).

Summary and conclusions

We observed a bicuculline-sensitive current in VB, but not RTN, neurons in the thalamus of the mouse. This tonic GABA<sub>A</sub> receptor-mediated current in VB neurons was increased by THIP and decreased by Zn<sup>2+</sup> but was unaffected by zolpidem or midazolam. The remarkable sensitivity of these GABA<sub>A</sub> receptors to very low concentrations of THIP (0.1 μM) is also observed in recordings from α<sub>4</sub>β<sub>3</sub>δ receptors expressed in HEK 293 cells. Triple labeling for α<sub>4</sub>, δ, and synaptophysin in cultured thalamic neurons showed that α<sub>4</sub> and δ subunit proteins are extensively co-localized and occur predominantly, but not exclusively, at extrasynaptic locations. Taken together, our data are consistent with the notion that the tonic current in VB neurons results from the persistent activation of extrasynaptic α<sub>4</sub>β<sub>3</sub>δ GABA<sub>A</sub> receptors by ambient levels of GABA in the perisynaptic space.

Acknowledgments

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References


