Adenosine A<sub>3</sub> Receptors Regulate Serotonin Transport via Nitric Oxide and cGMP*

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Many antidepressants inhibit 5-hydroxytryptamine (5HT) transport resulting in increased 5HT levels in the synapse. However, physiological regulation of neurotransmitter uptake has not been demonstrated. We have examined the effect of receptor-activated second messengers on the 5HT transporter in rat basophilic leukemia cells (RBL 2H3). Here, we show that activation of an A<sub>3</sub> adenosine receptor results in an increase of 5HT uptake in RBL cells, due to an increase in maximum velocity (V<sub>max</sub>). The A<sub>3</sub> adenosine receptor-stimulated increase in transport is blocked by inhibitors of nitric oxide synthase and by a cGMP-dependent kinase inhibitor. In fact, compounds that generate nitric oxide (NO) and the cGMP analog 8-bromo-cGMP mimicked the effect of A<sub>3</sub> receptor stimulation, suggesting that the elevation in transport occurs through the generation of the gaseous second messenger NO and a subsequent elevation in cGMP. Additionally, the 5HT transporter is differentially regulated by second messengers since direct activation of protein kinase C by phorbol esters decreases 5HT uptake by decreasing V<sub>max</sub>. Our results suggest that the changes in transport are due to a direct modification of the 5HT transporter, possibly by phosphorylation, which appears to alter the rate at which transport occurs. As the 5HT transporter in RBL cells is identical to that in neurons, our results suggest that analogous mechanisms may operate in the brain.

A major mechanism in the termination of the action of amino acid and monoamine neurotransmitters, including serotonin, is removal from the synapse by transporters present within the plasma membrane of presynaptic cells (1–4). In addition to neurons, the serotonin transporter is localized in various cell types in the periphery including platelets and lung endothelial cells as well (5). The serotonin transporter has been shown to be a site of interaction for antidepressant drugs such as fluoxetine (Prozac®), appetite suppressant drugs such as fenfluramine, and drugs of abuse such as 3,4-methylenedioxyamphetamine and cocaine (6–10). Recently the 5HT<sub>1</sub> transporter cDNA was cloned (11, 12) and found to belong to a superfamily of Na<sup>+</sup>/Cl<sup>-</sup>-dependent monoamine and amino acid neurotransmitter transporters, including those for dopamine and norepinephrine (13–15).

5HT transport inhibitors have been shown to alter brain function and behavior presumably by increasing the duration and concentration of 5HT present in the synapse (6, 16), suggesting that acute regulation of transport activity by membrane-bound receptors could alter serotonergic neurotransmission. Our studies and others have demonstrated an action of PKC on the 5HT transporter in platelets, endothelial cells, and RBL cells (17–19). The human serotonin transporter was found to be regulated by cAMP in a human choriocarcinoma cell line (JAR), through an increase in mRNA and protein levels (20). Receptor regulation of a glutamate transporter in primary astrocytes has been suggested but without characterization of the relevant second messengers involved and mechanism of regulation (21, 22). The rat basophilic leukemia cell line (RBL 2H3) provides a model system with cellular components similar to those involved in synaptic neurotransmission. We have taken advantage of the presence of both the 5HT transporter and the A<sub>3</sub> adenosine receptor in RBL 2H3 cells (23–25) to determine if serotonin transporter function can be directly modulated by a membrane-bound G-protein-coupled receptor.

EXPERIMENTAL PROCEDURES

Materials—Culture media (EMEM) was obtained from BioWhitaker, and penicillin, streptomycin, and fetal bovine serum were from Life Technologies, Inc. NECA, SNAP, L-NMMA, XAC, H-8, calmidazolium, and LY-83,583 were purchased from RBI. 8-Bromo-cGMP, methylene blue, hydroxyamine HCl, and L-arginine were purchased from Sigma. Fluoxetine was a gift from Eli Lilly. [H]Paroxetine, [H]Leucine, [H]Alanine, and the 125I-cGMP RIA kit were purchased from DuPont NEN.

Cell Culture—RBL 2H3 cells were maintained in EMEM containing 15% fetal calf serum, L-glutamine, and penicillin/streptomycin. Cells for assay were plated in 24-well culture plates in EMEM containing 2.5% fetal calf serum, L-glutamine, and penicillin/streptomycin. With continuing passage uptake levels in the RBL 2H3 cells occasionally declined. Differences in V<sub>max</sub> and K<sub>m</sub> between the NECA-treated experiments and PMA-treated experiments are due to the use of cells from different passages. For a given treatment (PMA or NECA), percent changes in uptake were the same regardless of passage number.

Uptake Assay—All drug treatments were done in the media. After drug treatment the medium was removed, and the cells were washed once and then placed in uptake buffer (25 mM HEPES, 4.8 mM KCl, 1 mM KH<sub>2</sub>PO<sub>4</sub>, 1.3 mM CaCl<sub>2</sub>, 1.2 mM MgSO<sub>4</sub>, 5.6 mM glucose, 125 mM NaCl, 1 mM sodium ascorbate, and 10 µM pargyline). 100 nM [3H]5HT was added to each well for 15 min. Nonspecific uptake was determined by adding 10 µM fluoxetine in Na<sup>+</sup>-free media for 10 min prior to the addition of [3H]5HT. Medium was aspirated from the wells, which were then washed three times with ice-cold uptake buffer. The cells were then lysed with 0.5 N NaOH and counted for tritium in liquid scintillation mixture.

[3H]Paroxetine Binding—10<sup>6</sup> cells/well were grown in six-well plates. After treatment the cells were washed once with uptake buffer and varying concentrations of ligand (0.05–2 nM) were added to each well. 10 µM fluoxetine was used to determine nonspecific binding. After a 2-h incubation at room temperature the solution was aspirated, and the cells were washed twice with 1 ml of ice-cold uptake buffer. 1 ml of 1% SDS was used to solubilize the cells. The lysate was then monitored for tritium. Attempts to do binding on whole cells in suspension resulted in a significantly lower specific signal, approximately 25% below that in

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The abbreviations used are: 5HT, 5-hydroxytryptamine; NO, nitric oxide; PKC, protein kinase C; RBL, rat basophilic leukemia; NECA, 5′,N′-carboxamidoadenosine; XAC, xanthine amine congener; N<sub>0</sub>-CPTA, N<sub>0</sub>-cyclopropyladenosine; PMA, phorbol 12-myristate 13-acetate; NOS, nitric oxide synthase; L-NMMA, N<sub>0</sub>-monomethyl-L-arginine acetate, SNAP, S-nitroso-N-acetylpenicillamine; RIA, radioimmunoassay; IP<sub>3</sub>, inositol trisphosphate.

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A

Fig. 1. Effect of adenosine agonists and modulators of cGMP on the uptake of [3H]5HT into RBL 2H3 cells. A, cells were treated with either 1 μM NECA (closed symbols) or vehicle (open symbols) for 15 min. Values are expressed as the mean of three experiments done in triplicate ± S.E. B, cells were treated for the indicated times with 1 μM NECA. Values are expressed as the mean of three experiments done in triplicate ± S.E. C, cells were treated with N°-cyclopentyladenosine and 8-bromo-cGMP for 15 min. The cells were treated with XAC for 5 min preceding the addition of NECA for a total of 20 min (solid bar). H-8 was applied 15 min before NECA for a total of 30 min (solid bar). The hatched bars indicate the effect of XAC and H-8 alone. Values are expressed as the mean of three experiments done in triplicate ± S.E. * indicates significantly different (p < 0.01) by Student's t test.

cells grown as monolayers in plates. A similar observation has been documented for the dopamine transporter (26).

cGMP RIA—RBL cells were plated in 12-well plates at 2.5 × 10^5 cells/well. Prior to assay the cells were incubated in Eagle's 2 medium with 20 mM HEPES with 1 mM CaCl_2 and 0.1 mM MgSO_4 for 30 min at 37 °C. NECA, XAC, and L-NAME were added for the same amount of time as in uptake experiments. The assay was terminated by the addition of 0.1 N HCl, 0.1 mM CaCl_2, and the plates frozen until assayed. cGMP was assayed by radioimmunoassay (New England Nuclear).

[3H]Alanine and [3H]Leucine Uptake—Uptake assay conditions and treatments were the same as that for [3H]5HT. Uptake was for 2 min with 10 nM [3H]alanine and 5 nM [3H]leucine, respectively.

RESULTS

Effect of NECA on [3H]5HT Uptake in RBL 2H3 Cells—Incubation of RBL cells with the adenosine receptor agonist NECA led to an increase in [3H]5HT uptake (Fig. 1A), demonstrating that the function of the 5HT transporter can be regulated by a membrane-bound G-protein-coupled receptor. The increase in uptake was due to an elevation in the V_max accompanied by an increase in the Michaelis-Menten constant for transport (K_m) (Table I). The adenosine receptor antagonist XAC fully blocked the effect of NECA (Fig. 1C) while another adenosine receptor agonist N°-cyclopentyladenosine increased
Effect of NECA on transport (Fig. 3). Each of the three compounds inhibited the effect of NECA on transport (Fig. 3). The maximal elevation of \( ^{3}H \)5HT uptake occurred within 10 min of the application of NECA and was followed by a gradual decline (Fig. 1B).

Effect of PKC Activation on \( ^{3}H \)5HT Uptake—Previous characterization of the second messenger pathways of the A3 adenosine receptor in RBL 2H3 cells demonstrated that the receptor was coupled to an elevation in IP_{3} and Ca^{2+} via a pertussis and cholera toxin-sensitive G-protein (28, 29). Elevations in intracellular calcium and IP_{3} could lead to the stimulation of various protein kinases such as PKC. In contrast to the effect of NECA, however, direct stimulation of PKC with 0.1 \( \mu \)M of the phorbol ester PMA decreased 5HT uptake by decreasing the \( V_{\text{max}} \) without a significant change in \( K_{\text{m}} \) (Table I). The inactive phorbol ester 4\( \alpha \)-phorbol 12-myristate 13-acetate had no effect on uptake (data not shown). The effect of PKC activation demonstrates that the 5HT transporter can be differentially regulated by intracellular second messengers.

Influence of Modulators of cGMP on 5HT Uptake—Since the A_{3} adenosine receptor in RBL 2H3 did not effect cAMP levels (28), and PKC had an opposing effect, we examined whether cGMP might mediate the effect of NECA on uptake. In fact, the cell permeant cGMP analog 8-bromo-cGMP mimicked the effect of NECA (Fig. 1C). The relatively selective cyclic nucleotide-dependent protein kinase inhibitor H-8 was found to block the effect of NECA on the 5HT transporter as well (Fig. 1C). Thus, it appeared that the increase in 5HT transport due to NECA was mediated by cGMP.

Effect of NOS Inhibitors on the NECA-induced Elevation in Uptake—Production of cGMP can be stimulated by the gaseous second messenger nitric oxide (30). NO production results from the activation of nitric oxide synthase (NOS) (31). The NOS inhibitors L-NAME and NMMA blocked the effect of NECA on uptake while having no effect themselves (Fig. 2A). The inhibition of NECA-stimulated NOS by L-NAME was partially reversed by 100 \( \mu \)M L-arginine, the natural substrate of NOS. NO can be generated directly by the NO donors SNAP and hydroxylamine. Both compounds increased the level of uptake with maximal stimulation occurring at 10 min, paralleling the time course of the effect of NECA on 5HT uptake (Fig. 2B). These two NO generating compounds produce NO by two distinct pathways (32) and therefore confirm a role for NO in stimulating 5HT uptake.

Effect of Calmidazolium, LY-83,583, and Methylene Blue on 5HT Uptake—Certain isoforms of NOS have been demonstrated to be dependent on calmodulin (which is blocked by calmidazolium) for the production of NO (31). Methylene blue has been shown to block the action of NO by scavenging NO, and/or by inhibiting soluble guanylyl cyclase (30) while LY-83,583 inhibits guanylyl cyclase (33). Therefore, we examined the effect of these compounds on A_{3} adenosine receptor activation of 5HT uptake. While all three compounds inhibited the effect of NECA on transport (Fig. 3), each of the three compounds also inhibited uptake when added alone. These data raise the possibility that tonic activation of the NO-cGMP pathway may regulate the activity of the 5HT transporter in the RBL 2H3 cell under basal conditions.

Effect of NECA and NO Generating Compounds on cGMP Levels—Direct measurement of cGMP levels after A_{3} receptor stimulation with NECA showed an increase in cGMP to 170% of basal levels. This effect was blocked by the antagonist XAC. The NOS inhibitor L-NAME blocked the NECA-induced increase in cGMP levels as well (Fig. 4A). SNAP produced a 2.5-fold elevation in cGMP with an EC_{50} of 12 \( \mu \)M, while hydroxylamine elevated cGMP levels by 2-fold with an EC_{50} of 30 \( \mu \)M (Fig. 4).

Binding of \( ^{3}H \)Paroxetine to RBL 2H3 Cells—The changes in uptake produced by NECA and PMA could be the result of changes in the number of transporters at the cell surface, reflecting regulated intracellular trafficking. Analogous mechanisms have been described for insulin regulation of glucose transport in adipocytes and PMA regulation of \( \gamma \)-aminobutyric acid transport in Xenopus oocytes (34, 35). However, the total number of \( ^{3}H \)paroxetine-binding sites (\( B_{\text{max}} \)) present on the cell surface was not altered by either PMA or NECA treatment (Table I). Binding in membrane homogenates was also not changed suggesting no significant degradation was occurring (data not shown). Therefore, changes in \( V_{\text{max}} \) were apparently not due to a change in transporter number.

Effect of Treatments on Other Uptake Systems—Changes in uptake could be due to the alteration of ion gradients as the transporter is dependent on Na^{+} and Cl^{-} or could possibly be generalized to all transporters in the RBL 2H3 cell. Therefore, we examined the effect of SNAP, 8-bromo-cGMP, and PMA on Na^{+}-dependent alanine uptake and Na^{+}-independent leucine uptake. These second messenger activators had negligible effect on the Na^{+}-dependent uptake of \( ^{3}H \)alanine, indicating that the changes in 5HT uptake following the same treatments were not due merely to an alteration in the Na^{+} gradient. The effect of these three agents on Na^{+}-independent \( ^{3}H \)leucine uptake was opposite to their effect on 5HT transport in that PMA increased while SNAP and 8-bromo-cGMP decreased \( ^{3}H \)leucine uptake, indicating that the observed changes in 5HT uptake were not generalized to all transporters in the RBL cell (Table II).

Uptake in Plasma Membrane Vesicles—To further examine if the changes in 5HT uptake were due to altered ion gradients, the effects of PMA, SNAP, and 8-bromo-cGMP on \( ^{3}H \)5HT up-

| Table I |
|-----------------|-----------------|
| \( ^{3}H \)5HT uptake | \( ^{3}H \) paroxetine binding |
| \( V_{\text{max}} \) | \( K_{m} \) | \( B_{\text{max}} \) | \( K_{d} \) |
| pmol/ml/min/well | \( \mu \)M | pmol/10^{6} cells | nm |
|-----------------|-----------------|
| Control | 0.97 ± 0.02 | 0.2 ± 0.01 | 1.4 ± 0.5 | 1.3 ± 0.5 |
| NECA | 1.7 ± 0.1* | 0.6 ± 0.1* | 1.2 ± 0.2 | 1.0 ± 0.1 |
| Control | 2.4 ± 0.2 | 0.5 ± 0.3 | 1.4 ± 0.5 | 1.3 ± 0.5 |
| PMA | 1.3 ± 0.2* | 0.4 ± 0.2 | 1.2 ± 0.5 | 1.0 ± 0.2 |

* significantly different than control (p < 0.01) by Student’s t test.
Effects of second messenger activators on \(\text{[^3H]}\)Alanine and \(\text{[^3H]}\)Leucine uptake in whole RBL 2H3 cells

The cells were preincubated with PMA for 1h and for 10 min with SNAP and 8-bromo-cGMP.

| Treatment      | \(\text{[^3H]}\)Alanine (% of control) | \(\text{[^3H]}\)Leucine (% of control) |
|---------------|--------------------------------------|--------------------------------------|
| PMA           | 89 ± 8                               | 120 ± 8*                             |
| SNAP          | 108 ± 8                              | 87 ± 6*                              |
| 8-Br-cGMP     | 107 ± 5                              | 79 ± 8*                              |

* significantly different than control \((p < 0.01)\) by Student's \(t\) test.

This likely the result of modification of the transporter itself. Our results with calmidazolium are in agreement with observations in the JAR cells. While these studies have suggested regulation of 5HT transport by receptor-mediated second messenger production, a single study has demonstrated the regulation of transport via direct stimulation of a G-protein-coupled receptor. Hansson and Ronnback (21, 22) have shown that glutamate transport can be regulated by \(\alpha\)-adrenergic receptor activation in primary astrocyte cultures. However, neither the relevant second messenger systems nor the mechanism of regulation was characterized.

The existence of the \(\text{A}_3\) adenosine receptor in the RBL 2H3 cell line has been clearly established by Northern blot analysis and radioligand binding (24). Activation of the receptor in these cells results in the elevation of calcium and IP\(_3\) levels via a pertussis and cholera toxin-sensitive G-protein (28). Activation of the receptor with NECA has been shown to potentiate IgE receptor-induced release of 5HT but does not induce release by itself (28). The \(\text{A}_3\) receptor was found to inhibit forskolin-stimulated cAMP levels in transfected cells but not in RBL cells (25, 28). Our finding that the \(\text{A}_3\) adenosine receptor couples to cGMP via NO demonstrates a previously uncharacterized second messenger pathway for this receptor. Activation of the receptor led to an increase in cGMP levels as measured by RIA and was blocked by an NOS inhibitor as well as by an \(\text{A}_3\) receptor antagonist.

We have demonstrated that the \(\text{A}_3\) receptor regulates the 5HT transporter via NO and cGMP by the following proposed pathway. Activation of the receptor leads to an increase in calcium levels which would be necessary for activation of NOS (31). Subsequent production of NO would lead to the activation of cGMP production by stimulating soluble guanylyl cyclase (30). cGMP would then activate cGMP-dependent kinase which would then phosphorylate either the transporter directly or some other closely associated protein. Evidence for this pathway is supported in several ways by our data. The response to receptor activation was blocked by inhibitors of NOS and by an inhibitor of cyclic nucleotide-dependent kinases as well as by the receptor antagonist XAC. The response was also blocked by two inhibitors of soluble guanylyl cyclase. NO donating compounds each increased cGMP and mimicked the effect of NECA on 5HT uptake. Direct application of the cGMP analog 8-bromo-cGMP itself to the RBL cultures produced similar results to those produced by the \(\text{A}_3\) adenosine receptor agonists. Our results suggest cGMP mediated phosphorylation which could effect transport by 1) direct phosphorylation of the transporter, 2) changes in the number of transporters at the cell surface, or 3) by altering ion gradients. However, \(\text{[^3H]}\)paroxetine binding indicates no apparent change in transporter number (Table I), and changes in ion gradients are not responsible for altering uptake (Tables II and III). Therefore, it is likely that direct phosphorylation of the transporter or a closely associated protein accounts for the observed changes. One cau-
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In and Kemp (47) The numbers in the large open circles represent the potential protein kinase sites. Black shaded circles indicate amino acids conserved among the Na+/Cl--dependent transporters, and gray shaded circles indicate amino acids conserved among the monoamine transporters. The consensus site number 8 within the triangle is conserved among the monoamine transporters.

Fig. 5. Potential consensus protein kinase sites on the 5HT transporter. Consensus sites were identified using sequences from Pearson among the Na+/Cl--dependent transporters, and gray shaded circles indicate amino acids conserved among the monoamine transporters. The consensus site number 8 within the triangle is conserved among the monoamine transporters.

...in interpreting [3H]paroxetine binding is the possibility that we are measuring total binding since paroxetine is likely to be membrane permeant. Fractionation of different membrane pools followed by [3H]paroxetine binding might address intracellular trafficking more directly.

The inhibition of basal 5HT uptake by the compounds calmidazolium, methylene blue, and LY-83,583, each alone, raises the interesting possibility that 5HT transport can be tonically regulated by cGMP levels in the RBL cell. The data suggest that calmidazolium may be blocking calmodulin from interacting with a constitutive NOS to produce NO while LY-83,583, and methylene blue might block the activation of guanylyl cyclase triggered by NO. However, we did not see changes in basal uptake due to the NOS inhibitors themselves and increased levels of L-arginine did not stimulate uptake, indicating that NOS activity was not playing a role in this case. The blockade of calmodulin could have a variety of effects on several different second messenger systems and enzyme activities. Alternatively, tonic activation of guanylyl cyclase could be occurring, perhaps via eicosanoids such as arachidonic acid metabolites (39) or other gaseous second messengers such as carbon monoxide (40, 41). Methylene blue and LY-83,583 would then decrease basal 5HT uptake by inhibiting basal guanylyl cyclase activity.

Our findings, together with previous evidence that second messenger activation can alter the rate of transport without effecting the level of transporters in various cell types (17, 18), support the proposition that second messengers may converge to alter 5HT uptake by phosphorylation of the transporter or a closely associated protein. The preparation of plasma membrane vesicles removes cytosolic factors and therefore suggests that changes are occurring at the level of the transporter or related protein in the membrane. In addition, these changes are stable, persisting over the course of vesicle preparation and the subsequent uptake assay. Potential PKC, cAMP-dependent, and cGMP-dependent protein kinase sites have been identified on the intracellular domains of the cloned rat and human 5HT transporters primarily on the terminal regions (11, 12, 36). The PKC site present on the second intracellular loop is conserved among the known members of the Na+/Cl--dependent gene family (15). Phosphorylation of the glutamate transporter by PKC has been recently demonstrated (42), suggesting that other transporters may be similarly affected. Phosphorylation of the monoamine transporters has yet to be demonstrated.

Since NO freely diffuses across membranes it may act both intracellularly and intercellularly (43) to rapidly increase 5HT uptake in the brain. While it is unknown whether A1 adenosine receptors are localized to neurons possessing the 5HT transporter, it is likely that other receptors that can couple to NO and/or cGMP are. In fact, Wouterspoon et al. (44) have recently demonstrated that NADPH-diaphorase staining (which labels NOS) and 5HT immunoreactivity were colocalized in neurons present in the medial dorsal raphe nucleus. Thus, 5HT transporters present on one population of cells (i.e. presynaptic neurons or glia) may be regulated by NO produced in another (i.e. postsynaptic neurons). As sequence analysis has demonstrated that the transporter present in RBL 2H3 cells is the same as that expressed in brain, second messenger signaling events that regulate 5HT transporter activity in RBL cells may be relevant to what occurs in the brain. Preliminary results from treatment of hippocampal and cortical synaptosomes with SNAP or 8-bromo-cGMP demonstrated an increase in [3H]5HT...
uptake (in the presence of reserpine), while treatment with PMA resulted in decreased levels of uptake. Our data in synaptosomes imply conserved mechanisms in the regulation of 5HT uptake between mast cells and the brain (neurons and glia).

As the action of 5HT in the central nervous system is termin-

ated primarily by uptake into presynaptic neurons or sur-

rounding glia, significant alterations in the rate of transport could result in physiologically significant changes in serotonin neurotransmission. A change in the rate of uptake by NO and cGMP could shorten the time during which a neurotransmitter could interact with pre- and post-synaptic receptors and may limit the range of diffusion of the transmitter to surrounding cells such as neurons and glia. Since the precise density and localization of transporters and receptors at the synapse have not been clearly delineated, it is difficult to estimate the precise change in the range of diffusion from the synapse and what effect this may have in vivo. In fact, it has been demonstrated that acute treatment with 5HT uptake inhibitors alters neuronal activity by affecting 5HT autoreceptors (45). Regulation of transporter activity in the brain could play a role in numerous processes including long term potentiation where cGMP and cGMP-dependent kinase have been shown to enhance long term potentiation (46). Other retrograde messengers such as arachidonic acid metabolites and carbon monoxide may also converge to regulate neurotransmitter uptake and neurotransmitter levels in the synapse (40, 41).

In summary, our data demonstrate that a neurotransmitter transporter can be rapidly regulated by a membrane-bound G-protein coupled receptor in RBL cells and that this occurs via NO and cGMP. In conjunction we have found that the A1 adenosine receptor in RBL cells is coupled to NO and cGMP. Furthermore, the function of the 5HT transporter can be differentially regulated by two distinct second messenger systems. Thus, alterations in the activity of transporters via receptor activation may result in significant changes in neurotransmitter concentrations in the synapse and its surroundings. These data raise the possibility that abnormal regulation of transporter function is involved in the etiology of psychiatric and neurological disorders.

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