Synaptically Evoked GABA Transporter Currents in Neocortical Glia

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INTRODUCTION

It has been well established that glial transporters play a role in the determination of the time course of a synaptic response following evoked release. Block of transporter action has been shown to increase the duration of synaptically evoked responses in numerous systems, presumably by increasing the duration of the neurotransmitter transient (Barbour et al. 1994; Isaacson et al. 1993; Mager et al. 1993; Mennerick et al. 1999; Otis et al. 1996; Overstreet et al. 1999; Thompson and Gähwiler 1992). In addition to the role transporters play in the regulation of synaptic events at the nerve terminal, they may be important in regulating “spillover” of transmitter from neighboring nerve terminals at certain synapses within the brain (Barbour et al. 1994; Isaacson et al. 1993; Rossi and Hanaman 1998). Thus neurotransmitter transporters may be critically important in the determination of the time course of synaptic responses, the termination of the actions of transmitter following release, and the regulation of excitability and excitotoxicity throughout the brain.

Numerous investigations into the function of glutamate transporters during synaptic transmission have been undertaken in recent years (Bergles and Jahr 1997, 1998; Diamond and Jahr 1997; Mennerick et al. 1999; Otis et al. 1997; Overstreet et al. 1999) and have resulted in a wealth of information regarding the function of these transporters during synaptic events. However, at present, much less is known about the function of GABA transport during synaptic transmission. It has been demonstrated that in the hippocampus, pharmacological block of GABA transport results in a notable increase in the time course of the GABA_A receptor-mediated response (Dingledine and Korn 1985; Dräguhn and Heinemann 1996; Isaacson et al. 1993; Roepstorff and Lambert 1992, 1994; Thompson and Gähwiler 1992). However, it is unclear whether these transporters have a neuronal or glial localization. In addition, it is not known whether the GABA transporter actively removes GABA during a synaptic event or serves as a buffer as has been suggested for the glutamate transporter (Diamond and Jahr 1997; Tong and Jahr 1994; but see Mennerick et al. 1999). In the cortex, glial uptake of GABA may serve to primarily regulate paracrine levels of GABA rather than participate in uptake of GABA during a synaptic event (Conti et al. 1998; Ribak et al. 1996; Sprefacico et al. 1993), although there is some evidence that glial GABA transporters in the cortex (Minelli et al. 1996) and elsewhere in the brain (De Biasi et al. 1998) are located at sites very close to release.

Currently, there are three cloned GABA transporters in the rat CNS: GAT1, GAT2, and GAT3 (Borden et al. 1992; Clark et al. 1992; Guastella et al. 1990; for review, see Borden 1996). GAT-1 expression within the cortex has been shown to be high and is localized to puncta and fibers and astrocytic processes as well as a few pyramidal cells; its expression has been shown to have a high correlation with the soma and proximal dendrites of unlabeled pyramidal cells, indicative of localization at inhibitory synapses (Conti et al. 1998; DeFelipe and González-Albo 1998; Durkin et al. 1995; Minelli et al. 1995). GAT-3 has been shown to be expressed primarily in glia (Clark et al. 1992; Durkin et al. 1995; Minelli et al. 1996; Yan and Ribak 1999); its expression in the cortex has been shown to be low in the adult (Minelli et al. 1996; Yan and Ribak 1999) and may be developmentally regulated (Jursky and Nelson 1999). GAT-2 expression is localized predominantly to the leptomeninges (Durkin et al. 1995), with some expression in cortical neurons and astrocytes (Conti et al. 1999). Information regarding the concentration time course of GABA following release is of considerable interest. The time course of evoked GABA_A receptor-mediated responses in the hippocampus has been shown to be regulated by transporters; this is indicative of an extended presence of GABA in the synaptic cleft following release. In addition, GABA is reported to diffuse, or “spill over” to neighboring terminals at sufficient concentration following evoked release to activate receptors at
these synapses, indicating that GABA has a relatively long extrasynaptic concentration time course. Finally, it is believed that GABA may remain bound to GABA_A receptors for an extended period of time following release, (Jones and Westbrook 1995), which may contribute to a relatively long extra-cellular concentration time course. The recording of GABA transporter currents from astrocytes during synaptic transmission could provide direct evidence of the time course of GABA following release as well as insight into the functional role of GABA uptake by neocortical astrocytes.

Here we report that electrophysiologically characterized neocortical astrocytes [co-] express at least two types of GABA transporters, GAT-1 and GAT-2/3, as well as glutamate transporters. Currents mediated by the GABA transporters are slow rising and long-lasting, contrasting what is observed for glutamate transporter currents, and are active during both single and repetitive stimulation.

METHODS

Preparation of brain slices

Experiments were performed on neocortical slices (300 μm) obtained from rats aged 7–28 days postnatal. Animals were anesthetized with a mixture of 1:3 xylazine/ketamine dissolved in an equal volume of saline by intraperitoneal injection (1.8. ml/kg). A section of the cortex was then rapidly removed by dissection and glued to the stage of a vibrating tissue chopper (Vibratome). The tissue was immediately immersed in ice-cold cutting solution composed of (in mM) 5 KCl, 1.25 KH₂PO₄, 26 NaHCO₃, 5 MgCl₂, 2 TEA-Cl, 20 sucrose, and 10 dextrose (320 mosM). Coronal sections of 300 μm thickness were cut and transferred to a holding chamber filled with artificial cerebrospinal fluid (ACSF) and kept at 35°C for 1 h. The ACSF was composed of (in mM) 130 NaCl, 3 KCl, 2 CaCl₂, 1.25 NaH₂PO₄, 26 NaHCO₃, 2 MgCl₂, and 10 dextrose, kept at pH = 7.3–7.4 by bubbling with carbogen (5% CO₂–95% O₂). After this initial period, the slices were stored at room temperature.

For recording, one slice was transferred to a submersion chamber mounted on a fixed stage under the objective of a moveable microscope. The slice was then submerged and perfused continuously with carbogenated ACSF (32–34°C) flowing at a rate of 2–3 ml/min. Initially, the slice was viewed at ×10 with DIC optics to allow for gross placement of stimulating and recording electrodes. Individual astrocytes in layers 2/3 and 5 were then visualized at high power using a ×63 long-distance water-immersion lens coupled with a ×0.5–2 zoom.

Whole cell recording and stimulation

Patch pipettes are pulled on a Flaming-Brown Horizontal puller using a multi-stage pull, to resistances of 2–5 MΩ. The standard patch pipette solution is composed of (in mM) 135 KCH₃SO₄, 2 MgCl₂, 5 KCl, 10 HEPES, 2 Na₂ATP, 0.5 Na-GTP, and 0.1 EGTA (pH = 7.2–7.3 with KOH; osM = 295). For some initial experiments (n = 16), CsCl was substituted for KCH₃SO₄ in an effort to decrease membrane conductance. Experiments done in CsCl had an average input resistance 25.58 ± 1.62 MΩ and an average resting membrane potential of −88.27 ± 2.89 mV. This was a modest increase in input resistance (17.2 ± 0.64 MΩ; see RESULTS). However, CsCl was not utilized for experiments included in this study due to concerns for the long equilibration times necessary as a consequence of cell coupling. For experiments using biocytin or Lucifer yellow, a fixed amount of the required compound was simply added to the above mixture to give a concentration of 0.025% wt/vol. The liquid junction potential is measured with respect to ACSF, and all membrane potential recordings are corrected accordingly (−10 mV).

Standard techniques were used to obtain whole cell recordings. Briefly, the electrodes were visually guided onto glial cell bodies while applying gentle positive pressure to the back of the pipette to prevent clogging. On contact with the cell, negative pressure was applied until a tight seal (>1 GΩ) is formed. Further negative pressure was applied until the underlying membrane is ruptured and whole cell configuration is established. Voltage-clamp was obtained through the use of an Axopatch 200A amplifier (Axon Instruments). Whole cell current (low-pass filtered at 2–5 kHz; 8-pole Bessel filter) and membrane potential were amplified and recorded on a video cassette recorder with pulse-code modulation (sampling rate: 20 kHz) for later analysis. All experiments were also recorded on-line and current traces were also digitized on-line by data-acquisition software (pCLAMP; Axon Instruments) and stored in computer memory.

Synaptic inputs to glial cells (and nearby neurons) were stimulated using a bipolar tungsten-stimulating electrode placed in the slice in the vicinity of the target cell (50–200 μm). Single stimuli were evoked using intensities of between 20 and 200 μA at a duration of 100 μs. Repetitive stimuli were evoked using the same parameters at a frequency of 100 Hz. All experiments were done at the cell resting membrane potential.

Identification/characterization of cells

Experimental cells were identified by visual inspection under a ×63 water-immersion lens coupled with a ×0.5–2 zoom (Fig. 1A), electrophysiological characterization of membrane properties, and labeling with Lucifer yellow or Alexa 594 in a number of cells and confirming ultrastructure post hoc. Neocortical astrocytes are known to be relatively small cells, with cell bodies in the range of 6–15 μm. This size criterion was the basis for the initial selection of an experimental cell; identity was also established electrophysiologically: astrocytes are characterized by their high negative resting membrane potentials (RMPs), low input resistances, and passive, linear current-voltage relationships (e.g., Bergles and Jahr 1997; Chvatal et al. 1995; D’Ambrosio et al. 1998; Schwartzkroin and Prince 1979; but see Bordey and Sontheimer 1998). Based on these electrophysiological criteria, we characterize the cells in this study as neocortical astrocytes.

In ~40 cells, Lucifer yellow or Alexa 594 was included in the pipette, and cells were examined following recording (via an epifluorescence attachment to the microscope) to confirm astrocyte identity via cell morphology. Under epifluorescent illumination, individual cells displayed multiple processes branching from the soma, characteristic of astrocytes (Kosaka and Hama 1986). In five slices in which we recorded a GAT transport current using a biocytin containing electrode, we saw dye coupling of >50 cells with astrocytic morphology similar to what was has previously been reported for the CA1 region of hippocampus (D’Ambrosio et al. 1998). Slices were processed with a standard DAB reaction (Horikawa and Armstrong 1988).

Application of drugs

Drugs were dissolved in distilled water and applied by bath perfusion with the exception of direct application of GABA, which was applied using focal ejection from a glass micropipette using a pico-spritzer (General Valve). All chemicals and drugs were obtained from Sigma Chemical with the exception of (25S)-3-[1(S)-(1S)-2-[3-Dichloro-phenyl]ethyl][amino-2-hydroxypropyl][phenylmethyl]phosphonic acid (CGP-55845) (gift of Ciba Geigy), 1-[2-[[Diphenylmethylenemimo][oxy]ethyl]-1,2,5,6-tetrahydro-3-pyridinencarboxylic acid hydrochloride (NO-711) (RBI), l-trans-Pyrrolidine-2,4-dicarboxylic acid (l-trans-PDC) and 1-(4,4-Diphenyl-3-butenyl)-3-piperidinecarboxylic acid
hydrochloride (SKF-89976A) (Tocris Cookson), and Alexa 594 and Lucifer yellow (Molecular Probes).

Methods of analysis

Anatomical characterization of astrocytes: for the purpose of anatomical characterization and confirmation of cell identity, a group of recordings was made with the inclusion of Lucifer yellow or Alexa 594 in the intracellular recording media. Cell identity was confirmed following the experiment using a UV source attached to the microscope.

Statistical analysis: for unpaired t-tests, \( P < 0.05 \) values were taken as a significant difference. Data are expressed in means \( \pm \) SE. Cells that did not respond to experimental manipulation were excluded from analysis.

RESULTS

Cellular properties of neocortical astrocytes

Recordings were made from 210 neocortical glial cells, in layer 5 as well as layer 2/3, using whole cell voltage-clamp protocols. Approximately 85% of recorded cells had RMPs in the range of \(-80 \text{ to } -95 \text{ mV} (\approx -90.4 \pm 0.31 \text{ mV}; \text{Fig. 1D}; \text{estimated EK} \approx -100 \text{ mV}), \text{a linear I-V relationship (Fig. 1, B and C)} \text{and low input resistances} (17.22 \pm 0.64). \text{In a minority of cells (\approx 15%), much higher input resistances} (162.0 \pm 28.4 \text{ M\Omega}), \text{outward rectification on depolarization, and more hyperpolarized average RMPs} (\approx -93.8 \pm 0.70 \text{ mV}) \text{were observed. This group of cells was excluded from further study since they rarely exhibited any response to even high-intensity synaptic stimulation (see DISCUSSION)}. \text{All cells included in this study displayed electrophysiological characteristics (and dye coupling with biocytin in 5 of 5 filled cells) consistent with other reports for astrocytes recorded from in vitro slice preparations (Bergles and Jahr 1997; D’Ambrosio et al. 1998; Schwartzkroin and Prince 1979), and are thus presumed to be astrocytes. For the purposes of experiments investigating the presence of GABA uptake currents, only cells with stable, high-negative resting membrane potentials (<-80 mV), linear current voltage relationships, and low input resistances were used.}
Identification of transporter uptake currents in neocortical astrocytes

We have identified the presence of putative GABA transporter currents in electrophysiologically characterized neocortical astrocytes in slices obtained from rats age 10–30 days old, using selective antagonists for the GAT transporters. The transportable substrate nipecotic acid is an effective blocker of GAT-1 with a much lesser degree of effectiveness at the GAT-2 and GAT-3 transporters. β-alanine, which is also transportable, acts competitively at both GAT-2 and GAT-3 (Borden et al. 1995b). In addition, the nonsubstrate antagonists SKF-89967A and NO-711 show a high selectivity for the GAT-1 transporter (Borden et al. 1994b; Sudzak et al. 1992), while the compound (s)-(-)-1-[2-[tris-(4-methoxyphenyl)methoxy]ethyl]-3-piperidinecarboxylic acid ((S)-SNAP-5114) is highly selective for the GAT-2 and GAT-3 transporters (Borden et al. 1994a). Direct application of GABA (1 mM; 50- to 100-ms pulse, 6–9 psi) via a puffer pipette in the presence of gabazine (10 μM), CGP-55845A (2 μM), and 6-cyano-7-nitroquinoxalene-2,3-dione (CNQX, 10 μM-to block epilepticiform discharges) resulted in an inward current with a peak amplitude of $-22.71 \pm 3.34$ pA, a 10/90% rise time of 789.9 ± 24.77 ms, and a 10/90% decay time of 2,201 ± 198.6 ms ($n = 3$). Application of the GAT-1 and GAT-2/3 transporter inhibitors NO-711 (20 μM) and β-alanine (250 μM) resulted in a 52.46 ± 1.85% inhibition of the peak and a 64.26 ± 3.51% inhibition of the area ($n = 3$; Fig. 2). GABA receptor antagonists were included in the puffer pipette, but transporter antagonists were not; this may account for the lack of 100% block by the transporter antagonists as some displacement of the antagonists may occur during the puff.

Direct stimulation to the surface of the neocortex via a bipolar tungsten-stimulating electrode resulted in large inward currents recorded at rest in neocortical astrocytes. Application of ionotropic glutamate receptor antagonists and GABAA and GABAB receptor antagonists [CNQX (10 μM), d,l-2-amino-5-phosphonopentanoic acid (d,l-AP5, 50 μM), bicuculline (10–20 μM) or gabazine (10 μM), and CGP-55845A (2 μM); present for all experiments] resulted in a substantial reduction in the size of the evoked current, presumably by reducing feed forward excitation within the slice. Responses were evoked with both single (Fig. 3A–D) and repetitive (Fig. 4) stimulation protocols. In ~70% of the cells tested, a significant (10–50%) portion of the current remaining following application of glutamate and GABA receptor antagonists was blocked by the GAT-1 transporter antagonists SKF89967A (100 μM), NO-711 (20 μM), or nipecotic acid (100–200 μM; Figs. 3 and 4).

As the results were similar with each of these antagonists, the data were pooled. The average reduction of the peak current for single stimuli-evoked responses was 25.97 ± 3.02% ($n = 11$), while the average reduction of the peak current for repetitive stimuli-evoked responses was 26.53 ± 1.95% ($n = 12$).

![Fig. 2](image2.png)

**Fig. 2.** Direct application of GABA to a neocortical astrocyte. GABA (1 mM) applied to a layer 5 neocortical astrocyte via a puffer pipette induces an inward current (control). Subsequent application of the transporter antagonists NO-711 and β-alanine results in a reduction of the GABA-evoked current. GABAA and GABAB antagonists were present in the pipette as well as the bath. Transporter antagonists were added to the bath only.

![Fig. 3](image3.png)

**Fig. 3.** Single stimulation evoked uptake currents in neocortical astrocytes. A single shock is delivered to the tissue within ~100 μM from the recorded cell. A and B: in the presence of glutamate and GABA receptor antagonists, a biphasic current is evoked (control). Application of the GAT-1 transporter antagonists NO-711 and SKF-89967A results in a partial block of the late phase of the evoked response. A digital subtraction of these currents to better illustrate the GAT-1 transporter current time course is shown in B. C and D: glutamate uptake component for the same cell as in A. Application of L-trans PDC (in the presence of the GABA uptake antagonists) causes a reduction in the early component of the evoked current. The digital subtraction (D), reveals the glutamate uptake current. As was observed in most cells, the glutamate uptake component showed a fast rise and decay, relative to the GABA uptake current. Application of L-trans PDC in this cell also causes an inward current (~190 pA). In this and in subsequent figures, offsets are zeroed out for presentation purposes.

![Fig. 4](image4.png)

**Fig. 4.** The GAT-1 transporter mediates a long-lasting current during repetitive stimulation. A and B: repetitive stimulation evokes a long-lasting uptake current in a neocortical astrocyte. A: 20 shocks at 100 Hz were applied to the tissue while recording from a layer 5 neocortical astrocyte (control). Application of the GAT-1 transporter antagonists NO-711 and SKF-89967A results in a reduction of the late phase of the current (GAT-1 inhibitors). B: digital subtraction from A, demonstrating the long time course of the GABA transporter current.
Both types of stimulation protocols induced a response that was sensitive to the uptake antagonists. GAT-1-mediated responses to a single shock were found to have primarily a slow phase (11/14 cells tested; as determined from digital subtractions; Fig. 3, A–D). The slow component had an average amplitude of \(-18.81 \pm 4.07\) pA, a 10/90% rise time of 471.9 \pm 100.8 ms (time to peak = 691.0 \pm 116.7), and a 10/90% decay time of 1,238 \pm 309.8 ms. Repetitive stimulation protocols (4–20 stimuli at 100 Hz) also yielded long-lasting GAT-1 transporter currents with an average amplitude of \(-32.44 \pm 12.46\) pA, a time to peak of 481.1 \pm 62.72 ms, and a 10/90% decay time of 3,841 \pm 500.3 ms (n = 12; Fig. 4, A and B).

Application of the compound \(\beta\)-alanine (250 \(\mu\)M), which is reported to act predominantly on the rat GAT-2 and GAT-3 transporters (Borden et al. 1995b; Clark and Amara 1994) also resulted in a reduction in the amplitude of the evoked response (Fig. 5). The magnitude of inhibition was 29.12 \pm 6.147\% (n = 7) at 250 \(\mu\)M for all stimulation protocols (1–10 stimuli at 100 Hz). Digital subtraction of the \(\beta\)-alanine-sensitive component (250 \(\mu\)M) revealed GAT transporter current with an average amplitude of \(-63.53 \pm 13.90\) pA, time to peak of 438.6 \pm 28.10 ms (n = 6), and a 10/90% decay time of 2,336 \pm 123.3 ms (n = 4). Application of a lower dose (50 \(\mu\)M) of \(\beta\)-alanine resulted in a significant slowing of the rise of the response (time to peak 623.0 \pm 31.90 ms; n = 3; P < 0.05). Such a result would be expected of a competitive antagonist; at nonsaturating doses, \(\beta\)-alanine would interfere with but not completely block the binding of GABA to the GAT-2/3 transporters, causing GABA to remain in the extracellular space for a greater length of time and to diffuse greater distances before being transported.

Sequential application of the GAT-1 and GAT-2/3 antagonists resulted in a summation inhibition in 7 of 11 cells tested, indicating that these transporters are likely present on the same astrocytes (but see DISCUSSION). \(\beta\)-alanine also induced an inward current (\(-49.95 \pm 12.16\) pA, n = 10), consistent with it acting as a substrate for the GAT transporters.

The presence of L-glutamate transport currents was also investigated using the L-glutamate transporter antagonists L-trans-PDC (100 \(\mu\)M) or L-threo-hydroxyaspartate (L-THA) (300 \(\mu\)M). In most cells tested, a fast rising and fast decaying response was observed (\(-23.60 \pm 2.93\) pA, 10/90% rise time of 5.98 \pm 0.38 ms; n = 13; Fig. 2, C and D). Interestingly, the fast glutamate uptake current was observed on cells that also responded to the GABA uptake antagonists and showed a dramatically different time course.

Because astrocytes are also efficient potassium (K\(^{+}\)) buffers, it is probable that a significant portion of the current not blocked by GABA and glutamate transporter antagonists is a K\(^{+}\) current. Block of synaptic transmission by application of 100 \(\mu\)M cadmium eliminated 61.43 \pm 3.05\% of the evoked current (Fig. 6, middle; n = 15). The remaining current was blocked by the K\(^{+}\) channel antagonist Ba\(^{2+}\) (1 \(\mu\)M; Fig. 6, top; n = 2), indicating it is likely a K\(^{+}\) current resulting from the accumulation of extracellular K\(^{+}\) extruded from nearby cells (Henn et al. 1972). In addition, application of 1 \(\mu\)M Ba\(^{2+}\) resulted in a large inward current in the astrocytes (\(-182.4 \pm 0.5\) pA; n = 2), as has been previously observed (e.g., Anderson et al. 1995; Ballanyi et al. 1987; Perillan et al. 1999; Ransom and Sontheimer 1995).

**DISCUSSION**

To our knowledge, this is the first study to provide direct measurement of a GABA transporter current during synaptic transmission in an intact brain slice preparation. Recordings from neocortical astrocytes demonstrate that the electrical properties of these cells are similar to those reported for astrocytes elsewhere in the brain and in culture. In addition, the glutamate transport currents recorded here have similar temporal characteristics to those in previous reports (Bergles and Jahr 1997, 1998; Bergles et al. 1997). However, the characteristics of GABA transport recorded here differ greatly from previous descriptions of glutamate transport and indicate a different functional role for GABA versus glutamate transport in these cells.

**Astrocyte characteristics**

In this study we recorded from two distinct populations of cells, both of which had characteristics of glial cells. The main group of cells had very low input resistances, high negative resting membrane potentials, linear current voltage relationships and dye coupling, consistent with previous reports of astrocyte properties from studies completed elsewhere in the brain (Bergles and Jahr 1997; D’Ambrosio et al. 1998; Schwartzkroin and Prince 1979). These cells constituted the large majority of cells (85%) in this study and all of the cells in which evoked transporter currents were studied. The second group of cells exhibited some of the electrophysiological prop-
One important observation in this study is that the time course of the GABA transporter currents mediated by GAT-1 and GAT-2/GAT-3 transporters, as determined by antagonist sensitivity. Results obtained using antagonists for both types of transporter currents were similar with the exception of the presence of an inward current upon application of β-alanine. This current is likely the result of the fact that β-alanine is a transportable substrate for the GAT transporters and elicits its antagonist actions in this manner. Although nipecotic acid is a GAT-1 transportable antagonist, for most experiments it was used in combination with SKF-89976A or NO−711, which are not; thus any transport of it would have been blocked.

One concern in a study in which the identity of the current depends on the selectivity of the antagonists being used is whether the transporter antagonists themselves have an effect on GABA_A receptors, and, conversely, whether the GABA_A receptor antagonists themselves have an effect on GABA transporters. Numerous studies have shown no effect of bicuculline on GAT-1 transporter currents (Bonanno and Raiteri 1987; Cammack and Schwartz 1993; Cory et al. 1994; Dong et al. 1994; Haugh-Scheidt et al. 1995; Takahashi et al. 1992). The effects of the GAT-1 transporter inhibitors SKF-89976a and NO−711 are also minimal on GABA receptors (Borden et al. 1994b; Sudzak et al. 1992). β-alanine is a partial agonist at the GABA_A receptor; however, in all experiments where it was applied, GABA_A (and GABA_B) receptors were blocked.

One important observation in this study is the time course of the GABA transporter currents was exceedingly long. A faster component was occasionally observed, but this component is difficult to interpret due to its small size and infrequent appearance. The late component, however, was present in all cells that demonstrated a GABA transporter current.

It is possible that the slow time course of the transporter current reflects the slow turnover rate of the transporter for GABA. It is estimated that for the GAT-1 transporter, a single transport cycle takes ~25 ms at ~90 mV (Hilgemann and Lu 1999; Lu and Hilgemann 1999a), during which one molecule of GABA and one net positive charge are co-transported into the cell (Kavanaugh et al. 1992; Lu and Hilgemann 1999a,b). Thus the slow rise and decay times obtained for the single stimulus data might simply reflect the slow turnover rate of the transporter. However, previous studies of glutamate transporters have shown that despite their slow reported turnover rates, the time course of the underlying current does accurately reflect the time course of glutamate in the extracellular space (Bergles et al. 1997). Evidence shows that at high negative membrane potentials, the rate-limiting step for the GAT-1 transport cycle is the actual transport of GABA itself; furthermore, the charge transfer takes place very rapidly, prior to the binding and transport of GABA (Hilgemann and Lu 1999; Lu and Hilgemann 1999a,b). Consequently, when GABA binds, it is translocated prior to any charge movement: in this study, the initial current recorded is actually occurring following the delay of one transport cycle. The current would then follow the time course of the translocation reactions, with an ~25-ms time constant (Hilgemann and Lu 1999). In this scenario, the slow rise and decay of the current would reflect the time course of extracellular GABA, as GABA diffuses away from the synaptic cleft, to the glial transporters. The GABA that does not bind initially would continue to diffuse until it encounters free transporters, resulting in a significant period of extracellular freedom and degree of diffusional distance.

Data obtained from the time course of the transporter currents support the notion of a large, continuous population of transporters located further from the synaptic cleft than glutamate transporters. The 10/90% rise time of the single stimulus-evoked GABA transporter currents was 471.9 ± 100.8 ms, while the 10/90% rise time of the glutamate transporter current was 5.98 ± 0.38 ms. Presuming that the glutamate transporter current reflects glutamate uptake at or near the synaptic cleft (as the rise time indicates), these results are suggestive of an extrasynaptic origin for the GABA transporter currents we recorded.

The time to peak of the single and repetitive stimulus-evoked GABA transporter currents had similar values (691.0 ± 116.7 vs. 481.1 ± 62.72 ms, respectively; P > 0.1, unpaired t-test), while the amplitudes (−18.81 ± 4.07 vs. −32.44 ± 12.46 pA) and 10/90% decay times (1,238 ± 309.8 and 3,841 ± 500.3 ms, respectively; P < 0.01) increased significantly with stimulus number. Time to peak was utilized due to the fact that stimulus artifacts interfered with 10/90% rise time measurements for repetitive stimuli data. The prolongation of the decay of the transporter current observed for repetitive verses single stimulus-evoked responses implies that when inputs are stimulated repetitively, excess GABA diffuses further to unoccupied transporters due to the slow recovery rate of the GAT-1 transporter and implies that GABA has a significant degree of extracellular freedom following release.

Transmitter expression

The demonstration that at least two types of GABA transporter currents, as well as glutamate transporter currents, can
be evoked while recording from the same astrocyte provides
strong evidence that all are present within the same cell (but
see following text). While binding studies have localized
GAT-1, GAT-2, and GAT-3 expression to astrocytic processes
within the cortex (Conti et al. 1998, 1999; Minelli et al. 1995),
this study strongly suggests that functional, co-localized GAT
transporters are expressed on these cells. While the presence of
the GAT-1 transporter can be confirmed from the use of
NO-711/SKF89976a, the identity of the β-alanine-sensitive
current cannot be established because both GAT-2 and GAT-3
transporters are blocked by β-alanine at higher doses (Borden
et al. 1995b) and the expression of both transporter subtypes
on neocortical astrocytes has been confirmed (Conti et al. 1999;
Minelli et al. 1996; Yan and RibaK 1999).

It is interesting to note that the magnitude of repetitive
stimulus-evoked GAT-1-mediated currents was on average
smaller than GAT-2/3-mediated currents (−32.44 ± 12.46 vs.
−63.53 ± 13.90 pA). This could be due to the fact that
β-alanine blocks the activity of both GAT-2 and GAT-3 trans-
porters. Alternatively, it could represent a difference of expres-
sion in the transporter subtypes. GAT-3 expression has been
reported to be low in the adult and may be developmentally
regulated (Liu et al. 1993; Minelli et al. 1996), whereas GAT-1
expression is reported to be higher in the adult than the neonate
(Yan et al. 1997). Recordings in this study were made on
animals primarily over the age of 15 days old, but <26
(19.39 ± 0.46; n = 146). It is possible that the difference
observed here is a consequence of a developmental regulation
of transporter expression. However, further work needs to be
done to clarify this issue.

The possible co-expression of glutamate transporters within
the same cell is of interest. The time course of the glutamate
transporter currents recorded here are comparable with those
reported elsewhere (e.g., Bergles and Jahr 1997) and are indi-
cative of a relatively proximal location to the excitatory
synaptic cleft. Conversely, the time courses of both subtypes of
GABA transporter currents were remarkably slower. If the
underlying current directly reflects the time course of GABA,
as discussed in the preceding text, then this implies that the
location of the transporters to their respective synapses is quite
different. It is more difficult to make inferences about the
localization of the subtypes of GAT transporters relative to
each other. The time courses of both are similar, indicating
they are likely at similar distances from the synaptic cleft,
although it is unclear whether they are located at the same sites
on the astrocyte, or at different sites and thus serve different
functions. It should be noted, however, that we observed dye
coupling and since astrocytes are known to display electrical
coupling (Kettenmann and Ransom 1988), the possibility ex-
ists that the presence of multiple transporter currents observed
here reflects the recording of those currents from more than one
cell simultaneously. The fact that GAT-1 and GAT-2/3 trans-
porter currents have similar amplitudes and time courses ar-
gues against this, but such a scenario cannot be ruled out.
Nevertheless, it is clear that GAT-1 and GAT-2/3 transporter subtypes are expressed on neocortical astrocytes and that they
play a prominent role in the removal of GABA from the
extracellular space following synaptic release.

The average block of the late evoked current with all GAT
antagonists present was −39%. There was a significant resid-
ual remaining in the presence of 100 μM cadmium (−39%),
indicating a current of nonsynaptic origin. Indeed, application
of 1 mM Ba2+ blocked the remaining current (n = 2), indicat-
ing that it is a K+ current (Fig. 6). Approximately 22% of
the current of synaptic origin remains unaccounted for (gluta-
mate transporters blocked a negligible portion of the late com-
ponent). The origin of this portion of the current is unknown.
Antagonism of the GAT transporters may be incomplete or
other subtypes of GABA transporters may be present on the
astrocytes that are insensitive to the antagonists used (e.g.,
Borden et al. 1995a; Conti et al. 1999). Alternatively, there is
the possibility that other neurotransmitter or peptide transport-
ers are present on the astrocytes, which are activated during
synaptic stimulation.

Functional considerations

Based on the data from this study, the function of GABA
transporters on neocortical astrocytes may be quite different
from that of glutamate transporters. The time course of the
GABA transporter currents recorded here are significantly
slower than glutamate transporter currents in the same cells and
indicates that GABA is remaining in the extracellular space for
a much longer period of time than does glutamate. The slow
rise time observed for the GABA transporter current addition-
ally indicates that GABA is taking a relatively long time to
reach the astrocytic GABA transporter population, although a
small delay would be expected because the transporter would
have to cycle through one transport cycle before a current is
generated.

The slow time course of astrocytic GABA transporter cur-
rents recorded here suggests that glial-based GABA transport-
ers may not play a significant role in sculpting the time course of
inhibitory events during low-frequency firing. The observa-
tion that application of GABA transport inhibitors increase
the time course of single evoked inhibitory responses in the hip-
ocampus (Dingledine and Korn 1985; Draguhn and Heine-
mann 1996; Isaacson et al. 1993; Roepstorff and Lambert
1994; Thompson and Gähwiler 1992), as well as in cortex
(unpublished observations), indicates that neuronal transport-
ers in the neocortex are likely to play a prominent role in the
modulation of the IPSC time course. The direct recording of
transporter currents from neurons during synaptic activity
would help to resolve this issue. However, due to the small size
of the currents and their presumable location at the presynaptic
terminal, accurate resolution of neuronal transporter currents
would likely be exceedingly difficult.

One question this study raises, of course, is whether the time
course of the transporter currents recorded is an accurate re-
fection of the time course of extracellular GABA. Evidence
suggests that the passage of current occurs immediately fol-
lowing the transport of GABA and that this current would then
follow the time course of the translocation reactions; however,
the relationship between the time course of the transporter
current and the time course of GABA in an intact preparation
may be different. Experiments using rapid perfusion tech-
niques on excised patches to directly investigate the temporal
dynamics of GAT transporter function would clarify this issue.
Due to the small size of the currents recorded here, such
experiments would necessitate the use of an expression system
and therefore are beyond the scope of this study.

So why are inhibitory postsynaptic currents so much shorter
in duration than the transport currents we recorded? For our experimental conditions, we estimate an upper limit increase (i.e., if the GABA transporters are blocked) in extracellular GABA concentration of ~6 mM in response to a single stimulus. This is in good agreement with estimates derived from microdialysis studies in cortex (e.g., During et al. 1995; Wang et al. 2001) as well as from in vitro slice preparations (e.g., Rossi and Hamann 1998). At a GABA concentration of 6 mM, most synaptic GABA_A receptors would likely enter into a desensitized state (Overstreet et al. 2000). However, the activation of GABA_A receptors less susceptible to desensitization (e.g., Saxena and Macdonald 1994, 1996) may occur via spillover. The observation that extrasynaptic GABA concentrations are capable of reaching micromolar concentration levels several milliseconds following release support the notion of crosstalk between neighboring synapses as has been reported elsewhere (Barbour and Häusser 1997; Brickley et al. 1996; Isaacson et al. 1993; Kullmann et al. 1996; Rossi and Hamann 1998).

Our estimate of 6 mM is based on the integral of the stimulus-evoked transporter current. The average integrated transport current measured was \(1.7 \times 10^{-10}\) coulombs \((n=3; 5\) stimuli at 100 Hz, from subtraction currents before and during saturating doses of GAT-1 and GAT-2/3 blockers\), indicating the transport of \(~2 \times 10^9\) GABA molecules per stimulus (each electron transferred represents 1 GABA molecule) (Lu and Hilgemann 1999a,b). Assuming (based on the low input resistances) that most of the transport current we measured originated from the cell to which our electrode was attached (and thus ignoring current contribution from coupled cells), the processes from a single astrocyte extend over a cortical volume of \(~3 \times 10^3\) \(\mu\)m (Privat et al. 1995), which represents \(~6 \times 10^3\) \(\mu\)l of extracellular space (using a volume fraction of 0.2) (Nicholson 1995). Thus if all GABA transport were blocked, the distributed extracellular concentration of GABA could reach \(~5–6\) mM following a single stimulus.

Our data imply that one function of GABA transport on neocortical astrocytes is to remove GABA from the extrasynaptic space during both low- and high-frequency firing of inhibitory interneurons. It is clear that during both single and repetitive stimuli, GABA transporters are active and do participate in the removal of GABA from the extracellular space, albeit on a much slower time scale than that of a single inhibitory postsynaptic potential (10/90% decay \(\approx 50\) ms in cortex) (Connors et al. 1988; van Brederode and Spain 1995; unpublished observations). The time course of the transporter currents implies that the location of the transporters is remote to the synaptic cleft and that GABA is remaining present in the extracellular space for a significant period of time, which would indicate that GABA is available to bind to GABA receptors as well. Such a scenario is supported by the work of Jones, Overstreet, and Westbrook (Jones and Westbrook 1995; Jones et al. 1999; Overstreet et al. 2000), who propose that synthaptically released GABA remains bound to GABA_A receptors for a several seconds following release.

Of course, glial GABA transporters may have other important functions as well, such as to supply a source of extracellular GABA via reversal of the GABA transporter (Wu et al. 2001) and to regulate paracrine GABA, as has been suggested by others (Conti et al. 1998; Minelli et al. 1996). The relatively distal localization of glial GABA transporters, as compared with glutamate transporters, suggested by the long transporter current time course is consistent with this hypothesis.

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