GABA Transporter-1 (GAT1)-Deficient Mice: Differential Tonic Activation of GABA\textsubscript{A} Versus GABA\textsubscript{B} Receptors in the Hippocampus

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INTRODUCTION

After its release, GABA is taken up both to terminate inhibitory transmission and for reuse by neurons and glia. Because inhibition of GABA uptake has antiepileptic, cognition enhancing, and neuroprotective effects (O’Connell et al. 2001), there is an extensive interest in physiological and clinical aspects of GABA uptake. Four distinct GABA transporters (GATs 1–3 and a betaine/GABA transporter) have been identified in mammalian tissues. The GATs have unique anatomical distributions in the rodent CNS, and the major subtype, GAT1 (Guastella et al 1990), is particularly abundant in areas rich in GABAergic neurons, such as the hippocampus, neocortex, cerebellum, and retina (Borden 1996). The GABA uptake process by GAT1 is electrogentic and is driven by Na\textsuperscript{+} influx in the ionic ratios of 1 GABA:2 Na\textsuperscript{+}:1 Cl\textsuperscript{−} (Cammack et al. 1994; Lester et al. 1994). Recently, GAT1 has been visualized in knock-in mice that express a mGAT1-green fluorescent protein fusion (mGAT1-GFP) (Chiu et al. 2002). The fluorescence is observed in axons and nerve terminals of GABAergic interneurons. At the ultrastructural level, GAT1 is found in inhibitory axons and nerve terminals (Conti et al. 1998; Minelli et al. 1995), and this organization is well suited for functions associated with GABA uptake. Interestingly, GAT1 protein has also been observed at sites away from GABAergic synapses such as bare astrocytic processes (Conti et al. 1998), and a GAT1 mRNA signal has also been detected in the pyramidal cell layer of the hippocampus (Frahm et al. 2000). These data suggest that GAT1 is present both at synaptic and extrasynaptic sites, where it lowers the ambient [GABA], near GABA\textsubscript{A} receptors and GABA\textsubscript{B} auto- and heteroreceptors (Mitchell and Silver 2000). According to present concepts, inhibition of the GABA uptake system leads to micromolar levels of ambient GABA in the extracellular space (Dalby 2000).

We have examined the functional role of GAT1 in GABA signaling by performing electrophysiological measurements in GAT1-deficient mice. In the course of generating the mGAT1-GFP strain, we constructed a strain that contains a 2.2-kb neo cassette in intron 14 of the GAT1-GFP fusion gene (Chiu et al. 2002). Although this gene has a theoretically intact set of exons, such large insertions often produce splicing errors and lead to decreased protein expression. We report here that homozygotes from this strain are so hypomorphic for GAT1 in hippocampal axons and terminals, that they are functional knockouts (KOs), and for convenience the strain will be termed GAT1 KO.

We examined GABA\textsubscript{A} receptor conductances and the regulatory functions of GABA\textsubscript{B} receptors in the GAT1 KO mice. We found several distinct alterations, which generally support the essential role of GAT1 in regulating transmission by both GABA\textsubscript{A} and GABA\textsubscript{B} receptors.

METHODS

Generation of GAT1-deficient mice

GAT1-deficient mice were generated as an intermediate in the construction of the mGAT1-GFP strain (Chiu et al. 2002). By homologous recombination, a 2.2-kb neo cassette was inserted into intron 14 of the GAT1-GFP fusion gene in ES cells (Fig. 1A). Blastocysts were injected into C57Bl/6J blastocysts to generate chimeric mice. Male chimeras were mated to C57Bl/6J females to generate GAT1 KO progeny. "*K. Jensen and C.-S. Chiu contributed equally to this work. Address for reprint requests and other correspondence: I. Mody, Dept. of Neurology, The David Geffen School of Medicine at UCLA, 710 Westwood Plaza, RNRC 3-155, Los Angeles, CA 90095-1769 (E-mail: mody@ucla.edu).
injected with recombinant ES cells and implanted into C57Bl6 females (Chiu et al. 2002). The strain is maintained on a C57Bl6 background and originally named intron 14-neo-mGAT1 (Fig. 1, B and C). These mice are hypomorphic for GAT1 expression in the brain such that they are functional KOs. Accordingly, this strain will be termed GAT1 KOs in this paper. Individual mice were genotyped as described earlier (Chiu et al. 2002).

Synaptosomal preparation and GABA uptake assay

Mice were anesthetized with halothane, and brains were dissected and collected on ice. Tissue (~25 mg) was homogenized in 20× (wt/vol) homogenization buffer (0.32 M sucrose, 0.1 mM EDTA, and 5 mM HEPES, pH 7.5; 1 ml) (Nagy and Delgado-Escueta 1984). The mixture was centrifuged at 1,000× g for 10 min. The supernatant was further centrifuged at 10,000× g for 20 min to produce the crude mitochondria pellet. The mitochondria pellet was washed once with 1 ml homogenization buffer. The particulate (synaptosome) fraction from the 10,000× g was suspended with 1 ml homogenization buffer.

For GABA uptake assays, 20 µl of the suspension was mixed with 280 µl of uptake buffer, which contained (in mM) 128 NaCl, 2.4 KCl, 3.2 CaCl2, 1.2 MgSO4, 1.2 KH2PO4, 10 glucose, and 25 HEPES, pH 7.5 (Lu et al. 1998) and incubated at 37°C for 10 min. Subsequently, 100 µl GABA and [3H]GABA (5 and 0.05 µM final, respectively) was added and incubated for additional 10 min. Uptake was terminated by placing the samples on ice, followed by two washes with uptake buffer containing same concentration of cold GABA at 10,000× g. The GAT1-specific inhibitor NO-711 (30 µM final) was included to measure the non-GAT1 uptake activity; the NO-711-sensitive fraction accounted for 75–85% of WT activity.

Brain slice preparation and electrophysiology

Wildtype littermates (WT) and GAT KO mice (P15–P25) were anesthetized with halothane before decapitation, and the brains were removed and placed into an ice-cold artificial cerebrospinal fluid (ACSF), in accordance with a protocol approved by the UCLA Chancellor’s Animal Research Committee. The ACSF contained (in mM) 126 NaCl, 26 NaHCO3, 1.25 NaH2PO4, 2.5 KCl, 2 CaCl2, 2 MgCl2, 10 d-glucose, 0.2 l-ascorbic acid, 1 pyruvic acid, and 3 kynurenic acid, pH 7.3, bubbled with 95% O2-5% CO2. The brain was glued to a platform, and 350-µm-thick slices were cut in the coronal plane with a Leica VT1000S vibratome. The slices were stored in bubbled ACSF at room temperature for ≥1 h until transferred individually to the recording chamber.

During experiments, the slices were perfused with ACSF at 1.5
ml/min at 32–33°C. CA1 pyramidal cells in hippocampus were visually identified [Zeiss Axioskope infrared differential interference contrast (IR-DIC) videomicroscopy, ×40 water-immersion objective]. Whole cell recordings were made using an Axopatch 200B amplifier (Axon Instruments, Union City, CA). Patch electrodes were pulled (Narishige PP-83, Tokyo, Japan) from borosilicate glass (1.5 mm OD; Garner, Claremont, CA) and filled with a solution containing (in mM) 140 CsCl, 2 MgCl₂, and 10 HEPES, titrated to a pH of 7.2 with CsOH (osmolarity: 275–290 mosmol/l). In a few experiments, MgATP was used instead of MgCl₂, which did not influence the GABA_A currents. When excitatory and inhibitory postsynaptic currents (EPSCs and IPSCs) were recorded simultaneously, the patch pipette contained (in mM) 135 Cs-glucuronate, 10 CsCl, 5 TEA, 0.1 EGTA, and 15 HEPES, titrated to pH 7.2 with CsOH. Postsynaptic GABA_A receptor-mediated currents were recorded with patch pipettes containing (in mM) 130 K-methylsulfate, 0.3 NaGTP, 2 MgATP, 10 KCl, and 10 HEPES, pH 7.2 with KOH. The resistances of the electrodes were between 3 and 6 MΩ when filled with solution. The pyramidal cells were recorded in voltage-clamp mode at a holding potential (V_hold) of −70 mV, unless otherwise indicated. The series resistance and whole cell capacitance were monitored repeatedly during the experiments. The series resistance was compensated by 70–85% using lag values of 7–8 μs, and recordings were discontinued if the series resistance increased by >50%. In a random sample of cells, the average precompensation series resistance was 13.4 ± 1.3 (SE) MΩ (n = 10; range: 7–19 MΩ). No differences were noted in the series resistances of recordings between different groups of cells. SR95531, furosemide, and CGP 62349 were dissolved in 50% DMSO, whereas TTX, NO-711, and CGP 62349 were dissolved in 10% normal goat serum (NGS), 0.3% triton in PBS, pH 7.5, at 4°C, followed by incubation with the primary antibody for 36–48 h at 4°C with rotational mixing. Primary antibodies and their dilutions were rabbit anti-GABA_A receptor α1 (Upstate; 1:100), guinea pig anti-GABA_A-R1α/b (Chemicon; 1:1,000), rabbit anti-glutamate decarboxylase (GAD) 65 (Chemicon; 1:1,000), rabbit anti-calbindin D28k (Swant; 1:2,500), rabbit anti-calretinin (Chemicon; 1:1,000), goat anti-parvalbumin (Swant; 1:2,500), and rabbit anti-vesicular GABA transporter (vGAT; Synaptic Systems; 1:100). After treating with primary antibody, the brain slices were washed with PBS containing 0.5% triton, followed by two washes with PBS. The slices were then incubated in solutions containing the appropriate rhodamine red-x-conjugated secondary antibodies. These secondary antibodies are goat anti-rabbit, or goat anti-guinea pig, or donkey anti-goat secondary antibodies (Jackson Laboratories; 1:200). After three washes with PBS, slices were rinsed with PBS and mounted with Vectashield (refractive index: 1.4577).

**Analysis of phasic and tonic GABA receptor-mediated currents**

Recordings were low-pass filtered (8-pole Bessel, Brownlee 210A) at 3 kHz and digitized on-line at 20 kHz using a PCI-MIO 16E-4 data-acquisition board (National Instruments, Austin, TX). Spontaneous and miniature synaptic events were detected with amplitude- and kinetics-based criteria (events were accepted when they exceeded a threshold of 6–8 pA for >0.5 ms) using custom-written LabView-5.1-based software (National Instruments) running on a Pentium III computer. Traces were imported into a custom-written analysis program, where signals were analyzed and averaged and amplitudes and kinetics measured. All IPSCs and EPSCs were also inspected visually, and sweeps were rejected or accepted manually.

Tonic GABA_A receptor-mediated currents were examined by injecting the selective GABA_A antagonist SR95531 into the slice chamber in a final concentration of 100–150 μM (30–35 μl of a 6- to 8-mM SR95531 solution; chamber volume: 1.8–2.0 ml) (Brickley et al. 1996). This SR95531 application will be referred to in text and figures as “SR95531 ≈ 100 μM.” When a tonic GABA_A current was present, this led to an outward shift in the holding current (E_h ≈ −50 mV). To analyze the holding current, we measured the mean current in 5-ms-long epochs at 100-ms intervals. Epochs were rejected if contaminating IPSCs were present as described earlier (Nusser and Mody 2002). The mean holding current was calculated in three 5-s-long periods (a, b, and c). a: 20 s before SR95531 application; b: immediately before SR95531 application; c: 20 s after the application. The tonic GABA_A current was calculated as c − b. The difference between b and a was used to judge the spontaneous baseline changes without adding drugs and is termed “no treatment” in Table 1. Tonic GABA_A receptor-mediated postsynaptic currents were quantified in a similar manner, using bath injection of 35–40 μl of a 0.5-mM CGP 62349 solution, which led to an estimated final concentration of 8–12 μM and is referred to as “CGP 62349 > 8 μM.” Although these drugs were added as a 30- to 40-μl highly concentrated bolus to the bath, the concentrations that reached the cells were more than an order of magnitude higher than that required for full block of the respective receptors.

Paired and unpaired t-test were performed in Microsoft Excel (v. 2000), whereas for comparing multiple means, Duncan’s multiple range test was employed using Statistica 6.0 software (StatSoft, Tulsa, OK). Significance level was set to P < 0.05, and data are expressed as means ± SE with n indicating the number of cells.

**Immunocytochemistry**

Mice were anesthetized with halothane and were perfused with 4% paraformaldehyde in PBS, pH adjusted to 7.6 with Na₂HPO₄. Brains were dissected and kept in 4% paraformaldehyde for 1 h at 4°C, then incubated with 25% sucrose in phosphate-buffered saline for ~20 h. The brains were embedded in optimal cutting temperature (OCT) medium (Sakura, Torrance, CA) for sagittal sections and sliced with a cryostat at 35 μm. Brain slices were stored in (in mM) 11 NaH₂PO₄, 20 Na₂HPO₄, 30% ethylene glycol, and 30% glycerol, pH 7.5, at −20°C.

Sections were incubated for 2 h at room temperature in a blocking solution [10% normal goat serum (NGS), 0.3% triton in PBS, pH 7.6], followed by incubation with the primary antibody for 36–48 h at 4°C with rotational mixing. Primary antibodies and their dilutions were rabbit anti-GABA_A receptor α1 (Upstate; 1:100), guinea pig anti-GABA_A-R1α/b (Chemicon; 1:1,000), rabbit anti-glutamate decarboxylase (GAD) 65 (Chemicon; 1:1,000), rabbit anti-calbindin D28k (Swant; 1:2,500), rabbit anti-calretinin (Chemicon; 1:1,000), goat anti-parvalbumin (Swant; 1:2,500), and rabbit anti-vesicular GABA transporter (vGAT; Synaptic Systems; 1:100). After treating with primary antibody, the brain slices were washed with PBS containing 0.5% triton, followed by two washes with PBS. The slices were then incubated in solutions containing the appropriate rhodamine red-x-conjugated secondary antibodies. These secondary antibodies are goat anti-rabbit, or goat anti-guinea pig, or donkey anti-goat secondary antibodies (Jackson Laboratories; 1:200). After three washes with PBS, slices were rinsed with PBS and mounted with Vectashield.

**Image analysis and bouton quantification**

Brain slices were imaged using a Leica TCS SP1 confocal microscope system. Images were taken with a 100× plan apochromatic objective, NA 1.4 (Leica, No. 506038). The pinhole was set at 152 μm as recommended by the manufacturer. The scan speed was 200 lines/s (slow mode) and the image size was 1,024 × 1,024 pixels. Each image was scanned with four repeats. The laser power was adjusted using the microscope’s acousto-optical tunable filter so that the fluorescence of a sample fell within the linear range of the detection system.

The Imaged package was used for analysis, and immunopositive structures were counted using the “analyze particle” function. The criterion for detectable fluorescence intensity was set to >2.5 times average intensity where little or no background fluorescence was included. Only structures >20 or 25 pixels (~0.2–0.24 μm²), depending on bouton size, were included. Counted structures were inspected visually at the end of each analysis. When several boutons were closely interspaced, fluorescence became confluent and the structures were counted as one. Such large particles were observed with similar density in WT and KO.

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RESULTS

GAT1 KO mice have little or no NO-711-sensitive GABA transport activity

Both heterozygous and homozygous GAT1 KO mice are viable and fertile. The coding region of mGAT1 in these mice contains a C-terminal GFP fusion, and the neo-deleted mGAT1-GFP strain described previously has nearly normal GAT1 activity (Chiu et al. 2002). In the strain described in this paper, measurements of [3H]GABA uptake confirmed the lack of GAT1 activity. Crude synaptosome preparations from hippocampus of the GAT1 KO homozygotes showed <2% NO-711 sensitive [3H]GABA uptake activity (0.17 ± 0.08 pmol/10 min/mg) compared with WT littermates (17.1 ± 1.4 pmol/10 min/mg), and heterozygotes displayed intermediate activity (10.9 ± 0.4 pmol/10 min/mg; Fig. 1D). The NO-711-insensitive [3H]GABA uptake measured in hippocampal synaptosomes was unaffected in GAT1 KO animals or in heterozygotes (Fig. 1D), indicating that GABA uptake mediated by other transporters was not upregulated to compensate for the absence of GAT1 function.

GAT1 KO mice show increased tonic GABA_A currents in CA1 pyramidal cells

To assess the functional changes in GAT1 KO mice, we made whole cell recordings from hippocampal CA1 pyramidal cells in GAT1 KO and WT slices. First, we examined the influence of GAT1 deletion on phasic and tonic GABA_A receptor-mediated currents. Neurons loaded with CsCl were clamped at a V_hold of −70 mV in the presence of the broad-spectrum ionotropic glutamate receptor antagonist kynurenic acid (3 mM). Phasic currents appeared as spontaneous IPSCs (sIPSCs), and in both WT and GAT1 KO mice, sIPSCs were blocked by the GABA_A receptor antagonist SR95531 (>100 μM) (Fig. 2, A and B). In GAT1 KO slices, SR95531 also revealed a tonic, steady GABA_A receptor-mediated current of 45.8 ± 11 (n = 5) versus 4.9 ± 2.3 pA in WT (P < 0.05, n = 4). These tonic currents were measured by assessing the holding current every 100 ms in 5-ms-long epochs (see METHODS).

GABA-uptake inhibitors produce tonic GABA_A currents in WT slices

In WT slices, a tonic GABA_A current (mean: 25.2 ± 6.0 pA, n = 5, Fig. 2C, middle) could be recorded in the presence of the specific GAT1 inhibitor NO-711 (10 μM). Further increasing the NO-711 concentration to 50 μM in WT slices, reduced the tonic current by 50% to 12.6 ± 3.5 pA (n = 4, Fig. 2C, right) consistent with a possible GABA_A receptor blocking effect of the uptake blocker (Overstreet et al. 2000). The potentiating effect of NO-711 (10 μM) on the tonic current was absent in GAT1 KO slices demonstrating that NO-711-sensi-

FIG. 2. GAT1 KO mice display a large tonic GABA_A receptor-mediated current. A: in WT slices, spontaneous IPSCs (sIPSCs) were recorded in Ca1-loaded CA1 pyramidal cells (V_hold = −70 mV). Injection of the GABA_A antagonist SR95531 into the bath (>100 μM) blocked the sIPSCs; B: in GAT1 KO slices, SR95531 blocked the sIPSCs but also abolished a steady inward current (~85 pA in this example). This current reflects a tonic GABA_A receptor-mediated conductance. C: to illustrate the tonic GABA_A currents, the holding current of 3 representative WT pyramidal cells is plotted vs. time. In the WT control cell (left), the SR95531-sensitive tonic current was ~5 pA. Addition of 10 μM NO-711 produced a tonic GABA_A current of 15 pA in this example (middle), while 50 μM NO-711 caused no further increase. D: in a cell in an untreated GAT1 KO slice, a tonic current of ~80 was observed (left). The tonic current was strongly reduced by TTX (1 μM; middle). Furosemide (0.6 mM) reduced the tonic current by ~60% (right) compared with the untreated GAT1 KO slice.
tive GABA uptake was not functional in these animals. Perfusion of NO-711 (10 μM) slightly reduced the tonic current in GAT1 KO slices (mean: 38.1 ± 13.7 pA, n = 7, not shown), consistent with a blocking action of NO-711 on the GABA_A receptors responsible for the tonic current. This confounding effect of NO-711 could be overcome by adding a small concentration of agonist. The tonic current observed in GAT1 KOs could be reproduced in WT slices perfused with the combination of 10 μM NO-711 and 0.8 μM GABA: this treatment induced a tonic current of 45.4 ± 21 pA (n = 4, not shown) comparable to that observed in GAT1 KOs.

Modulation of tonic GABA_A currents in GAT1 KOs by furosemide and TTX

Next, we asked whether the tonic GABA_A conductance in GAT1 KOs could be modulated. We tested whether the increased GABA levels in the GAT1 KO mice originated from action potential-dependent release. TTX (1 μM) reduced the tonic GABA_A current by 63% to 17.0 ± 4.9 pA compared with 45.8 pA without TTX (P < 0.05, n = 4; Fig. 2D, middle). This result indicates that presynaptic action potentials are partially responsible for the GABA release that elevates [GABA]_o. In GAT1 KO slices, furosemide (0.6–1 mM), which blocks a subset of postsynaptic GABA_A receptors with distinct subunit compositions (Jackel et al. 1998), reduced the tonic GABA_A currents by 62% to 18.4 ± 3.5 pA (n = 8, P < 0.05; Fig. 2D, right). The effects of 0.6 or 1 mM furosemide on tonic currents were indistinguishable, and furosemide did not affect s- or mIPSCs (following text). A summary of the tonic currents under various treatments is shown in Table 1.

sIPSCs are not altered in GAT1 KO mice

As summarized in Table 2, sIPSCs were not significantly different in GAT1 KO mice when compared with those recorded in WT mice (Fig. 3, A and B). Their amplitudes were 36.6 ± 2.6 pA in WT (n = 6) and 54.3 ± 13 pA in GAT1 KO (n = 6, P > 0.05). Application of NO-711 (10 μM; n = 5) or NO-711 (10 μM) + GABA (0.8 μM; n = 4) in WT slices did not significantly affect sIPSC amplitudes, frequencies, or waveforms. Furosemide also failed to affect sIPSC amplitudes.

Table 2. Properties of spontaneous inhibitory postsynaptic currents (sIPSCs) in CA1 pyramidal cells

<table>
<thead>
<tr>
<th>sIPSCs</th>
<th>WT</th>
<th>WT + NO-711</th>
<th>WT + NO-711 + 0.8 μM GABA</th>
<th>GAT1 KO</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amplitude, pA</td>
<td>36.6 ± 2.6</td>
<td>48.0 ± 6.7</td>
<td>35.7 ± 4.8</td>
<td>54.3 ± 13</td>
</tr>
<tr>
<td>Frequency, Hz</td>
<td>24.1 ± 4.7</td>
<td>15.9 ± 4.5</td>
<td>22.2 ± 4.9</td>
<td>16.8 ± 5.8</td>
</tr>
<tr>
<td>Rise time (10–90%), μs</td>
<td>331 ± 55</td>
<td>330 ± 38</td>
<td>303 ± 32</td>
<td>369 ± 47</td>
</tr>
<tr>
<td>Decay constant, ms (weighted)</td>
<td>4.0 ± 0.2</td>
<td>4.0 ± 0.3</td>
<td>3.6 ± 0.2</td>
<td>4.1 ± 0.2</td>
</tr>
<tr>
<td>No. of cells</td>
<td>6</td>
<td>5</td>
<td>4</td>
<td>6</td>
</tr>
</tbody>
</table>

Properties of sIPSCs in CA1 pyramidal cells in WT slices, in WT slices perfused with NO-711 (10 μM), and in GAT1 KO slices. GABA_A receptor-mediated sIPSCs were recorded in Cl^-loaded cells at a V_hold of −70 mV. No significant changes were observed.

FIG. 3. sIPSCs are similar in WT and GAT1 KO mice. A: sIPSCs recorded in Cl^-loaded pyramidal cells in the presence of kynurenic acid (3 mM). B: the average of 50 sIPSCs in WT and GAT1 KO (right). In the GAT1 KO pyramidal cell, the sIPSC waveform was not different from that in WT, although the sIPSCs occurred on a noisier baseline.
in GAT1 KOs (38.6 ± 4.2 pA, n = 11), frequencies (21.4 ± 5.2 Hz, n = 11), 10–90% rise times (285 ± 38 μs, n = 5), or the weighted decay time constants (4.4 ± 0.36 ms, n = 5). These values are not significantly different from the GAT1 KO control data in Table 2 (P > 0.05).

**mIPSCs have reduced frequencies in GAT1 KO mice**

mIPSCs recorded in the presence of TTX (1 μM) had similar 10–90% rise times, decay time constants, and amplitudes in WT (n = 9) and GAT1 KO pyramidal cells (n = 9, P > 0.05; Table 3 and Fig. 4). Yet, the frequency of mIPSCs in GAT1 KO animals was reduced to about one-third of control frequencies (6.4 ± 1.6 Hz in GAT1 KO compared with 17.8 ± 2.1 Hz in WT; P < 0.01). One possible explanation for the finding of reduced mIPSC frequency, but no reduction in amplitudes, is that the GAT1 KO slices have a reduced number of functioning GABAergic synapses onto pyramidal cells. Another reason for the change in frequency could have been a differential detection of mIPSCs by our analysis software, resulting from a noisier baseline (more tonic current) in GAT1 KO cells. This second possibility is highly unlikely as the average peak-to-peak noise as well as the SD of the baseline current were not statistically different between the recordings from WT and GAT1 KO slices (Table 3). This contrasts with the tonic inhibition-dependent increase in baseline variance recorded in small compact neurons such as dentate gyrus granule cells (Stell and Mody 2002). However, in the larger and leakier pyramidal cells the tonic current is only a small fraction of the total holding current (13.8% in GAT KO and 9.7% in WT + 10 μM NO-711), and therefore it does not significantly contribute to the baseline variance. Furthermore, the lowest amplitude sIPSCs detected by the analysis software were also comparable between WT and GAT1 KO (12.8 ± 0.6 vs. 13.8 ± 0.7 pA, P > 0.05), and the fraction of the events found in the low-amplitude bins were not different between the two preparations (e.g., see lowest 25% of the events in the cumulative probability plots of amplitudes depicted in Fig. 4C). Nevertheless, it might have been possible for two nonsignificant effects (i.e., a slight reduction in amplitude and a small increase in detection threshold) to combine and artificially yield a significant reduction in mIPSC frequency. To examine this possibility, we have modeled such a combined effect by generating log-normal distributions that fit the amplitude distributions of mIPSCs in WT neurons. Next, we shifted the mean to lower amplitudes by 10%, and the detection threshold was shifted toward larger amplitudes by 8%, corresponding to the experimental values obtained in GAT1 KO slices. In such simulations, the number of detected events was reduced by <10%. Therefore we conclude that combined small shifts in amplitude and detection threshold are not responsible for the dramatic 64% reduction in mIPSC frequency in the GAT1 KO animals.

**Lack of tonic postsynaptic GABA B receptor activation**

The results so far indicate that slices from GAT1 KO mice have elevated ambient [GABA] o levels, which persistently activate postsynaptic GABA B receptors. Perfusion of NO-711 and GABA (0.8 μM) onto WT slices mimicked this condition. In the presence of such elevated [GABA] o, we also expected postsynaptic GABA B receptors to be tonically activated in CA1 pyramidal cells. We tested this hypothesis using K-methylsulfate-filled pipettes to record from neurons voltage-clamped at −50 mV while the GABA B receptor antagonist CGP 62349 was injected into the bath to yield a final concentration of >8 μM (Fig. 5A). Surprisingly, neither NO-711 (10 μM, n = 2) nor NO-711 plus 0.8 μM GABA (n = 3) induced any detectable postsynaptic GABA B

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**TABLE 3. Properties of GABA A-receptor-mediated miniature IPSCs (mIPSCs) in CA1 pyramidal cells**

<table>
<thead>
<tr>
<th>mIPSCs</th>
<th>WT</th>
<th>GAT1 KO</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amplitude, pA</td>
<td>37.5 ± 3.1</td>
<td>34.1 ± 2.9</td>
</tr>
<tr>
<td>Frequency, Hz</td>
<td>17.8 ± 2.1</td>
<td>6.4 ± 1.6**</td>
</tr>
<tr>
<td>Baseline peak-to-peak, pA</td>
<td>10.80 ± 0.78</td>
<td>9.73 ± 0.58</td>
</tr>
<tr>
<td>Baseline SD, pA</td>
<td>4.61 ± 0.2</td>
<td>4.06 ± 0.23</td>
</tr>
<tr>
<td>10–90% rise time, μs</td>
<td>298 ± 11</td>
<td>280 ± 9</td>
</tr>
<tr>
<td>Weighted decay time constant, ms</td>
<td>4.6 ± 0.2</td>
<td>4.6 ± 0.2</td>
</tr>
<tr>
<td>No. of cells</td>
<td>9</td>
<td>9</td>
</tr>
</tbody>
</table>

Properties of mIPSCs in CA1 pyramidal cells of WT and GAT1 KO slices. mIPSCs were recorded in the presence of 1 μM TTX at a Vhold of −70 mV. **P < 0.01.
The results indicate that postsynaptic GABA<sub>B</sub> receptors are not activated by the levels of GABA that activate GABA<sub>A</sub> receptors to produce the tonic current.

**Presynaptic GABA<sub>B</sub> receptor tone is lost in GAT1 KO mice**

In light of the abundance of presynaptic GABA<sub>B</sub> receptors (Schuler et al. 2001), we next examined a possible tonic activation of such receptors by ambient GABA in WT and GAT1 KO slices. CA1 pyramidal cells were recorded with Cs-glucuronate-filled pipettes at V<sub>hold</sub> between −20 and −30 mV, where inward sEPSCs and outward sIPSCs of roughly similar amplitudes could be observed (Fig. 6A). Under the conditions of such reduced driving forces, the detectable events had frequencies of about 1/10 of those recorded under control conditions at two to three times the driving force (Stell and Mody 2002). In WT slices in the absence of NO-711, CGP 62349 (>8 μM) caused a large increase in sIPSC frequency from 1.5 ± 0.3 to 4.9 ± 2.3 and 5.7 ± 0.3 Hz at 20 and 30 s, respectively, after CGP application (n = 4, P < 0.01). The mean sIPSC amplitudes increased also from 26.1 ± 2.7 to 31.0 ± 2.5 pA in CGP 62349 (P < 0.05, n = 4). The frequency of sEPSCs changed less dramatically from a control frequency of 1.7 ± 0.4 to 3.1 ± 1.3 Hz at 20 s and to 3.7 ± 1.9 Hz at 30 s after CGP 62349 (n = 4, P > 0.05), without a change in mean current.

**FIG. 5.** Tonic GABA<sub>B</sub> receptor-mediated postsynaptic currents are not present in WT or GAT1 KO CA1 pyramidal cells. A: no postsynaptic outward currents were present in WT as tested by the GABA<sub>B</sub> receptor antagonist CGP 62349 (>8 μM). This experiment was performed in the presence of NO-711 (10 μM). In an untreated GAT1 KO slice (right), a tonic postsynaptic GABA<sub>B</sub> current was also absent. The pyramidal cells were recorded with a K-based intracellular solution-filled electrode and held at −50 mV. Slices were perfused with kynurenic acid (3 mM) and picrotoxin (50 μM) to block ionotropic glutamate and GABA<sub>A</sub> receptors, respectively. B: baclofen (10 μM) induced a robust GABA<sub>B</sub> receptor-mediated current, which was revealed during antagonism by CGP 62349 (>8 μM). The GABA<sub>B</sub> current reached 55 pA in the exemplar WT cell and 60 pA in the GAT1 KO cell. Scale bars apply to all traces in A and B. C: summary of postsynaptic GABA<sub>B</sub> currents in CA1 pyramidal cells. The WT data consist of the pooled results from 5 cells where NO-711 (n = 2) or NO-711 + 0.8 μM GABA (n = 3) was present. No tonic GABA<sub>B</sub> currents were observed (1.3 ± 1.4 pA, n = 5). When NO-711 and GABA were replaced by 10 μM baclofen, the GABA<sub>B</sub> currents reached 50.4 ± 13.9 pA (n = 9). For GAT1 KO slices, the GABA<sub>B</sub> current reached 1.3 ± 1.4 pA (n = 3) in the absence and 41.2 ± 5.9 pA (n = 7) in the presence of baclofen (10 μM). The baclofen-induced currents were not different between WT vs. GAT1 KO (P > 0.05). Error bars indicate SE.

**FIG. 6.** A presynaptic GABA<sub>B</sub> tone is lost in GAT1 KO mice. A: simultaneous recordings of sEPSCs (inward currents) and sIPSCs (outward currents) using Cs-glucuronate pipettes. V<sub>hold</sub> was about −30 mV to obtain similarly sized inward and outward currents. Inset: the events on a faster time scale (I: sIPSC, E: sEPSC). After injection of CGP 62349 (>8 μM), sIPSCs increased in frequency and amplitude, whereas sEPSCs did not change. B: the increase in sIPSC frequency after the CGP 62349 injection was abolished by 6,7-dinitroquinoxaline-2,3-dione (DNQX; 10 μM) and APV (50 μM), which block glutamatergic excitation. This indicates that tonically active GABA<sub>B</sub> receptors control the presynaptic network in WT. C: in GAT1 KO slices, no increases in the frequencies of sIPSCs or sEPSCs were observed after CGP 62349 application.
amplitudes (24.8 ± 3.1 pA in control vs. 25.9 ± 2.5 pA in CGP 62349). The increase in sIPSC frequency produced by the GABAB receptor antagonist in WT slices was abolished by blocking glutamatergic excitation with 6,7-dinitroquinoxaline-2,3-dione (DNQX; 10 μM) and t-APV (50 μM). Twenty and 30 s after CGP application, the normalized sIPSC frequencies were 89.7 ± 6.0 and 86.7 ± 11.7% of control, respectively (n = 4, P > 0.05). This finding is consistent with a powerful GABAB receptor-dependent control of interneuron activation by excitatory synapses in WT slices.

In contrast, in GAT1 KO slices with excitation intact blockade of GABAB receptors failed to increase the frequency of sIPSCs. The sIPSC frequency was 1.9 ± 0.3 Hz during the control period and remained steady at 2.0 ± 0.4 and 1.7 ± 0.6 Hz, respectively, at 20 and 30 s after CGP 62349 (n = 5, P > 0.05). For sEPSCs, the frequency during the control period was 1.8 ± 0.5 Hz, and 2.0 ± 0.5 Hz and 2.2 ± 0.6 Hz, at 20 and 30 s after CGP 62349 (P > 0.05). To circumvent the possibility that marginally detectable sIPSCs were rendered below detection threshold by the activation of presynaptic GABAB receptors, we repeated the experiments in Cl−-loaded cells, with glutamatergic excitation blocked by kynurenic acid (3 mM) and a high driving force (Vhold = −70 mV). Under these conditions, i.e., in the absence of an excitatory drive onto interneurons, blocking GABAB receptors had no significant effect (P > 0.05; paired t-test) on the frequencies of the sIPSCs in either WT (n = 8) or GAT-1 KO (n = 5) slices when the frequency of the events during a control period was compared with 10-s periods measured 20 and 30 s after CGP 62349 was added to the bath. To illustrate these results, the normalized frequencies were plotted in the graphs of Fig. 7, A–D. The histogram in Fig. 7E depicts the normalized frequencies in a window of 15–30 s after the CGP 62349 application.

**GAT1 KO mice have normal numbers of GABAergic interneurons and synapses**

The decrease in quantal GABA release and loss of pre-synaptic GABAB receptor function prompted us to perform histological stainings of the hippocampus. Using fluorescence immunocytochemistry, we stained for calbindin, calretinin, and parvalbumin, proteins that label various subtypes of GABAergic interneurons. The expression of these proteins was intact in the GAT1 KO mice as was the number of interneurons labeled by these markers (not shown). Immunostainings were also performed for the α1-subunit of GABA_A receptors (Fig. 8, A and B), GABA_A-R1 receptors (C and D), GAD-65 (E and F), and for the vesicular GABA transporter vGAT (G and H). Total levels of fluorescence were similar within 20%. For GABAB receptors, glutamic acid decarboxylase 65 kDa (GAD65) and vGAT, punctate staining was observed; and we measured the density of positive structures that were >20 or 25 pixels (~0.2–0.24 μm²). The immunocytochemistry indicated no significant differences between the density of GABA synapses and receptors of WT and GAT1 KO mice (Fig. 8I).

**DISCUSSION**

Uptake of neurotransmitters is presumed to critically influence synaptic transmission in the CNS (Genton et al. 2001; Spinks and Spinks 2002). While many experimental studies have addressed the acute effects of uptake inhibition, we generated GAT1 KO mice where the GABA uptake in the brain has been chronically impaired during the entire life span. In these animals, some of our findings may have been predicted from the known acute effects of GAT1 inhibitors. These include an increased GABA_A receptor-mediated tonic current in principal cells (Frahm et al. 2001; Nusser and Mody 2002) and the lack of change in amplitude and decay of mIPSCs (Isaacson et al. 1993). The present study confirms this lack of effect without complications from the additional pharmacological effects of the GAT1 blockers previously employed.

Other effects were not expected. First, there is a decrease in the frequency of quantal GABA miniature release. Second, a presynaptic GABAB tone is present in WT slices but not in the GAT1 KO. These findings indicate that chronically elevated
levels of [GABA]o alter the presynaptic receptor function and quantal transmitter release in the brain.

**Generation of GAT1-deficient mice**

The GAT1 KO strain described in this paper differs from a previously described mGAT1-GFP knock-in mouse: the present strain contains a floxed 2.2-kb neo selection cassette in intron 14, which precedes the final coding exon of mGAT1 (Chiu et al. 2002). A large insertion in an intron often interferes with splicing so that the resulting protein is expressed at subnormal levels (Labarca et al. 2001; Single et al. 2000; Wang et al. 1999). This paper exploits the unusual, but not unprecedented, fact that the insertion virtually eliminates protein function: GAT1-mediated GABA-transport was absent in hippocampal synaptosomes of GAT1 KO mice (2% of control). Interestingly, in the absence of GAT1-mediated NO-711-sensitive GABA transport, other GABA transport (insensitive to NO-711) did not become upregulated in GAT1 KO mice.

**Increased GABAₐ tone in GAT1-deficient mice**

Previous electrophysiological studies showed that the decay of electrically evoked inhibitory postsynaptic currents and potentials are prolonged by GAT1 inhibition in hippocampal and neocortical brain slices (Engel et al. 1998; Ling and Benardo 1998; Roepstorff and Lambert 1994; Thompson and Gähwiler 1992). On the other hand, the kinetics of mIPSCs, which reflect the quantal GABAergic transmission, are normally not shaped by GABA uptake (Isaacson et al. 1993; Overstreet et al. 2000). Thus the GABA uptake process influences synaptic transmission when several nerve terminals are concurrently stimulated. Recent studies using dual recordings suggest that the activation of GAT1 after evoked transmission depends on the specific GABAergic pathway and requires a close anatomical spacing between the inhibitory boutons (Overstreet and Westbrook 2003).

When we examined the spontaneous GABAergic activity in brain slices, we found that GAT1 deficiency led to a tonic GABAₐ receptor-mediated background conductance in the hippocampus. This finding is mimicked in WT slices by acute blockade of GAT1 with GAT1 blockers such as NO-711 (Bai et al. 2001; Frahm et al. 2001; Nusser and Mody 2002; Overstreet and Westbrook 2001). In the GAT1 KO animals, we can exclude the possibility that in whole cell recordings the tonic current was caused by locally enhanced GABA levels through the reverse operation of GAT1 (Wu et al. 2003).
operation of another GABA transporter, e.g., GAT3 can also be excluded, as NO-711-insensitive GABA transport remained unaltered in GAT1 KOs. Instead, similar to cerebellar granule cells, the tonic conductance most likely arises from extrasynaptic receptors activated by ambient GABA levels (Brickley et al. 1996, 2001).

In our experiments, the majority of the tonic current was blocked by furosemide, while the IPSCs were not affected by this drug. Because furosemide preferentially blocks α4- and α6-containing receptors (but the latter are not expressed in hippocampus) (Korpi et al. 2002), the furosemide-sensitive tonic current in CA1 pyramidal cells could be mediated by α4-containing receptors, which may form αβ- or αβy2 channels (Bencsits et al. 1999). The furosemide-insensitive portion of the tonic current could arise from α5-containing receptors, which are strongly expressed in the CA1 pyramidal cells (Pirker et al. 2000) and are thought to be localized extrasynaptically (Brunig et al. 2002; Crestani et al. 2002).

What are the GABA levels in the slice?

Given that the tonic current is driven by [GABA]o, what are then the approximate levels of GABA in the slice surface where we record? Perfusion of 0.8 μM GABA and 10 μM NO-711 yielded a similar tonic current in WT slices and in untreated GAT1 KO slices. Thus if the postsynaptic sensitivity is similar in these animals, we can suggest that [GABA]o in GAT1 KO slices is ~1 μM. Processes in addition to GAT1 might control GABA levels near receptors. We found that NO-711-sensitive uptake accounts for 75–80% of the uptake in crude synaptosomes from WT tissue, implying that non-GAT1 uptake may produce 20–25% of WT uptake in the GAT1 KO brain. It is unnecessarily pessimistic to assume that this remaining activity produces an error of >20–25% in our estimate of 0.8–1 μM GABA.

At such steady-state [GABA]o, the data of Overstreet et al. (2000) led one to expect that 15–20% of GABA receptors would desensitize at the synapse, producing a corresponding decrease in s- or mIPSC amplitudes (Overstreet et al. 2000). We detected no change in IPSC amplitudes when GABA uptake was blocked, either chronically in the GAT1 KO animals or acutely during perfusion of NO-711. Similar to the results obtained here an increased [GABA]o subsequent to vigabatrin administration was found to decrease mIPSC frequency without changing their amplitudes (Wu et al. 2003). The discrepancy between our data and those of Overstreet et al. (2000) may be due to species differences or to another unknown factor that renders the synaptic receptors in our mouse preparation less sensitive to desensitization. Even if synaptic GABA receptors do not desensitize, one might expect GAT1 block or knockdown to reduce apparent IPSC amplitude via a second mechanism. In this second mechanism, synaptic receptors are again exposed to low levels of GABA contributing to the generation of the large tonic conductance, and synaptic receptors are locally saturated in response to a quantum of GABA, so that the mIPSC grows from an altered baseline to a constant peak. That clearly elevated [GABA]o led to no significant differences in the amplitudes of s- and mIPSCs is best explained by the idea that synaptic GABA receptors have low affinity and are therefore not activated by the low ambient concentrations of [GABA]o in the slice (Stell and Mody 2002). Instead, it is likely that extrasynaptic GABA_A receptors generate the tonic conductance (Mody 2001), but the molecular identity of these receptors in various cell types remains to be determined. It is interesting to note that interneurons themselves may have a substantial tonic conductance, albeit of different pharmacological properties than that of principal cells (Semyanov et al. 2003). In light of the possible excitatory and inhibitory actions of GABA on interneurons (Chavas and Marty 2003), it is difficult to speculate how an enhancement of the tonic conductance on interneurons might impact the overall network excitability in GAT1 KOs. An excitatory GABA action on some interneurons may thus explain the normal sIPSC frequency in GAT KOs when extracellular GABA levels are elevated.

Decreased GABA_B tone in GAT1-deficient mice

Extracellular GABA concentrations sufficient to activate the high-affinity GABA_A receptors responsible for generating the tonic current did not activate postsynaptic GABA_B receptors in both WT and GAT1 KO mice. Yet, functional GABA_B receptors were clearly present on pyramidal cells in both genotypes because baclofen induced a robust outward current, known to be mediated by GIRK2-containing channels (Luscher et al. 1997). It has been reported that 1 μM GABA activates postsynaptic GABA_A receptors and strongly activates presynaptic GABA_B receptors to depress GABA release, while it does not activate postsynaptic GABA_B receptors (Yoon and Rothman 1991). These results fit with our observation that ambient GABA levels in the slices, even in the absence of a GAT1-mediated uptake, preferentially activate presynaptic but not postsynaptic GABA_B receptors. However, these data are in contrast with findings in expressed GABA_B1 and R2 subunits, which couple efficiently to GIRK channels, and as little as 200 nM GABA is sufficient to induce a robust K+ current (Jones et al. 1998). Thus it is plausible that postsynaptic GABA_B receptors in brain slices are under some modulatory control that will reduce their responsiveness to GABA.

We have also studied the tonic activation of presynaptic GABA_B receptors that could contribute to lowering the frequency of sIPSC and sEPSC in pyramidal cells. This presynaptic GABA_B tone was detected in WT slices where [GABA]o is low, possibly in the range of a few hundred nanomolars. Because ultrastructural studies have demonstrated a high density of presynaptic GABA_B receptors on excitatory terminals (Kulik et al. 2002), the activation of presynaptic GABA_B receptors is expected to alter the release of both glutamate and GABA. Indeed, in WT animals, we found an increased frequency of sEPSCs and sIPSCs after application of a GABA_B receptor antagonist. However, it has to be kept in mind that the GABA_B antagonist might affect sIPSC frequency via two distinct mechanisms: a direct effect on the inhibitory terminals and soma and an indirect action by changing the excitatory drive onto interneurons. A relief from the GABA_B tone would cause an increased glutamate release onto interneurons, leading to increased interneuronal firing. In support of this possibility, CGP 62349 did not have an effect when the WT interneurons were pharmacologically isolated by DNQX and APV. The direct glutamatergic inputs onto pyramidal cells were less influenced by presynaptic GABA_B receptors. Therefore at least in our preparation, ambient GABA may predominantly stimu-
late GABA_{B} heteroreceptors located on glutamatergic connections onto GABAergic interneurons (McBain et al. 1999). Elucidating these connections and their regulation requires direct recordings from interneurons (Lei and McBain 2003), followed by a comprehensive anatomical classification (Hájos and Mody 1997).

[GABA]_{o} is likely to be many times higher in GAT1 KO slices compared with WT. Yet a presynaptic GABA_{B} tone could not be revealed in the GAT1 KO animals. Previous studies showed that 21 days of oral treatment with the GAT1 blocker tiagabine causes no changes in the GABA_{A} or GABA_{B} receptor binding in the mouse hippocampus (Thomsen and Suzdak 1995). Similarly, our immunochemoical studies in mice lacking GAT1-mediated GABA uptake revealed no changes in the numbers of GABA_{A} and GABA_{B} receptors or in the level of vGAT- or GAD65-containing inhibitory nerve terminals. Our data thus indicate that prolonged high extracellular GABA concentrations decreased the function of the pathway underlying presynaptic GABA_{B} control of sIPSC frequency, most likely without a change in receptor number. The molecular basis for this effect is unknown, but there are several precedents for the idea that prolonged activation of other G-protein-coupled receptors results in altered signaling pathways controlled by the receptors. G-protein-coupled receptors desensitize during prolonged exposure to agonist then recover over days subsequent to agonist removal (Wetherington and Lambert 2002). It is established that desensitization can involve phosphorylation, receptor endocytosis, and/or reduced gene expression (Ferguson 2001). Another possibility is that levels of endogenous RGS proteins, which determine the magnitude of G-protein-mediated presynaptic inhibition (Chen and Lambert 2000), may have been altered by the prolonged agonist exposure. Regardless of the mechanism, our findings are consistent with a novel plasticity of the function of presynaptic GABA_{B} receptors.

Decreased quantal GABA_{A}-mediated transmission

The reduced frequency of mIPSCs in GAT1 KOIs was an unexpected finding, particularly in light of their unaltered amplitudes, and kinetics, and the lack of change in postsynaptic GABA_{A} receptors and presynaptic GABAergic markers. The decreased frequency is unlikely to have been caused by activation of GABA_{B} receptors because sIPSCs recorded with CsCl electrodes were not depressed by a presynaptic GABA_{B} tone (Fig. 7D), and mIPSCs are usually quite resistant to GABA_{B} activation (Otis and Mody 1992; Overstreet and Westbrook 2001). Moreover, a chronic elevation in extracellular GABA levels by pretreatment of slices with the GABA transaminase inhibitor vigabatrin reduces the frequency of mIPSCs in a GABA_{B} receptor-independent manner (Overstreet and Westbrook 2001). Because the number of GABAergic boutons, as inferred from the vGAT staining, did not change in GAT1 KOIs, a likely explanation for the reduction in mIPSC frequency is a reduced rate of quantal release at individual nerve terminals. This could be due to a reduction in the number of docked vesicles, or to upstream mechanisms, such as a reduced spontaneous Ca^{2+} release from intracellular stores (Llano et al. 2000). To test these possibilities further, it is necessary to examine the Ca^{2+} sensitivity of mIPSCs and to perform quantitative ultrastructural studies on subpopulations of GABAergic terminals. Reductions in mIPSC frequency also occur in experimental epilepsy (Hirsch et al. 1999) and may represent a general type of plasticity when ambient GABA levels are high.

Conclusions

The deficiency of GABA uptake in GAT1 KO mice chronically elevates ambient GABA levels. In the hippocampus, this leads to an increase in a GABA_{A} receptor-mediated tonic conductance, which may control cell excitability, chloride homeostasis, or cell volume (Mody 2001). Future studies will have to determine how prolonged increases in extracellular GABA levels, achieved here by GAT1 deficiency, decrease quantal GABA release, and presynaptic GABA_{B} receptor function, as these changes are likely to have profound influences on hippocampal network function.

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