GAT-3 Transporters Regulate Inhibition in the Neocortex

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Submitted 24 April 2005; accepted in final form 24 August 2005

Kinney, Gregory A. GAT-3 Transporters regulate inhibition in the neocortex. J Neurophysiol 94: 4533–4537, 2005. First published August 31, 2005; doi:10.1152/jn.00420.2005. The role of GAT-3 transporters in regulating GABA receptor-mediated inhibition was examined in the rat neocortex using an in vitro slice preparation. Pharmacologically isolated GABA A receptor-mediated responses were recorded from layer V neocortical pyramidal cells, and the effects of SNAP-5114, a GAT-3 GABA transporter-selective antagonist, were evaluated. Application of SNAP-5114 resulted in a reversible increase in the amplitude of an evoked GABA A response in most cells examined, although no effect on the decay time was observed. Examination of the spontaneous output of inhibitory interneurons revealed a reversible increase in the frequency and amplitude of spontaneous inhibitory synaptic currents as a consequence of GAT-3 inhibition. This effect of GAT-3 inhibition on spontaneous inhibitory events was action potential-dependent because no such increases were observed when SNAP-5114 was applied in the presence of TTX. These results demonstrate that GAT-3 transporters regulate inhibitory interneuron output in the neocortex. The increase in inhibitory interneuron excitability resulting from application of SNAP-5114 suggests that inhibition of GAT-3 transporter function results in a reduction in ambient GABA levels, possibly by a reduction in carrier-mediated GABA release via the GAT-3 transporter.

INTRODUCTION

Factors that govern GABA levels in the extracellular space are of significant interest toward understanding the mechanisms by which GABAergic inhibition controls excitability. Extensive studies in the hippocampus have demonstrated that the GAT-1 transporter actively removes GABA from the synaptic and extracellular space, controlling the magnitude/time course of both GABA A (Dingledine and Korn 1985; Thompson and Gahwiler 1992) and GABA B (Scanziani 2000; Solis and Nicoll 1992; Thompson and Gahwiler 1992) responses. In the neocortex, Ling and Benardo (1998) demonstrated similar effects for GAT-1, suggesting that similar mechanisms govern synaptic GABA in the neocortex.

GABA transporters also regulate tonic inhibition (Chiu et al. 2005; Jensen et al. 2003; Nusser and Mody 2002; Rossi et al. 2003; Semyanov et al. 2003), a slow form of GABAergic inhibition that regulates excitation and neuronal output (Hausser and Clark 1997; Semyanov et al. 2003). Recent work by Richerson’s group (Wu et al. 2003) suggests that the release of GABA via GABA transporter reversal may be integral in maintaining GABA levels responsible for activating tonic inhibition in the hippocampus. In addition, work by Raiteri et al. (2002) suggests that reversal of the GABA transporter may occur during certain pathological conditions. However, while these reports provide evidence that GABA transporters are capable of reversing and activating a tonic form of GABAergic inhibition, currently it is unknown which GABA transporters are releasing GABA and whether GABA transporters are integral in maintaining tonic GABA levels in situ.

Most studies to date have focused on the role of the GAT-1 transporter in regulating GABA levels. However, the GAT-3 transporter is expressed in several areas of the mammalian brain, primarily in glia, indicating that the GAT-3 transporter may be a candidate for the regulation of paracrine GABA (Durkin et al. 1995; Minelli et al. 1996). Due to the lack of a selective GAT-3 antagonist, studies of GAT-3 transporter function at the cellular level have been limited to glia (Kinney and Spain 2002) or have involved indirect methods of disabling the GAT-3 transporter (Hamann et al. 2002); nevertheless these studies have indicated a role for GAT-3 transporters in regulating synaptic and tonic GABA.

In this study, I take advantage of a selective antagonist of the GAT-3 transporter, SNAP-5114 (Borden et al. 1994; Dalby 2000), to investigate the role of GAT-3 transporters in regulating GABAergic inhibition in the neocortex. I focus on the role of GAT-3 transporters in regulating synaptic input onto layer V neocortical pyramidal cells. Due to the reported glial, nonsynaptic localization of the GAT-3 transporter in the neocortex, it was expected that GAT-3 inhibition would alter the level of tonic inhibition a pyramidal cell receives. Although a consistent change in the tonic conductance of neocortical pyramidal cells was not observed, a significant increase in inhibitory interneuron output was observed, pointing to reduced inhibition onto inhibitory interneurons as a result of GAT-3 antagonism. These results suggest that GABA levels are reduced by block of the GAT-3 transporter, possibly via a reduction in GAT-3 carrier-mediated GABA release. These results demonstrate that GAT-3 transporters actively regulate the level of inhibition a layer V neocortical pyramidal cell receives.

METHODS

Tissue preparation and recordings were performed as described previously (Kinney and Spain 2002). Artificial cerebrospinal fluid (ACSF) was composed of (in mM) 130 NaCl, 3 KCl, 2 CaCl 2, 1.25 NaH 2 PO 4, 26 NaHCO 3, 2 MgCl 2, and 10 dextrose and kept at pH = 7.3–7.4 by bubbling with carbogen (5% CO 2/95% O 2).

Whole-cell recordings were made from layer V pyramidal cells visualized under DIC optics, using 2–4 MΩ pipettes filled with (in mM) 135 KCH 3 SO 4, 2 MgCl 2, 5 KCl, 10 HEPEs, 2 Na 2 ATP, 0.5 Na-GTP, and 0.1 EGTA (pH = 7.2 w/KOH; osM ≈ 285; standard patch solution). GABA A currents were recorded using a high internal Cl − patch solution that contained (in mM) 130 CsCl, 1 CsCl, 3.45
Cs-BAPTA, 10 HEPES, 5 Mg-ATP, and 10 QX-314 (pH = 7.2 w/CsOH; osM = 285). For each solution, liquid junction potential was calculated with respect to ACSF, and membrane potential recordings were corrected.

Recordings in voltage-clamp were obtained through the use of a Multiclamp 700a amplifier (Axon). Whole cell current (low-pass filtered at 2–5 kHz) and membrane potential were amplified and digitized on-line by data-acquisition software (pCLAMP; Axon) and stored in computer memory. Access resistance was continuously monitored for each cell, and recordings were terminated/discarded when a significant (>10%) increase occurred.

Synaptic inputs to pyramidal cells were stimulated using a bipolar tungsten-stimulating electrode placed in the vicinity of the target cell (100–500 μm) using intensities of 20–125 μA at 100 μs. Experiments were performed at −60 mV unless otherwise noted.

Drugs were dissolved in distilled water, DMSO (<0.1%), or 0.5N NaOH (<0.1%) and applied by perfusion. All chemicals and drugs were obtained from Sigma Chemical with the exception of CGP-55845A, d,l-2-amino-5-phosphopentanoic acid d,l-AP5, 6-cyano-7-nitroquinoxalene-2,3-dione (CNQX), and SNAP-5114 (Tocris).

**Statistical analysis**

Changes in mean amplitude, 10/90% rise, 10/90% decay, and area of evoked responses were evaluated using a Student’s paired t-test (P < 0.05 taken as significant). Cells that exhibited a change >10% were considered to have responded. Data are expressed in mean ± SE. Spontaneous inhibitory postsynaptic currents (sIPSCs) and miniature IPSCs (mIPSCs; 300-1,500 events) were detected and analyzed using Mini Analysis Program (Synaptosoft). A Mann Whitney U test (P < 0.05) was used to evaluate changes in s- and mIPSC amplitude and interval.

**RESULTS**

A set of experiments was completed recording from layer V pyramidal cells in current clamp recording conditions, at −55 mV (standard patch solution; see METHODS) in the absence of neurotransmitter antagonists, to evaluate the effect of GAT-3 block on mixed excitatory/inhibitory inputs to neocortical pyramidal cells. In 6/9 cells, application of SNAP-5114 (20 μM) resulted in a significant, reversible reduction in the amplitude (33.33 ± 3.08% reduction; n = 6; P < 0.001) of the evoked depolarizing response; the area was also significantly reduced (38.54 ± 8.01% reduction; n = 7; P < 0.005; Fig. 1A). Overall, when all cells were pooled, SNAP-5114 induced a 23.33 ± 5.55% decrease in amplitude (n = 9; P < 0.005).

To test for a direct reduction in excitatory transmission caused by application of SNAP-5114, isolated AMPA excitatory postsynaptic potentials (EPSPs) were recorded (standard patch solution) in the presence of 5 μM gabazine, 50 μM d,L-AP5, and 2 μM CGP55845a to block GABA_A, N-methyl-D-aspartate (NMDA), and GABA_B receptors, respectively. In 3/3 cells, application of 20 μM SNAP-5114 resulted in no significant change to the evoked AMPA EPSP (Fig. 1B; 0.933 ± 6.134% increase; n = 3).

These results suggested a selective enhancement of inhibitory neurotransmission was resulting in an overall reduction in the dominant excitatory response. To test for this, pharmacologically isolated GABA_A IPSCs were recorded from neocortical pyramidal cells, using a high Cl− internal solution (see METHODS). These experiments were performed in the presence of 10 μM CNQX, 50 μM d,L-AP5, and 2 μM CGP55845a to block AMPA, NMDA, and GABA_B receptor-mediated responses, respectively. Application of the GAT-3 GABA transporter antagonist SNAP-5114 (20 μM) resulted in a significant, reversible increase in the amplitude to the evoked IPSC in most cells examined (39.64 ± 6.00% increase; n = 7/10; P < 0.001; Fig. 1A). When all cells were pooled, a 26.76 ± 7.78% increase (n = 10; P < 0.01) was observed. Although a modest change to the 10–90% decay was observed in two cells (22 and 25% increase), overall, no consistent effect on the decay time (0.16 ± 5.5% increase; n = 10) was seen.

An increase in inhibitory output seemed a plausible mechanism underlying the observed increase in IPSC amplitude. To test this, pharmacologically isolated sIPSCs were recorded from neocortical pyramidal cells (in the presence of 10 μM CNQX, 50 μM d,L-AP5, and 2 μM CGP55845a). Two to 4 min of data was collected for each cell, and the corresponding sIPSCs were analyzed for frequency (inter-event interval) and amplitude. In 4/7 cells, application of SNAP-5114 resulted in a significant decrease in the inter-event interval (P < 0.05) and a significant increase in the amplitude of sIPSCs (P < 0.05; Mann Whitney U test; Fig. 2A–C). When data from responding cells were pooled, a significant decrease in average interval (25.11 ± 5.3% decrease; n = 4; P < 0.05) and a significant increase in average amplitude (11.3 ± 0.90% increase; n = 4; P < 0.05) were observed (Mann-Whitney U test; Fig. 2D). When all data were pooled, an 18.0 ± 5.7% decrease in average interval was observed (n = 7; P < 0.05; Mann Whitney U test).

These results indicate that inhibitory interneuron output has increased, possibly as a result of an increase in inhibitory interneuron excitability. It was hypothesized that GAT-3 blockade may be reducing the level of GABA reaching inhibitory interneurons by reducing GABA release via the GAT-3 transporter, thus increasing interneuron excitability. This would imply that the effect on sIPSCs described in the preceding text is action potential dependent. Consequently, in the

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**FIG. 1.** SNAP-5114 causes a reduction in an excitatory postsynaptic potential, no change to an isolated AMPA receptor-mediated evoked response, and a reversible enhancement of a GABA_A current. A: application of 20 μM SNAP-5114 results in a decrease in the amplitude of an evoked excitatory postsynaptic potential (no antagonists present). B: application of 20 μM SNAP-5114 causes no changes to an isolated AMPA receptor-mediated response. C: evoked synaptic GABA_A receptor-mediated responses in control conditions, during application of 20 μM SNAP-5114, and after wash, illustrating the reversible enhancement of inhibition mediated by SNAP-5114.
absence of action potential firing, no effect should be observed. To test this hypothesis, the preceding experiments were repeated in the presence of TTX (1 μM). Slices were bathed in 1 μM TTX for 10 min prior to recording baseline activity, which generally reduced sIPSC activity in a given cell by ~10–20%. Application of SNAP-5114 (20 μM), in the presence of TTX, did not result in a significant decrease in interevent interval or increase in amplitude of mIPSCs in any of eight cells tested. Overall, an *increase* in interevent interval (14.5 ± 4.97; n = 8) and a *decrease* in amplitude (4.3 ± 0.83; n = 8) were observed (Fig. 2E).

**Discussion**

To this author’s knowledge, this is the first study to date examining the role of GAT-3 transporters on GABA<sub>₆</sub> receptor-mediated synaptic responses. This study clearly demonstrates the importance, in the neocortex, of a previously overlooked GABA transporter subtype. At baseline conditions, the impact that GAT-3 transporters have on inhibitory and excitatory neurotransmission in the neocortex appears to be at least as significant as the more heavily studied GAT-1 transporters. The results of this study indicate that GAT-3 transporters are actively suppressing inhibition in the neocortex under normal conditions. Block of GAT-3 transporters resulted in a significant, reversible enhancement of inhibitory neurotransmission in most cells examined. An assessment of the spontaneous output of inhibitory interneurons in the neocortex revealed an increase in both the frequency and amplitude of such output as a consequence of GAT-3 inhibition. This result suggests an increase in the excitability of inhibitory interneurons is occurring as a result of GAT-3 antagonism. This was confirmed when inhibitory interneuron output was shown to be unaffected by GAT-3 antagonism in the absence of action potential firing.

A plausible mechanism underlying the observed increase in inhibitory interneuron activity is that SNAP-5114 block of the GAT-3 transporter results in a reduction in nonvesicular release of GABA and a consequential decrease in tonic inhibition of inhibitory interneurons. Currently the source of GABA underlying tonic inhibition in the mammalian brain has not been unequivocally demonstrated, although nonvesicular release of GABA has been demonstrated in the hippocampus and has been shown to activate tonic inhibition on hippocampal pyramidal cells under certain circumstances (Richerson and

![FIG. 2. Application of SNAP-5114 results in a reversible increase in inhibitory interneuron output that is action potential dependent. A: sample traces of spontaneous inhibitory postsynaptic currents (sIPSCs) from control (top), during application of 20 μM SNAP-5114 (middle), and after washout of SNAP-5114 (bottom). B and C: cumulative probability histograms for event amplitude (B) and interevent interval (C) for control (black traces), during application of SNAP-5114 (red traces), and after washout (light gray traces) of SNAP-5114, graphically illustrating the overall effect on frequency and amplitude for this cell. In this cell, application of SNAP-5114 significantly increased event amplitude, and significantly decreased inter-event interval (*P < 0.001; Mann Whitney U test), causing a shift in the cumulative probability curves. D: in the absence of TTX, spontaneous inhibitory synaptic currents are significantly increased in both frequency (interevent interval) and amplitude when GAT-3 transporters are blocked (P < 0.05; Mann Whitney U Test). E: in the presence of TTX, a converse and nonsignificant reduction in the frequency and amplitude of miniature inhibitory synaptic currents is observed upon block of GAT-3 transporters.]
Wu 2003; Wu et al. 2003). Carrier-mediated GABA release via the GAT-3 transporter is a putative source of GABA underlying tonic inhibition: GAT-3 transporters are present in the neocortex primarily on glia and may be located at sites remote to the synaptic cleft (Minelli et al. 1996, 2003). In the cerebellum, GABA receptors underlying tonic GABAergic inhibition are thought to be localized extrasynaptically (Nusser et al. 1995, 1998); thus release of GABA from glia might be an effective mechanism of maintaining tonic inhibition.

Alternative and more complicated scenarios could of course underlie the effects of GAT-3 inhibition. The GAT-3 transporter may be actively removing GABA from the extracellular space following synaptic release (Kinney and Spain 2002) or acting as a regulatory mechanism for tonic GABAergic inhibition (Rossi et al. 2003). In the absence of GAT-3 transport, excess extracellular GABA may be causing a GABA depolarization (Staley et al. 1995) or alternatively may be suppressing a set of recurrent inhibitory interneurons. Clearly, direct recordings from inhibitory interneurons are needed to better understand the mechanism of action of the GAT-3 transporter.

It is of course possible that SNAP-5114 has a nonspecific action on neurotransmission or neuronal excitability. However, observations in this study argue against such nonspecific actions: SNAP-5114 had no effect on excitatory neurotransmission and SNAP-5114 had no effect in the presence of TTX. These observations argue against a general nonspecific action of this compound on neurotransmitter release, GABA$_A$ receptor sensitivity, or on neuronal excitability.

When EPSPs were examined in the absence of any neurotransmitter antagonists, a significant reduction in the size of the depolarization was observed (Fig. 1B) on GAT-3 transporter blockade, presumably due to an enhancement of underlying inhibition. The suppression of excitation observed here could presumably curtail the excitatory output of neocortical pyramidal cells, and serve as a suppressive tool for seizure activity. Enhancement of inhibitory interneuron output may underlie the suppressive action of this compound in animal models of epileptic activity (Dalby 2000) and points to the need for further studies investigating the actions of GAT-3 transporters in the neocortex and elsewhere in the mammalian brain.

Of considerable interest is the possibility that GAT-3 transporters are actively releasing GABA into the extracellular space under “normal” conditions, in an in vitro slice preparation, and that this nonvesicular GABA release is dynamically modulating inhibitory interneurons in the neocortex. Interestingly, the actions of this putative nonvesicular GABA release appear to be limited to inhibitory interneurons. Layer V pyramidal cells receive significant inhibitory input from layer II/III, so it is possible that the actions of GAT-3 reported here reflect activity in layer II/III. Thus in the neocortex, there may be laminar differences in how inhibition is controlled, just as there are laminar differences in the expression of inhibition (van Brederode and Spain 1995). Further studies recording directly from neocortical inhibitory interneurons as well as layer II/III pyramidal cells are necessary to better understand the function of the GAT-3 transporter in the neocortex and its role in regulating neocortical inhibition. Currently the source of GABA underlying tonic inhibition in the brain is unknown, although studies in the hippocampus suggest that GABA trans-


