GAT-1 and Reversible GABA Transport in Bergmann Glia in Slices

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Barakat, L., and A. Bordey. GAT-1 and reversible GABA transport in Bergmann glia in slices. J Neurophysiol 88: 1407–1419, 2002; 10.1152/jn.01002.2001. Although glial GABA uptake and release have been studied in vitro, GABA transporters (GATs) have not been characterized in glia in slices. Whole cell patch-clamp recordings were obtained from Bergmann glia in rat cerebellar slices to characterize carrier-mediated GABA influx and efflux. GABA-induced inward currents at −70 mV that could be pharmacologically separated into GABA_A receptor and GAT currents. In the presence of GABA_A, receptor blockers, mean GABA-induced currents measured−48 pA at −70 mV, were inwardly rectifying between −70 and +50 mV, were inhibited by external Na⁺ removal, and were diminished by reduction of external Cl⁻. Nontransportable blockers of GAT-1 (SKF89976-A and NNC-711) and a transportable blocker of all the GAT subtypes (nipecotic acid) reversibly reduced GABA-induced transport currents by 68 and 100%, respectively. A blocker of BGT-1 (betaine) had no effect. SKF89976-A and NNC-711 also suppressed baseline inward currents that likely result from tonic GAT activation by background GABA. The substrate agonists, nipecotic acid and β-alanine but not betaine, induced voltage- and Na⁺-dependent currents. With Na⁺ and GABA inside the patch pipette or intracellular GABA perfusion during the recording, SKF89976-A blocked baseline outward currents that activated at −60 mV and increased with more depolarized potentials. This carrier-mediated GABA efflux induced a local accumulation of extracellular GABA detected by GABA_A receptor activation on the recorded cell. Overall, these results indicate that Bergmann glia express GAT-1 that are activated by ambient GABA. In addition, GAT-1 in glia can work in reverse and release sufficient GABA to activate nearby GABA receptors.

INTRODUCTION

Henn and Hamberger (1971) suggested that glia were capable of removing transmitters such as γ-aminobutyric acid (GABA) that overflow from the synaptic cleft. This pioneer work on GABA uptake was rapidly followed by a substantial number of studies on the ability of glia to take up GABA in vitro (Iversen and Kelly 1975). It is now clear that GABA influx into glia and in particular astrocytes is mediated via specific high-affinity transporters (Schousboe and Westergaard 1995). Four types of high-affinity GABA transporters (GATs), each with a distinct structure, pharmacology, and distribution have recently been cloned. In rat and human, these GATs are named GAT-1, BGT-1, GAT-2, and GAT-3 (Borden et al. 1992, 1995a; Guastella et al. 1990). Of these, BGT-1 transports both GABA and the osmolyte betaine (Matskevitch et al. 1999). Prior to the cloning of distinct GATs, based on pharmacological studies it was unclear whether there were distinct GABA-uptake systems in neurons and in glia (Larsson et al. 1988; Smith et al. 1992). It is now clear that the so-called “neuronal” GATs are expressed in astrocytes both in cultured cells and in brain tissue (Borden 1996; Borden et al. 1995b; Kanner and Bendahan 1990). Immunoreactivity for GAT-1/2/3 has been reported in astrocytes from different brain regions (Conti et al. 1999; De Biasi et al. 1998; Minelli et al. 1996; Morara et al. 1996; Ribak et al. 1996; Yan and Ribak 1998). It has also become evident that astrocytes in brain tissue and in culture express different GAT subtypes (Borden 1996), thus confounding the extrapolation of data from uptake studies in vitro to the role of glial GATs in intact brain. Thus there is a critical need for characterizing GATs in astrocytes in situ as well as for other transporter and receptor systems (Kimelberg et al. 2000). While GATs are generally viewed as permitting influx of GABA, they also have the ability to work in reverse depending on internal Na⁺ concentration and membrane depolarization (Cammack and Schwartz 1993; Cammack et al. 1994; Risso et al. 1996). Although transporter-mediated GABA release has been shown from astrocytes (Gallo et al. 1991; Levi and Gallo 1995; Yee et al. 1998), the membrane depolarization at which GABA efflux occurs has not been investigated in astrocytes.

To our knowledge, GAT currents have not been characterized in astrocytes in slices. Immunohistochemical studies show that the specialized astrocytes of the cerebellum, the Bergmann glia, possess GAT-1 on their soma and processes (Morara et al. 1996; Swan et al. 1994) that encapsulate GABAergic synapses on Purkinje cells (Castejon 1990). Thus we ask the two following questions: do Bergmann glia indeed possess functional GATs in slices and in particular the fast transport cycle GAT subtype GAT-1 and can GATs in Bergmann glia work in reverse and mediate GABA efflux? To answer these questions, whole cell patch-clamp recordings were obtained from Bergmann glia in cerebellar slices. In the presence of GABA receptor blockers, every recorded Bergmann glial cell displayed inward currents in response to pressure application of GABA that were Na⁺ and voltage-dependent and sensitive to various GAT blockers. The pharmacological profile of the GABA responses indicates that GAT-1 is expressed in Bergmann glia. Finally, intracellular injection of GABA induces an efflux of GABA that can be detected at and above holding potentials of −40 mV and activates GABA_A receptors.

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METHODS

Slice preparation

Cerebellar slices were prepared as previously described (Bordey and Sontheimer 2000; Muller et al. 1994). Briefly, 15- to 30-day-old Sprague Dawley rats were anesthetized using pentobarbital (50 mg/kg) and decapitated. A rapid craniotomy that removed the occipital bone and mastoid processes allowed the cerebellum to be quickly detached, removed and chilled (0–4°C) in 95% O2-5% CO2 saturated artificial cerebrospinal fluid (ACSF) containing (in mM) 125 NaCl, 3 KCl, 2 CaCl2, 2 MgCl2, 25 NaHCO3, and 10 glucose. Next, the cerebellum was glued (cyanocrylate glue) to the stage of a vibratome containing 3 mM MgATP and 0.2 mM NaGTP was used. Osmolality of all solutions was adjusted to 305 mosM, respectively. The osmolarity of all solutions was maintained by compensating the transients of a small (5 mV) 10-ms hyperpolarization step. The capacitance reading of the amplifier was used as value for the whole cell capacitance. Capacitive and leak conductances were not subtracted. Junction potentials of 4 and 14 mV with a KCl and KGlucuronate-rich solutions were not corrected. Peak currents were determined using Clampfit (Axon Instruments), and statistical values (mean ± SD, with n being the number of cells tested) were evaluated with a statistical graphing and curve-fitting program (Origin, MicroCal). Statistical comparison of means was performed with Student’s t-test.

Both bath and pressure applications were used. Receptor and transporter inhibitors were diluted in ACSF and applied by a rapid bath application system. Receptor and transporter substrate agonists were pressure applied by a computer-controlled pressure ejection system (2-channel picorspritzer, General Valve). They were diluted in ACSF, in which HEPES replaced NaHCO3 and pH was adjusted to 7.4 by NaOH. When NaCl was replaced by choline or another chemical, similar changes were performed in the pressure pipette solution. The pressure ejection pipettes were standard unpolished patch-electrodes with resistances of 6–8 MΩ for local agonist application and were just above the slice. The applied pressure was between 3 and 5 psi. For applying two drugs to the same cell, a theta glass with one distinct drug in each compartment was used.

INTRACELLULAR PERFUSION OF A GABA TRANSPORTER BLOCKER DURING THE RECORDING

Intracellular perfusion of a GABA transporter blocker was performed as previously reported by others for single or multiple drugs application (Tang et al. 1990). We used a straight pipette holder with a perfusion port (EH-U2, E. W. Wright). Through the perfusion port, a polyethylene tube (0.86 mm ID and 1.27 mm OD) was introduced sufficiently far to reach well into the patch pipette solution. A 1-ml syringe containing the LY-filled intracellular solution to be perfused during the recording was connected to the polyethylene tube via an elongated and thinned plastic pipette tip. Before adding the patch pipette, positive pressure was manually applied to fill up the tube all away to the end, remove air bubbles, and visualize eflux of solution. Then, after applying negative pressure to prevent any solution leakage but without adding an air bubble to the end of the tube, the patch pipette was inserted into the holder. To perfuse the LY-filled solution containing either GABA or SKF89976-A, a positive pressure was manually applied to add sufficient solution to double the volume in the patch pipette (about 20 µl). The concentrations of GABA and SKF89976-A were double to obtain the intended final concentrations in the cell.

Whole cell recordings and drug application

Whole cell patch-clamp recordings were obtained as previously described (Bordey and Sontheimer 2000). Patch pipettes were pulled from thin-walled borosilicate glass (1.55 mm OD; 1.2 mm ID; WPI, TW150F-40) on a PP-83 puller (Narishige, Japan). Pipettes had resistances of 6–8 MΩ when filled with the following solutions (in mM): 140 KCl or 140 CsCl or 134 K-glucuronate and 6 KCl when noted; 1.0 CaCl2; 2.0 MgCl2; 10 ethylene glycol-bis-(aminoethyl ether)-N,N,N′,N′-tetraacetic acid (EGTA); and 10 HEPES. pH was adjusted to 7.2 with NaOH, which introduced Na+ at a concentration of 2.5 mM. For some recordings with a KCl-based solution and for all of the recordings with a CsCl-based solution, the intracellular solution containing 3 mM MgATP and 0.2 mM NaGTP was used. Osmolarities of the intracellular and extracellular solutions were 295–300 and 305–310 mosM, respectively. The osmolarity of all solutions was measured with a vapor pressure osmometer 5500 (Wescor) and was adjusted by addition of water or Na-mannitol. In some experiments, extracellular Na+ was replaced by an equimolar amount of choline or Li+. To test GABA transport reversal, GABA (10 mM) and HEPES-Na (10 mM) replaced equimolar amount of intracellular KCl and HEPES, respectively. When voltage steps were applied to the recorded cell, 5 mM Cs+ and 40 mM TEA was added to the extracellular solution in exchange for an equimolar amount of Na+ to suppress voltage-activated and time-dependent outward K+ currents.

Cell identification

Images of cells visually chosen for recordings were archived using a LG3 frame grabber (Scion) for later (off-line) comparison to LY fills. After recordings, slices were transferred to a fixation medium containing 4% paraformaldehyde in phosphate-buffered saline (PBS). The next day, slices were washed three times in PBS for 1 h, were incubated for 15 min with 1% Triton X-100, 5% normal goat serum (NGS, Sigma) in PBS, and then were incubated for 1 h in the blocking solution 0.2% triton, 5% NGS in PBS. Slices were then incubated with Cy3-conjugated antibody against GFAP (Sigma, dilution 1:500) for 2 h at room temperature in the blocking solution. Slices were then washed in PBS and mounted on glass coverslips with fluorescent mounting medium (Vecastield, Vector) and were viewed on an Olympus microscope (BX51). Images were captured with a DVC color camera and printed on an Epson color printer. No bleed-through was observed between the Texas red/Cy3 and FITC fluorescence channels in control experiments.

Chemicals were purchased from Sigma (Saint Louis, MO), unless otherwise noted.
RESULTS

Whole cell recordings were obtained from 143 visually identified Bergmann glia in cerebellar slices of 15- to 30-day-old rats. This age period corresponds to the final maturation of inhibitory synapses onto Purkinje cells from basket and stellate cells (Crepel et al. 1981). Every recorded cell was filled with LY and identified as a Bergmann glial cell by a typical morphology characterized by a small soma size (8–12 μm) and three or more long, parallel processes extending in the molecular layer toward the pial surface (de Blas 1984; Reichenbach et al. 1995). Figure 1A shows a representative example of a LY-filled Bergmann glial cell. Recorded cells with such morphology stained positive for glial fibrillary acidic protein (GFAP), identifying them as astrocytes (Eng 1985) (Fig. 1B).

In addition, cells recorded with KCl- and K-Gluconate-based intracellular solutions had a characteristically low mean input resistance of 60.4 ± 22.4 (SD) MΩ, (n = 125), a mean hyperpolarized resting membrane potential (V_R) of −76.9 ± 8.3 mV (n = 125), and lacked current-induced action potentials under current clamp (data not shown). Mean cell capacitance (C_m) was 45.3 ± 18.3 pF (n = 125). Because no significant difference for these parameters was observed between both intracellular solutions, values were pooled. The mean input resistance of the cells recorded in 5 mM Cs/40 mM TEA was 110.8 ± 13.4 MΩ (n = 17). These values were determined in the first 3 min of whole cell recording.

Bergmann glia possess pharmacologically distinct GABA transporters

GABA ACTIVATES BOTH GABAA RECEPTORS AND TRANSPORTERS. Bergmann glia were recorded at a holding potential of −70 mV unless otherwise noted. Pressure applications (puff) of GABA (500 μM, 5 s) induced large inward currents of −585.9 ± 259.0 pA (n = 19, Fig. 1, Ca and Da). These currents activated within 5 s, decayed to 70% of the peak value while GABA was still present in the bath, and were often accompanied by an increase in noise (Fig. 1, Ca and Da). Such currents are typical of GABA responses in Bergmann glia (Muller et al. 1994) and other astrocytes (Fraser et al. 1997). To identify the nature of the GABA-induced currents, we bath applied antagonists of GABA receptors (GABARs). Bath application of picrotoxin (PTX, 500 μM; Fig. 1Cb) or bicuculline (250 μM, Fig. 1Db), two blockers of GABAA Rs, reversibly reduced GABA-induced currents by 93.6 ± 4.1%, (n = 29, the percentage block with both blockers was pooled). The residual currents were small in amplitude, did not decrease in amplitude while GABA was still present, and were not accompanied by an increase in noise. Such residual currents were observed in all the tested

FIG. 1. GABA activates GABAA receptors and transporters in Bergmann glia in situ. A: photograph of a Lucifer-yellow-filled Bergmann glial cell in a 250 μm slice from a 16-day-old rat. B: anti-GFAP antibody labeling superimposed on the cell shown in A. C: a puff of GABA (500 μM) induced an inward current (a) reduced by bath application of 500 μM picrotoxin (PTX, b), a blocker of GABAAc receptors. The PTX-insensitive current was not affected by 100 μM phaclofen, a blocker of GABABRs (c). Recordings were obtained with a KCl-based internal solution. D: a puff of GABA (500 μM) induced an inward current (a) reduced by bath application of 250 μM bicuculline (b). E: a puff of isoguvacine (500 μM) induced an inward current (a) that was completely blocked by bicuculline (b). Recordings in (D and E) were obtained with a CsCl-based internal solution. F: a puff of picrotoxin (PTX)- and phaclofen-insensitive current was not affected by 2 mM Ba2+ (F) and 1 μM TTX (G). All the recordings were performed from a holding potential of −70 mV.

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cells. The PTX- and bicuculline-insensitive GABA-induced currents were unaffected by the GABA_A_R antagonist phaclofen (100 μM; −50.5 ± 8.2 pA in control and −51.1 ± 8.0 pA in phaclofen, n = 7; Fig. 1Cc; data not shown for currents in the presence of bicuculline). For further characterization of GABA-induced inward currents, phaclofen and PTX were routinely applied in the external solution. PTX was used instead of bicuculline because PTX blocks both GABA_A_R and GABA_C_R channels in the presence of bicuculline. For further characterization of GABA-induced currents in other cell types in vitro (Dong et al. 1994; Kav...mann glia using either a CsCl-based intracellular solution or a KCl-based intracellular solution with ATP + GTP. The mean residual current amplitude was −47.4 ± 17.9 pA (n = 49/49) and −46.0 ± 13.7 pA (n = 27/27) without and with ATP + GTP in a KCl-based intracellular solution, respectively (Fig. 1C). This mean amplitude was similar to the mean current amplitude of −47.3 ± 7.1 pA (n = 16) when the cells were recorded with a CsCl-based intracellular solution containing ATP (Fig. 1D). Replacement of CsCl for KCl did not reduce the magnitude of GAT currents, which was expected because K+ is not required for GAT activity (Attwell and Mobbs 1994; Cammack and Schwartz 1993). These GABA-induced currents insensitive to PTX, bicuculline, and phaclofen resemble GAT currents in other cell types in vitro (Dong et al. 1994; Kavanaugh et al. 1992; Risso et al. 1996), including retinal Müller glia (Biedermann et al. 1994; Zhao et al. 2000). Puffs of isoguvacine (500 μM) induced inward currents that were completely blocked by bicuculline (200 μM), showing that GABA_A_R antagonists are efficient at fully blocking GABA_A_R activation (n = 3) in our recording conditions (Fig. 1E). These GABA-induced currents were also unaffected by 2 mM Ba^{2+} or Cs^{+} (−51.7 ± 5.2 pA in control and −53.1 ± 5.7 pA in Ba^{2+} or Cs^{+}, n = 4, data for Ba^{2+} and Cs^{+} were pooled; Fig. 1F for Ba^{2+}, data not shown for Cs^{+}). This result rules out the possibility that K^+ which could be released by surrounding Bergmann glia depolarized by GABA via GATs, generates the current by passing through inwardly rectifying K^+ channels in the recorded Bergmann glial cell (Bordey and Sontheimer 2000). In addition, residual GABA-induced currents were unaffected by 1 μM tetrodotoxin (TTX, −49.0 ± 10.8 pA in control and −49.0 ± 11.2 pA in TTX, n = 3, Fig. 1G) and an extracellular solution containing 0 Ca^{2+}/1 mM EGTA (n = 2, data not shown). This result rules out the possibility that glutamate, which could be released following depolarization of presynaptic terminals by GAT activation (Bonanno et al. 1993), induces the current by activating receptors on the recorded Bergmann glial cell (Bergles et al. 1997; Feigenspan and Bornmann 1994; Muller et al. 1996).

Every cloned GABA transporter depends on Na^+ and Cl^- for the uptake of GABA (Borden et al. 11992, 995a; Guastella et al. 1990). Figure 2A shows that when choline or Li^+ was substituted for external Na^+ (n = 14), PTX- and phaclofen-insensitive GABA currents were reversibly and completely blocked, identifying them as GAT currents. Lowering extracellular Cl^- from 136 to 11 mM (NaCl replaced with Na-glucuronate) reduced GAT currents by 63.0% (n = 4, data for Ba^2+ and Cs^+ were pooled; Fig. 2B). A 50% change in extracellular Cl^- concentration did not yield any detectable reduction in GABA-induced GAT currents.

In all the tested cells, GABA induced a transport current. To
determine whether GAT amplitudes were different before and after the completion of the GABAergic synaptic connections onto Purkinje cells at postnatal day (P) 23 in the rat (Rosina et al. 1999), we divided our mean GAT current amplitudes into two age groups P15-P22 and P24-P30. Mean GAT current amplitudes were $-45.7 \pm 15.2$ pA ($n = 41$) and $-48.6 \pm 16.6$ pA ($n = 26$) in cells from P15 to P22 and P24 to P30 rats, respectively. These values were not significantly different.

**VOLTAGE DEPENDENCE OF GABA-INDUCED GAT CURRENTS.** When we gradually changed the holding potential of the recorded Bergmann glial cell from $-70$ to $+50$ mV, GABA-induced GAT currents decreased progressively in amplitude when recorded with either a KCl-based intracellular solution (Fig. 2C; $n = 5$) or a CsCl-based intracellular solution ($n = 3$, data not shown). The current-voltage ($I-V$) relationship rectified inwardly and did not reverse for positive membrane potentials (Fig. 2D, $i$ and $i_{cl}$ in KCl- and CsCl-based solutions, respectively, mean $\pm$ SE are shown in the graphs). Such $I-V$ curves are expected for GATs under the recording conditions used (Biedermann et al. 1994; Cammack and Schwartz 1993; Dong et al. 1994). All together these data suggest that GABA-induced currents in the presence of GABA$_A$/GABA$_B$/GABA$_C$ blockers are generated by GABA being taken up into Bergmann glia by GABA transporters.

**PHARMACOLOGICAL CHARACTERIZATION OF GAT CURRENTS.** Four distinct GATs have been cloned thus far, termed GAT-1, -2, and -3 and BGT-1 in rats. GAT-1 displays a distinct pharmacological profile from the other GATs (Borden et al. 1992, 1995a; Guastella et al. 1990). We first tested nipeptic acid and $\beta$-alanine, which are competitive blockers of GAT-1/2/3 subtypes (Krogsgaard-Larsen and Johnston 1975; Liu et al. 1993) and GAT-2/3 subtypes (Borden et al. 1992; Liu et al. 1993), respectively. Bath application of nipeptic acid (100 $\mu$M) and $\beta$-alanine (100 $\mu$M) induced baseline inward currents that averaged $-51.0 \pm 2.2$ pA ($n = 11/11$) and $-26.1 \pm 11.0$ pA ($n = 8/8$), respectively, as shown by the deviation of the holding current with the GAT substrate agonist from the control (Fig. 3, Ab, dashed line). In the presence of bath-applied $\beta$-alanine, GABA induced additional inward current (Fig. 3B). Nipeptic acid and $\beta$-alanine occluded 100% ($n = 11$, Fig. 3A) and $57.1 \pm 5.3\%$ ($n = 8$, Fig. 3B) of the maximal GABA-induced GAT currents, respectively. GAT currents were unaltered by betaine, a competitive blocker of BGT-1 ($-51.0 \pm 9.2$ pA in control and $-50.4 \pm 9.4$ pA with betaine, $n = 5$; data not shown). We then tested the effects of two specific nontransportable blockers of GAT-1 SKF89976-A and NNC-711 (Borden et al. 1994). Bath application of SKF89976-A (100 $\mu$M; Fig. 3D) and NNC-711 (10 $\mu$M; Fig. 3C) reversibly reduced GAT currents in every studied cell by $67.9 \pm 4.4\%$ ($n = 19$; % block by each blocker was similar and data were thus pooled). The reduction of GAT current by $\beta$-alanine and their incomplete block by GAT-1 blockers suggest that GAT-1 and another GAT subtypes are expressed in Bergmann glia.
Interestingly, these GAT-1 blockers induced outward currents in 63% of the tested glia even though these compounds are nontransportable blockers of GAT-1. These outward currents averaged 10.5 ± 2.7 pA (n = 12). This result suggests that SKF89976-A and NNC-711 block an inward current due to either a tonic activation of GATs by ambient levels of GABA and/or a leakage conductance associated with the transporter (Cammack and Schwartz 1993, 1996; Mager et al. 1996). Such a leakage conductance has been observed in the absence of GABA, and the current is carried by Na⁺ ions. To help distinguish between these two possibilities, we used an extra-cellular solution containing 0 Ca²⁺/1 mM EGTA/1 mM TTX to prevent synaptic Ca²⁺-dependent GABA release and thus reduce levels of background GABA. In this condition, SKF89976-A applied by pressure (Fig. 7A before intracellular GABA perfusion) or perfused intracellularly (Fig. 7B) never induced any current in Bergmann glia (n = 6).

To further characterize GAT currents in Bergmann glia, we pressure applied different substrate agonists and studied the Na⁺ and voltage dependence of the resulting currents. GABA and a substrate agonist (each 500 μM) were applied in succession on the same cell using a theta glass pressure pipette. The mean inward currents induced by nipecotic acid and GABA were not significantly different (P = 0.02) and averaged −42.9 ± 7.1 and −50.8 ± 4.7 pA, respectively (n = 8/8, Fig. 4A). Similarly, mean inward currents induced by β-alanine and GABA were not significantly different and averaged −47.4 ± 5.8 and −51.6 ± 7.0 pA (n = 4/4), respectively (Fig. 4B).

**FIG. 5.** Voltage dependence of nipecotic acid- and β-alanine-induced GAT currents. A and B: records of nipecotic acid (A) - and β-alanine (B) - induced currents obtained at different holding potentials from −70 to +50 mV. C and D: mean I-V curves of the currents shown in (A, ■) and (B, ●), respectively. E: records of nipecotic acid-induced currents obtained under control conditions and following increasing concentrations of GABA in the bath (1, 5, 10, and 20 μM). Recorded cells were held at −30 mV. F: plot of the peak amplitudes of nipecotic acid-induced outward currents (n = 3) as a function of the bath-applied GABA concentrations.
β-alanine was pressure applied in the presence of bath-applied taurine (100 μM) to block taurine transporters because β-alanine is also a substrate agonist of taurine transporters (Liu et al. 1992; Smith et al. 1992). A puff of betaine did not induce any inward currents (n = 11, Fig. 4C). When Na⁺ was replaced by choline, currents induced by nipecotic acid (Fig. 4A, n = 3) and β-alanine (Fig. 4B, n = 3) were reversibly blocked, suggesting that both substrate agonists induced transport currents. Consistent with this idea we found that in the absence of PTX, nipecotic acid- and β-alanine-induced currents were not affected by the subsequent application of PTX (n = 5, data not shown). The voltage dependence of β-alanine- and GABA-induced GAT currents was similar (Figs. 5B and 2C, respectively). β-alanine-induced currents were strictly inwardly rectifying up to +50 mV (Fig. 5D). In contrast nipecotic acid-induced inward currents were followed by outward currents that are detectable at and above −30 mV [Fig. 5A, see outward currents above baseline (•–•)]. Only outward currents were observed at +30 and +50 mV (Fig. 5, A and C). We questioned whether the outward currents following nipecotic acid-induced inward currents could be explained by nipecotic acid-induced heteroexchange of GABA pressure applied onto the same cell (data not shown). To support the interpretation of nipecotic acid-induced GABA heteroexchange, slices were incubated with increasing concentrations of bath applied GABA prior to pressure applications of nipecotic acid. When the cells were held at −30 mV, outward currents following nipecotic acid-induced inward currents were detectable (n = 3, Fig. 5E). In addition, the amplitude of these outward currents was dependent on the concentration of bath-applied GABA (Fig. 5, E and F). Together these data demonstrate the presence of functional GAT-1 in Bergmann glia in situ.

GAT-mediated GABA efflux from Bergmann glia

For all of the following experiments, seal and whole cell recordings were performed initially in a normal external solution and subsequently, in an external solution containing 0 Ca²⁺/1 mM EGTA/1 μM TTX, to block Ca²⁺-dependent neuronal GABA efflux. TEA (40 mM)/5 mM Cs⁺/100 μM phaclofen was also bath applied to block K⁺ currents and GABAA_R activation.

GATS CAN WORK IN REVERSE IN BERGMANN GLIA RECORDED WITH 10 mM INTRACELLULAR GABA. Bergmann glia were held at −20 mV and recorded with a KCl-based intracellular solution (isotonic Cl⁻) containing 10 mM GABA and 12.5 mM [Na⁺]. In the presence of PTX, a puff of SKF89976-A (500 μM) induced a mean inward current of −17.3 ± 3.5 pA (n = 5/5, Fig. 6A), suggesting that SKF89976-A blocked an outward current due to transport reversal at −20 mV. SKF89976-A-sensitive currents were not observed between −60 and −40 mV, were outward at −20 mV and up, and increased in amplitude when the cell was further depolarized (Fig. 6B, ♣). These data suggest that GAT-1 can work in reverse. However, to verify that GABA efflux was accompanied Na⁺ efflux, we wonder whether GABA could activate GABAA_R on the same cells. We thus repeated the same experiments in the absence of extracellular PTX. In this condition, a puff of SKF89976-A (500 μM) induced a smaller mean inward current that was −9.0 ± 2.6 pA in amplitude (n = 3 of 5 cells, Fig. 6B). In this condition (symmetrical Cl⁻ concentrations), GABAA_R-mediated currents are inward at −20 mV and likely reduce the apparent amplitude of outward GAT currents. In addition, at depolarized potentials, it is likely that GABAA_Rs are desensitized because of carrier-mediated GABA efflux and ambient GABA accumulation. GABAA_R desensitization would explain the lack of effect of PTX on outward currents at depolarized potentials. Together these results suggest that GABAA_Rs is activated by GABA released via depolarization-induced transport reversal.

ACTIVATION OF GABAA_Rs ON GABA TRANSPORT REVERSAL. In the following experiments, we intracellularly perfuse GABA during the recording. Intracellular GABA and Na⁺ concentrations were then about 10 and 12.5 mM, respectively (see METHODS). In addition, we recorded cells with a K-gluconate intracellular solution to impose a Nernst equilibrium potential for Cl⁻ ions at −61 mV (intracellular Cl⁻ concentration of 12 mM). In this condition, GABAA_R-mediated currents were outward at and above −60 mV like transport reversal-mediated currents. GABA- and isoguvacine-induced currents reversed at −61.4 ± 7.3 mV (n = 8; range −53 to −70 mV; because no significant difference was observed between the agonists values were pooled). On intracellular perfusion of GABA in

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**FIG. 6.** SKF89976-A-sensitive baseline reverse transport current. Bergmann glia were recorded with an internal solution containing GABA and Na⁺ at 10 mM each and in the presence of PTX, phaclofen, 0 Ca²⁺/1 mM EGTA/1 μM TTX/40 mM TEA/5 mM Cs⁺ in the bath. A: SKF89976-A-induced inward currents recorded at different holding potentials. These inward currents resulted from the blockade of baseline currents from the reverse transport of GABA. B: I-V curves of the outward current blocked by SKF89976-A with (♦) and without (□) 500 μM PTX. SKF89976-A was pressure applied at 500 μM.
Bergmann glia recorded at −20 mV, an outward current of 22.7 ± 3.9 pA (n = 4) developed (Fig. 7). These outward currents were reversibly reduced by a puff of SKF89976-A (500 μM, n = 4, Fig. 7A) or bicuculline (200 μM, n = 4, data not shown). The initial decrease in the outward current amplitude (Fig. 7A, large arrow) observed approximately 20 s after the current onset may be due to GABA-A receptor desensitization as suggested in Fig. 6. On intracellular perfusion of a LY-filled solution without Na⁺ or GABA, a puff of SKF89976-A or bicuculline did not induce any current (n = 8). LY was routinely added in the intracellularly perfused solution but not in the recording solution to confirm that the perfused solution was diffusing into the cell. LY did diffuse into the cell in all experiments of this type (data not shown). We used an additional control to verify that the intracellularly perfused drug was being effectively perfused inside the recorded cell. Intracellular perfusion of 20 μM SKF89976-A progressively reduced and blocked GABA-induced GAT currents without blocking GABA-A receptor-mediated currents (n = 4, Fig. 7B).

To prevent GABA-A receptor desensitization due to GABA efflux and ambient GABA accumulation, we studied GABA efflux-mediated GABA-A receptor activation by applying 150-ms voltage steps. Voltage steps (20-mV increment) were applied from −100 to +40 mV from a holding potential of −70 mV. In response to voltage steps, Bergmann glia generated transient (capacitative) and small steady-state currents (Fig. 8A). These voltage steps were applied before and after the following successive experimental conditions: intracellular perfusion of GABA during the recording (Fig. 8A), bath application of PTX to block GABA-A receptors (Fig. 8B), and after washout of PTX bath application of SKF89976-A to block GAT-1 and thus to block GABA efflux and GABA-A receptor activation (Fig. 8C). The action of each drug is summarized in Fig. 8. Intracellular perfusion of GABA (see previous paragraph) induced an increase in the steady-state currents without affecting capacitative currents (Fig. 8B, compare with A). Point-by-point subtraction of the currents in the presence and absence of intracellularly isolated outward currents that averaged 23.3 ± 6.0 pA at −20 mV (n = 5, Fig. 8E). This mean amplitude is similar to that of the outward currents obtained on intracellular GABA perfusion in cells held at −20 mV (Fig. 7A). The I-V curves of these currents show that they are voltage-dependent and outwardly rectifying, appear near −60 mV, and could be clearly detected at −40 mV (Fig. 8E, right). Subsequent bath application of PTX (10 μM) induced a decrease in the steady-state outward currents (Fig. 8C). The subtracted currents (traces with PTX from traces without PTX) isolated PTX-sensitive currents that represent GABA-A receptor-mediated outward currents and averaged 11.9 ± 4.2 pA at −20 mV (n = 4, Fig. 8F). The GABA-A receptor-mediated currents were outward at −20 mV as expected in our intracellular recording conditions imposing an E_cl of −61 mV (internal [Cl⁻] of 12 mM). Subtracting the control traces (Fig. 8A, no internal GABA) from the traces with internal GABA and PTX in the bath (Fig. 8C) isolated transporter-mediated outward currents that averaged 11.6 ± 1.7 pA at −20 mV (n = 4, Fig. 8G). The outward current induced by intracellular GABA perfusion (Fig. 8E) is thus composed of transport- and receptor-mediated currents, and each represents 50% of the total outward current in our recording conditions. The I-V curves of receptor- and transporter-mediated outward currents show that they are voltage-dependent and outwardly rectifying and can be clearly detected at −40 mV (Fig. 8F, right). Similarly, subsequent application of SKF89976-A after washing out PTX also reduced steady-state outward currents (Fig. 8D). SKF-89976-A

FIG. 7. Intracellular GABA perfusion during the recording to study GABA transport reversal. A: recorded cells were held at −20 mV to allow GABA transport reversal. GABA (20 mM) was intracellularly perfused during the recording as indicated by the arrow. Before intracellular GABA perfusion SKF89976-A did not affect baseline currents. After intracellular GABA perfusion, an outward current developed that displayed a transient peak of 40 pA (as shown by a thick head) and then plateau at about 25–30 pA. This outward current was reduced by pressure application of 500 μM SKF89976-A. B: 500 μM GABA was pressure applied from a holding potential of −70 mV under different conditions: in the absence of PTX, in the presence of 500 μM PTX as indicated by the bar, following the intracellular perfusion of 20 μM SKF89976-A as indicated by the arrow, and after washout of PTX. Intracellular perfusion of SKF89976-A resulted in a progressive reduction of GAT currents, and after washout of PTX, intracellular SKF89976-A resulted in a progressive reduction of GABA currents as indicated by the arrow, and after washout of PTX. Intracellular perfusion of 20 μM SKF89976-A progressively reduced and blocked GABA-induced GAT currents without blocking GABA-A receptor-mediated currents (n = 4, Fig. 7B).

FIG. 8. Voltage step records for GABA efflux and GABA-A receptor activation. A–D: current traces following 20-mV increment voltage steps of 150 ms applied from −100 to +40 mV from a holding potential of −70 mV under control conditions (A) and after intracellular perfusion of 20 mM GABA (B). Following intracellular GABA perfusion, the cells were recorded in the presence of PTX (C) and SKF89976-A (D) following washout of PTX. E: traces in A were subtracted from traces in B to reveal GABA efflux currents and their respective I-V curve (right). F: traces in C were subtracted from traces in B to reveal GABA-A receptor-mediated currents and their respective I-V curve. G: traces in C were subtracted from traces in A to reveal GAT-mediated efflux currents and their respective I-V curve. H: traces in D were subtracted from traces in B to reveal current due GAT-1 mediated GABA efflux and their respective I-V curve. Experiments were performed in the presence of phaclofen and 0 Ca²⁺/1 mM EGTA/1 μM TTX/40 mM TEA/5 mM Cs⁺. Bergmann glia were recorded with a K-glucuronate-based intracellular solution. I: scheme describing GABA efflux and the resulting GABA-A receptor activation on the recorded cell. On intracellular perfusion of GABA, GABA leaves the cell via GAT-1 reversal and activates GABA-A receptors. Both actions generate outward GABA currents displayed in E. Part of these outward GABA currents are PTX sensitive (GABA-A receptor-mediated GABA efflux) and PTX insensitive (G), which represent GABA efflux currents via GAT reversal. SKF89976-A blocks currents mediated by GAT-1 transporter reversal and the currents due to GABA-A receptor activation.
GABA TRANSPORTERS IN BERGMANN GLIA IN SITU

A  Control

B  Intracellular GABA

C  GABA in + PTX out

D  GABA in + SKF89976-A out

E  B-A: GABA currents

F  B-C: PTX-sensitive currents

G  C-A: GAT currents

H  B-D: SKF89976-A-sensitive currents

I  Intracellular GABA perfusion

GABA

GABA_A Rs  (PTX sensitive)

GAT-1

GAT-2/3

(SKF89976-A sensitive)
sensitive currents that represent GAT-1 transport reversal- and GABA_A-R-mediated currents averaged 19.9 ± 8.4 pA at −20 mV (n = 4, Fig. 8H). Before intracellular perfusion of GABA, bath application of PTX did not affect the steady-state currents (n = 3; data not shown), demonstrating that PTX did not have a nonspecific action. These data suggest that PTX-sensitive currents are due to GABA transport reversal-mediated GABA efflux that activates GABA_ARs on the recorded Bergmann glial cell.

Because of the nonideal electrical properties of Bergmann glia due to their extensive processes and low input resistance, we obtained recordings of GAT currents from glial cell body that had been pulled out from the slice. This procedure will allow us to more accurately determine the threshold activation of carrier-mediated efflux current. Once Bergmann glia were recorded, their cell body was pulled out of the slice. Two or three very short processes of about 4–6 μm each stayed attached to the soma. In this condition, the mean cell membrane capacitance was 18.7 ± 3.6 pF (n = 4) and the mean cell input resistance was 220.8 ± 27.0 MΩ. We monitored GAT reversal by applying voltage steps as described for Fig. 8. Voltage steps were applied every 10 mV instead of 20 mV with GABA in the recording pipette instead of being intracellular perfused during the recording. In three of four cells, we observed SKF80076-A-sensitive outward currents (Fig. 9D) that activated at −60 mV and were clearly detectable at −50 mV with a mean value of 15.6 ± 0.8 pA (Fig. 9E).

**DISCUSSION**

In the present study, we report for the first time direct measurements of GABA transporter currents in an intact system and electrophysiological characterization of these currents in Bergmann glia. In particular, our data show that Bergmann glia in situ possess functional GABA transporters in particular GAT-1 that are likely activated by ambient levels of extracellular GABA and Bergmann glial GABA transporters can mediate electrogenic GABA efflux that results in the activation of nearby GABA_A receptors.

**Bergmann glia express multiple and functional GABA transporter subtypes**

**ISOLATION OF FUNCTIONAL GATS IN BERGMANN GLIA.** Our data showed that a large portion of GABA-induced currents were sensitive to PTX and bicuculline and thus mediated by GABA_A-R and perhaps GABA_B-R activation. GABA_A-R properties in Bergmann glia were characterized in a previous report (Muller et al. 1994) and were not further analyzed in the present study. In the presence of blockers for all GABARs, GABA-induced currents were blocked by removal of external Na^+, identifying them as currents due to GAT activation (Borden 1996). The lack of difference between GAT current amplitudes before and after the GABAergic network completion suggests that Bergmann glia express GAT-1 earlier than the completion of the synaptic connections onto Purkinje cells. However, we cannot rule out that there may be a spatial redistribution of GATs on the glial membrane. These GAT currents were also dependent on external Cl^− concentration and voltage as previously reported (Borden 1996). In the absence of internal GABA and Na^+, no outward currents could be detected at depolarized potentials as reported from studies on GAT-1 expressed in oocytes (Kavanaugh et al. 1992; Mager et al. 1993) and on GATs in retinal horizontal cells (Cammack and Schwartz 1993; Dong et al. 1994) and Müller glia (Biedermann et al. 1994; Zhao et al. 2000) in vitro. In addition, a 0 current was obtained at and above +30 mV. The uptake of one molecule of GABA is coupled to the uptake of two Na^+ and one Cl^− ions into the cell. The concentrations of GABA and cotransported ions and the stoichiometry of GATs determine the reversal potential of GAT currents. To predict the reversal potential of GAT currents assuming the standard stoichiometry for GATs, the following simple transport model has been used:

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**FIG. 9.** Voltage step records for GABA efflux recorded in the soma. A–C: current traces following 10-mV increment voltage steps of 150 ms applied from −100 to −20 mV from a holding potential of −70 mV. The cells were recorded with an internal solution containing GABA + Na^+ each at 10 mM. The cells were recorded under control conditions (A), in the presence of SKF89976-A (B), and following wash out of SKF89976-A (C). D: traces in B were subtracted from traces in A to reveal GAT-1 transport reversal-mediated currents. E: I-V curve of traces in D.
Bergmann glia express functional GAT-1 but not BGT-1 and suggest that they co-express some unidentified GAT isoforms. ARE GLIAL GATS ACTIVATED BY BACKGROUND GABA? SKF89976-A blockade of a basal inward current suggests either tonic activation of GAT-1 by ambient GABA or an uncoupled (leakage) current associated with the transporters in the absence of GABA (Cammack and Schwartz 1993, 1996; Cammack et al. 1994). The action of SKF89976-A on baseline currents is likely due to tonic activation of GATs by ambient GABA for two reasons: SKF89976-A did not have any effect when applied in an extracellular solution containing 0 Ca²⁺/1 mM EGTA/1 μM TTX to prevent synaptic release of GABA and GABA can reach 10–100 μM at the synaptic cleft (Kammers and Werblin 1992), which is sufficient to activate nearby GATs known to have a high affinity for GABA (EC₅₀ of 4.7–7 μM for GAT-1) (Guastella et al. 1990; Kavanaugh et al. 1992). The possibility that an undetectable leakage current was also present cannot be excluded.

Glial GATs can mediate electrogenic GABA efflux

To study GABA transport reversal, we either recorded cells with a pipette containing internal GABA and Na⁺ or we perfused GABA + Na⁺ intracellularly during recordings to obtain a final concentration of 10 mM GABA and 12.5 mM Na⁺. In both conditions, pressure application of SKF89976-A blocked an outward current of about 20 pA at −20 mV. An uncoupled Na⁺ leakage current through GATs has been reported in the absence of GABA due to a channel mode of the transporters (Cammack and Schwartz 1993, 1996). Because we used a high GABA concentration of 10 mM that is thought to prevent this leakage current (Cammack and Schwartz 1993), it is unlikely that the SKF89976-A-mediated outward current blockade results from the blockade of an uncoupled leakage current. Thus these data suggest that GATs were working in reverse on internal (GABA + Na⁺) perfusion and glial cell depolarization. However, to ensure that GABA was indeed released, we verified whether adjacent GABAₐRs on the recorded cell were activated during GABA transport reversal. To obtain I-V curves of carrier-mediated GABA efflux, we studied GABA transport reversal by applying voltage steps. This protocol also prevented GABAₐR desensitization that might have occurred following extracellular GABA accumulation with the cell held at depolarized potentials. The I-V curve of intracellular GABA-induced outward currents showed that these currents were voltage-dependent and outwardly rectifying, activated near −60 mV, and were clearly detectable at −50 mV (Fig. 9). Intracellular GABA-induced outward currents were also reduced by SKF89976-A and PTX. These data confirm that transporter-mediated GABA efflux on Bergmann glial cell depolarization activated GABAₐRs upon the recorded cell. As expected for GABAₐR and GAT activation, PTX- and SKF89976-A-sensitive-currents were voltage-dependent and increased in amplitude when the cell was depolarized (equilibrium potential for Cl⁻ being −61 mV). We did not observe any GABAₐR-mediated inward currents at or less than −60 mV. This result is consistent with the absence of GABA efflux at hyperpolarized potentials. I-V curves also show that sufficient GABA was released above −60 mV to activate nearby GABAₐRs. The observation of transporter-mediated GABA efflux is in good agreement with Schwartz’s study on retinal
neurons in vitro (Schwartz 1987). Schwartz could detect transporter-mediated GABA efflux currents at −60 mV, and such currents measured about 20 pA at −20 mV and were outwardly rectifying.

**Could GABA transporters work in reverse under physiological conditions?**

A 12.5 mM concentration of intracellular Na⁺ is near physiological because astrocytes have been shown to have about 11–16 mM intracellular Na⁺ in vitro (Rose and Ransoms 1996). Regarding intracellular GABA concentration, astrocytes are thought to contain very little endogenous GABA and to quickly metabolize labeled exogenous GABA (Bardakdjian et al. 1979). However, immunoreactivity for GABA has been observed in Bergmann glia (Benagiano et al. 2000). Glial GABA can be synthesized by two pathways, one from glutamate via glutamate decarboxylase present in Bergmann glia (Martínez-Rodriguez et al. 1993) and the other from putrescine via diamine oxidase observed in astrocytes from other brain regions (Laschet et al. 1992; Seiler et al. 1979). Consistent with this idea, it has been reported that astrocytes can synthesize and secrete GABA (Albrecht et al. 1998; Bowery et al. 1975; Pearce and Dutton 1981; Yee et al. 1998). In particular, peripherial glial cells and astrocytes surrounding the optic nerve have been reported to have sufficient GABA to release and subsequently activate receptors on neurons (Bowery et al. 1975; Yee et al. 1998). Thus far our results show that GAT-1 can mediate GABA efflux from Bergmann glia under near physiological ionic conditions. One of the next intriguing questions is whether Bergmann glia have sufficient GABA either newly taken up or endogenously synthesized in their thin processes to release and activate nearby receptors.

**Functional implications of GABA transporters in Bergmann glia**

Arguments in favor of a role of glial GABA transporter on synaptic activity come from previous studies: Bergmann glia have been shown to possess GAT-1 on their radial processes ensheathing Purkinje cell somata and dendrites (Morara et al. 1996; Swan et al. 1994); the estimated cycle time of GAT-1 has been reported to be 10 ms, which is near the decay time of GABA, synaptically released and its affinity for GABA (5–10 μM) could control the time course of transmission near GABA, receptors (half-maximal concentration estimated at 10–100 μM) (Kamermans and Werblin 1992; Mager et al. 1993); and previous studies have shown that β-alanine, which inhibits GAT-2 and -3 that are primarily expressed in astrocytes (Borden 1996), affects GABAergic synaptic activity in hippocampal slices (Druguhn and Heinemann 1996) and neuronal GABA responses in cervical ganglia (Bowery et al. 1975). These results suggest that both neuronal and also glial GATs have the ability to contribute to the removal of GABA from synaptic clefts. The next step would be to determine the relative contribution of GATs in glia or neurons to the removal of synaptic GABA. It is also interesting to note that distinct GABA transporter subtypes that have different affinities for GABA (Borden 1996) would allow GABA concentrations in the extracellular space to be spatially modulated by glia.

Overall, our study that characterizes GABA transport in Bergmann glia in situ is an important step in defining the role of GABA transporters in a neuroglial network.

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