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# Evidence for significant contribution of a newly identified monoamine transporter (PMAT) to serotonin uptake in the human brain

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PMAT, plasma membrane monoamine transporter
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SSRI, selective serotonin reuptake inhibitor
CNS, central nervous system
MDCK, Madin–Darby canine kidney

ABSTRACT

The high affinity serotonin transporter (SERT) constitutes the principal pathway for removal of serotonin (5-HT) from extracellular fluid of brain, but evidence indicates that other transporters may also be involved in this process. We recently reported the cloning of a novel plasma membrane monoamine transporter (PMAT), which is abundantly expressed in the human brain and avidly transports 5-HT [Engel K, Zhou M, Wang J. Identification and characterization of a novel monoamine transporter in the human brain. J Biol Chem 2004;279:50042–9]. In this study, we evaluated whether PMAT contributes to total human brain uptake of 5-HT using a hybrid depletion approach in *Xenopus laevis* oocytes. We also examined whether PMAT interacts with selective serotonin reuptake inhibitors (SSRIs) using MDCK cells stably expressing recombinant human PMAT. Microinjection of total human brain poly(A)$^+$ mRNA into oocytes elicited ~2.5–3-fold increase in 5-HT uptake. Pre-hybridization of poly(A)$^+$ mRNA with PMAT or SERT antisense oligonucleotides significantly reduced mRNA-induced 5-HT uptake. An additive inhibitory effect was observed when poly(A)$^+$ mRNA was co-hybridized with both PMAT and SERT antisense oligonucleotides. In contrast, mRNA-induced 5-HT uptake was not affected by pre-hybridization with sense oligonucleotides. These data suggest that functional transcripts of PMAT are present in the human brain, and the PMAT transporter may be significantly involved in brain uptake of 5-HT. All five tested SSRIs inhibited PMAT with $IC_{50}$ values ranging from 11 to 116 μM, which are much greater than clinically encountered concentrations, suggesting that PMAT activity is minimally affected by SSRI therapies.



## 1. Introduction

Serotonin (5-hydroxytryptamine, 5-HT) is a neurotransmitter in the central nervous system (CNS) that influences a multitude of brain functions, including autonomic function, motor activity, hormone secretion, cognition, and complex processes associated with affection, emotion, and reward [1,2]. Functional deficiency in 5-HT signaling has been implicated in the pathophysiology of various depressive syndromes [2–4]. A major goal of antidepressant therapy is to restore 5-HT

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signaling by elevating extracellular 5-HT levels in the brain [4,5]. Consequently, genes involved in 5-HT clearance, such as the serotonin reuptake transporter (SERT) and the monoamine oxidases (MAOs), have long been considered as drug targets for the treatment of depression [5,6]. For example, the widely used selective serotonin reuptake inhibitors (SSRIs) exert their pharmacological effects by specifically blocking SERT-mediated 5-HT reuptake.

Transporter-mediated cellular uptake plays a key role in determining the intensity and duration of 5-HT signaling [4,7]. Earlier studies suggested that two different transport systems for 5-HT may exist, a high affinity-low capacity process and a low affinity-high capacity process, which are termed uptake$_1$ and uptake$_2$, respectively [8–10]. The uptake$_1$ system for 5-HT is now known to be mediated by the $Na^+/Cl^-$-dependent, high affinity transporter, SERT. SERT is primarily expressed in serotonergic neurons and represents the major pathway for presynaptic 5-HT clearance [4,7]. The low affinity uptake$_2$ system is $Na^+$-independent, displays broad substrate specificity towards monoamine neurotransmitters, and has not been well characterized [8,11]. Recent cloning work suggests that the uptake$_2$ system may consist of multiple transporters, including members of the organic cation transporters (OCT) family and the plasma membrane monoamine transporter (PMAT) recently identified in our laboratory [12,13]. These non-SERT transporters may play a role in 5-HT clearance in specific brain regions and/or when extracellular concentrations of 5-HT reach high levels. Uptake$_2$ transporters have also been proposed as potential drug targets for developing antidepression agents with improved efficacy [14].

The human PMAT cDNA encodes a protein of 530 amino acid residues with 11 putative transmembrane domains [12]. When expressed in mammalian cells or *Xenopus laevis* oocytes, PMAT exhibits typical uptake$_2$ characteristics, mediating $Na^+$-independent, low affinity and high capacity transport of monoamine neurotransmitters [12]. Among the monoamine neurotransmitters, PMAT has the highest affinity towards 5-HT ($K_m$ = 114 μM), as compared to dopamine, norepinephrine, and epinephrine ($K_m$ ranging from 330 to 15,000 μM) [12]. In cells stably expressing PMAT and SERT, the apparent affinity of recombinant PMAT for 5-HT is much lower than that of SERT ($K_m$, 114 versus 0.5 μM) [12,15]. However, PMAT also has a much larger transport capacity ($V_{max}$), resulting in roughly comparable uptake efficiencies ($V_{max}/K_m$) to SERT in heterologous expression systems [12,15]. Consistent with its transport function for monoamine neurotransmitters, PMAT mRNA is most strongly expressed in the human brain, and is widely distributed in the CNS [12]. Currently, the *in vivo* significance of PMAT in brain clearance of 5-HT in humans or intact animals is unknown as most of our previous studies were performed using cDNA transfected heterogenous express systems. In this study, we used an antisense hybrid depletion approach in *X. laevis* oocytes to investigate the functional significance of PMAT in the brain. This method has been widely used to evaluate the relative importance of a specific transporter to total tissue uptake [16–20]. Because our data suggested a significant role of PMAT in total brain 5-HT uptake, potential pharmacological interactions between the SSRIs and PMAT have also been investigated.

## 2. Materials and methods

### 2.1. Chemicals

[$^3$H]5-HT (specific radioactivity, 27.1 Ci/mmol) was from Perkin-Elmer Life Sciences Inc. (Boston, MA). [$^3$H]$MPP^+$ (specific radioactivity, 39.3 Ci/mmol) was from American Radiolabeled Chemicals Inc. (St. Louis, MO). Fluoxetine, paroxetine, and fluvoxamine were from Sigma (St. Louis, MO). Citalopram and sertraline were obtained from Cenrilliant Corporation (Round Rock, TX).

### 2.2. cRNA and mRNA expression in X. laevis oocytes

PMAT cDNA [12] was subcloned into an oocyte expression vector pOX under the control of T3 promoter using *Hin* dIII and *Xba* I cloning sites. The correct orientation and the sequence of the PMAT cDNA were verified by restriction analysis and direct DNA sequencing. Human SERT cDNA under the control of the T7 promoter in pBluescript-SKII was a kind gift of Dr. Randy D. Blakely (Vanderbilt School of Medicine, Nashville, TN, USA). The SERT coding region is flanked 5′ by alfalfa mosaic virus and 3′ by *Xenopus* β-globin UTRs [21]. Plasmids were linearized with *Xba* I and the cRNA was synthesized in the presence of $^{m7}$GpppG using the mCAP RNA Capping kit (Stratagene, La Jolla, CA) with T3 or T7 polymerase. The purity and integrity of *in vitro* synthesized cRNA were verified by RNAase-free agarose gel electrophoresis. Oocytes were harvested from *X. laevis* (NASCO, Fort Atkinson, WI) and defolliculated as described previously [12]. Healthy stages V and VI oocytes were injected with either 50 nl of cRNA (0.8 μg/μl) or water (control) using an automatic nanoliter injector Nanoject II (Drummond, Broomall, PA). Injected oocytes were maintained in modified Barth's medium (88 mM NaCl, 1 mM KCl, 0.82 mM $MgSO_4$, 0.4 mM $CaCl_2$, 0.33 mM $Ca(NO_3)_2$, 2.4 mM $NaHCO_3$ and 10 mM HEPES/Tris, pH 7.4) at 18 °C. For mRNA expression, human brain poly(A)$^+$ mRNA, pooled from eight adult brains, was obtained from BD Clontech (Palo Alto, CA) and injected into oocytes (40 ng/oocyte). Injected oocytes were incubated at 18 °C for 4–5 days before uptake assays.

### 2.3. Hybrid depletion

Three pairs of nucleotides (21–22 nt) corresponding to different PMAT coding regions were designed based on the sequence of RNA probes used in a previous RNA interference (RNAi) study [12]. These oligonucleotides (oligos) demonstrated high specificity towards PMAT when blasted using the human genome database. For SERT, two pairs of nucleotides (18–21 nt) were designed based on the RNAi study of Thakker et al., where they were shown to be effective in blocking SERT expression [22]. The locations and sequences of oligos used in this study were shown in Fig. 1 and Table 1. Oligos were synthesized and purified by HPLC at Invitrogen (Grand Island, NY). Antisense-mediated hybrid depletion was performed using a method modified from Nakai et al. [19]. Briefly, human brain poly(A)$^+$ mRNA pooled from eight adult brains (0.8 μg/μl) was denatured at 65 °C for 5 min in 50 mM NaCl RNAase-free solution containing antisense or sense (control) oligos (1.5 ng/oocyte). The mixture was then annealed at 42 °C for

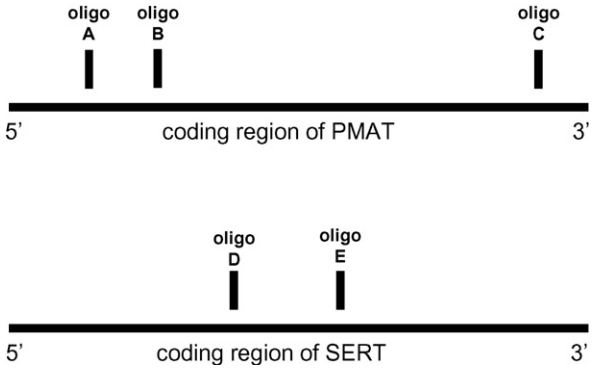


**Fig. 1 – Illustration of positions of PMAT and SERT oligos used in hybrid depletion study.**

30 min, cooled on ice, and injected into oocytes (50 nl/oocyte). After 4–5 days incubation at 18 °C, uptake assays were performed. The specificity and potency of the antisense oligos were also tested by prehybridizing cRNA (0.8 μg/μl) with sense or antisense oligos.

### 2.4. Transport assays in X. laevis oocyte

Uptake assays were performed at 25 °C in transport buffer (100 mM NaCl, 2 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$ and 10 mM HEPES, pH 7.4). Oocytes were washed with 2 ml room temperature transport buffer and then incubated in 250 μl transport buffer containing a [$^3$H]-labeled ligand for 20–60 min. At the end of incubation, uptake was terminated by removing the incubation medium. Oocytes were then rapidly washed five times with 3 ml ice-cold transport buffer. Individual oocyte was then solubilized in 10% SDS and the radioactivity was quantified by liquid scintillation counting.

### 2.5. PMAT interaction with SSRIs

Madin–Darby canine kidney (MDCK) cell lines stably transfected with PMAT cDNA or the pcDNA3 vector were used in these studies [12]. Cells were maintained in minimum essential medium (Gibco BRL, Grand Island, NY) containing 10% fetal bovine serum (Gibco BRL, Grand Island, NY) and G418 (500 μg/ml). For uptake studies, cells were plated (105 cells/well) in 24-well plates and allowed to grow at 37 °C for 2–3 days until confluent. Growth medium was aspirated and each well was rinsed with Krebs–Ringer–Henseleit (KRH) buffer (5.6 mM glucose, 125 mM NaCl, 4.8 mM KCl, 1.2 mM $KH_2PO_4$, 1.2 mM $CaCl_2$, 1.2 mM $MgSO_4$ and 25 mM HEPES, pH 7.4). Cells were then incubated in KRH buffer for 15 min at 37 °C in the presence of SSRIs at various concentrations (0–1000 μM). Transport assays were performed at 37 °C by incubating cells in KRH buffer containing [$^3$H]$MPP^+$ (0.1 μM) for 1 min in the absence (control) or presence of SSRIs. Uptake was terminated by aspirating the reaction mixture and washing the cells three times with ice-cold KRH buffer. Cells were then solubilized and the radioactivity was quantified by liquid scintillation counting. Protein content in each well was measured using a BCA protein assay kit (Pierce, Rockford, IL) and the uptake in each well was normalized to the corresponding protein content.

### 2.6. Statistical analysis

Each experiment was repeated at least three times. Results are expressed as mean ± S.E. ($n$ = 8–12) for uptake in oocytes or mean ± S.D. ($n$ = 3) for uptake in MDCK cells. Statistical significance was determined by unpaired Student's $t$-test or two-way ANOVA test using the software STATAT 9.0 (College Station, TX). The $IC_{50}$ was determined by nonlinear least-squares regression fitting as described previously [12].

**Table 1 – Sequences and positions of oligonucleotides used for hybrid depletion**

| Oligonucleotide | Sequence | Position[a] |
|---|---|---|
| PMAT | | |
| A | | |
| Sense | 5′-GGCGTAGTGATGAGCTTCACC-3′ | |
| Antisense | 5′-GGTGAAGCTCATCACTACGCC-3′ | +60 to +80 |
| B | | |
| Sense | 5′-CAACAGCTTCATCACGGACGTG-3′ | |
| Antisense | 5′-CACGTCCGTGATGAAGCTGTTG-3′ | +254 to +275 |
| C | | |
| Sense | 5′-CACCATGACCGTGTCCTACATG-3′ | |
| Antisense | 5′-CATGTAGGACACGGTCATGGTG-3′ | +1454 to +1475 |
| SERT | | |
| D | | |
| Sense | 5′-CACTGGCAACTGCACCAATTA-3′ | +840 to +860 |
| Antisense | 5′-TAATTGGTGCAGTTGCCAGTG-3′ | |
| E | | |
| Sense | 5′-TAGCTACAACAAGTTCAA-3′ | +1169 to +1186 |
| Antisense | 5′-TTGAACTTGTTGTAGCTA-3′ | |

[a] Nucleotide numbers (positions) are relative to the translation start site.

## 3. Results

### 3.1. Functional expression of PMAT and SERT in oocytes and validation of oligos

PMAT cRNA was synthesized and injected into oocytes. Uptake was performed using [$^3$H]5-HT (1 μM). Compared to water-injected oocytes, oocytes injected with PMAT cRNA exhibited ~6-fold increase in 5-HT uptake (Fig. 2a). Pre-hybridization of PMAT cRNA with sense oligos (A–C) had no inhibitory effect on PMAT-mediated 5-HT uptake. In contrast, pre-hybridization of cRNA with antisense oligos (A–C) reduced 5-HT uptake levels to those observed in water-injected oocytes (Fig. 2a). These data suggest that the antisense oligos effectively blocked PMAT expression in oocytes. Similarly, oocytes injected with SERT cRNA exhibited a large increase (~70-fold) in 5-HT uptake (20 nM) (Fig. 2b). Replacement of $Na^+$ in the uptake buffer with mannitol abolished SERT activity. Pre-hybridization of SERT cRNA with sense oligos (D and E) had no inhibitory effect. In contrast, pre-hybridization of antisense oligos (D and E) almost completely blocked SERT-mediated 5-HT uptake. These data demonstrated the effectiveness and specificity of antisense oligos in blocking PMAT and SERT expression in oocytes.

### 3.2. Effect of PMAT antisense oligos on 5-HT uptake in oocytes injected with total human brain poly(A)$^+$ mRNA

Total human poly(A)$^+$ mRNA was injected into *X. laevis* oocytes. After 4–5 days incubation, mRNA-induced 5-HT uptake (1 μM) was measured (Fig. 3a). Compared with water-injected oocytes, oocytes injected with human brain poly(A)$^+$ mRNA exhibited ~2.5-fold increase in 5-HT uptake (Fig. 3a). Pre-hybridization of mRNA with the PMAT sense oligos had no effect on mRNA-induced 5-HT uptake, which was defined as the difference in 5-HT uptake between water- and mRNA-injected oocytes. In contrast, pre-hybridization of poly(A)$^+$ mRNA with PMAT antisense oligos, which specifically block the expression of PMAT in oocytes, resulted in 40–60% reduction of mRNA-induced 5-HT uptake (Table 2). These data suggest that functional PMAT transcripts are present in the human brain, and PMAT may contribute significantly to total brain 5-HT uptake.

### 3.3. Effect of SERT antisense oligos on 5-HT uptake in oocytes injected with total human brain poly(A)$^+$ mRNA

The effect of SERT oligos on poly(A)$^+$ mRNA-induced 5-HT uptake (1 μM) was also examined under identical conditions. As shown in Fig. 3b, pre-hybridization of total human brain poly(A)$^+$ mRNA with SERT-sense oligos did not affect mRNA-induced 5-HT uptake significantly. In contrast, a 60–70% reduction in 5-HT uptake was observed in oocytes injected with mRNA pre-hybridized with SERT-antisense oligos (Table 2). Importantly, compared to oocytes injected with mRNA treated with single antisense oligo (oligo B or oligo E), 5-HT uptake was significantly decreased in oocytes injected with mRNA co-hybridized with both anti-PMAT and anti-SERT oligos (oligo B + oligo E) (Fig. 3b). The 5-HT uptake in those oocytes is not significantly different from that in water-injected oocytes, suggesting that poly(A)$^+$ mRNA-induced 5-HT uptake was completely abolished by pre-hybridization with both SERT- and PAMT-antisense oligos (Fig. 3b). The sense oligos, either alone or in combination, did not have any inhibitory effect. All together, these data suggest that besides

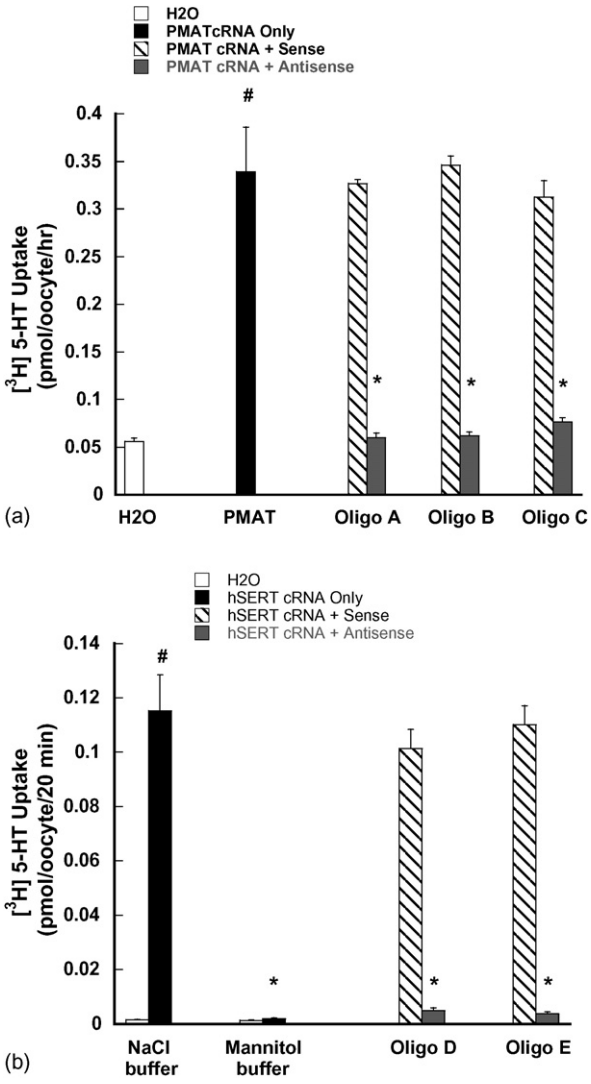


**Fig. 2 – Effect of PMAT and SERT sense and antisense oligos on 5-HT uptake in cRNA-injected oocytes. (a) Oocytes were injected with 50 nl water (open bars), 40 ng PMAT cRNA only (solid bars), 40 ng PMAT cRNA pre-hybridized with 1.5 ng sense oligos (shaded bars) or 40 ng PMAT cRNA pre-hybridized with 1.5 ng antisense oligos (gray bars). Uptake of 1 μM [$^3$H]5-HT was performed after 1 h incubation at 25 °C. (b) Oocytes were injected with 50 nl water (open bars), 40 ng SERT cRNA only (solid bars), 40 ng SERT cRNA pre-hybridized with 1.5 ng sense oligos (shaded bars) or 40 ng SERT cRNA pre-hybridized with 1.5 ng antisense oligos (gray bars). Uptake of 20 nM [$^3$H]5-HT was performed after 20 min incubation in $Na^+/Cl^-$ buffer or mannitol buffer at 25 °C. Each bar represents the mean ± S.E. ($n$ = 8–10). #Significantly different from water injected oocytes ($p < 0.001$); *significantly different from cRNA injected oocytes ($p < 0.001$).**

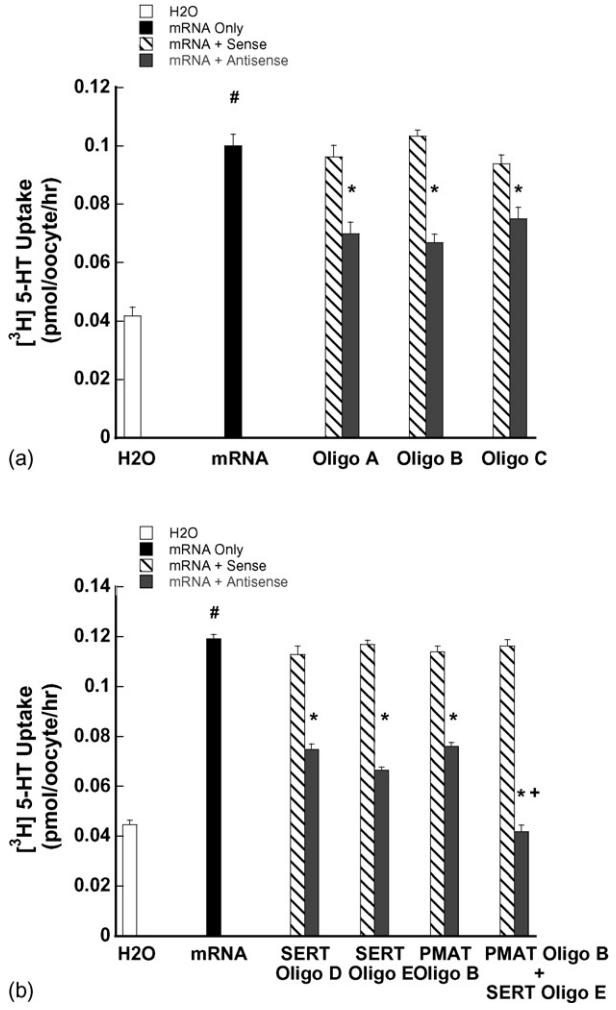


**Fig. 3 – Effect of sense and antisense oligos on 5-HT uptake in human brain poly(A)$^+$ mRNA-injected oocytes. (a) Oocytes were injected with 50 nl water (open bars), 40 ng human brain poly(A)$^+$ mRNA only (solid bars), 40 ng human brain poly(A)$^+$ mRNA pre-hybridized with 1.5 ng PMAT sense oligos (shaded bars) or 40 ng human brain poly(A)$^+$ mRNA pre-hybridized with 1.5 ng PMAT antisense oligos (gray bars). Uptake of 1 μM [$^3$H]5-HT were performed during 1 h exposure at 25 °C. (b) Oocytes were injected with 50 nl water (open bars), 40 ng human brain poly(A)$^+$ mRNA only (solid bars), 40 ng human brain poly(A)$^+$ mRNA pre-hybridized with 1.5 ng SERT sense oligos (shaded bars) or 40 ng human brain poly(A)$^+$ mRNA pre-hybridized with 1.5 ng SERT antisense oligos (gray bars). Uptake of 1 μM [$^3$H]5-HT was performed during 1 h exposure at 25 °C. For the double hybrid depletion, oocytes were injected with 40 ng human brain poly(A)$^+$ mRNA pre-hybridized with 1.5 ng SERT antisense oligo E and 1.5 ng PMAT antisense oligo B. Each bar represents the mean ± S.E. ($n$ = 8–10). #Significantly different from water injected oocytes ($p < 0.001$); *significantly different from mRNA injected oocytes ($p < 0.001$); +significantly different from oocytes injected with mRNA hybridized with single antisense oligo ($p < 0.01$).**

**Table 2 – Effect of antisense oligonucleotides on 5-HT uptake in oocytes injected with total human brain poly(A)$^+$ mRNA**

| [$^3$H]5-HT uptake (pmol/oocyte 60 min) | |
|---|---|
| PMAT | |
| Water | 0.0418 ± 0.002 |
| mRNA | 0.1000 ± 0.004 |
| mRNA + sense A | 0.0962 ± 0.004 |
| mRNA + antisense A | 0.0699 ± 0.004* |
| mRNA + sense B | 0.1030 ± 0.002 |
| mRNA + antisense B | 0.0668 ± 0.003* |
| mRNA + sense C | 0.0939 ± 0.003 |
| mRNA + antisense C | 0.0751 ± 0.004* |
| SERT | |
| Water | 0.0445 ± 0.002 |
| mRNA | 0.1190 ± 0.002 |
| mRNA + sense D | 0.1130 ± 0.003 |
| mRNA + antisense D | 0.0749 ± 0.002* |
| mRNA + sense E | 0.1170 ± 0.002 |
| mRNA + antisense E | 0.0666 ± 0.001* |
| mRNA + sense B (PMAT) + sense E (SERT) | 0.1160 ± 0.003 |
| mRNA + antisense B (PMAT) + antisense E (SERT) | 0.0418 ± 0.008* |

Hybrid depletion was performed as described in Section 2 with human brain poly(A)$^+$ mRNA. Data are means ± S.E. ($n$ = 8–10). *$p < 0.001$ vs. corresponding mRNA injected oocytes.

SERT, PMAT may also play a significant role in 5-HT uptake in the human brain (Table 2).

### 3.4. *PMAT interaction with SSRIs*

SERT is a well-established target for many antidepressant drugs, including the SSRIs. Our hybrid depletion data suggest

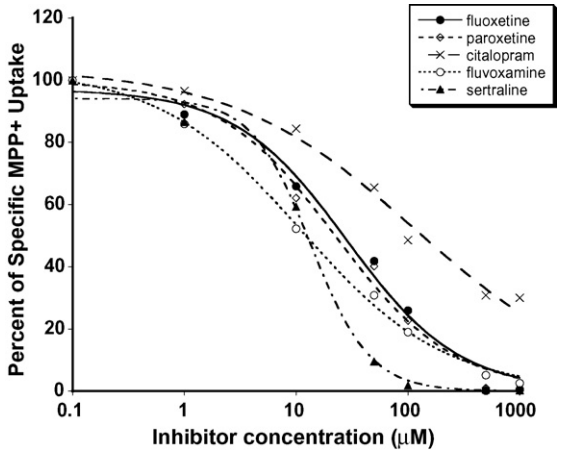


**Fig. 4 – Effect of SSRIs on PMAT-mediated [$^3$H]MPP$^+$ uptake in MDCK cells. Transport was measured in PMAT-transfected cells and vector-transfected cells (control) with 0.1 μM [$^3$H]MPP$^+$. Inhibitors were present during 15 min preincubation and 1 min incubation periods. The PMAT-specific uptake was calculated by subtracting the transport activity in the control cells. Each value represents the mean ± S.D. ($n$ = 3).**

**Table 3 – $IC_{50}$ values of various SSRIs towards human PMAT vs. estimated free drug concentrations in human brain**

| SSRI | $IC_{50}$ towards PMAT (μM) | Estimated brain free drug concentration[a] (μM) |
|---|---|---|
| Fluoxetine | 28.39 ± 7.22 | 0.13–0.44 |
| Paroxetine | 22.46 ± 5.68 | 0.02–1.32 |
| Citalopram | 116.67 ± 13.09 | 0.18–1.33 |
| Fluvoxamine | 11.00 ± 1.88 | 0.14–3.73 |
| Sertraline | 13.54 ± 1.67 | 0.009–0.087 |

[a] Values are calculated as steady state plasma concentrations of total drug times plasma unbound fraction using data from [22,23], assuming that unbound brain concentrations of SSRIs approximate their unbound plasma concentrations [32,33].

that besides SERT, PMAT may also play a role in 5-HT uptake in the brain. To investigate whether PMAT is a hidden drug target for the SSRIs, we tested the inhibitory effect of five commonly marketed SSRIs on PMAT. All inhibition studies were carried out using a non-metabolizable PMAT substrate, $MPP^+$, at a low substrate concentration (0.1 μM). All five SSRIs inhibited PMAT at micromolar concentrations (Fig. 4). The calculated $IC_{50}$ values of these SSRIs, along with their estimated free drug concentrations at steady state, are summarized in Table 3. The most potent inhibitors are fluvoxamine and sertraline, followed by paroxetine and fluoxetine, and then by citalopram. The concentrations of SSRIs required to produce an inhibitory effect on PMAT are much higher than the steady state free drug concentrations commonly encountered clinically (Table 3) [23,24], suggesting that the activity of PMAT is not affected by the SSRIs at clinically used dosages.

## 4. Discussions

In this study, we employed an antisense hybrid depletion approach in *X. laevis* oocytes to explore the functional significance of a newly cloned monoamine transporter PMAT in the human brain. Previously, the hybrid depletion approach has been used to estimate the relative contribution of a number of individual membrane transporters to tissue-specific uptake of a ligand [16–20]. This relatively simple and straightforward method allows for the evaluation of gene function in human tissues that cannot be easily studied in genetic knockout models. In our study, total poly(A)$^+$ mRNA isolated from human brain was injected into *X. laevis* oocytes to allow the expression of the full spectrum of genes in the brain. The mRNA-induced 5-HT uptake was measured in transport assays, and the effect of PMAT-specific antisense oligos was investigated. To ensure specificity, we included sense oligos as controls and designed both PMAT and SERT oligos based on sequences that were shown to be effective and specific in previous RNAi studies [12,22]. Our results showed that pre-hybridization of total human brain poly(A)$^+$ mRNA with PMAT antisense substantially reduced mRNA-induced 5-HT uptake, suggesting that functional PMAT transcripts are present in the human brain, and PMAT may be significantly involved in total brain uptake of 5-HT.

The current data provided evidence of a significant role of PMAT in brain 5-HT uptake, which are in line with our previous findings that recombinant PMAT efficiently transports 5-HT and PMAT mRNA is abundantly expressed in the human brain. While more studies employing chemical or genetic knockout models are necessary to further elucidate the specific roles of PMAT in 5-HT signaling pathway, several animal studies appear to provide evidence of the importance of non-SERT mediated 5-HT uptake in the brain [25–28]. For example, significant 5-HT uptake activity has been reported in certain neuronal tissues from SERT knockout mice [26,27]. It has also been demonstrated that in hypothalamus, corpus callosum and optic nerves of rat brain, 5-HT uptake under $Na^+$-free condition accounted for about 20% of total uptake, and fluoxetine, a potent inhibitor of SERT, only caused a 57% decrease in 5-HT uptake [28]. Most recently, a study reported that local perfusion of decynium 22, a high affinity inhibitor of OCT and PMAT ($K_i$ = 0.1 μM) [29], resulted in a significant, dose-dependent increase in extracellular 5-HT level in rat dorsomedial hypothalamus where SERT is minimally expressed [25]. Therefore, PMAT, together with the OCTs, may play a significant role in 5-HT clearance in brain regions where expression of SERT is low or when SERT function is pharmacologically inhibited, such as chronic use of antidepressants.

It is generally believed that the majority (>95%) of released 5-HT is taken up by the high affinity transporter SERT in the brain [30]. Thus, it seems somewhat surprising that anti-SERT oligos only produced 60–70% inhibition on 5-HT uptake in our hybridization studies. Furthermore, anti-PMAT oligos generated a significant effect (40–60% reduction) in mRNA-mediated 5-HT uptake. This observation may be explained by concentration differences of SERT and PMAT mRNA in the total brain mRNA pool. In the brain, SERT mRNA is known to be highly localized to serotonergic neurons in median and dorsal raphe nuclei and the caudal linear nucleus [4,31]. In our study, poly(A)$^+$ mRNA isolated from whole brain homogenate was used, in which the concentrations of SERT may have been greatly diluted. In contrast, our previous Northern blot showed that PMAT mRNA is abundantly expressed in the human brain in all regions [12]. The relatively high copy numbers of PMAT mRNA in total poly(A)$^+$ mRNA may explain the significant inhibitory effect of anti-PMAT oligos.

There are major intrinsic limitations of the hybrid depletion method. For instance, membrane protein expression levels in the oocytes may not be proportional to those in original tissues due to the intrinsic species difference in protein translation efficiency. Furthermore, post-translational protein processing between brain cells and *Xenopus* oocytes may be different, which may result in differences in binding affinities to 5-HT or drugs between native brain PMAT and recombinant PMAT produced in oocytes. Nevertheless, these encouraging results from hybrid depletion studies warrant further investigation of the *in vivo* significance of native PMAT in the brain.

A significant portion of individuals undergoing depression and anxiety treatment exhibit delayed or poor response to the SSRIs, which are thought to exert their pharmacological effects by specifically blocking SERT. We investigated the potential interactions of PMAT with the SSRIs using a stably transfected mammalian cell line. All five commonly marketed SSRIs, including fluoxetine (Prozac), sertraline (Zoloft), citalopram, fluvoxamine and paroxetine, inhibited PMAT with $IC_{50}$ values 3–4 orders greater than those of SERT [15,32]. At clinically encountered concentrations in the brain (low nanomolar range) (Table 3) [23,24], SSRIs do not affect the function of PMAT. If PMAT indeed plays a significant role in brain clearance of 5-HT *in vivo*, it would suggest that inhibition of this 5-HT transporter may lead to an increase in brain 5-HT levels independent of the SERT pathway. Thus, PMAT may potentially represent a new target for antidepressant drug discovery.

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