Identification and Characterization of a Novel Monoamine Transporter in Human Brain

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Running title: cloning of a novel brain monoamine transporter
SUMMARY

Precise control of monoamine neurotransmitter levels in the extracellular fluids of the brain is critical in maintaining efficient and robust neurotransmission. High affinity transporters in the SLC6A family function in removing monoamines from the neurosynaptic cleft. Emerging evidence suggests that these transporters are only one part of a system of transporters that work in concert to maintain brain homeostasis of monoamines. Here we report the cloning and characterization of a new human plasma membrane monoamine transporter, PMAT. The PMAT cDNA encodes a protein of 530 amino acid residues with 10-12 transmembrane segments. PMAT is not homologous to known neurotransmitter transporters, but exhibits low homology to members of the equilibrative nucleoside transporter family. When expressed in MDCK cells and Xenopus laevis oocytes, PMAT efficiently transports serotonin ($K_m=114$ µM), dopamine ($K_m=329$ µM), and the neurotoxin 1-methyl-4-phenylpyridinium (MPP+) ($K_m=33$ µM). In contrast, there is no significant interaction of PMAT with nucleosides or nucleobases. PMAT-mediated monoamine transport does not require Na$^+$ or Cl$^-$, but appears sensitive to changes in membrane potential. Northern blot analysis showed that PMAT is predominantly expressed in the human brain and widely distributed in the central nervous system. These studies demonstrate that PMAT may be a novel low affinity transporter for biogenic amines which under certain conditions might supplement the role of the high-affinity transporters in the brain.
INTRODUCTION

In the central nervous system (CNS), monoamine neurotransmitters, including dopamine, serotonin and norepinephrine, control a variety of physiological, behavioral and endocrine functions (1,2). Monoamine-mediated neurotransmission is also critically involved in a number of brain pathological processes such as Parkinson’s disease, depression, schizophrenia, and drug addiction (1,2). A key step that determines the intensity and duration of monoamine signaling is the transport of released monoamines into brain cells. This process is carried out by cell surface transporters that transport monoamines into presynaptic nerve terminals or neighboring glial cells, where they can be recycled through repackaging into secretory vesicles or degraded by intracellular enzymes (3-5).

Uptake of released monoamines into presynaptic neurons is mainly carried out by a family of Na$^+$ and Cl$^-$ dependent high affinity plasma membrane transporters, which includes the dopamine transporter (DAT), the serotonin transporter (SERT), and the norepinephrine transporter (NET) (3-5). These transporters, which share high sequence similarity and belong to the solute carrier 6A (SLC6A) family, are the known targets for many psychostimulants, antidepressants and neurotoxins (3-5).

Several lines of evidence suggest that in addition to the SLC6A high affinity transporters (i.e., DAT, SERT and NET), the brain expresses other transporters to regulate extracellular monoamine levels. First, a number of studies have described CNS monoamine uptake activities that exhibit properties distinct from the SLC6A high affinity transporters (6-9). Examples of such properties include Na$^+$-independency (7,8), lower substrate affinity (6,7,9), or insensitivity to inhibitors of the high affinity transporters (7-
9). Second, several studies have reported specific dopamine or serotonin uptake in brain regions that do not express SERT or DAT (10-12). Finally, recent studies from DAT and SERT knockout mice also provide indirect evidence for the existence of alternative monoamine transporters in the brain (9,13). While mechanisms, such as diffusion (14), heterogenic uptake (15), or uptake by organic cation transporters (OCTs) (9,12), have been proposed to explain these observations, an alternative could be that the brain expresses other monoamine transporters that have not yet been identified at the molecular level.

In this study, we report the cloning and characterization of a novel plasma membrane monoamine transporter, PMAT. We show that PMAT is molecularly distinct from the classic monoamine transporters, abundantly expressed in the human brain and efficiently transports monoamine neurotransmitters. Our findings provide new insights into the complexity and potential compensatory mechanisms of regulating monoamine neurotransmitter levels in the brain.

**EXPERIMENTAL PROCEDURES**

*Cloning of PMAT*—Using BLAST search at the National Center for Biotechnology Information, we identified a hypothetical ENT-like protein predicted by the human genome annotation project. Primers were designed to amplify a cDNA fragment from human kidney by RT-PCR. 5'- and 3'- rapid amplification of cDNA ends (RACE) were used to obtain the flanking region sequences as described previously (16). A pair of primers (5'-GAGAGGCTGCCATGGGCTCCGTGGGGAGC-3' and 5'-CGGTCCTCGGAGGACTTTGCAGAACTTCAGTCC-3') flanking the open reading
frame were used to isolate the full-length cDNA from human kidney and brain by RT-PCR. BLAST was used in database searching, and the Vector NTI Suite 8 software (InforMax, Frederick, MD) was used to analyze nucleotide and protein sequences.

*Northern Analysis*—Human multiple tissue northern blots (Clontech, Palo Alto, CA) were hybridized to a random-primed [\(^{35}\)S]dCTP-labeled cDNA fragment of PMAT for 16 h at 68°C in PerfectHyb Plus hybridization buffer (Sigma, St. Louis, MO) and washed under high stringency conditions (0.5 X SSC, 0.1% SDS at 68°C).

*YFP Construct and Fluorescence Microscopy*—PMAT cDNA was subcloned into BglII and XbaI sites of the yellow fluorescence vector pEYFP-C1 (Clontech, Palo Alto, CA) and transfected into MDCK cells. Empty pEYFP-C1 vector was transfected into MDCK cells as a control. YFP positive cells were purified using the FACS Vantage SE flow cytometry sorter (BD Bioscience, San Jose, CA). Sorted cells were seeded on a chambered cover glass and visualized with a Leica Spectral confocal microscope.

*Expression and Functional Characterization in MDCK Cells*—PMAT cDNA was subcloned into pcDNA3 vector (Invitrogen, Carlsbad, CA) and transfected into MDCK cells using Lipofectamine (Invitrogen, Carlsbad, CA). Stably transfected cell lines were obtained by G418 selection and cultured in Minimum Essential Medium containing 10% fetal bovine serum and G418 (200 µg/ml). For the transport experiments, cells were plated in 24-well plates and allowed to grow for 3 days. Transport assays were performed at 37°C in Krebs-Ringer-Henseleit (KRH) buffer (5.6 mM glucose, 125 mM NaCl, 4.8 mM KCl, 1.2 mM KH\(_2\)PO\(_4\), 1.2 mM CaCl\(_2\), 1.2 mM MgSO\(_4\), 25 mM HEPES,
pH 7.4) containing a [³H]-labeled ligand. Uptake was terminated by washing the cells three times with ice-cold PBS buffer, and cells were solubilized and the radioactivity was quantified by liquid scintillation counting. Ascorbic acid (100 µM) and pargyline (10 µM) were present in experiments with monoamines to prevent oxidation. Sodium dependency was assessed in KRH buffer with isotonic replacement of NaCl with N-methyl-D-glucamine, whereas Cl⁻ dependency was assessed in KRH buffer with chloride salts replaced by corresponding gluconate salts at equivalent molarity. Protein content in each uptake well was measured using a BCA protein assay kit (Pierce, Rockford, IL) and the uptake in each well was normalized to its protein content. The experiments were performed in triplicates and repeated at least three times, and results from the representative experiments are expressed as mean ± S.D. Statistical significance was determined by Student’s t-test. Kinetic parameters were determined by nonlinear least-squares regression fitting as described previously (17).

Expression in Xenopus laevis Oocytes—cRNA of PMAT was synthesized and injected into defolliculated oocytes as described previously (17). Uptake was measured on groups of 8-10 oocytes 2-3 days post-injection at 25°C in transport buffer (100 mM NaCl, 2 mM KCl, 1 mM CaCl₂, 1 mM MgCl₂, and 10 mM HEPES, pH 7.4) containing a [³H]-labeled ligand. Uptake was terminated by washing oocytes 5 times with ice-cold buffer. Individual oocytes were then solubilized in 10% SDS and the radioactivity was quantified by liquid scintillation counting.

RNA Interference Study—siRNAs were synthesized by Dhharmacon (Lafayette, CO) and Ambion (Austin, TX). The siRNA duplex with the following sense and antisense sequence was used: 5’-CAGCUUCAUCACGGACGUGtt-3’ and 5’-
CACGUCCGUGAUGAAGCUGtt-3’. Sense and antisense RNAs were annealed following the manufacturer’s recommended procedures. The human astrocytoma cell line A172 (American Type Culture Collection, Rockville, MD), and PMAT- and vector-transfected MDCK cells were cultured in DMEM medium containing 10% fetal bovine serum. A172 cells were plated in 24-well plates at 3 X 10⁴ cells/well, PMAT- and vector-transfected MDCK cells at 8 X 10⁴ cells/well. Cells were grown for 24 h, then transfected with siRNA (40 pmol/well) using lipofectamine and OPTI-MEM I reduced serum medium (Invitrogen, Carlsbad, CA). Silencing was examined 48-72 h after transfection by uptake assays as described above. Control cells (i.e. mock cells) were treated with lipofectamine and OPTI-MEM I reduced serum medium without the siRNA. Semi-quantitative RT-PCR was performed to examine PMAT mRNA expression using the following PMAT specific primers: (5’-CGGGCGTGATGATCTCTCTGAGCCGCATC-3’ and 5’-GGTTGAACACAGCCATGATGAGGATGGGCA-3’). GAPDH primers (5’-CGTATTGGGCGCCTGGTCACCAGGGCTGCT-3’ and 5’-TTGAGGGCAATGCCAGCCCCAGCGTCGAAG-3’) were used as an internal control. Amplification conditions were 94°C for 1 min, 55°C for 1 min, and 72°C for 1 min. PMAT amplification was performed for 20 cycles for PMAT-transfected MDCK and 36 cycles for A176 cells and vector-transfected MDCK cells. GAPDH amplification was performed for 20 cycles for all samples.

RESULTS

Cloning of PMAT—We initially identified PMAT from the draft human genome database due to its sequence homology to equilibrative nucleoside transporters (ENTs) (18,19). Using 5’- and 3’-RACE and RT-PCR, we isolated a 2,112 base pair complementary DNA from human kidney poly (A)+ RNA. The open reading frame encodes a protein of 530
amino acids with a calculated molecular mass of 58 kDa (Fig. 1a). A BLAST search revealed that PMAT is not highly homologous to any known proteins and only exhibits low sequence identity (~20%) to mammalian ENTs (SLC29A) (Fig. 1b). The PMAT gene was previously named human equilibrative nucleoside transporter 4 (hENT4) and assigned to chromosome 7p22.2 (18,20,21). The deduced protein sequence of PMAT is identical to the hENT4 protein predicted from the human genomic DNA (accession number BK000627) (20). The protein sequence is also identical to that of an hENT4 clone (accession number BC047592) recently isolated from a human brain cDNA library (21) except for three amino acid substitutions at positions 79 (Glu/Val), 124 (Lys/Asn) and 429 (Thr/Pro). Gene orthologs of PMAT were also found in mouse (20) and rat (Wang, unpublished observation). Hydropathy analysis predicted the presence of 10-12 membrane-spanning domains in PMAT. An 11 transmembrane domain model is proposed in Fig. 1c. There are one potential N-linked glycosylation site (Asn-523), several putative protein kinase C phosphorylation sites (Ser-6, -262, -268, -306, -476 and Thr-338), and one cAMP-dependent protein kinase phosphorylation site (Thr-199).

Tissue Distribution—Expression of PMAT in various human tissues was examined by high stringency Northern blotting. A strong, single hybridization signal of 2.9 kilobase (kb) was detected in adult human brain and skeletal muscle (Fig. 2). Weak hybridization signals were also observed in kidney, heart and liver. In the human brain, strong hybridization signals were found in all regions tested (cerebellum, cerebral cortex, medulla, occipital pole, frontal and temporal lobes, and putamen) and in spinal cord (Fig. 2), suggesting that PMAT is widely distributed in the CNS. A BLAST search of human
EST database also found PMAT expression in various brain regions and in cells of neuronal and glial origins.

Membrane Localization—To determine whether PMAT is a plasma membrane or an intracellular transporter, we designed an expression construct that fused the yellow fluorescence protein (YFP) to the N-terminus of PMAT. When expressed in MDCK cells and observed by confocal microscopy, the fusion protein was primarily localized to the plasma membrane (Fig. 3), suggesting that PMAT is a plasma membrane protein.

Substrate Specificity—To ascertain the functional properties of PMAT, we used MDCK cells to establish a cell line stably expressing PMAT and screened a variety of radio-labeled compounds for uptake activity. Despite its sequence similarity to the equilibrative nucleoside transporters, PMAT does not significantly transport nucleosides and nucleoside analogs such as uridine, cytidine, thymidine, adenosine, inosine, guanosine, and azidothymidine (Fig. 4). There was also no significant uptake of nucleobases (e.g. uracil, cytosine, adenine, hypoxanthine) or nucleotides (e.g. ATP). Instead, cells expressing PMAT exhibit a high transport activity for [3H]-labeled MPP⁺ (Fig. 4), a neurotoxin that causes Parkinson’s disease by selectively damaging dopaminergic nerve terminals. PMAT-mediated MPP⁺ uptake is time and concentration dependent with an apparent Michaelis constant ($K_m$) of $33 \pm 7 \ \mu M$ and a maximal transport rate ($V_{max}$) of $2.8 \pm 0.1$ nmol per min per mg protein (Fig. 5).

To further define the substrate specificity of PMAT, we performed competition studies using [3H]MPP⁺ as the substrate. Several biogenic amines (serotonin, dopamine,
norepinephrine, epinephrine, histamine) significantly inhibited the \([^{3}\text{H}]\text{MPP}^+\) uptake (Fig. 6). The neurotransmitter acetylcholine also marginally inhibited PMAT-mediated \([^{3}\text{H}]\text{MPP}^+\) uptake. In contrast, various nucleosides, nucleobases, the neurotransmitter GABA and other endogenous compounds exhibited no inhibitory effect (Fig. 6). Prototypical substrates of OCTs (e.g. TEA and guanidine) and the organic anion transporters (e.g. PAH) also did not significantly inhibit PMAT transport activity. These data indicate that PMAT mainly functions as a transporter for biogenic amines.

We next tested whether biogenic amines are indeed substrates for PMAT by directly testing the uptake of radio-labeled compounds. At a substrate concentration of 1 \(\mu\)M and an incubation time of 1 min, PMAT-expressing MDCK cells showed 13- and 20-fold higher serotonin and dopamine uptake than control cells (Fig. 7a). A 4- to 5-fold increase was also seen for norepinephrine and epinephrine. The increase in acetylcholine uptake is only about 1.4 fold, suggesting that this neurotransmitter is a poor substrate for PMAT (Fig. 7a). To confirm that the observed enhanced uptake in MDCK cells was due to heterologous expression of PMAT, we also expressed PMAT in \(X.\ laevis\) oocytes. Compared to water-injected oocytes, oocytes injected with PMAT cRNA exhibited significantly enhanced uptake of MPP\(^+\), serotonin, and dopamine (Fig. 7b), further demonstrating that PMAT is a \textit{bona fide} transporter for monoamines.

Because of the sequence similarity of PMAT to the ENT nucleoside transporters, the possibility that ENTs may as well transport monoamines was investigated. Cells stably transfected with the prototypic ENT transporter, hENT1 (18,22), showed no transport activity for MPP\(^+\) (data not shown), suggesting that there is no significant substrate overlap between PMAT and the ENTs. Together these data demonstrated that
PMAT is functionally distinct from nucleoside transporters and has evolved, from the *ENT* gene family perhaps, to handle biogenic amines.

**Transport Kinetics**—The transport kinetics of PMAT towards various monoamines was determined from initial rate studies ([Fig. 8](#)). PMAT-mediated monoamine uptake was saturable and the kinetic parameters (*K*<sub>m</sub> and *V*<sub>max</sub>) are summarized in [Table 1](#) and are compared with those of MPP<sup>+</sup>. Among the compounds tested, PMAT has the highest affinity for MPP<sup>+</sup>, followed by serotonin and dopamine, and then by norepinephrine and epinephrine ([Table 1](#)). Under the same experimental conditions, the apparent maximal transport velocities (*V*<sub>max</sub>) are significantly higher for the low affinity substrates. When the transport efficiency (*V*<sub>max</sub>/*K*<sub>m</sub>) was calculated and normalized to MPP<sup>+</sup>, PMAT exhibited comparable transport efficiencies for MPP<sup>+</sup>, serotonin and dopamine, but much lower efficiencies for norepinephrine and epinephrine ([Table 1](#)). The apparent affinities (*K*<sub>m</sub>) of PMAT for serotonin and dopamine are 2-3 orders of magnitude lower than those of SERT and DAT. However, compared to SERT or DAT stably expressed in MDCK or LLC-PK1 cells (23,24), the *V*<sub>max</sub> values of PMAT-mediated serotonin or dopamine transport are also 2-3 orders higher than the *V*<sub>max</sub> values of SERT or DAT. These data suggest that PMAT is a low affinity but high capacity transporter for serotonin and dopamine.

**Ion Dependency**—To determine the bioenergetics of PMAT-mediated transport, we investigated the effect of Na<sup>+</sup> and Cl<sup>−</sup> gradients and membrane potential on PMAT-mediated MPP<sup>+</sup> uptake. Replacement of Na<sup>+</sup> or Cl<sup>−</sup> in the uptake medium had no effect on uptake activity ([Fig. 9a](#)), suggesting that PMAT-mediated transport does not involve a
Na\(^+\) and Cl\(^-\) cotransport mechanism. In contrast, depolarization of cells with increased extracellular K\(^+\) strongly reduced MPP\(^+\) uptake (Fig. 9b), indicating that transport is driven by the inside negative membrane potential. To exclude the possibility that the effect of K\(^+\) on PMAT could be a specific ionic effect, we conducted MPP\(^+\) uptake experiments in the presence of 10 mM barium with a normal extracellular K\(^+\) concentration. Barium is an effective potassium channel blocker that can greatly reduce membrane potential in MDCK cells (25). As shown in Fig. 9c, barium substantially reduced PMAT-mediated MPP\(^+\) uptake at a low, normal extracellular K\(^+\) concentration. These data suggest that the inhibitory effect of high external K\(^+\) on MPP\(^+\) uptake was unlikely due to a specific ion effect, but rather related to changes in membrane potential.

Interaction with Transport Inhibitors—To define the pharmacological properties of PMAT, we tested the sensitivity of PMAT to a number of known inhibitors of SERT, DAT, NET, and OCTs (Fig. 10). The calculated inhibition potencies (\(K_i\)) are summarized in Table 2. Among the inhibitors tested, the cyanine dye decynium-22, an OCT inhibitor, had the highest inhibition potency (\(K_i = 0.10 \, \mu M\)). Yet, another potent OCT inhibitor, corticosterone, had no inhibitory effect at concentrations as high as 200 \(\mu M\). The selective DAT inhibitor, GBR12935, inhibited PMAT with a \(K_i\) of 7.9 \(\mu M\). The antidepressant drug fluoxetine (Prozac), a selective SERT inhibitor, and the tricyclic antidepressant desipramine, a potent inhibitor of both NET and SERT, only inhibited PMAT-mediated uptake at micromolar concentrations (\(K_i = 22.7\) and 32.6 \(\mu M\), respectively). The inhibition potencies of these inhibitors towards PMAT are 2-3 orders of magnitude lower than those for DAT, SERT or NET, suggesting that PMAT is
relatively insensitive to the high affinity inhibitors of the SLC6A monoamine transporters.

RNA Interference Study—To determine whether PMAT indeed plays a role in monoamine uptake in cells of brain origin, we performed small interfering RNA (siRNA) studies in cultured human A172 astrocytoma cells. This cell line was chosen because it was derived from human brain and we detected significant endogenous expression of PMAT mRNA by RT-PCR. Several double-stranded RNAs (21 bp) corresponding to different regions of PMAT cDNA were synthesized and tested for their ability to knockdown PMAT expression in PMAT-transfected MDCK cells. Transfection of cells with one siRNA resulted in a significant and reproducible reduction in PMAT activity as judged by serotonin uptake (~63% decrease), with a corresponding reduction in PMAT mRNA expression level in transfected MDCK cells (Fig. 11). This siRNA was then used in A172 cells to test its effect on cellular uptake of serotonin. In parallel with a reduced PMAT mRNA expression level in siRNA treated A172 cells (Fig. 11a), a consistent decrease in serotonin uptake, ranging from 30-40%, was observed in cells treated with siRNA (Fig. 11b). These data indicate that the PMAT protein is produced in A172 cells and is functional in this cell line of human glial origin. However, because A172 is a cultured cancer cell line, the results obtained thus may not reflect what happens in vivo.

DISCUSSION

In this study, we identified and characterized a novel human plasma membrane monoamine transporter that is abundantly expressed in the human brain and skeletal
muscle. One surprising feature of PMAT is that the transporter is not related to known transporters that interact with monoamine neurotransmitters (i.e. SLC6A and OCTs), but belongs to the equilibrative nucleoside transporter family. Our data clearly demonstrated that unlike the existing ENT members that mainly transport nucleosides and their structural analogs, PMAT specifically transports biogenic amines but does not interact with nucleosides and related compounds. The strong expression of PMAT mRNA in brain and skeletal muscle, tissues that are highly innervated by monoaminergic neurons, is consistent with its functional property.

The characteristics of PMAT, such as Na\(^+\)-independency, low affinity, and relative insensitivity to inhibitors of DAT, SERT and NET, coincide with earlier findings that described non SLC6A-mediated monoamine uptake activities in the brain (6-9). Thus, PMAT may be responsible for some of these activities. Our results may also explain monoamine transport activities in brain regions that do not express the SLC6A high affinity transporters or in mice where these transporters are deleted by gene knockout. Additionally, the significant expression of PMAT in skeletal muscle suggests that this transporter may also play a role in sympathetic clearance of norepinephrine.

The functional properties of PMAT seem to complement those of the SLC6A monoamine transporters. While SERT, DAT and NET function as Na\(^+\) and Cl\(^-\) dependent high affinity transporters, PMAT-mediated transport is Na\(^+\) and Cl\(^-\) independent and is of low affinity and high capacity in nature. It has been suggested that synaptic concentrations of neurotransmitters can transiently reach high concentrations in the millimolar range (26), where the high affinity transporters would be saturated. Under such conditions, PMAT, which saturates at much higher concentrations, may play an
important role in neurotransmitter transport. Additionally, under conditions where the classic SLC6A transporters are inhibited pharmacologically, such as chronic use of antidepressants, PMAT may serve as a backup system for monoamine clearance.

However, it is critical to point out that we have no direct evidence that PMAT participates in monoamine inactivation in brain or skeletal muscle. It is also possible that PMAT functions as a transporter for an unknown substrate that has not been assayed yet. Further investigations such as extensive substrate screening, detailed brain localization and gene knockout studies are needed to elucidate the in vivo role of PMAT.

Previously, members of the OCT family (SLC22A) have been shown to transport monoamines in a Na\(^+\)-independent manner (27). A prominent member is OCT3, also known as the extraneuronal monoamine transporter (EMT). OCT3 transports epinephrine and norepinephrine with low affinities, but does not transport serotonin or dopamine well (28,29). OCT3 is mainly expressed in the placenta, heart and liver, and only weakly expressed in the human brain (28,30). Nevertheless, OCT3 may contribute to epinephrine and norepinephrine transport in localized brain regions. Taken together, the CNS seems to employ a complex transport system, consisting of both high affinity transporters (DAT, SERT, NET) and low affinity transporters (PMAT and OCT3), to transport monoamines over a wide concentration range.

Brain disorders such as Parkinson’s disease, depression and drug addiction affect millions of people worldwide. While the exact causes are unclear, the progression and treatment of these disorders are often associated with alteration of monoamine neurotransmitter levels in the CNS. Thus, the identification of a novel transport
mechanism for monoamine neurotransmitters may provide opportunities to develop new drugs for the treatment of brain disorders.
FOOTNOTES

1 The abbreviations used are SLC, solute carrier; MPP+, 1-methyl-4-phenylpyridinium; CNS, central nervous system; DAT, dopamine transporter; SERT, serotonin transporter; NET, norepinephrine transporter; ENT, equilibrative nucleoside transporter; RT, reverse transcriptase; RACE, rapid amplification of cDNA ends; OCT, organic cation transporter.

2 The nucleotide sequence for PMAT cDNA has been deposited in the GenBank database under the Accession Number AY485959.

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REFERENCES


FIGURE LEGENDS

**Fig. 1.** Sequence analysis of PMAT. a, Amino acid sequence of PMAT. The putative transmembrane domains (I-XI) are underlined. b, Phylogenetic tree of human PMAT and the human equilibrative nucleoside transporters. The percent amino acid identity between PMAT and the related human proteins hENT1 (accession number NM_004955), hENT2 (NM_001532) and hENT3 (NM_018344) is shown to the right. c, Predicted topology of PMAT in the membrane. The TopPred method (31) was used for prediction. Circles represent individual amino acids. Branched lines indicate potential N-linked glycosylation site. PMAT has six consensus sites for protein kinase C phosphorylation (filled black circles) and one cAMP-dependent protein kinase phosphorylation site (filled gray circle).

**Fig. 2.** Northern blot analysis of human PMAT mRNA transcripts in different human tissues and in various regions of the human brain.

**Fig. 3.** Confocal microscopy imaging of MDCK cells expressing YFP and a YFP fusion construct of PMAT.

**Fig. 4.** Uptake of $[^3\text{H}]$-labeled compounds by MDCK cells transfected with vector (open bars) or PMAT cDNA (solid bars). Cells were incubated for 1 min at 37°C with 1 μM of radiolabeled substrates. Hypo, hypoxanthine; Uri, uridine; AZT, azidothymidine; Ade, adenosine. In the uptake studies of uridine (Uri) and adenosine (Ade), a second set of
experiments were performed in the presence of 10 µM nitrobenzylthioinosine (NBMPR). NBMPR is a potent nucleoside transport inhibitor that inhibits endogenous nucleoside uptake activity in MDCK cells. At 10 µM, NBMPR has little inhibitory effect on PMAT.

**Fig. 5.** Uptake of $[^3]$H]MPP$^+$ by PMAT-transfected MDCK cells. a, Time course of MPP$^+$ (1 µM) uptake by PMAT and vector-transfected (control) cells. Vector-transfected cells (open circles) and PMAT-transfected cells (solid circles) were incubated at 37°C. b, Concentration-dependent transport of $[^3]$H]MPP$^+$. The PMAT-specific uptake was measured at 37°C for 1 min and calculated by subtracting the transport activity in control cells. Inset, Eadie-Hofstee plot.

**Fig. 6.** Effect of various compounds on $[^3]$H]MPP$^+$ uptake by PMAT-transfected cells. PMAT-transfected cells were incubated for 10 min with 1 µM $[^3]$H]MPP$^+$. All compounds were present at 500 µM during preincubation and incubation periods. Hypo, hypoxanthine; 5-HT, serotonin; DA, dopamine; NE, norepinephrine; EPI, epinephrine; ACh, acetylcholine; GABA, $\gamma$-aminobutyric acid; TEA, tetraethylammonium; PAH, p-aminohippuric acid. The values were expressed as percentages of $[^3]$H]MPP$^+$ uptake by PMAT-transfected cells without an inhibitor. Data are mean ± S.D. (n = 3). * significantly different from the uptake in the absence of an inhibitor ($p < 0.001$).

**Fig. 7.** Uptake of radiolabeled neurotransmitters and MPP$^+$ at 1 µM. a, Vector-transfected MDCK cells (open bars) and PMAT-transfected cells (solid bars) were incubated for 1 min at 37°C. b, Uptake of radiolabeled compounds by PMAT expressed in *Xenopus*.
laevis oocytes. Oocytes were injected with water (open bars) or PMAT cRNA (solid bars). Uptake assays were performed 2-3 days post injection at 1 µM substrate concentration. Each value represents the mean ± SE from 8-10 oocytes. 5-HT, serotonin; DA, dopamine; NE, norepinephrine; EPI, epinephrine; ACh, acetylcholine.

**Fig. 8.** Concentration-dependent transport of a, serotonin, b, dopamine, c, norepinephrine and d, epinephrine. PMAT-transfected cells and vector-transfected (control) cells were incubated for 1 min at 37°C. The PMAT-specific uptake was calculated by subtracting the transport activity in control cells. Insets, Eadie-Hofstee plots. The Eadie-Hofstee plot of epinephrine appeared not consistent with a single uptake mechanism and was thus resolved into two components.

**Fig. 9.** a, Na⁺ and Cl⁻ dependence of MPP⁺ uptake (1 µM). Vector-transfected cells (open bars) and PMAT-transfected cells (solid bars) were incubated for 1 min at 37°C. NaCl in the standard uptake solution was replaced by equal molar amounts of the indicated salts. NMDG, N-methyl-D-glucamine. b and c, Influence of membrane potential on PMAT-mediated MPP⁺ uptake (1 µM). Vector-transfected cells (open bars) and PMAT-transfected cells (solid bars) were incubated for 1 min at 37°C. In b, KRH buffers containing 5 µM of valinomycin with different compositions of potassium and sodium were used. In c, Ba²⁺ was added to block the potassium channels. To avoid precipitation of barium by sulfate and phosphate in the KRH buffer, a chloride salt-based buffer (5 mM glucose, 145 mM NaCl, 3 mM KCl, 1 mM CaCl₂, 0.5 mM MgCl₂, 5 mM HEPES, pH 7.4) with different compositions of potassium and sodium was used.
Fig. 10. Inhibition of specific 5-HT uptake in PMAT-transfected cells by decynium-22 (◊), GBR12935 (Δ), fluoxetine (○), desipramine (●), and corticosterone (▲). Transport was measured in PMAT-expressing cells and vector-transfected cells (control) at 1 min with 0.1 µM [³H]5-HT. Inhibitors were present during preincubation and incubation periods. The PMAT-specific uptake was calculated by subtracting the transport activity in control cells. Each value represents the mean ± S.D. (n = 3).

Fig. 11. RNAi mediated reduction in mRNA expression and serotonin uptake. a, Effect of siRNA on PMAT mRNA expression. Vector- and PMAT-transfected MDCK cells and human A172 astrocytoma cells were mock transfected or transfected with siRNAs and analyzed for PMAT mRNA expression by semi-quantitative RT-PCR. GAPDH primers were used as an internal control. PMAT amplification was performed for 20 cycles for PMAT-transfected MDCK and 36 cycles for A172 cells. GAPDH amplification was performed for 20 cycles for all samples. b, Uptake of radiolabeled serotonin at 1 µM. Mock transfected cells (empty bars) and cells transfected with siRNAs (solid bars) were incubated for 1 min at 37°C. Each bar represents the mean ± S.D. (n = 3). * significantly different from mock cells (p < 0.05).
Table 1. Kinetic constants of PMAT

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<th>Substrate</th>
<th>$K_m$ (µM)</th>
<th>$V_{max}$ (nmol/min/mg protein)</th>
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<tr>
<td>MPP⁺</td>
<td>33 ± 7</td>
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<td>Serotonin</td>
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</tbody>
</table>

Values are given as mean ± S.D. (n = 3). $V_{max}/K_m$ values were expressed as relative values, setting $V_{max}/K_m = 1$ for MPP⁺.
Table 2. Inhibition potencies ($K_i$) of various compounds.

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>$K_i$ (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Decynium-22</td>
<td>0.10 ± 0.03</td>
</tr>
<tr>
<td>GBR12935</td>
<td>7.9 ± 1.0</td>
</tr>
<tr>
<td>Fluoxetine</td>
<td>22.7 ± 6.1</td>
</tr>
<tr>
<td>Desipramine</td>
<td>32.6 ± 2.7</td>
</tr>
<tr>
<td>Corticosterone</td>
<td>450.5 ± 76.5</td>
</tr>
</tbody>
</table>

Values are given as mean ± S.D. (n = 3).
Figure 1

a

MGVSQGQLEEPSVADTPVRMSPTFDHSQLEHAAEAAQQG13RAGVPAFTDVTLD5EPVQHAIYFAMLLAQCVFLLPYNSFITVVDV1L5KYP

b

ehENT1 (18.9%)

hENT2 (19.7%)

hENT3 (20.0%)

PMAT

c

IN

OUT

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Figure 2

PMAT 2.9 kb
β-Actin 2.0 kb, 1.8 kb
Figure 4

Uptake (pmol per mg protein per 1 min)

- MPP+
- Uracil
- Cytosine
- Adenine
- Hypo
- Uri
- Uri + NBMPR
- Cytidine
- Thymidine
- AZT
- Ade
- Ade + NBMPR
- Inosine
- Guanosine
- ATP
Figure 5

(a) 

[\text{\[^{3}H\]MPP\(^{+}\] uptake (pmol per mg protein)}

\begin{align*}
0 & \quad 5 & \quad 10 & \quad 15 & \quad 20 & \quad 25 & \quad 30 \\
\text{Time (min)} & \quad \text{pmol per mg protein} \\
0 & \quad \text{Control} & \quad \text{PMAT}
\end{align*}

(b) 

[\text{\[^{3}H\]MPP\(^{+}\] uptake (n mol per mg protein per min)}

\begin{align*}
0 & \quad 200 & \quad 400 & \quad 600 & \quad 800 & \quad 1,000 & \quad 1,200 \\
\text{MPP\(^{+}\] concentration (µM)} & \quad \text{n mol per mg protein per min} \\
0 & \quad 0.5 & \quad 1.0 & \quad 1.5 & \quad 2.0 & \quad 2.5 & \quad 3.0
\end{align*}
Figure 7

(a) Uptake (pmol per mg protein per min)

(b) Uptake (pmol per oocyte per hr)
Figure 8

![Diagram](image-url)

**a** 5-HT concentration (µM) vs. [3H]-5-HT uptake (nmol per mg protein per min)

**b** Dopamine concentration (µM) vs. [3H]-dopamine uptake (nmol per mg protein per min)

**c** Norepinephrine concentration (mM) vs. [3H]-NE uptake (nmol per mg protein per min)

**d** Epinephrine concentration (mM) vs. [3H]-epinephrine uptake (nmol per mg protein per min)
Figure 9

(a) [的数据

(b) [的数据

(c) [的数据
Figure 10

Percent specific $[^3H]5$-HT uptake

Inhibitor concentration (µM)
Figure 11

(a) Gel electrophoresis showing expression of GAPDH and PMAT in PMAT-transfected MDCK cells, vector-transfected MDCK cells, and A172 cells. The bands for GAPDH (875 bp) and PMAT (564 bp) are indicated.

(b) Graph showing uptake of [3H]5-HT (pmol per mg protein per min) in PMAT-transfected MDCK cells, vector-transfected MDCK cells, and A172 cells. The bars indicate the mean ± SEM, and the * symbol indicates a statistically significant difference.