Regulation of $\gamma$-Aminobutyric Acid (GABA) Transporters by Extracellular GABA*

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$\gamma$-Aminobutyric acid (GABA) transporters on neurons and glia at or near the synapse function to remove GABA from the synaptic cleft. Recent evidence suggests that GABA transporter function can be regulated, although the initial triggers for such regulation are not known. One hypothesis is that transporter function is modulated by extracellular GABA concentration, thus providing a feedback mechanism for the control of neurotransmitter levels at the synapse. To test this hypothesis, GABA uptake assays were performed on primary dissociated rat hippocampal cultures that endogenously express the cloned rat brain GABA transporter GAT1. In both experimental systems, extracellular GABA induces chronic changes in GABA transport that occur in a dose-dependent and time-dependent manner. In addition to GABA, ACHC and nipecotic acid, both substrates of GAT1, up-regulate transport; GAT1 transport inhibitors that are not transporter substrates down-regulate transport. These changes occur in the presence of blockers of both GABA$_{\text{A}}$ and GABA$_{\text{B}}$ receptors, occur in the presence of protein synthesis inhibitors, and are not influenced by intracellular GABA. Surface biotinylation experiments reveal that the increase in transport is correlated with an increase in surface transporter expression. This increase in surface expression is due, at least in part, to a slowing of GAT1 internalization in the presence of extracellular GABA. These data suggest that the GABA transporter fine-tunes its function in response to extracellular GABA and would act to maintain a constant level of neurotransmitter at the synaptic cleft.

GABA* transporters are members of a large family of Na$^+$-dependent neurotransmitter reuptake proteins, located on the plasma membrane of neurons and glia, that function in part to determine neurotransmitter levels in the synaptic cleft (1). Demonstration of a physiological role for GABA transporters comes from experiments involving specific GABA uptake inhibitors; these inhibitors prolong the decay phase of GABA$_{\text{A}}$ receptor-mediated post-synaptic potentials (2) and both prolong the decay phase and increase the magnitude of responses mediated by the G protein-coupled GABA$_{\text{A}}$ receptor (2–4). GABA transporters also play a physiological role in toad and catfish horizontal cells where calcium-independent GABA efflux through the transporter is a principal mode of neurotransmitter release (5). GABA transporters also have a pathophysiological role. There is decreased calcium-independent GABA release in the affected hippocampus of temporal lobe epileptics. This decrease in transporter-mediated efflux is correlated with fewer GABA transporters and is hypothesized to result in decreased inhibitory tone (6).

Not only can GABA transporters regulate neuronal signaling, transport itself can be regulated. This is true for GABA transporters and other members of this family as well (for review see Refs. 7 and 8). Functional modulation occurs through a variety of second messengers such as kinases, phosphatases, arachidonic acid, and pH. These factors may act directly on the transporter protein (e.g. by phosphorylation; see Refs. 9–12) or by regulating the interaction of the transporter with other synaptic proteins, such as syntaxin (13). A recurring theme is that the regulation occurs through changes in the number of functional surface transporters (14–16).

The data are few regarding the physiological signals that trigger functional transporter regulation. In rat basophilic leukemia cells, the maximum velocity of serotonin transport is increased upon adenosine receptor activation (17). Serotonin transport is increased in platelets following stimulation of histamine receptors (18). Increases in glutamate transport in primary astrocyte cultures are prevented by antagonists of $\alpha$-amino-3-hydroxy-5-methylisoxazole-4-propionic acid/kainate receptors (19); this result is consistent with the hypothesis that extracellular transmitter levels, signaled by receptor activation, feed back to up-regulate transporter function. Several investigations have shown regulation of transporters following interactions of the transporter with transporter antagonists. Heterologously expressed norepinephrine transporters are down-regulated following long term (>3 days) treatment with the norepinephrine transporter antagonist desipramine, perhaps through changes in protein expression and/or transporter turnover (20). For GABA transporters, chronic treatment with the GAT1-specific transporter inhibitor tiagabine down-regulates GABA transporter expression in brain tissue (21), although whether this is due to inhibition of the transporter directly or to spillover of GABA onto GABA receptors, and subsequent receptor-mediated signaling effects, is not known.

In the present report, we show that both agonists and antagonists of the GABA transporter can trigger long term changes in GABA transporter function, and we identify the mechanism underlying these changes. This effect occurs in several different cell systems including hippocampal cells that endogenously express the transporter. We show that transporter expression increases with extracellular GABA concentration and occurs on a time scale of minutes, consistent with...
the idea that transporter function might be regulated in order to maintain constant neurotransmitter levels at the synapse. Furthermore, we show that the modulation occurs through the action of GABA on the transporter directly (i.e. it is not mediated by GABA receptors) and that the regulation of transporter expression levels is due to a net change in the rate of transporter internalization.

**EXPERIMENTAL PROCEDURES**

**Cell Culture**—Primary hippocampal cultures were prepared from postnatal day 0-3 rats by mincing tissue in α-MEM supplemented with cysteine, glucose, and 100 units of papain (Sigma or Worthington). Tissue was incubated for 20 min at 37 °C followed by gentle trituration, dilution, and plating onto poly-l-lysine-coated glass coverslips. To obtain pure neuronal cultures, mixed cultures were treated for 48 h with 10 μM cytosine arabinoside (Sigma); treatment was initiated 24 h after plating. Astrocyte cultures were prepared as described (22). Cells were plated onto untreated 24-well plates and maintained in Earle’s MEM supplemented with 10% fetal bovine serum.

1F9 cells (CHO cells stably expressing GAT1; see Ref. 23) were maintained in α-MEM supplemented with 5% fetal bovine serum, t-glutamine, and penicillin/streptomycin. Transfections were carried out using LipofectAMINE (Life Technologies, Inc.) in Opti-MEM I (Life Technologies, Inc.). The lipid/DNA mixture was incubated with the cells for 5 h; cells were then rinsed and re-fed with complete media. Stable transformants were obtained by selection in 500 ng/ml G418 (Life Technologies, Inc.).

**3H/GABA Uptake Assays**—Pre-assay drug incubations were performed in HBSS. Preincubation solutions were continually perfused to maintain constant extracellular drug concentrations. Following preincubation, cells were rinsed three times in 1× HBSS and allowed to equilibrate for 10 min in the final wash. Buffer was then exchanged with control HBSS or drug-containing HBSS. GABA was added to initiate the assay. The final [3H]GABA concentration of the assay solution was 100 nM; the total GABA concentration of the assay solution was 30 μM. In order to minimize changes in transporter expression during the assay, assay times were 5 min. The assay was terminated by rapidly rinsing the cells 3 times with 1× HBSS, followed by solubilization in 300 μl of 0.001-0.005% SDS at 37 °C for 2 h. Aliquots were used for scintillation counting and to determine protein concentrations. Statistical analyses of the uptake data were performed using SPSS. Two-sample comparisons were made using t-tests; multiple comparisons were made using one-way analysis of variances followed by Tukey’s honestly significant difference post hoc test.

**Biotinylation Experiments**—Biotinylation experiments were performed essentially as described (14, 16). Cells were grown in 60-mm tissue culture dishes to 80% confluence. The cells were rinsed twice with 37 °C phosphate-buffered saline/CaCl2/MgCl2 (in mM: 138 NaCl, 2.7 KCl, 1.5 KH2PO4, 9.6 Na2HPO4, 1 MgCl2, 0.1 CaCl2, pH 7.4). The cells were next incubated with 2 ml of a solution containing 1 mg/ml sulfo-NHS biotin (Pierce) in phosphate-buffered saline/CaCl2/MgCl2 for 20 min at 4 °C with gentle mixing. The biotinylation solution was removed by two washes in phosphate-buffered saline/CaCl2/MgCl2 plus 100 mM glycerine and quenched in this solution by incubating the cells at 4 °C for 45 min with gentle shaking. The cells were lysed with 1 ml of RIPA buffer (in mM: 100 Tris-Cl, pH 7.4, 150 NaCl, 1 mM EDTA, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 1 μg/ml leupeptin, 1 μg/ml aprotinin, 250 μM phenylmethylsulfonyl fluoride) at 4 °C for 60 min. The cell lysates were centrifuged at 20,000 × g at 4 °C for 60 min. The supernatant fractions (300 ml) were incubated with an equal volume of ImmunoPure Immobilized Monomeric Avidin beads (Pierce) at room temperature for 60 min. The beads were washed three times with RIPA buffer, and absorbed proteins were eluted with SDS sample buffer (62.5 mM Tris-Cl, pH 6.8, 2% SDS, 100 mM β-mercaptoethanol) at room temperature for 30 min.

**Western Analyses**—Analysis was performed on aliquots 1) taken prior to incubation with beads (total cell lysate), 2) of the supernatant fraction after adsorption and centrifugation (intracellular fraction), and 3) of the bead eluate (biotinylated fraction). Western blotting was carried out using anti-GAT1 antibody 346J (13) as described (24) and visualized using ECL reagents (Amersham Pharmacia Biotech). Monoclonal anti-actin antibodies (Sigma) were used to normalize protein levels in each fraction. Immunoreactive bands were scanned and quantitated with ImageQuant (Molecular Dynamics).

**RESULTS**

To examine the effect of extracellular GABA on chronic changes in GABA uptake, primary neuronal cultures from neonatal rat hippocampus were preincubated in 100 μM GABA for 1 h prior to assay. The results of this experiment are shown in Fig. 1A. Compared with control cultures not incubated in GABA, treatment of these cultures with extracellular GABA resulted in a greater than 2-fold increase in GABA uptake measured in subsequent transport assays. This increase in transport did not occur in experiments in which extracellular GABA was replaced by glutamate or by glycine (data not shown). These data suggest that the modulation of uptake is due to a GABA-mediated process. The inclusion of SKF89976A, a high affinity inhibitor (25) of the rat brain GABA transporter GAT1 (26), during the assay reduced GABA uptake by greater than 90% both in control cultures and cultures pretreated with GABA. These data suggest that the majority of GABA transport mediated in these neuronal cultures, and the subsequent modulation by GABA, occurs via GAT1. However, the effect is not specific to GAT1 expressed in neuronal cultures. Fig. 1B shows that a similar modulation in GABA uptake following extracellular GABA treatment occurs in primary rat hippocampal astrocyte cultures. Treatments with SKF89976A reveal that the effects on astrocyte cultures are mediated primarily by GAT1 as well. To eliminate the possibility that the increase in GABA transport following GABA preincubation might simply be due to an increase in the velocity of transport (because of higher GABA concentrations inside the pretreated cells), we performed the assays using 30 μM GABA, a concentration that is saturating for GAT1-mediated transport.

Fig. 1, C and D, shows that the increase in transport following extracellular GABA treatment is both concentration-dependent and time-dependent. A logistic fit to the concentration-response data estimates an EC50 for up-regulation by GABA to be approximately 5 μM, which is comparable to the KM values for GABA uptake in both brain tissue (27, 28) and in cells heterologously expressing GAT1 (23, 26, 29). Additionally, maximal up-regulation of uptake occurred with 30 min of pretreatment with 100 μM GABA. Twelve-hour treatment with extracellular GABA resulted in no further increases in uptake than that seen at 30 min (data not shown).

The evidence that the EC50 of GABA necessary for up-regulation is comparable to the KM of the transporter for GABA suggested the hypothesis that the up-regulation was closely related to a transporter-mediated process. To add support to this hypothesis, experiments were performed (i) using other substrates and an antagonist of the transporter, and (ii) inhibitors of GABA receptors. The results of these experiments are shown in Fig. 2. Fig. 2A shows concentration-response curves for neuronal cultures treated with two GABA transport substrates, nipecotic acid and ACHC, and the GAT1 antagonist SKF89976A. Pretreatment of neurons for 1 h prior to assay with either nipecotic acid or ACHC resulted in an up-regulation in GABA transport, with estimates of EC50 values for up-regulation of approximately 9 and 72 μM, respectively. SKF89976A caused a down-regulation of transport, similar to that previously reported for the GAT1 antagonist tiagabine (21), with an EC50 for down-regulation of approximately 1 μM. To rule out the possibility that the decrease in transport seen following incubation with SKF89976A was due to acute inhibition of uptake during the assay (i.e. to rule out the possibility that SKF89976A was not washed off prior to assay), transport in untreated cells was compared with cells that were treated with SKF89976A for 5 min and then rinsed and assayed. No difference between these two groups was seen (data not shown). These results provide further evidence to support the
hypothesis that the transport regulation is a transporter-mediated process. Namely, \( EC_{50} \) values for both up-regulation and down-regulation for each compound tested are comparable to the \( K_m \) values for transport estimated for these compounds in previous GABA transporter investigations (30, 31).

Because nipeotic acid, ACHC, and SKF89976A prevent the uptake of GABA competitively, it was possible that some of the transport regulation was due to GABA receptor-mediated action from neuronally released GABA. To test this hypothesis, GABA-mediated up-regulation was examined in the presence of bicuculline and phaclofen, inhibitors of GABA\(_A\) and GABA\(_B\) receptors, respectively. These results are shown in Fig. 2B. Pretreatment of cultures with these drugs, at concentrations that are routinely used to eliminate receptor-mediated responses in hippocampal cells, failed to inhibit the up-regulation. In addition, pretreatment of cultures with cycloheximide, a protein synthesis inhibitor, failed to alter the GABA-mediated up-regulation; these latter results are not surprising given the 30-min time course over which transport is altered (see Fig. 1D).

Given the data from hippocampal neurons suggesting that regulation of transport is mediated through GAT1, an expression system was sought that would mimic the endogenous phenomenology and that would permit a detailed characterization of the mechanisms underlying the regulation. Therefore, the experiments were repeated in 1F9 cells, a mammalian cell line stably expressing GAT1. The results of such experiments are shown in Fig. 3. As in neuronal cultures, pretreatment of 1F9 cells with 100 \( \mu M \) extracellular GABA resulted in a greater than 2-fold increase in subsequent GABA uptake. The other two transporter substrates, nipeotic acid and ACHC, similarly increased transport. The GAT1 antagonist SKF89976A, incubated alone or with submaximal concentrations of transporter substrates, reduced subsequent GABA transport. Similar results were obtained using a PC12 cell line stably expressing GAT1 (data not shown). These data demonstrate that GAT1 regulation occurs similarly in cells that endogenously express the transporter and in heterologous expression systems and support the idea that the regulation is a transporter-mediated effect. Given that there is unlikely to be extracellular GABA in these cultures in the absence of that exogenously applied, these data strongly suggest that the change in transport is not due to spillover of GABA onto GABA receptors.

Chronic changes in uptake induced by transporter-interacting compounds could be produced, in general, either by altering the turnover rate of individual transporters or by altering the number of functional transporters. By analogy with receptor binding experiments, data obtained from saturation experiments are often used to distinguish between these two possibilities as follows: changes in the maximum velocity of transport (\( V_{\text{max}} \)) are indicative of changes in the number of transporter-binding sites, and changes in affinity (\( K_m \)) are indicative of changes in the function of individual transporters. Saturation analysis was performed on 1F9 cells preincubated in control solution or solution containing GABA or SKF89976A. The results of this experiment are shown in Fig. 4A. Eadie-Hofstee transformations (not shown) of the saturation data revealed \( V_{\text{max}} \) values of 424 pmol/min/mg of protein (untreated cells), 302 pmol/min/mg of protein (SKF89976A-treated cells), and 842 pmol/min/mg of protein (GABA-treated cells). \( K_m \) values, which were not significantly affected by the treatments were 5.3, 5.9, and 5.4 \( \mu M \) for untreated, SKF89976A-treated, and GABA-treated cells, respectively. These alterations in \( V_{\text{max}} \)
are consistent with changes in the number of functional transporters.

To test this hypothesis directly, GAT1 immunoreactivity following biotinylation of surface proteins was examined in 1F9 cells preincubated with either GABA or SKF89976A. These data are shown in Fig. 4B. As shown both in the representative immunoblot and in the graph of densitometry measurements, preincubation of cells with GABA caused an increase in the amount of GAT1 immunoreactivity in the biotinylated fraction, the fraction corresponding to the surface population of transporters. This increase in surface immunoreactivity was correlated with a decrease in intracellular GAT1 immunoreactivity. Pretreatment of cells with SKF89976A resulted in a decrease in surface GAT1 immunoreactivity and a corresponding increase in intracellular GAT1 labeling. Two control experiments support these findings. First, the intracellular cytoskeletal pro-

Fig. 2. Regulation of GABA uptake by GABA transporter agonists and antagonists. A, dose-dependent changes in GABA transport. Neuronal cultures were preincubated for 1 h in control medium or medium containing various concentrations of nipeotic acid (open circles), ACHC (open squares), or SKF89976A (filled circles). Data are from two experiments, six wells/concentration. Mean GABA uptake under control conditions was 430 fmol/min/mg of protein. B, GABA receptor antagonists and protein synthesis inhibitors do not affect GABA-mediated up-regulation. Drug concentrations (in μM) are shown below the abscissa; neuronal cultures were preincubated for 30 min. Data are from two experiments, four wells/condition/experiment. Mean GABA uptake under control conditions was 404 fmol/min/mg of protein.

Fig. 3. Regulation of transport by transporter agonists and antagonists is recapitulated in mammalian cells stably expressing the rat brain GABA transporter GAT1. Drug concentrations (in μM) are shown below the abscissa; cells were preincubated for 1 h. Data are from three experiments, four wells/condition/experiment. Mean GABA uptake under control conditions was 1839 fmol/min/mg of protein. The experiment was repeated twice with similar results. B, changes in surface GAT1 immunoreactivity as assessed by surface biotinylation. 1F9 cells were preincubated for 1 h prior to biotinylation in control medium or medium containing 100 μM GABA or 10 μM SKF89976A. Representative immunoblot shows GAT1 immunoreactivity in total cell lysates, intracellular, and biotinylated fractions. Quantitation of GAT1 immunoreactivity is shown in the graph for cells preincubated in control solution (open bars), GABA (filled bars), and SKF89976A (hatched bars). Data are from densitometry measurements made from three separate experiments and plotted with respect to controls from within the given fraction. Experimental conditions that resulted in a significant change (p < 0.05) from control values are denoted by the asterisk.

Fig. 4. Regulation by extracellular GABA and SKF89976A is due to changes in surface levels of GAT1. A, saturation analysis of GABA-mediated and SKF-mediated changes in transport. 1F9 cells were preincubated for 1 h in control medium (open squares) or medium containing various concentrations of GABA (open circles) or SKF89976A (filled circles). Data shown are from one experiment, six wells/concentration. Mean GABA uptake under control conditions was 430 fmol/min/mg of protein. The experiment was repeated twice with similar results. B, changes in surface GAT1 immunoreactivity as assessed by surface biotinylation. 1F9 cells were preincubated for 1 h prior to biotinylation in control medium or medium containing 100 μM GABA or 10 μM SKF89976A. Representative immunoblot shows GAT1 immunoreactivity in total cell lysates, intracellular, and biotinylated fractions. Quantitation of GAT1 immunoreactivity is shown in the graph for cells preincubated in control solution (open bars), GABA (filled bars), and SKF89976A (hatched bars). Data are from densitometry measurements made from three separate experiments and plotted with respect to controls from within the given fraction. Experimental conditions that resulted in a significant change (p < 0.05) from control values are denoted by the asterisk.
tein actin was not labeled by the biotinylation reagent, suggesting that only surface proteins were being labeled; and second, immunoreactive bands were not seen in untransfected CHO cells immunoblotted with the GAT1 antibody (data not shown). The immunoblot data correlate well with the functional changes in uptake suggesting that compounds that interact with the transporter act to alter the number of cell-surface transporters. Furthermore, the evidence that the amount of transporter immunoreactivity in total cell lysates was unchanged by GABA or SKF89976A treatment supports the data showing regulation of the transporter in the presence of cycloheximide (see Fig. 2B) and suggests that the modulation is due to a redistribution of transporters rather than due to synthesis of new transporter protein.

The data obtained in the presence of GABA receptor blockers and the results from experiments using substrates of the transporter that do not activate GABA receptors strongly suggest that the mechanism of transporter regulation is not through a GABA receptor-mediated process. Another possibility is that the amount of GABA (or related compounds) present intracellularly regulates transporter redistribution. This hypothesis is consistent with up-regulation of GABA transport by transporter substrates and down-regulation of GABA transporters in the presence of SKF89976A. To test this hypothesis, 1F9 cells were transfected with another GABA transporter, GAT3, and experiments were performed in the presence of extracellular GABA and SKF89976A. If regulation is due to the amount of intracellular GABA, then GABA uptake by GAT3 in the presence of SKF89976A (which blocks GAT1 with approximately 200-fold higher affinity than GAT3) should increase GAT1 expression. On the other hand, if the regulation signal is a GAT1-mediated process directly, then the presence of SKF89976A should result in a down-regulation of GAT1 expression (as shown in Fig. 4). The result of this experiment is shown in Fig. 5. The concentration of SKF89976A was chosen such that the majority of GAT1 would be inhibited, whereas GAT3 inhibition would be minimal. Cells expressing both GAT1 and GAT3 show less inhibition by SKF89976A than cells expressing GAT1 alone when SKF89976A is included in the assay. This decrease in inhibition of uptake is consistent with GABA transport occurring through GAT3. Biotinylation experiments (see immunoblot) revealed that surface GAT1 expression was reduced following preincubation with SKF89976A. Since intracellular GABA should have been accumulating via GAT3 during this time, intracellular GABA levels do not appear to determine the regulation of the transporter. Rather, the down-regulation of the transporter in the presence of SKF89976A in these cells is consistent with an interaction of compounds with the transporter directly. It is interesting to note that preincubation of GAT1/GAT3-expressing cells with both GABA and SKF89976A caused a slight increase in transport compared with untreated cells. Since surface GAT1 expression was reduced by this treatment, such an increase is likely due to an increase in GAT3 expression.

The redistribution of GAT1 from intracellular locations to the plasma membrane in the presence of extracellular GABA suggested that the modulation might be occurring by changes in the rates of transporter turnover. To test this hypothesis, 1F9 cells were preincubated for 1 h with a control solution or a solution containing 100 μM GABA, surface-biotinylated, and then processed at various time points after biotinylation. The results of this experiment are shown in Fig. 6A. Control cultures showed an approximately 50% decrease in surface GAT1

![Fig. 5. Regulation of surface GAT1 levels by extracellular GABA is not due to intracellular GABA accumulation. Drug concentrations (in μM) are shown below the abscissa; values underlined indicate that the drug was added only during the assay. CHO cells expressing either GAT1 alone (open bars) or both GAT1 and GAT3 (filled bars) were preincubated for 1 h in control medium or medium containing GABA or GABA and SKF89976A. Data are from two separate experiments, four wells/condition/experiment. Mean GABA uptake in GAT1 expressing cells under control conditions was 1764 fmol/min/mg of protein; mean GABA uptake in GAT1/GAT3-expressing cells was unchanged by GABA or SKF89976A treatment supports the data showing regulation of the transporter in the presence of cycloheximide (see Fig. 2B) and suggests that the modulation is due to a redistribution of transporters rather than due to synthesis of new transporter protein.

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immunoreactivity (GAT1 immunoreactivity of the biotinylated fraction) over the 2-h experiment. In contrast, the decrease in surface GAT1 immunoreactivity was significantly slowed in GABA-treated cultures. Greater than 80% of the immunoreactivity remained at 2 h. Although these data strongly support the idea that the interaction of the transporter with substrates slows the internalization of the transporter, these data do not rule out the possibility that there is also an increase in the rate of transporter insertion into the membrane as well.

To determine whether a similar mechanism occurs with the endogenous GABA transporter, these time course biotinylation experiments were repeated in hippocampal neurons. Cultures were preincubated for 1 h with a control solution or a solution containing 100 μM GABA, surface-biotinylated, and then processed at various time points after biotinylation. The results of this experiment are shown in Fig. 6B. Once again, the evidence suggests that the interaction of the transporter with substrates slows transporter internalization.

DISCUSSION

Neurotransmitter transporters exhibit a number of functional properties that enable them to influence extracellular neurotransmitter levels. For example, unitary transport rates are dependent on substrate concentrations, with half-maximal effective concentrations for transport occurring in the sub-micromolar to low micromolar range (1, 32). Additionally, transporters operate in reverse and non-vesicular efflux of transmitter will contribute to ambient extracellular transmitter levels. Transmitter efflux through the transporter may be related to pathophysiological conditions (6, 33, 34) but is also a principal mode of neurotransmitter release in some systems (5). Neurotransmitter transporter expression can be regulated, and such changes in the number of functional transporters will also contribute to the control of synaptic neurotransmitter levels. In the present report, we show that GABA transporter substrates and antagonists can interact with the GABA transporter to regulate direct transporter uptake through alterations in surface transporter expression. These data strongly suggest a feedback mechanism in which transporters use extracellular neurotransmitter levels as a signal for the dynamic control of neurotransmitter levels at the synapse.

GABA transporter function is modulated by protein kinase C both in cells that endogenously express the transporter (13, 35) and in heterologous expression systems (24, 36, 37). The results from a majority of these studies show changes in the maximum velocity of transport, consistent with the hypothesis that transporter expression levels are altered. More direct measures, including subcellular fractionation (24) and estimating functional GABA transporter number by transporter-specific charge movements (15), support this hypothesis. One mechanism by which protein kinase C mediates its effect on GAT1 is by regulating the interaction of GAT1 with components of the docking and fusion apparatus (13). The evidence that GABA-mediated decreases in GAT1 function also result in a change in surface GAT1 molecules raises the possibility that such SNARE proteins are also involved in this form of GAT1 internalization. SNARE proteins have been implicated in the internalization and externalization of the type 4 glucose transporters (for review, see Ref. 38).

There appear to be multiple mechanisms even by which transporter substrates and/or antagonists can influence transporter function and expression. mRNA levels in brain for serotonin (39, 40) and dopamine (41) transporters are reduced following long term (>24 h) transporter antagonist treatments or by removal of substrate (42), although whether this significantly alters transporter protein levels (42) is unclear. In the present experiments it is unlikely that changes in mRNA or protein levels are mediating the regulation because (i) the time course of the modulation is on the order of minutes, (ii) it occurs in the presence of protein synthesis inhibitors, and (iii) GAT1 protein levels, although redistributed, appear to be unchanged. The triggers for these effects on mRNA levels are unclear since in vivo blockade of transporters likely causes increased receptor-mediated signaling as well.

Mammalian cells expressing norepinephrine transporters also show reduced uptake following 3-day transporter antagonist incubation, and this occurs in the absence of changes to mRNA levels (20). Whether this net internalization of norepinephrine transporters occurs by a mechanism similar to that of GAT1 in unknown; the much slower time course suggests that this could be a different form of transporter regulation. Experiments are under way to examine changes in GAT1 mRNA and protein levels following long term (>3 day) substrate and antagonist treatments. We are also examining whether other neurotransmitter transporters are regulated in a manner similar to GAT1. Although there have not been other reports of transporter regulation by extracellular neurotransmitter levels on the time scale of minutes, such changes may be easily overlooked because the time course of the regulation occurs on a time scale comparable to that typically used to assay uptake (i.e., 15–60 min). Thus, assays used to assess control levels of uptake will be confounded by concomitant changes in transporter expression.

There are two well characterized systems in which agonist-induced and antagonist-induced signals produce changes in rates of internalization. These are nicotinic acetylcholine receptors (43) and G protein-coupled receptors (for review, see Ref. 44). Although the phenomenology of these processes is well described, the mechanisms underlying the down-regulation in surface expression are not well understood. How rates of transporter internalization are altered by substrate interaction is not known, although the opposite effects that occur in the presence of antagonists raise the possibility that the internalization signal is influenced by transporter conformation. One possibility is that the process of substrate transport alters the interaction of GABA transporter with SNARE proteins (e.g. syntaxins) that act to sequester the transporter in a non-functional state (13), thus shifting the balance of surface and internalized transporter pools.

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