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REVIEW

Regulated trafficking of neurotransmitter transporters: common notes but different melodies

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Abstract

The activity of biogenic amine and amino acid neurotransmitters is limited by presynaptic and astrocytic Na^+-dependent transport systems. Their functional importance is underscored by the observation that these transporters are the targets of broad classes of psychotherapeutic agents, including antidepressants and stimulants. Early studies suggested that the activity of these transporters can be fine tuned by a number of different signaling pathways. In the past five years, several groups have provided compelling evidence that changing the cell surface availability of these transporters contributes to this fine tuning. This regulated trafficking can result in rapid (within minutes) increases or decreases in the plasma membrane expression of these transporters and is independent of transcriptional or translational control mechanisms. Many of the same signaling molecules, including protein kinase C (PKC), tyrosine kinase, phosphatidylinositol 3-kinase (P13-K), and protein phosphatase, regulate the transporters for different neurotransmitters. In addition to these classical receptor activated pathways, transporter substrates also regulate activity and cell surface expression of these transporters. In fact, some of the transporters form complexes with signaling molecules. Given the functional and genetic similarities of these transporters, it is not surprising that the same signaling molecules regulate their trafficking, but except for the molecules, the actual effects on individual transporters are remarkably different. It is as if the same musical notes have been rearranged into several different melodies.

Keywords: dopamine, GABA, glutamate, protein kinase C, serotonin, transporter.

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The identification of processes that inactivate putative neurotransmitters after release into the extracellular space was one of several criteria used to support neurotransmitter status of many small molecules. During the late 1960s and early 1970s, these inactivation mechanisms were identified and characterized. Although extracellular metabolism contributes to the inactivation of certain neurotransmitters (e.g. acetylcholine and peptides), plasma membrane transporters play a far more significant role in terminating the activity of most of the small molecule neurotransmitters (biogenic amines and amino acids). These transporters utilize the electrochemical energy derived from the inward movement of Na^+ to drive the intracellular accumulation of neurotransmitter.

In the early 1990s, many of these transporters were cloned, and two broad gene families were identified. The first includes the norepinephrine, dopamine, serotonin and γ-aminobutyric acid transporters. The second family, which shares no significant sequence homology with the first, includes several different glutamate transporters (for a review see Amara 1992; Blakely 1992; Kanai *et al*. 1993). The cloning of these transporter proteins provided the tools required to study transcriptional and post-translational regulation in a variety of experimental systems.

It should not be surprising that molecules with the potential to dramatically modulate both the temporal and spatial characteristics of synaptic transmission are

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Abbreviations used: DAT, dopamine transporter; EAAC1, excitatory amino acid carrier 1; GABA, γ-aminobutyric acid; GLAST, glutamate-aspartate transporter; GLT-1, glutamate transporter-1; GLUT4, glucose transporter subtype 4; MDCK, Madin-Darby canine kidney; PI3-K, phosphatidylinositol 3-kinase; PKC, protein kinase C; PDGF, platelet-derived growth factor; PP2A, protein phosphatase 2 A; SERT, serotonin transporter; L-*trans*-PDC, L-*trans*-pyrrolidone-2,4-dicarboxylate.

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dynamically regulated. Several early studies suggested that the activity of many of these transporters can be rapidly regulated by various signaling molecules. Although frequently accompanied by a change in maximal velocity, it is possible that these effects could be due to a change in the electrochemical gradients required for transport, a change in the catalytic efficiency/turnover number of the transporters, or a change in the numbers of transporters at the plasma membrane. The ability to alter the cell surface expression of membrane proteins is an evolutionarily conserved feature of many biological systems (for a review see Hicke 1999). One well-studied example involves the GLUT4 subtype of glucose transporter. After release into the circulation, insulin causes a dramatic redistribution of this transporter from an intracellular compartment to the cell surface resulting in increased clearance of glucose by muscle and adipose tissue (for a review see Czech and Corvera 1999; Pessin *et al*. 1999; Simpson *et al*. 2001). Another example includes the regulated trafficking of membrane receptors, including two large families of receptors, G-protein coupled receptors and growth factor receptors (for recent discussion see Hicke 1999; Laporte *et al*. 2000; Bhatnagar *et al*. 2001). A relatively common theme for regulation of these receptors has emerged. Frequently, binding of agonist accelerates internalization through endocytotic pathways thereby decreasing the responsiveness of the cell to subsequent exposure to agonist, although there are exceptions with 'paradoxical' regulation (Roth *et al*. 1998).

This review will describe recent studies that indicate that the activity and cell surface expression of four different neurotransmitter transporters can be rapidly regulated. An overview of the methods used to study the cell surface expression of transporters will be first provided. The effects of specific signaling molecules on the cell surface expression of the GABA, serotonin, dopamine, and glutamate transporters will be described. These transporters are regulated by many of the same signaling molecules, including protein kinase C (PKC), tyrosine kinase-linked pathways, and protein phosphatase. In addition, substrates for the individual transporters regulate transporter activity and cell surface expression. Given the similarities of these systems, one might expect common themes to emerge. So far common signals have been identified, but these signals have somewhat different effects on individual transporters. At the end of the review, some of the similarities and differences will be summarized.

For those interested readers, there are also examples of regulated trafficking of both the norepinephrine and glycine transporters (Apparsundaram *et al*. 1998; Gerrlings *et al*. 2001). The author wishes to apologize for not including these transporters in the discussion and for not adequately acknowledging many studies that demonstrate acute regulation of transporter activity.

Strategies used to follow cell surface expression of transporters

Several different strategies have been used to follow the cellular redistribution of the various transporters. Probably the most commonly employed technique involves the covalent modification of cell surface proteins with a membrane impermeant biotinylation reagent. After biotinylation, cells are lysed and the biotinylated/cell surface proteins are batch extracted using avidin coated sepharose beads. The levels of transporter expression in the cell lysate (total protein), in the biotinylated (cell surface), and the non-biotinylated (intracellular) fractions are analyzed by western blot (Daniels and Amara 1998). With this technique, it is possible to test for potential cell lysis by following the biotinylation of intracellular proteins, such as actin or calnexin (Qian *et al*. 1997; Davis *et al*. 1998). Another commonly used complementary technique is the use of immunocytochemistry combined with confocal microscopy to qualitatively visualize a redistribution of transporter protein (for an example see Davis *et al*. 1998; Daniels and Amara 1999). With some transporters, it is possible to use ligands that only bind to the cell surface transporters of intact cells, but will bind to all transporters in a lysed cellular preparation. With this strategy, a change in binding to intact cells with no change in total binding implies a redistribution (for examples see Pristupa *et al*. 1998; Doolen and Zahniser 2001). Finally, it is possible to separate intracellular vesicles from the plasma membrane using differential centrifugation. The amount of immunoreactivity in each fraction is then evaluated by western blot. This strategy has been used with both *Xenopus* oocytes and with cell cultures (for examples see Corey *et al*. 1994; Melikian and Buckley 1999).

In this review, the model systems used to study the regulation are identified, because it appears that some affects are dependent on the cellular context. It is not clear if these differences are related to the levels of protein expression, the presence or absence of specific subtypes of signaling molecules, or other differences related to cell biology (for example expression in polarized vs. non-polarized cells). It seems safest to use a couple of different model systems, including primary cultures to verify that effects are not specific to one single model system. Of course, the observation that some effects may depend on the model system could provide strategies to isolate specific proteins that are required for regulation of these transporters. In the sections below, the effects of various signaling molecules on the cell surface expression of specific transporters will be described.

GABA transporters

There are five closely related transporters that mediate the Na^+-dependent accumulation of GABA, betaine, and taurine

(for a review see Borden 1996; Nelson 1998). Three of these transporters, GAT1-3 are thought to mediate the clearance of GABA in brain. GAT1 has been the focus of several studies performed by Mike Quick and colleagues showing that PKC, transporter substrates, and tyrosine phosphorylation alter the cell surface expression and activity of the GAT1 subtype of GABA transporter (for a review see Beckman and Quick 2000).

PKC-dependent regulation of GAT1

Activation of PKC has multiple effects on GAT1-mediated transport activity. In *Xenopus* oocytes injected with GAT1 cRNA, activation of PKC with an intracellular injection of phorbol ester causes a rapid (within minutes) redistribution of the transporter from an intracellular compartment to the cell surface and an increase in transport activity (Corey *et al*. 1994; Quick *et al*. 1997). In primary cultures that endogenously express GAT1 and in mammalian cells transfected with GAT1, bath application of phorbol esters has the opposite effect, decreasing GAT1 cell surface expression and activity (Beckman *et al*. 1999). It is not clear why different effects are observed in the two systems, but it is possible that the coupling of signaling pathways to vesicle trafficking in oocytes is different from that observed in mammalian cells. Given the remarkable evolutionary conservation of many other important biological pathways, it seems unlikely that there would be fundamental differences of this type. Instead, it seems more likely that intracellular injection of phorbol ester may explain the difference. Quick and colleagues have found that bath application of phorbol esters decreases GAT1 mediated transport activity in *Xenopus* oocytes, but the effects of bath application on GAT1 cell surface expression were not examined (Quick *et al*. 1997). Since phorbol esters interact with several targets including subtypes of PKC and non-PKC targets (for a review see Kazanietz *et al*. 2000), it is possible intracellular and bath application results in interaction with, or activation of, different combinations of these targets.

At present, it seems likely that the physiologically relevant effect of PKC activation is to decrease GAT1 cell surface expression since one assumes that the cellular milieu in primary cultures is more likely to mimic that observed *in vivo*. This notion is further supported by the observation that agonists for several different G-protein coupled receptors, including muscarinic, glutamatergic and serotonergic receptors decrease GABA transport activity in primary cultures of neurons (Beckman *et al*. 1999). The effects of muscarinic receptor activation are blocked by inhibition of PKC and are accompanied by a decrease in GAT1 cell surface expression. Therefore, different neurotransmitters that activate receptors coupled to PKC could theoretically decrease GAT1 mediated activity presumably altering GABAergic transmission.

At least some of the effects of PKC on GAT1 are dependent on SNARE proteins, including syntaxin 1A. In oocytes, both syntaxin and synaptophysin appear to be necessary for the phorbol ester-dependent redistribution of GAT1 to the plasma membrane, suggesting that these SNARE proteins are required for the fusion of GAT1 containing vesicles to the oocyte plasma membrane (Quick *et al*. 1997). Activation of PKC also increases a direct interaction between syntaxin 1A and GAT1; this interaction dramatically decreases the catalytic efficiency (turnover number) of the transporters (Beckman *et al*. 1998; Deken *et al*. 2000). In neuronal cultures, cleavage of syntaxin with botulinum toxin decreases cell surface expression of GAT1 but also increases GAT1 transport activity (Deken *et al*. 2000; Horton and Quick 2001). Therefore, synataxin 1A may be required for trafficking of GAT1 to the cell surface but may also form a complex with the transporter to decrease the catalytic efficiency.

Regulation of GAT1 by substrates

Pre-incubation with GABA causes an increase in GABA transport activity in primary neuronal cultures and Chinese hamster ovary cells stably transfected with GAT1 (Bernstein and Quick 1999). In neuronal cultures, the increased activity is sensitive to the GAT1-specific inhibitor SKF8997A, suggesting GABA increases the activity of GAT1 and not the activity of one of the other GABA transporters expressed in these cultures. This notion is further supported by the observation that this effect is correlated with an increase in cell surface expression of GAT1. These effects are mimicked by other transporter substrates but not by GABA receptor agonists. Furthermore, SKF8997A blocks the GABA-induced increase in transporter activity. Together, these data provide compelling evidence that the effects are mediated through an interaction of GABA with the transporter and not a GABA receptor. There are two possible explanations for this effect. A signaling molecule could either be activated by an intracellular accumulation of GABA or the interaction of GABA with the transporter could activate a signaling pathway. To determine if an intracellular accumulation of GABA is sufficient to cause this effect, CHO cells were transfected with both GAT1 and GAT3. In these cotransfected cells, the GAT1 specific inhibitor blocks the GABA-induced increase in activity and cell surface expression. As GAT3 still accumulates GABA under these conditions, it appears that a direct interaction of GABA with the GAT1 transporter is required to increase GAT1 mediated activity and GAT1 cell surface expression.

Regulation of GAT1 by tyrosine kinase linked pathways

More recently, the same group has demonstrated that tyrosine kinase linked signaling pathways regulate GAT1 (Law *et al*. 2000). Brain-derived neurotrophic factor causes an increase

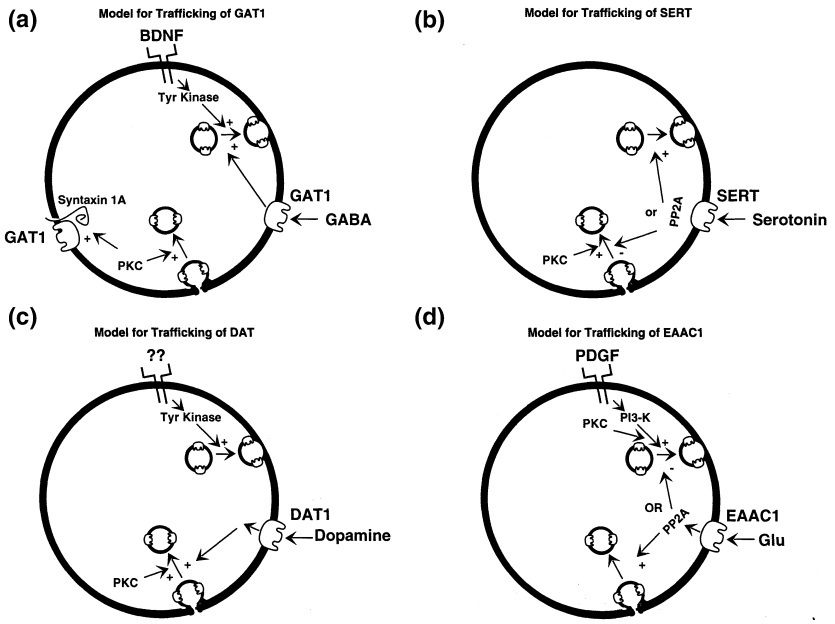

Fig. 1 Schematic representation of regulated trafficking of GAT1 (a), SERT (b), DAT (c) and EAAC1 (d). To simplify each of these models, an increase in cell surface expression is illustrated as an increase in the rate of translocation of transporters from a subcellular compartment to the cell surface and a decrease in cell surface expression is illustrated as an increase in the rate of endocytosis of the transporter. In most studies, steady state levels of the transporters have been measured and either kinetic step could be affected.

in both the activity and cell surface expression of GAT1, and inhibition of tyrosine kinase blocks this effect. Pre-incubation with either genistein or K252a (inhibitors of tyrosine kinase) decreases GABA transport activity and inhibition of protein-tyrosine phosphatase with pervanadate increases transport activity, suggesting that there is basal activation of these pathways in the absence of exogenous brain-derived neurotrophic factor. A genistein-induced decrease in transport activity is also observed in CHO cells stably transfected with GAT1 and is associated with a redistribution of the transporter from the plasma membrane to an intracellular compartment. These effects are correlated with changes in tyrosine phosphorylation of GAT1. Mutation of all five of the potential intracellular tyrosine phosphorylation sites does not appear to disrupt transporter function but eliminates tyrosine phosphorylation and regulation by tyrosine kinase inhibitors. These data suggest that direct tyrosine phosphorylation is required for this regulated trafficking of GAT1, but not for normal insertion of GAT1 in the plasma membrane. This may imply that there are two distinct intracellular pools of GAT1: one that is constitutively trafficked to the plasma membrane and another that is redistributed in response to activation of tyrosine kinase. The effects of inhibition of tyrosine kinase are additive with the effects of PKC inhibition and are not blocked by a PKC antagonist, suggesting that two distinct signaling pathways regulate the cell surface expression of GAT1.

Summary of GAT1 regulation

Together these studies provide compelling evidence that the activity of the GAT1 subtype of GABA transporter can be dynamically up- and down-regulated by several different stimuli (see Fig. 1a). Activation of PKC apparently has two additive effects that can decrease activity. The first is associated with a decrease in cell surface expression; the second is associated with an increase in GAT1-syntaxin 1A complexes and a decrease in catalytic efficiency. GAT1 substrates and growth factor receptor agonists both increase the activity and cell surface expression of GAT1.

Serotonin transporters

At present, it appears that a single gene product mediates the Na^+-dependent clearance of extracellular serotonin. This serotonin transporter (SERT) is regulated by multiple signaling pathways (for a review see Blakely *et al*. 1998; Blakely and Bauman 2000). As is observed for the GAT1 subtype of GABA transporter, the cell surface expression of SERT is regulated by PKC and by transporter substrates. In addition, SERT is regulated by protein phosphatase 2A which directly complexes with the transporter.

PKC-dependent regulation of SERT

A mammalian cell line (human embryonic kidney 293) stably transfected with SERT has been used to study several different aspects of regulation of transporter activity and cell surface expression. In this system, activation of PKC causes a rapid (maximal within 30 min) decrease in activity (Qian *et al*. 1997). This effect is largely attributed to a decrease in the V_{max} for serotonin transport and is correlated with a decrease in the amount of biotinylated (cell surface) transporter with no change in the total amount of SERT expression, suggesting that a change in cell surface expression is responsible for the decrease in transport activity.

In a subsequent study, the same group demonstrated that PKC activation is associated with an increase in phosphorylation of SERT (Ramamoorthy *et al*. 1998). Activation of PKC causes phosphorylation of a band of the appropriate molecular weight that is immunoprecipitated from cells stably transfected with SERT but not from control cells. This observation and other controls strongly suggest that the band observed is SERT. Both a time course and a concentration response curve for PKC activation reveal a close correlation between the effects on activity and on protein phosphorylation. In the same study, the investigators demonstrate that two different inhibitors of protein phosphatase 2A (PP2A), okadaic acid and calyculin A, also decrease serotonin transport activity and increase SERT phosphorylation. At present, it is not clear if this effect is associated with a change in SERT cell surface expression. Interestingly, the effects of PKC activation and PP2A inhibition on both activity and SERT phosphorylation are additive. In addition, the effects of PP2A inhibitors are not blocked by inhibitors of PKC, Ca^{2+}/calmodulin-dependent kinase, or protein kinase A. Together these studies suggest that at least two different phosphorylation events are associated with changes in serotonin transporter activity. One of these phosphorylation events is mediated by PKC; the second phosphorylation event is dependent on a kinase that has not yet been identified.

Regulation of SERT by substrates

This group also found that serotonin transporter substrates have an impact on both phosphorylation and cell surface expression of SERT (Ramamoorthy and Blakely 1999). Serotonin and other transporter substrates (amphetamine and fenfluramine) inhibit PMA-stimulated phosphorylation of SERT. These effects are not mimicked by serotonin receptor agonists, but are blocked by serotonin transport inhibitors, including cocaine and the tricyclic antidepressants (imipramine, paroxetine and citalopram). These data provide compelling evidence that the effect of serotonin is mediated through the serotonin transporter. Pre-loading the cells with high concentrations of serotonin does not have the same effect as simultaneous incubation with serotonin, indirectly suggesting that an interaction of serotonin with the extracellular surface of the transporter is required. Interestingly, the EC_{50} for inhibition of SERT phosphorylation by serotonin was nearly 10-fold lower than the K_m for serotonin transporter activity. This implies that the coupling of transporter to this effect may depend on different conformational changes in the transporter than are required for the transport of substrate (i.e. independent of substrate translocation). Finally, this group demonstrated that serotonin blocks the PMA-induced internalization of SERT. Together, these data suggest that this interaction of serotonin with the transporter may provide a homeostatic mechanism to prevent PKC-dependent internalization of SERT when extracellular levels of serotonin approach the K_m value.

More recently, the same group has examined the possibility that SERT may directly interact with PP2A and that this interaction may be regulated by many of the same signaling events that regulate activity and cell surface expression of SERT (Bauman *et al*. 2000). They found that PP2A immunoreactivity can be immunoprecipiated with two different anti-SERT antibodies. The immunoprecipitate contains functional phosphatase activity that can be blocked by okadaic acid. To exclude the possibility that the association of SERT and PP2A might be an artifact related to the expression system, the investigators demonstrated this association in native brain tissues that express SERT. Interestingly, activation of PKC or inhibition of protein phosphatase abolishes the interaction between SERT and PP2A. Finally, serotonin prevents PKC from causing disassembly of the complex. Together, these data provide evidence for an interaction between SERT and PP2A that is dependent on many of the same factors that regulate the activity, cell surface expression, and phosphorylation of SERT. Although it implies that serotonin activates PP2A, this cannot easily be tested pharmacologically because the PP2A inhibitor, okadaic acid, increases SERT phosphorylation (Ramamoorthy and Blakely 1999) and decreases SERT activity (Sakai *et al*. 1997) in the absence of substrate.

Summary of SERT regulation

These data are consistent with the following model (see Fig. 1b). Under 'baseline' conditions, SERT may exist as a complex with PP2A. Upon activation of PKC, the transporter and/or an accessory protein are phosphorylated, PP2A dissociates from the complex, and SERT cell surface expression decreases. When the transporter is occupied by substrate, the effects of PKC activation are prevented, presumably serving as a homeostatic mechanism to prevent down-regulation of SERT when extracellular serotonin levels are elevated.

Dopamine transporters

Like serotonin, the clearance of extracellular dopamine is apparently mediated by a single gene product. The activity and cell surface expression of the dopamine transporter (DAT) are decreased in response to PKC activation. DAT also appears to be regulated by substrates and tyrosine kinase linked pathways, but the effects are somewhat different than might be predicted from the studies of GAT1 and SERT.

PKC-dependent regulation of DAT

In a series of studies, activation of PKC was shown to acutely decrease DAT activity and increase transporter

phosphorylation (for a recent report see Huff *et al*. 1997). Several different groups have reported similar effects of PKC on DAT activity and have provided compelling evidence that this effect is associated with internalization of the transporter protein in different cellular systems including *Xenopus* oocytes (Zhu *et al*. 1997; Pristupa *et al*. 1998; Daniels and Amara 1999; Melikian and Buckley 1999). At present there is no direct evidence that phosphorylation of DAT is required for this internalization. In fact, mutation of all serine/threonine residues within classical PKC consensus sequences does not eliminate PKC-dependent regulation or internalization of DAT, but does abolish PKC-dependent phosphorylation of DAT (Chang *et al*. 2001). This may suggest that direct transporter phosphorylation is not required for DAT internalization, and that a scaffolding protein, such as PICK1, may be the target of phosphorylation (Torres *et al*. 2001). As is observed with GAT1, activation of a subtype of metabotropic glutamate receptor, mGluR5, results in a PKC-dependent inhibition of DAT mediated activity (Page *et al*. 2001), providing a functional link between cell surface receptors and DAT activity.

Two different groups have examined the intracellular fate of DAT to determine if it is targeted for degradation or available for recycling to the plasma membrane. In Madin-Darby canine kidney (MDCK) cells stably transfected with DAT, activation of PKC decreases dopamine transport activity to approximately 30% of control levels and causes a pronounced decrease in cell surface expression of DAT epitope tagged with green fluorescent protein (Daniels and Amara 1999). This group used several strategies to develop evidence that the decrease in the cell surface expression is related to an acceleration of endocytosis, rather than a slowing of the redistribution of the transporter from a subcellular compartment to the cell surface. Expression of a dominant negative variant of dynamin 1 blocks the PKC-dependent internalization of DAT, suggesting that endocytosis occurs via the clathrin pathway. Finally, they developed evidence that internalized transporters are targeted for lysosomal degradation rather than being recycled to the plasma membrane.

Melikian and Buckley have also examined the effects of PKC activation on DAT using PC12 cells, but concluded that the transporters could be recycled to the plasma membrane (Melikian and Buckley 1999). As was observed in other cellular systems, activation of PKC caused a dramatic decrease in DAT mediated activity and cell surface expression. Using subcellular fractionation techniques and organelle specific markers, this group concluded that DAT is internalized through an endosomal pathway and that activation of PKC increases the appearance of DAT in an endosomal recycling compartment. These studies suggest that internalized DAT is recycled back to the plasma membrane in this system. At present, it seems possible that both pathways of internalization are available for DAT and that cellular context or basal activation of signaling pathways may influence the fate of internalized transporters.

Tyrosine kinase-dependent regulation of DAT

As observed for GAT1, the activity of DAT is decreased by inhibitors of tyrosine kinase using *xenopus* oocytes as a model system (Doolen and Zahniser 2001). This effect is associated with decreases in the V_{max} for dopamine transport and in the binding of a ligand that should selectively bind to DAT located in the plasma membrane. This study suggests that activation of growth factor receptors linked to tyrosine kinase may physiologically increase dopamine transporter activity and that there is basal activation of this pathway in the absence of exogenous growth factors in this system.

Substrate- and PP2A-dependent regulation of DAT

Daniels and Amara reported that dopamine or cocaine have no effect on cell surface expression of DAT in MDCK cells (Daniels and Amara 1999). In addition, they found that dopamine has no effect on the PKC-dependent internalization. In a more recent study, dopamine and other substrates (amphetamine) caused an internalization of DAT in human embryonic kidney 293 cells stably transfected with DAT (Saunders *et al*. 2000). These effects were blocked by transport inhibitors such as cocaine, providing fairly strong evidence that these effects were mediated through an interaction of dopamine with the transporter. At present, it is unclear why different results were obtained in these two studies, but there is evidence that substrates may also down-regulate DAT activity *in vivo* (for example, see Metzger *et al*. 2001). Interestingly, as is observed for SERT, PP2A can be immunoprecipitated from striatal homogenates with anti-DAT antibodies, suggesting that DAT may functionally couple to PP2A (Bauman *et al*. 2000).

Summary of DAT regulation

Activation of PKC causes a decrease in DAT mediated activity and cell surface expression (See Fig. 1c). Substrates appear to be capable of regulating DAT activity and cell surface expression, but the effect is opposite to that observed for GAT1 or SERT. Since this effect of substrates is not observed in all systems studied, it is possible that an additional protein(s) is required. There is also evidence that inhibition of protein tyrosine kinase decreases DAT activity and cell surface expression. Finally, two studies suggest that activation of PKC accelerates endocytosis of the transporter rather than slowing the rate of delivery of the transporter to the cell surface. The ultimate fate of internalized transporter, whether it is targeted for degradation or recycled to the plasma membrane, may be influenced by factors that have not yet been identified.

Glutamate transporter regulation

Five Na^+ dependent high affinity glutamate transporters have been identified (for a review see Sims and Robinson 1999; Danbolt 2001). In the CNS, expression of two of these transporters, GLT-1 and GLAST, is generally restricted to glial cells. Two of the transporters, EAAC1 and EAAT4, are neuronal and EAAT5 is thought to be restricted to retina. At least some of these transporters are also observed in various peripheral tissues, and there is evidence for variants related to alternate mRNA splicing. Recent studies indicate that these transporters are regulated by the same signaling pathways that regulate GAT1, SERT, and DAT, but there are many differences.

PKC-dependent regulation of glutamate transporters

C6 glioma have been used as a model system to study the regulation of EAAC1 activity and cell surface expression. This cell line endogenously expresses EAAC1 and none of the other glutamate transporters (Dowd *et al*. 1996; Palos *et al*. 1996). We have found that activation of PKC rapidly (within minutes) increases both the activity and cell surface expression of EAAC1 in this cell line (Dowd and Robinson 1996; Davis *et al*. 1998). In *Xenopus* oocytes, there is preliminary evidence that direct activation of PKC or activation of a metabotropic glutamate receptor that couples to PKC activation may cause a decrease in EAAC1 activity (Trotti *et al*. 1998, 2001). Therefore, as is observed with GAT1 (see above) activation of PKC may have opposite effects on EAAC1 cell surface expression that appear to depend on the model system being employed to study trafficking. There are two lines of evidence that PKC may increase EAAC1 cell surface expression in mammalian systems *in vivo*. First, in primary cultures of neurons and astrocytes, a model system that presumably reflects the intracellular milieu observed *in vivo*, activation of PKC increases the cell surface expression of EAAC1 (Munir *et al*. 1997; Robinson laboratory unpublished observations). Secondly, a large intracellular pool of EAAC1 is observed *in vivo* and is not observed for the other glutamate transporters (Rothstein *et al*. 1994; Kugler and Schmitt 1999), suggesting that this protein is available for mobilization to the cell surface *in vivo*.

PKC activation has varied effects on the GLT-1 subtype of glutamate transporter. In Hela cells stably transfected with GLT-1, activity and phosphorylation is increased by activation of PKC (Casado *et al*. 1993). In stably transfected MDCK cells, activation of PKC decreases GLT-1 mediated activity (Carrick and Dunlop 1999). We did not observe any effects of PKC activation on GLT-1 mediated activity in a number of different cell lines (Tan *et al*. 1999). More recently, we have examined the effects of PKC activation on GLT-1 mediated activity and cell surface expression in C6 glioma stably transfected with GLT-1 and in primary cultures that endogenously express GLT-1. In both systems, activation of PKC rapidly decreases GLT-1 cell surface expression (Kalandadze *et al*. 2000; unpublished observations). Since the effects of PKC activation are opposite for GLT-1 and EAAC1 when they are both expressed in C6 glioma, it seems likely that differences in primary amino acid sequence will ultimately explain these differences. In preliminary studies, we have begun to identify structural domains of these transporters required for trafficking using a chimera based strategy (Kalandadze *et al*. 2000).

Several groups have studied the effects of PKC activation on GLAST mediated transport activity with contradictory results showing that activation of PKC increases, decreases, or has no effect on GLAST activity (Casado *et al*. 1991; González and Ortega 1997; Daniels and Vickroy 1999; González *et al*. 1999; unpublished observations). There is also evidence that the transporter is not internalized in response to activation of PKC (Conradt and Stoffel 1997). In preliminary studies, we observe a loss of GLAST immunoreactivity with activation of PKC in astrocyte cultures, but it is not clear that the mechanisms are similar to those observed for transporters that are internalized in response to activation of PKC (Susarla *et al*. 2001).

Tyrosine kinase-, phosphatidylinositol 3-kinase-, and growth factor-dependent regulation of EAAC1

The regulated trafficking of the insulin-responsive GLUT4, subtype of glucose transporter depends on the receptor tyrosine kinase, phosphatidylinositol 3- kinase (PI3-K), and other downstream signaling molecules (for a review see Czech and Corvera 1999; Pessin *et al*. 1999; Simpson *et al*. 2001). In an analogous fashion, platelet-derived growth factor (PDGF) rapidly increases EAAC1-mediated transport activity in C6 glioma (Sims *et al*. 2000). This increase in activity is associated with an increase in cell surface EAAC1. Although PDGF activates phospholipase Cγ which can lead to PKC activation (Valenzuela *et al*. 1997), the effect of PDGF on EAAC1 activity and cell surface expression are not blocked by a PKC antagonist. In many cellular systems, PDGF also activates other signaling molecules, including tyrosine kinase, mitogen-activated protein kinase and PI3-K. Although inhibitors of tyrosine kinase and PI3-K block the PDGF-induced increase in transport and cell surface expression, inhibition of mitogen-activated protein kinase has no effect. These data suggest that activation of tyrosine kinase and PI3-K is necessary for PDGF-induced regulation of EAAC1 and that the signaling pathways involved in EAAC1 trafficking are analogous to those that regulate GLUT4 trafficking.

Although inhibition of PKC has no effect on the PDGF-induced regulation of EAAC1, the effects of PKC activation and PDGF are not additive. This suggests that PKC and

PDGF regulate EAAC1 trafficking by independent but converging signaling pathways. It is possible that there is a finite intracellular pool of EAAC1 that is available for mobilization by these signaling pathways and that both PDGF and PKC cause maximal redistribution of EAAC1. It also seems possible that the effects of PKC may depend on activation of PI3-K, since an inhibitor of PI3-K completely blocks the PKC-dependent redistribution of EAAC1, and partially attenuates the increase in transporter activity (Davis *et al*. 1998).

Substrate-dependent regulation of glutamate transporters

Pre-incubation with glutamate causes an increase in the activity of the GLAST subtype of glutamate transporter (Duan *et al*. 1999). The increase in activity occurs rapidly (within minutes) and is accompanied by an increase in transporter V_{max}. Ionotropic glutamate receptor agonists do not mimic this effect, and antagonists for the ionotropic or metabotropic glutamate receptors do not block the effect of glutamate. In examining the effects of other transporter substrates, this group made the curious observation that D-aspartate mimics the effect of L-glutamate, but L-*trans*-pyrrolidone-2,4-dicarboxylate (L-*trans*-PDC) does not. L-*trans*-PDC does, however, block the effect of glutamate. This suggests that only a subgroup of transporter substrates couple to the increase in transport activity. This observation contrasts with those made for SERT where it appears that all substrates tested are equally effective at blocking transporter phosphorylation (Ramamoorthy and Blakely 1999). At present, the best explanation for the lack of an effect of L-*trans*-PDC is that it is a less efficient substrate than either D-aspartate or L-glutamate. In a different study, we found that pre-incubation with glutamate causes an increase in transporter activity through a mechanism that is consistent with an interaction with the transporters, but is not accompanied by an increase in the cell surface expression of GLT-1, GLAST, EAAC1 or EAAT4 (Munir *et al*. 2000). It seems possible that differences in the cultures used for these studies may explain these results. For example, it is possible that substrate-induced trafficking of GLAST is only observed when another signaling molecule is activated, as is observed for EAAC1 (see below).

Glutamate also increases the cell surface expression of the EAAT4 subtype of transporter in a glioma cell line, BT4C (Gegelashvili *et al*. 2000). In these studies, both D-aspartate and L-*trans*-PDC increased EAAT4 cell surface expression suggesting that this effect is mediated through an interaction with the transporter.

The effects of glutamate on the cell surface expression of EAAC1 have recently been examined in our laboratory. In preliminary studies, we have found that glutamate blocks the redistribution of EAAC1 induced by either PKC activation or by PDGF (Gochenauer *et al*. 2000 unpublished observations). This effect is not mimicked by glutamate receptor agonists, but is mimicked by L-aspartate. Although two other transporter substrates, L-*trans*-PDC or D-aspartate, do not mimic the effect of glutamate, they block the effect of glutamate. Therefore, it seems highly likely that these effects are mediated through a direct interaction with the transporter, but that not all transporter substrates are capable of causing this effect. Using a similar strategy to that employed for GAT1 (Bernstein and Quick 1999), we have developed evidence that an intracellular accumulation of glutamate is not sufficient to block the effect of PKC activation (unpublished observations). In an earlier study, we found that glutamate causes a slight reduction in EAAC1 cell surface expression in primary neuronal cultures (Munir *et al*. 2000), but we suspect that this effect was related to basal activation of signaling pathways that induce redistribution of the transporter. With the exception of this study, glutamate has no effect on EAAC1 cell surface expression in the absence of a stimulus that induces redistribution of the transporter. This suggests that glutamate is effectively serving as a 'coincidence detector' to block redistribution of the transporter. Studies of the potential mechanisms of this effect are currently underway. So far, we have found that okadaic acid blocks this effect of glutamate, suggesting that the transporter is functionally coupled to PP2A activation.

Summary of glutamate transporter regulation

Studies to date have provided evidence that the cell surface expression of at least four of these transporters can be regulated. The cell surface expression of a postsynaptic neuronal transporter, EAAC1, is increased by activation of PKC and by activation of growth factor receptor activated pathways, tyrosine kinase/PI3-K (see Fig. 1d). Somewhat surprisingly, a subset of substrates block this redistribution through a pathway that is apparently dependent on PP2A activation. Activation of PKC decreases the cell surface expression of the glial transporter, GLT-1. Substrates increase the cell surface expression of GLAST and EAAT4.

Recent studies have shown that two of the glutamate transporters, EAAT3 (the human homolog of EAAC1) and EAAT4, are regulated by interactions with novel proteins (Jackson *et al*. 2001; Lin *et al*. 2001). Although these interacting proteins do not regulate cell surface expression of the transporters, the presence of these molecules suggests that these transporters may exist in larger complexes with structural and signaling molecules.

Conclusions

During the last few years, several groups have developed evidence that neurotransmitter transporters are frequently regulated by changing the cell surface expression. The

Table 1 Effects of signaling molecules/substrates on the cell surface expression of neurotransmitter transporters

Transporter	PKC Activation	Inhibition of tyrosine kinase	Activation of growth factor rec.	Substrate
Gat1	↓	↓	↑	↑
SERT	↓	ND	ND	↓ (Requires PKC Act.)
DAT	↓	↓	ND	↓, –
EAAC1	↑	ND	↑	↓ (Requires PKC Act. or PDGF)
GLAST	ND	ND	ND	↑, –
EAAT4	ND	ND	ND	↑
GLT-1	↓	ND	ND	ND

↓ or ↑ indicate a decrease or increase in cell surface expression, respectively. ↑, – indicates that there are reports of a change and a report of no effect. ND indicates not yet reported. Requires PKC activation indicates that no effect is observed in the absence of PKC activation. For EAAC1, an effect of substrate is observed when either PKC is activated or cells are treated with PDGF. Act., activation; rec., receptor.

advantage of this type of regulation is that it can occur within minutes and avoids the potentially time consuming process of RNA transcription and translation. While PKC, growth factor activated pathways, and substrates regulate many of these transporters, the effects of these molecules on different transporters can vary dramatically (see Table 1) and the observations made to date raise several interesting questions.

PKC activation generally decreases the cell surface expression of these transporters, with the exception of the EAAC1 subtype of glutamate transporter, which is increased in response to activation of PKC. It is not clear if alterations in cell surface expression are the result of increasing the rates of endocytosis or decreasing redistribution of the transporters from a subcellular compartment to the plasma membrane. If the effects are mediated through internalization, one needs to determine if the transporters are recycled back to the plasma membrane or are targeted for degradation. Given that DAT is degraded in one system and enters a recycling endosome in another (see above), it seems possible that this cellular decision could be regulated by the cellular milieu or may depend on activation of other cellular signals. A regulated fate would provide additional opportunities to fine tune the duration of the effects of reducing the numbers of transporters at the plasma membrane, presumably recycling the transporters would be faster than synthesizing new transporters. At present, there is very little evidence that that transporter phosphorylation is required for regulation by PKC. If it isn't, it implies that other proteins are required for these effects.

There is also evidence that growth factor receptor signaling pathways can increase the cell surface expression of many of the transporters, but in some cases it appears that these effects many be constitutively active in absence of added growth factor. Interestingly although the effects of PKC activation on GAT1 and EAAC1 are quite different, growth factor signaling increases the cell surface expression of both of these transporters.

Extracellular neurotransmitter also appears to regulate the cell surface expression of many of these transporters. In the simplest scheme, one would think that this would provide a homeostatic mechanism and that increases in extracellular transmitter would increase cell surface expression of the transporter. This would be analogous to agonist dependent internalization of receptors which presumably serves to limit the impact of prolonged receptor activation. With the transporters, substrates appear to have very different effects. For example, the cell surface expression of GAT1 increases in response to substrate, while substrate causes DAT to decrease. For two of the transporters, SERT and EAAC1, substrate has no effect on the cell surface expression under basal conditions, but blocks the PKC-induced redistribution. For both of these transporters, the implication is that coupling of the transporter to PP2A mediates this effect of substrate. If correct, it is remarkable that this form of coincidence detection is preserved in these genetically unrelated transporters that are regulated in opposite directions by PKC.

Understanding the mechanisms that contribute to the regulation of neurotransmitter transporters has the potential to impact on many different areas of neuroscience research and drug use. These transporters control both the spatial and temporal distribution of neurotransmitter after release into the extracellular space. These transporters are the targets of many different clinical drugs and the sites of action of drugs of abuse. As this relatively young field continues to mature, it will be interesting to learn more about the mechanisms of this regulation and the impact of these diverse types of regulation on normal and abnormal brain function.

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