Selective Transport of Monoamine Neurotransmitters by Human Plasma Membrane Monoamine Transporter and Organic Cation Transporter 3

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Running title: Functional Comparison of hPMAT and hOCT3

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Abbreviations: CNS, central nervous system; SLC, solute carrier family; D22, decynium-22 (1,1’-Diethyl-2,2’-cyanine); DAT, dopamine transporter; GUSB, beta-glucuronidase; MPP+, 1-methyl-4-phenylpyridinium; NET, norepinephrine transporter; OCT, organic cation transporter; SERT, 5-HT transporter; TEA, tetraethylammonium

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Abstract

The plasma membrane monoamine transporter (PMAT) and organic cation transporter 3 (OCT3) are the two most prominent low-affinity, high-capacity (i.e. uptake2) transporters for endogenous biogenic amines. Using the Flp-in® system, we expressed hPMAT and hOCT3 at similar levels in HEK293 cells. Parallel and detailed kinetics analysis revealed distinct and seemingly complementary patterns for the two transporters in transporting monoamine neurotransmitters. hPMAT is highly selective towards serotonin (5-HT) and dopamine, with the rank order of transport efficiency (Vmax/Km) being: dopamine, 5-HT >> histamine, norepinephrine, epinephrine. The substrate preference of hPMAT towards these amines is substantially driven by large (up to 15 folds) distinctions in its apparent binding affinities (Km). In contrast, hOCT3 is less selective than hPMAT towards the monoamines, and the Vmax/Km rank order for hOCT3 is: histamine > norepinephrine, epinephrine > dopamine> 5-HT. Interestingly, hOCT3 demonstrated comparable (≤2-fold difference) Km towards all amines, and distinctions in Vmax played an important role in determining its differential transport efficiency towards the monoamines. Real-time RT-PCR revealed that hPMAT is expressed at much higher levels than hOCT3 in most human brain areas, whereas hOCT3 is selectively and highly expressed in adrenal gland and skeletal muscle. Our results suggest that hOCT3 represents a major uptake2 transporter for histamine, epinephrine and norepinephrine. hPMAT, on the other hand, is a major uptake2 transporter for 5-HT and dopamine, and may play a more important role in transporting these two neurotransmitters in the central nervous system.
Introduction

The monoamines, including the catecholamines (dopamine, epinephrine, norepinephrine), serotonin (5-HT) and histamine, are a group of important neurotransmitters and neurohormones. Monoamines regulate a wide array of physiological, behavioral, cognitive and endocrine functions in central and peripheral nervous systems (Carlsson, 1987; Greengard, 2001). The actions of released monoamine neurotransmitters are terminated by plasma membrane transporters that actively remove the transmitters from the extracellular space. Two distinct transport systems, named uptake$_1$ and uptake$_2$, are responsible for the clearance of monoamines (Grundemann et al., 1998; Eisenhofer, 2001). Uptake$_1$ consists of Na$^+$ and Cl$^-$ dependent, high affinity transporters in the Solute Carrier 6 family and includes the 5-HT transporter (SERT, SLC6A4), the dopamine transporter (DAT, SLC6A3) and the norepinephrine transporter (NET, SLC6A2) (Blakely et al., 1994; Torres et al., 2003). Predominantly expressed on the nerve endings of monoaminergic neurons, uptake$_1$ transporters are the major mechanism for clearing released transmitters from the synaptic cleft. They are also the targets for numerous clinically significant agents such as antidepressants and drugs of abuse (Amara and Kuhar, 1993; Torres et al., 2003).

The uptake$_2$ was originally characterized as a Na$^+$ and Cl$^-$-independent, low-affinity, high-capacity transport system in peripheral tissues such as heart and smooth muscle cells (Iversen, 1971; Bonisch et al., 1985; Eisenhofer, 2001). Historically, uptake$_2$ has been associated with monoamine metabolism and was proposed to play a backup role in monoamine uptake (Grundemann et al., 1998; Eisenhofer, 2001). However, emerging data suggest that these transporters may be actively involved in various monoamine signaling pathways and may
represent promising targets for neuropsychiatric and neurodegenerative disorders (Schildkraut and Mooney, 2004; Zhou et al., 2007; Cui et al., 2009; Daws, 2009).

The molecular identity of uptake2 was unclear until recently. Molecular cloning work, including those from our laboratory, suggests that uptake2 consists of multiple organic cation transporters with broad substrate selectivity (Grundemann et al., 1998; Wu et al., 2000; Engel et al., 2004; Daws, 2009). In particular, the organic cation transporter 3 (OCT3, also termed extraneuronal monoamine transporter) and the plasma membrane monoamine transporter (PMAT) are the two most prominent uptake2 transporters for endogenous monoamines (Grundemann et al., 1998; Wu et al., 2000; Engel et al., 2004; Daws, 2009). OCT3 (SLC22A3) and PMAT (SLC29A4) both transport a broad range of organic cations, including monoamine neurotransmitters and the prototypical organic cations 1-methyl-4-phenylpyridinium (MPP+) and tetraethylammonium (TEA) (Grundemann et al., 1998; Kekuda et al., 1998; Engel et al., 2004; Engel and Wang, 2005). OCT3- and PMAT-mediated monoamine transport show classic uptake2 characteristics, such as Na\(^+\) and Cl\(^-\) independency, low substrate affinity but high transport capacity (Wu et al., 1998; Engel et al., 2004). Both transporters are highly sensitive to inhibition by the isocyanine compound, decynium 22 (Hayer-Zillgen et al., 2002; Engel et al., 2004). However, OCT3 is highly sensitive to corticosterone whereas PMAT is not (Hayer-Zillgen et al., 2002; Engel et al., 2004). OCT3 and PMAT have been reported to be expressed in the brain and a number of peripheral tissues (Slitt et al., 2002; Engel et al., 2004). In rodent brains, in situ hybridization and immunolocalization work suggested that Oct3 and Pmat are expressed in neuronal cells in many brain regions (Amphoux et al., 2006; Dahlin et al., 2007;
Gasser et al., 2009). Oct3 is also reported to be expressed in astroglial cells (Cui et al., 2009; Gasser et al., 2009).

The large overlaps in substrate specificity and tissue distribution of OCT3 and PMAT raise important questions regarding their specific contribution to monoamine clearance in vivo. Although both transporters have been characterized in several studies (Grundemann et al., 1998; Grundemann et al., 1999; Wu et al., 2000; Hayer-Zillgen et al., 2002; Engel et al., 2004; Engel and Wang, 2005), comprehensive and parallel analyses have not been performed to compare their substrate selectivity and transport kinetics for endogenous monoamines. Furthermore, while the expression patterns of Oct3 and Pmat have been studied in rat and mouse brains (Amphoux et al., 2006; Dahlin et al., 2007; Vialou et al., 2007), little is known regarding their relative expression levels in various human brain areas. The current study is designed to compare the intrinsic transport efficiencies of human PMAT and human OCT3 for endogenous monoamines. Using the Flp-in® system, we stably expressed hPMAT and hOCT3 at comparable levels in HEK293 cells, and compared their transport kinetics towards monoamines. We then determined and compared the expression levels of the two transporters in various human brain regions and tissues. Finally we evaluated the contribution of each transporter to 5-HT clearance through mouse brain synaptosome uptake studies.
Materials and Methods

Materials. [\(^3\)H]MPP\(^+\) (85 Ci/mmol) was purchased from American Radiolabeled Chemicals, Inc. [\(^3\)H]5-HT (28 Ci/mmol), [\(^3\)H]dopamine (51.3 Ci/mmol), [3H]epinephrine (77 Ci/mmol), [\(^3\)H]norepinephrine (5.3 Ci/mmol), [\(^3\)H]histamine (14.2 Ci/mmol), and [\(^14\)C]tetraethyammonium (3.5 mCi/mmol) were purchased from Perkin Elmer. Non radiolabeled chemicals were purchased from Sigma-Aldrich. Cell culture media and reagents were from Invitrogen. Cell culture plastic wares were from Beckton-Dickinson or Corning, Inc.

Cell culture. Flp-in HEK293 cells were purchased from Invitrogen and maintained in D-MEM (high glucose) supplemented with 10% FBS, 2 mM L-glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin and 100 µg/ml zeocin. Flp-in HEK293 cells stably transfected with hPMAT or hOCT3 were maintained in D-MEM (high glucose) supplemented with 10% FBS, 2 mM L-glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin and 150 µg/ml hygromycin. Cells were cultured in a 37°C humidified incubator with 5% CO\(_2\). For better attachment of cells, all cell culture plastic surfaces were pre-treated with 0.01% poly L-ornithine (MW 30,000 \(\sim\) 70,000)/phosphate buffered saline solution before plating.

Generation of HEK293 cell lines stably expressing hPMAT and hOCT3 at isogenic locations. To generate HEK293 cell lines stably expressing hPMAT or hOCT3 at isogenic locations, the Flp-in\(^{\circledR}\) system from Invitrogen was used. This system uses Flp-recombinase to mediate integration of the transfected gene into the Flippase Recognition Target (FRT) site in the Flp-in host cells. Human PMAT (SLC29A4) cDNA was previously cloned (Engel et al., 2004). It was amplified using Pfu Ultra polymerase (Stratagene) with the primers 5’-
TATGGATCCGAGAGGCTGCCATGGGCTCCGT-3’ and 5’-AATGCGGCCGCCAGTGGGCGGGGCTGGCTCAGA-3’ and ligated into the BamHI/NotI sites of pcDNA5/FRT vector (Invitrogen). To clone hOCT3 (SLC22A3) cDNA, human skeletal muscle total RNA (Clontech) was reverse-transcribed using Superscript III reverse transcriptase (Invitrogen) according to manufacturer’s instruction. hOCT3 cDNA was amplified from the cDNA pool with the primers 5’-AATAGGATCCGCCACCATGCCCCCTCCTTTGACGA-3’ and 5’-GAGGCTCGAGTCCTGGATAGCTCCTTCTTTCTGTC-3’ and ligated into the BamHI/XhoI sites of pcDNA5/FRT vector. Inserts of both hPMAT and hOCT3 expression vectors were sequenced and aligned with NCBI reference sequences (AY485959.1 and NM_021977.2) to ensure fidelity. The encoded amino acid sequences of hPMAT and hOCT3 are identical to the published protein sequences (Kekuda et al., 1998; Engel et al., 2004). The pcDNA5/FRT empty vector, hPMAT or hOCT3 expression vectors were then co-transfected with pOG44 expressing the Flp-recombinase into the Flp-in HEK293 cell line from Invitrogen, which contains only one FRT site at a defined genomic locus. Transfected cells were selected by hygromycin B treatment (150 µg/ml) and expanded. All stably transfected cell lines were cultured in the presence of hygromycin B (150 µg/ml) and cells of 10-30 passages were used for this study.

Immunostaining of Flp-in hPMAT and hOCT3 cell lines. Flp-in hPMAT and hOCT3 cells were grown on Lab-Tek II CC2 Chamber Slide System (Nalgene Nunc International) pre-treated with poly L-ornithine for 2~3 days until confluent. Cells were rinsed twice with DPBS (Dulbecco’s Phosphate Buffered Saline) and fixed for 30 min at room temperature with 4% paraformaldehyde, rinsed twice with DPBS and incubated in 50 mM NH₄Cl in DPBS for 15 min.
to quench the fixative. Cells were then permeabilized with 0.2% Triton X-100 in DPBS for 10 min followed by 90-min incubation with blocking buffer (10% FBS, 0.1% Triton X-100 in DPBS) to block nonspecific binding sites. Cells were incubated with primary antibodies diluted in blocking buffer overnight at 4°C with constant shaking. PMAT polyclonal antibody directed against the C-terminal amino acids 469-482 of human PMAT protein was previously reported by our lab (Dahlin et al., 2007) and diluted 1:200. OCT3 polyclonal antibody (C-14) was obtained from Santa Cruz Biotechnology, Inc. and used at 1:100 dilution. After incubating with primary antibodies, cells were washed 3 times with DPBS containing 0.05% Triton X-100 and incubated with fluorescence conjugated secondary antibodies for 1 hr (Alexa fluor 488 goat anti-rabbit for PMAT, Alexa fluor 555 donkey anti-goat for OCT3, both from Invitrogen and diluted 1:1000 in blocking buffer). Cells were washed 3 times and observed under a Nikon fluorescence microscope with imaging capabilities.

**Taqman real time RT-PCR for quantification of hPMAT and hOCT3 mRNA transcripts.**

Flp-in HEK293 cells total RNA were extracted using RNeasy mini kit (Qiagen). Total RNA from various human tissues were purchased from Clontech Laboratories, Inc. The RNA samples were prepared from normal non-diseased human tissues pooled from varying numbers of subjects (see supplemental Table 1 for details). Total RNA (2~4 µg) were reverse transcribed to first-strand cDNA using Superscript III reverse transcriptase (Invitrogen) according to manufacturer’s protocol. Taqman real-time PCR reagents, supplies, primers and probes for hPMAT (SLC29A4), hOCT3 (SLC22A3) and GUSB (beta glucuronidase) were purchased from Applied Biosystems. All primers and probes for respective cDNAs were validated by Applied Biosystems. The primers were designed to span adjacent exons so that genomic DNA will not
be amplified (hPMAT and hOCT3, exons 9~10; GUSB, exons 11~12). Taqman real-time PCR reactions were setup and run according to manufacturer’s protocols on an Applied Biosystems 7900HT fast realtime PCR system. Ten to 100 nanograms of cDNA were used per well in a total volume of 25 µl on 96-well clear top real time PCR plate. All samples were run in triplicates. For absolute quantification, standard curves for hPMAT and hOCT3 were generated on each plate using serial dilutions of the cDNA expression vectors with pre-determined copy numbers. For relative quantification, GUSB standard curve was generated using serial dilutions of Flp-in HEK293 cell cDNA. GUSB was selected as the reference gene because previous reports suggested its expression level is more stable than other commonly used housekeeping genes such as beta-actin and GAPDH (Fink et al., 2008; Romanowski et al., 2008). Absolute or relative amounts for each cDNA were calculated by plotting Log(amount) against Ct (threshold cycle) values on a semi-log plot. A linear relationship between Log(amount) and Ct was indicated by each standard curve.

**Uptake assays in cultured cells.** Flp-in pcDNA5 control cells, hPMAT and hOCT3 cells were plated in 24-well plates and allowed to grow for 2~3 days to reach 80~90% confluency. Transport assays were performed at 37°C in KRH buffer (5.6 mM glucose, 125 mM NaCl, 4.8 mM KCl, 1.2 mM KH2PO4, 1.2 mM CaCl2, 1.2 mM MgSO4, 25 mM HEPES, pH 7.4) containing known concentrations of substrates with radio-labeled tracer compounds. Uptake was terminated by washing the cells three times with ice-cold KRH buffer. Cells were then solubilized with 0.5 ml of 1 N NaOH at 37°C for 2 hrs, neutralized with 0.5 ml of 1 N HCl, and 0.4 ml of the lysates were used for liquid scintillation counting. Protein concentrations in the lysates were measured using a BCA protein assay kit (Pierce) and the uptake in each well was
normalized to its protein content. Transporter-specific uptake was calculated by subtracting the background uptake in Flp-in pcDNA5 cells. All uptake assays were performed in triplicates.

**Synaptosome preparation and uptake experiments.** Mouse brain synaptosomes were prepared using a method described by Zhu *et al.* (Zhu et al., 2006). Briefly, adult mice of both sexes (2-4 months old) were euthanized in a CO₂ chamber. Whole brain was quickly removed and homogenized on ice in a wheaton homogenizer with 5 ml of ice-cold sucrose homogenization buffer (320 mM sucrose, 5 mM HEPES, pH 7.4). The homogenate was centrifuged at 1000 g, 4°C for 10 min to remove nuclear debris. The supernatant was centrifuged again at 16,000 g, 4°C for 20 min. The synaptosome pellet was washed twice in 20 ml ice-cold synaptosome uptake buffer (130 mM NaCl, 10 mM d-glucose, 1.3 mM KCl, 2.2 mM CaCl₂, 1.2 mM MgSO₄, 1.2 mM KH₂PO₄, 10 mM HEPES, 0.1 mM ascorbate, pH 7.4) and resuspended in 6 ml of uptake buffer. For uptake assays, 100 µl of synaptosome preparations were pre-incubated with 100 µl of synaptosome uptake buffer with or without inhibitors for 10 min in a 37°C shaking water bath. To initiate the uptake, 100 µl of monoamine substrates spiked with [³H]-radiolabeled tracers were added to the synaptosomes. The final concentrations of all monoamine substrates were 0.5 µM. The inhibitors used in the uptake buffer were: citalopram (10 µM); GBR12909 (1 µM), desipramine (1 µM), decynium-22 (10 µM), and corticosterone (100 µM). After 5 min incubation at 37°C with gentle shaking, the uptake was terminated by diluting the synaptosome mixture in 5 ml ice-cold uptake buffer, followed immediately by filtration through Whatman GF/B glass microfiber filters on a vacuum filtration manifold. The filters were then washed with ice-cold uptake buffer, transferred to scintillation vials and immersed in scintillation fluid overnight before counting. Non-specific monoamine binding to filters was obtained by
setting up parallel assays without adding synaptosomes. Specific monoamine uptake of synaptosomes was calculated by subtracting non-specific binding counts.

**Data analysis.** Data points with error bars indicate mean ± S.D. for independent triplicates. All experiments were repeated 2~3 times. Where applicable, $p$ values were obtained through Student’s t-test. Data groups are considered significantly different if $p < 0.05$. The kinetics data were fitted in GraphPad Prism® software with the Michaelis-Menten equation $V = V_{\text{max}} S / (K_m + S)$, where $V$ is specific uptake rate, $S$ is substrate concentration, $V_{\text{max}}$ is the maximal uptake rate and $K_m$ is the Michaelis constant. All data obtained good fittings with $R^2 > 0.95$. 
Results

Stable expression of hOCT3 and hPMAT in Flp-in HEK293 cells. To directly compare the intrinsic transport efficiency \( (V_{\text{max}}/K_m) \) of hOCT3 and hPMAT towards monoamines, it is important to know the protein expression level of each transporter in the system. This is because the \( V_{\text{max}} \) measured from cell uptake studies is a product of intrinsic catalytic efficiency \( (K_{\text{cat}}) \) of a single transporter and the number of transporters \( (E_{\text{total}}) \) in the system (i.e. \( V_{\text{max}} = K_{\text{cat}} \times E_{\text{total}} \)). Currently, there are no methods to directly quantify the number of hOCT3 or hPMAT expressed on the cell surface. Because of the lack of purified hOCT3 or hPMAT protein as normalization standard, it is also not feasible to quantify protein expression using immunoblotting methods. One approach to overcome this problem is to generate cell lines that express these transporters at similar levels so that the \( V_{\text{max}} \) values are directly comparable to infer their intrinsic transport efficiency. To this end, we chose the Flp-in\(^\circledR\) system which utilizes Flp-recombinase to integrate an expressed gene into a defined genomic locus (Weichart et al., 2006; Nourian et al., 2008). The commercially supplied Flp-in HEK293 cell line is engineered to contain a single integration site. Stably transfected cells only contain a single copy of the transporter cDNA integrated at the same genomic locus and under the control of the same CMV promoter, resulting in consistent and comparable expression of the transfected gene. After transfection and hygromycin selection, we obtained stable cell lines for hOCT3 and hPMAT. Taqman real-time PCR was used to quantify the copy numbers of hOCT3 and hPMAT transcripts in the two cell lines. As shown in Figure 1A, hOCT3 and hPMAT transcripts were expressed at similar levels in the two transfected cell lines. Endogenous expression of hOCT3 and hPMAT mRNA in HEK293 cells was negligible (data not shown). Immunostaining with hOCT3- or hPMAT-specific antibodies
showed that the corresponding transporters were expressed on plasma membranes with uniform distribution in nearly every cell (Figure 1B). Since some background staining was observed for OCT3 immunostaining in control cells, additional western blot analysis was performed. The data confirmed that the hOCT3 protein was specifically expressed in hOCT3 cDNA-transfected but not in pcDNA5 vector-transfected cells (Supplemental Figure 1). To evaluate the expression stability with culture time, MPP+ (1 µM) uptake activity was measured in all subsequent uptake assays using cells of 10-30 passages. In all uptake experiments, absolute MPP+ transport rates were consistent with no more than 20% batch-to-batch variations (data not shown). These data suggest that we have obtained two human cell lines that stably express hOCT3 and hPMAT at similar levels on the cell surface.

Monoamine uptake by hOCT3 and hPMAT. To assess the activity of hOCT3 and hPMAT in the stable cell lines, parallel uptake was carried out in vector control, hOCT3 and hPMAT cells for endogenous monoamines (5-HT, dopamine, norepinephrine, epinephrine, histamine) and two model organic cations, MPP+ and TEA. All compounds were used at a final concentration of 1 µM. Compared to vector-transfected cells, significantly enhanced uptake was observed for all seven tested compounds in both hOCT3- and hPMAT-expressing cells (Figure 2). Both transporters demonstrated a high activity towards MPP+ but a very low activity for TEA. For the endogenous amines, the transport activities of hOCT3 were higher for histamine, epinephrine and norepinephrine than for dopamine and 5-HT. For hPMAT, an opposite pattern showing higher dopamine and 5-HT transport activity was observed.

Transport Kinetics. To determine the kinetic basis for the differential uptake activity of hOCT3 and hPMAT, initial rate studies were carried out. To define the initial rate period for
each substrate, time-dependent uptake was first carried out. The initial phase, where uptake rates increase linearly with time, varied for the test compounds and lasted about 2-5 min (Figure 3). Uptake was linear within 2 min for all compounds, and 2 min incubation time was therefore used in kinetic studies except for TEA. Due to the low TEA uptake activities for both hOCT3 and hPMAT, TEA kinetic study was extended to 5 min, which is still within the linear range (Figure 3), to increase signal-to-noise ratio. Metabolism of monoamines was also negligible as the monoamine oxidase inhibitor pargyline (10 µM) had no effect on uptake rates (data not shown).

We then performed parallel kinetic analysis for each substrate in vector, hOCT3- and hPMAT-expressing cells. Specific uptake was obtained by subtracting background uptake in vector-transfected cells. The data were fitted to the Michaelis-Menton equation (Figure 4) and the kinetic parameters were summarized in Tables 1 and 2. For MPP+, a probe substrate transported by all organic cation transporters, hOCT3 and hPMAT showed similar apparent binding affinity (Km = 166 ± 11 vs. 111 ± 3 µM), but the Vmax was somewhat higher for hOCT3, resulting in ~ 2-fold higher transport efficiency (Vmax/Km) than hPMAT. For TEA, the Vmax/Km values are similar between the two transporters, and the transport efficiency was only 3% of their transport efficiency toward MPP+. Interestingly, the mechanism underlying the low TEA transport efficiency appears to be different. For hPMAT, this was mainly due to a much reduced affinity (80-fold lower) in spite of a 2-fold increase in Vmax. However, for hOCT3, a reduced (5.7-fold) Vmax and a lower affinity (5.5-fold) both contributed.

For the endogenous monoamines, a seemingly complementary pattern was observed. For hOCT3, the rank order of Vmax/Km is histamine > norepinephrine, epinephrine > dopamine > 5-HT (Table 1). For hPMAT, the Vmax/Km rank order is: dopamine, 5-HT >> histamine,
norepinephrine, epinephrine (Table 2). Compared to hOCT3, hPMAT is much more selective towards 5-HT and dopamine as its $V_{\text{max}}/K_m$ for these two amines are 5-7 times greater than other amines. In contrast, only 2- to 4.6-fold difference in $V_{\text{max}}/K_m$ was observed for hOCT3 for its favored versus unfavored amines (Table 1). Interestingly, like TEA, the mechanisms underlying the monoamine selectivity of hOCT3 and hPMAT appeared quite different. For hPMAT, a distinction in $K_m$ was a primary driving force, and the transporter displayed up to 15-fold differences in $K_m$ but no more than 3-fold differences in $V_{\text{max}}$ for its favored amines (dopamine, 5-HT) versus the unfavored ones (histamine, norepinephrine, epinephrine) (Table 2). In contrast to hPMAT, the substrate preference of hOCT3 was not readily observable in its apparent binding affinity ($K_m$), as the transporter exhibited no more than 2-fold differences in its $K_m$ values towards all amines. Differences in $V_{\text{max}}$ contributed significantly in determining the monoamine preference (as measured by $V_{\text{max}}/K_m$) of hOCT3 (Table 1).

Quantification of hPMAT and hOCT3 transcripts in various human tissues and brain regions. Our analysis in heterologous expression system revealed distinct transport kinetic properties of hPMAT and hOCT3 towards endogenous monoamines. The relevance of these two transporters in clearing a specific monoamine in vivo will depend on their intrinsic transport efficiency for the amine and the abundance of the transporter in the relevant tissue. Using Taqman real-time PCR assay, we quantified the copy numbers of hPMAT and hOCT3 transcripts in mRNA pooled from normal human tissues (see Supplemental Table 1 for details). The absolute copy numbers of hPMAT and hOCT3 mRNA in 10 ng of total RNA as well as their relative levels normalized to GUSB are shown in Figure 5. In the nine tested human brain areas, hPMAT expression in general was much higher than hOCT3. hPMAT transcripts are
particularly abundant in the cerebral cortex, hippocampus, substantia nigra, medulla oblongata, and cerebellum. In the peripheral organs, adrenal gland and skeletal muscle highly and selectively expressed hOCT3, whereas pancreas selectively expressed hPMAT. In heart and small intestine, the two transporters were expressed at similar levels.

**Monoamine uptake in mouse brain synaptosomes.** To further explore the relevance of PMAT and OCT3 in brain monoamine uptake, we examined the uptake of the monoamine neurotransmitters 5-HT, dopamine and norepinephrine in synaptosomes prepared from mouse whole brain homogenates. To reveal uptake2 activities, the following well-established specific inhibitors were used to suppress uptake1 transporters: citalopram for SERT (Torres et al., 2003), GRB12909 for DAT (Andersen, 1989), and desipramine for NET (Bymaster et al., 2002). Previous studies demonstrated that decynium-22 (D22) is a potent inhibitor for both human PMAT and OCT3, whereas corticosterone potently inhibits OCT3 but not PMAT (Grundemann et al., 1998; Hayer-Zillgen et al., 2002; Engel et al., 2004). The concentrations of the inhibitors were chosen based on their reported K_i or IC_{50} values, and their effects on PMAT and OCT3 were first tested in the stably transfected cells. As expected, the uptake1 inhibitors citalopram (10 µM), GBR12909 (1 µM), and desipramine (1 µM) had negligible effect (less than 10% inhibition) on hPMAT- or hOCT3-mediated MPP+ uptake (Figure 6). On the other hand, corticosterone (100 µM) almost completely suppressed hOCT3 activity without affecting PMAT. D22 (10 µM) effectively inhibited both hOCT3 and hPMAT (Figure 6). These results were consistent with previous reports and justified the concentrations of the inhibitors used in our synaptosome uptake studies. We then examined the uptake of the monoamine neurotransmitters 5-HT, dopamine and norepinephrine in mouse brain synaptosomes. As shown in Figure 7A,
citalopram reduced synaptosome 5-HT uptake by ~50%, consistent with SERT playing a major role in brain 5-HT clearance. Co-inhibition of citalopram with D22 inhibited 5-HT uptake by ~70%, suggesting that non-SERT mediated 5-HT uptake may account for 20% of total synaptosome uptake. Surprisingly, D22 alone inhibited 5-HT uptake by 60%, suggesting that decynium-22 may also substantially affect SERT at the concentration used. In contrast, corticosterone had no effect on synaptosome 5-HT uptake. Similar inhibitory patterns were obtained for dopamine and norepinephrine uptakes (Fig. 7B and 7C). The DAT inhibitor GBR12909 inhibited synaptosomal dopamine uptake by more than 50%. D22 alone inhibited dopamine uptake by 65%. Co-inhibition of D22 with GBR12909 produced 85% inhibition. Corticosterone, alone or with GBR12909, had no specific or additive effect. For norepinephrine (Fig. 7C), the NET inhibitor desipramine inhibited synaptosomal norepinephrine uptake by about 45%. D22 alone only inhibited norepinephrine uptake by about 25%. Co-inhibition of desipramine with D22 produced 60% inhibition. Corticosterone still had no effect on norepinephrine uptake.
Discussion

The removal of released monoamine neurotransmitters through transporters is a major mechanism to inactivate the chemical messengers. Studies suggest that in addition to the high-affinity, low-capacity uptake\textsubscript{1} transporters, low-affinity, high-capacity uptake\textsubscript{2} transporters also play a significant role in regulating monoamine neurotransmission (Iversen, 1971; Eisenhofer, 2001). Uptake\textsubscript{2} transporters (e.g. PMAT and OCT3) may represent promising drug targets for neuropsychiatric disorders (Schildkraut and Mooney, 2004; Zhou et al., 2007; Daws, 2009). However, before these transporters can be further explored as drug targets, it is crucial to know the relative contribution of PMAT and OCT3 in clearing specific monoamines in vivo, which is directly related to their intrinsic transport efficiencies and expression levels at the physiological sites of interest.

The transport activities of OCT3 or PMAT towards monoamines have been evaluated previously in several discrete studies (Grundemann et al., 1998; Grundemann et al., 1999; Engel et al., 2004; Engel and Wang, 2005; Amphoux et al., 2006). However, results from these studies are difficult to compare because transporter expression levels vary in different systems and kinetic data are not available for some monoamines. Furthermore, discrepancies exist in published data. For example, the reported K\textsubscript{m} of hOCT3 for norepinephrine varied more than 5 fold (Grundemann et al., 1998; Amphoux et al., 2006; Koepsell et al., 2007), probably due to prolonged incubation time used in some studies. Therefore, in the current study, we aimed to comprehensively and rigorously analyze the transport kinetics of hOCT3 and hPMAT towards monoamines under well-controlled conditions.
Using the Flp-in® system, we isogenically expressed hPMAT and hOCT3 in HEK293 cell lines at comparable transcript levels (Figure 1). During the course of our study, the transport activities of both hPMAT and hOCT3 were stable as measured by MPP+ uptake (<20% variation, data not shown). All studies were done at a short incubation time within the linear range (Figure 3). Our analysis revealed complementary substrate preference of hPMAT and hOCT3 for the monoamine neurotransmitters. hPMAT exhibits strong preference for dopamine and 5-HT whereas hOCT3 favors histamine, norepinephrine and epinephrine. The two transporters differ markedly in their magnitudes of selectivity. The $V_{\text{max}}/K_{m}$ values of hPMAT for 5-HT and DA are 5-7 times greater than for other monoamines (Table 2). In contrast, hOCT3 shows only 2-4.6 fold difference in $V_{\text{max}}/K_{m}$ for monoamines. The mechanisms underlying their selectivity are also different. For hPMAT, the substrate preference is readily observable in its $K_{m}$, suggesting that initial substrate binding is an important step for hPMAT selectivity. In contrast, hOCT3 exhibits less than 2-fold variations in its $K_{m}$ values towards various monoamines. Distinction in $V_{\text{max}}$ contributes significantly to differences in $V_{\text{max}}/K_{m}$, suggesting that turnover rates are more important in the monoamine selectivity of hOCT3. MPP+ and TEA are two probe substrates for all organic cation transporters (Koepsell et al., 2007). MPP+ is efficiently transported by hOCT3 and hPMAT with comparable $K_{m}$. Notably, hPMAT transports dopamine and 5-HT almost as effectively as MPP+ (Table 2). hOCT3, however, transports its most favored monoamine, histamine, only at 50% efficiency of MPP+. We also found that TEA is a poor substrate for both hOCT3 and hPMAT with transport efficiencies only 3% of that of MPP+.

The complementary kinetic profiles of hPMAT and hOCT3 suggest that the two transporters may have distinct functions in monoamine uptake in vivo. To further evaluate the
roles of these transporters in vivo, we quantified the mRNA copy numbers in selected human tissues and brain regions by real-time RT-PCR (Figure 5). In the CNS, hPMAT transcripts significantly outnumber hOCT3 in eight of the nine tested regions, suggesting that hPMAT is the major uptake\textsubscript{2} transporter in the CNS. Consistent with our previous findings in mouse brain (Dahlin et al., 2007), hPMAT is highly expressed in brain areas innervated with serotonergic and dopaminergic fibers such as the cerebral cortex, hippocampus, substantia nigra and cerebellum. However, contrary to our previous northern blot data, which showed high hPMAT hybridization signal in skeletal muscle (Engel et al., 2004), we only detected low expression of hPMAT in this tissue by real-time RT-PCR. The reason for this discrepancy could be due to inter-individual variations in gene expression. Also, our previous study may have overestimated the abundance of hPMAT in skeletal muscle because the mRNA loading control (\(\beta\)-actin) was overly saturated in that study. Similar to this study, we did not detect significant expression of Pmat in mouse skeletal muscle (Dahlin et al., 2007). Our results showed that hOCT3 is selectively and highly expressed in the adrenal gland and skeletal muscle. The medulla of adrenal gland is the body’s major source of circulating epinephrine and norepinephrine, which elicit the fight-or-flight response (Ungar and Phillips, 1983). In the skeletal muscle, adrenergic signaling through \(\alpha\)- and \(\beta\)-adrenoceptors controls important physiological functions including muscle blood flow and metabolism (Lynch and Ryall, 2008). The high expression of hOCT3 in these organs may serve to take up excess circulating epinephrine/norepinephrine after their release.

The apparent affinities of hPMAT and hOCT3 towards the monoamine neurotransmitters are much lower than the uptake\textsubscript{1} transporters. The \(K_m\) values of hPMAT and hOCT3 towards monoamines are in the \(\mu\)M to mM range (Tables 1 and 2) whereas the \(K_m\) values of uptake\textsubscript{1}
transporters determined in heterologous expression systems are in the sub-micromolar range (Daws, 2009). The in vivo concentrations of monoamine neurotransmitters fluctuate and vary significantly in different brain areas. The extracellular concentrations of the monoamine are highest at its site of release and plummet as the transmitter diffuses away (Bunin and Wightman, 1999). For example, while the concentrations of extracellular 5-HT in mouse striatum and frontal cortex were determined to be in the low nanomolar range by microdialysis (Mathews et al., 2004), its concentration at the synaptic cleft was estimated to be as high as 6 mM (Bunin and Wightman, 1998), which far exceeds the $K_m$ of SERT (0.1-0.4 µM). Instead, 5-HT is estimated to diffuse more than 20 µm away from the synaptic cleft, where its concentration falls into the nanomolar range and 5-HT is recycled by SERT localized in the perisynaptic area (Bunin and Wightman, 1998; Bunin and Wightman, 1999). While the precise localization of PMAT or OCT3 in neurons is unknown, it is possible that they participate in the clearance of monoamines at high concentration areas and/or after repeated stimulation of monoaminergic neurons. Clearance by uptake2 transporters may also predominate when uptake1 transporters are inactivated pharmacologically, such as under antidepressant treatment. It may not be unusual that the brain employs both high-affinity and low-affinity uptake systems to regulate a wide concentration range of certain endogenous molecules. A recent analysis of $Slc$ gene expression in the mouse brain also demonstrated the co-expression of Na⁺-dependent, high affinity transporters as well as Na⁺-independent, low affinity transporters for other endogenous compounds such as choline, nucleosides, and glucose (Dahlin et al., 2009).

As an exploratory study, we also tested the relative contribution of Pmat and Oct3 to monoamine clearance in mouse brain synaptosomes using chemical inhibitors (Figure 7).
Citalopram, GBR12909 and desipramine reduced synaptosome 5-HT, dopamine and norepinephrine uptake by 45%~60%, consistent with uptake1 transporters playing a major role in brain monoamine clearance. Co-inhibition of D22 with uptake1 inhibitors suppressed monoamine uptake by 60%~85%, suggesting that uptake2 activities may account for 15%~35% of total uptake. The much greater inhibitory effect of D22 alone for 5-HT and dopamine uptake suggests that this compound may also inhibit SERT and DAT to certain degrees. In contrast, the OCT3-specific inhibitor corticosterone had no inhibitory effect on synaptosome uptake of any of the monoamines. These data suggest that PMAT may play a more important role in mediating uptake2 activities in the brain. However, we can not exclude the role of Oct3 in brain monoamine uptake as there are limitations in the synaptosome uptake studies. Synaptosome studies mainly detect uptake activities in neuronal cells. Oct3 is expressed in astroglial cells (Cui et al., 2009) and could be important for monoamine uptake in these cells. In addition, our studies used synaptosomes prepared from whole brain homogenates, and thus may not be able to detect Oct3 activities in specific brain areas or microstructures where its expression is high.

In summary, we examined side-by-side the transport kinetics of hPMAT and hOCT3 for endogenous monoamines in a well controlled expression system. Our data demonstrate complementary substrate preference and distinct tissue expression patterns for hOCT3 and hPMAT. Our results suggest that hPMAT is the major uptake2 transporter for 5-HT and DA in the CNS, whereas hOCT3 represent the major uptake2 transporter for histamine, norepinephrine and epinephrine in peripheral organs. To explore uptake2 transporters as potential drug targets, the distinct roles of these transporters in human physiology should be considered.
References


Footnotes

a) This work was supported by National Institutes of Health National Institute of General Medicine Sciences [Grant GM066233].

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c) We acknowledge Dr. Horace Ho for his contribution to the OCT3 western blot in Supplemental Figure 1.
Figure Legends

**Figure 1.** (A) Quantification of hOCT3 and hPMAT mRNA levels in stably transfected Flp-in HEK293 cells. Total RNA from Flp-293-pcDNA5, hOCT3 and hPMAT cells were extracted and reverse transcribed. hOCT3 and hPMAT mRNA copy numbers were determined by Taqman real-time RT-PCR using specific primers and probes. Diluted hOCT3 and hPMAT expression vectors with known copy numbers were used as standards to determine absolute mRNA copy numbers. (B) Localization of hOCT3 and hPMAT in Flp-in HEK293 cells stably transfected with hPMAT or hOCT3. Cells transfected with pcDNA5 vector were used as control. Confluent cells were immunostained with anti-OCT3 or anti-PMAT primary antibodies and Alexa fluor conjugated secondary antibodies. Images were taken under fluorescent microscope with corresponding filters.

**Figure 2.** Uptake of MPP+, TEA and monoamines by hOCT3 and hPMAT. Flp-293 pcDNA5, hOCT3 and hPMAT cells were run in parallel with substrate concentrations at 1 µM.

**Figure 3.** Time-dependent uptake of MPP+, TEA and monoamines by hOCT3 and hPMAT. Uptake experiments were conducted as described in Materials and Methods for up to 30 minutes. Time points were chosen within the linear uptake phases (which range between 2-5 minutes) to determine kinetics parameters. The concentrations of substrates are: MPP+ 1 µM, dopamine 10 µM, 5-HT 10 µM, norepinephrine 20 µM, epinephrine 20 µM, histamine 10 µM, TEA 20 µM.
Figure 4. Concentration-dependent uptake for MPP+, TEA, and monoamines in hOCT3 and hPMAT cells. Uptake for a specific substrate was carried out in parallel in Flp-293 pcDNA5, hOCT3 and hPMAT cells. Uptake in pcDNA5 cells were subtracted from hOCT3 or hPMAT cells to obtain transporter-specific uptake. Uptake time points were 5 min for TEA and 2 min for all other substrates.

Figure 5. hOCT3 and hPMAT mRNA levels in various human tissues and brain regions. Total RNA from human tissues were reverse-transcribed to cDNA. hOCT3, hPMAT and hGUSB transcripts levels were quantified using Taqman real-time RT-PCR. (A), absolute copy numbers of hOCT3 and hPMAT mRNA in 10 ng of total RNA; (B), hOCT3 and hPMAT mRNA levels normalized to hGUSB.

Figure 6. Effects of inhibitors used in synaptosome uptake studies on MPP+ uptake mediated by hOCT3 and hPMAT. Flp-293 pcDNA5, hOCT3 and hPMAT cells were run in parallel with MPP+ concentrations at 1 µM and in the absence or presence of inhibitors. NT: no treatment; Cit: citalopram (10 µM); Cort: corticosterone (100 µM); D22: decynium-22 (10 µM); GBR: GBR12909 (1 µM); Des: desipramine (1 µM). Data were expressed as percentage of MPP+ uptake obtained from cells with no treatment.
Figure 7. Uptake of 0.5 µM of 5-HT (A), dopamine (B) and norepinephrine (C) in adult mouse brain synaptosomes in the presence or absence (no treatment) of various inhibitors. Cit: citalopram (10 µM); Cort: corticosterone (100 µM); D22: decynium-22 (10 µM); GBR: GBR12909 (1 µM); Des: desipramine (1 µM). *p<0.05 compared to No Treatment (NT); **p<0.05 compared to citalopram or GBR12909 or desipramine alone.
Table 1. Summary of Kinetic Parameters for hOCT3.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>(K_m) (µM)</th>
<th>(V_{max}) (pmol/mg protein/min)</th>
<th>(V_{max}/K_m) (µl/mg protein/min)</th>
<th>(V_{max}/K_m) relative to MPP+</th>
</tr>
</thead>
<tbody>
<tr>
<td>MPP+</td>
<td>166 ± 11</td>
<td>15,664 ± 821</td>
<td>94.7 ± 1.1</td>
<td>100%</td>
</tr>
<tr>
<td>TEA</td>
<td>921 ± 161</td>
<td>2,760 ± 92</td>
<td>3.03 ± 0.43</td>
<td>3.2%</td>
</tr>
<tr>
<td>Dopamine</td>
<td>1,033 ± 127</td>
<td>22,676 ± 484</td>
<td>22.1 ± 3.2</td>
<td>23%</td>
</tr>
<tr>
<td>5-HT</td>
<td>988 ± 264</td>
<td>11,562 ± 3,109</td>
<td>11.7 ± 0.2</td>
<td>12%</td>
</tr>
<tr>
<td>Norepinephrine</td>
<td>923 ± 172</td>
<td>30,134 ± 2,674</td>
<td>32.9 ± 1.3</td>
<td>35%</td>
</tr>
<tr>
<td>Epinephrine</td>
<td>458 ± 37</td>
<td>12,760 ± 605</td>
<td>28.0 ± 3.6</td>
<td>30%</td>
</tr>
<tr>
<td>Histamine</td>
<td>641 ± 24</td>
<td>34,604 ± 47</td>
<td>54.0 ± 2.1</td>
<td>57%</td>
</tr>
</tbody>
</table>

Concentration-dependent uptake for MPP+, TEA, and monoamines were carried out in parallel in Flp-293 pcDNA5 and hOCT3 cells. Uptake in pcDNA5 cells were subtracted from hOCT3 cells to obtain hOCT3-specific uptake. Uptake time points were 5 min for TEA and 2 min for all other substrates. The kinetics data were fitted in GraphPad Prism® software with the Michaelis-Menten equation. Data represent Mean ± S.D. for 2~3 independent experiments.
Table 2. Summary of Kinetic Parameters for hPMAT. Data represent Mean ± S.D. for 2~3 independent experiments.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>$K_m$ (µM)</th>
<th>$V_{max}$ (pmol/mg protein/min)</th>
<th>$V_{max}/K_m$ (µl/mg protein/min)</th>
<th>$V_{max}/K_m$ relative to MPP+</th>
</tr>
</thead>
<tbody>
<tr>
<td>MPP+</td>
<td>111 ± 3</td>
<td>6,557 ± 942</td>
<td>59.2 ± 10.0</td>
<td>100%</td>
</tr>
<tr>
<td>TEA</td>
<td>8,759 ± 3,175</td>
<td>15,246 ± 3,023</td>
<td>1.79 ± 0.30</td>
<td>3.0%</td>
</tr>
<tr>
<td>Dopamine</td>
<td>406 ± 48</td>
<td>22,402 ± 3,166</td>
<td>55.1 ± 1.3</td>
<td>93%</td>
</tr>
<tr>
<td>5-HT</td>
<td>283 ± 40</td>
<td>14,194 ± 2,381</td>
<td>50.1 ± 1.4</td>
<td>85%</td>
</tr>
<tr>
<td>Norepinephrine</td>
<td>1,078 ± 107</td>
<td>8,822 ± 1,323</td>
<td>8.16 ± 0.41</td>
<td>14%</td>
</tr>
<tr>
<td>Epinephrine</td>
<td>951 ± 59</td>
<td>7,252 ± 195</td>
<td>7.65 ± 0.68</td>
<td>13%</td>
</tr>
<tr>
<td>Histamine</td>
<td>4,379 ± 679</td>
<td>42,374 ± 4,098</td>
<td>9.72 ± 0.57</td>
<td>16%</td>
</tr>
</tbody>
</table>

Concentration-dependent uptake for MPP+, TEA, and monoamines were carried out in parallel in Flp-293 pcDNA5 and hPMAT cells. Uptake in pcDNA5 cells were subtracted from hPMAT cells to obtain hPMAT-specific uptake. Uptake time points were 5 min for TEA and 2 min for all other substrates. The kinetics data were fitted in GraphPad Prism® software with the Michaelis-Menten equation. Data represent Mean ± S.D. for 2~3 independent experiments.
Figure 1

A

![Graph showing mRNA/hGUSB (arbitrary units) for hOCT3 and hPMAT cells.]

B

- pcDNA5 cells
- hOCT3 cells
- hPMAT cells

Anti-PMAT

Anti-OCT3
Figure 2

The graph shows the uptake (pmol/mg protein/min) of various substances over time, comparing pcDNA5, hOCT3, and hPMAT conditions. The substances include MPP+, TEA, Dopamine, 5-HT, Norepinephrine, Epinephrine, and Histamine.
Figure 3

**MPP+**

- pcDNA5
- hPMAT
- hOCT3

**TEA**

- pcDNA5
- hPMAT
- hOCT3

**Dopamine**

- pcDNA5
- hPMAT
- hOCT3

**5-HT**

- pcDNA5
- hPMAT
- hOCT3

**Norepinephrine**

- pcDNA5
- hPMAT
- hOCT3

**Epinephrine**

- pcDNA5
- hPMAT
- hOCT3

**Histamine**

- pcDNA5
- hPMAT
- hOCT3
Figure 5

A

B

mRNA copies per 10 ng RNA

Adrenal gland, Heart, Skeletal Muscle, Small Intestine, Pancreas, Spinal Cord, Cerebellum, Substantia Nigra, Cerebral Cortex, Medulla Oblongata, Hippocampus, Caudate Nucleus, Nucleus Accumbens, Pons

mRNA/hGUSB (arbitrary units)

Adrenal gland, Heart, Skeletal Muscle, Small Intestine, Pancreas, Spinal Cord, Cerebellum, Substantia Nigra, Cerebral Cortex, Medulla Oblongata, Hippocampus, Caudate Nucleus, Nucleus Accumbens, Pons
Figure 6

The figure shows the MPP+ uptake with different treatments. The treatments include NT, Cit, Cort, D22, GBR, and Des. The x-axis represents the different treatments, and the y-axis represents the MPP+ uptake percentage. Two categories are compared: hOCT3 and hPMAT. The bars indicate the percentage uptake, with error bars showing the variability.
Figure 7

(A) 5-HT Uptake (pmol/mg protein/min)

(B) Dopamine Uptake (pmol/mg protein/min)

(C) Norepinephrine Uptake (pmol/mg protein/min)
Selective Transport of Monoamine Neurotransmitters by Human Plasma Membrane Monoamine Transporter and Organic Cation Transporter 3

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Journal Title: The Journal of Pharmacology and Experimental Therapeutics.

Supplemental Figure 1. Western blot of membrane proteins from hOCT3- and vector-transfected Flp-in HEK293 cells.

hOCT3   pcDNA5

Method. Confluent cells were washed twice with 3 ml of ice-cold phosphate-buffered saline (PBS), pH 8.0. Biotinylation was carried out on ice by incubation with 1 ml of PBS containing a membrane-impermeable biotinylation reagent NHS-SS-biotin (0.5 mg/ml, Thermo Scientific). After two successive 20-min incubations on ice with freshly prepared NHS-SS-biotin and gentle shaking, cells were briefly rinsed with 3 ml of PBS containing 100mM glycine. Cells were then incubated on ice with the same solution for 20 min to quench the unreacted NHS-SS-biotin. Cells were then solubilized on ice by incubating in 1 ml of lysis buffer (10 mM Tris, 150 mM NaCl, 1 mM EDTA, 0.1% SDS, 1% Triton X-100, protease inhibitors phenylmethylsulfonyl fluoride 200 µg/ml, leupeptin 3µg/ml, pH 7.4) for 1 h. Fifty microliters of streptavidin-agarose beads was then added to the supernatant to isolate cell membrane protein, which are subjected to Western blot using OCT3 antibody (1:1000 dilution), followed by horseradish peroxidase-conjugated goat anti-rabbit IgG (1:10,000 dilution).
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**Supplemental Table 1.** List of RNA tissue pools used for real-time PCR quantification of hPMAT and hOCT3 expression levels

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Subject Number</th>
<th>Gender</th>
<th>Age Range</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adrenal gland</td>
<td>62</td>
<td>both</td>
<td>15-61</td>
</tr>
<tr>
<td>Heart</td>
<td>3</td>
<td>male</td>
<td>30-39</td>
</tr>
<tr>
<td>Skeletal muscle</td>
<td>2</td>
<td>both</td>
<td>43 &amp; 46</td>
</tr>
<tr>
<td>Small intestine</td>
<td>5</td>
<td>both</td>
<td>20-61</td>
</tr>
<tr>
<td>Pancreas</td>
<td>1</td>
<td>male</td>
<td>35</td>
</tr>
<tr>
<td>Spinal cord</td>
<td>22</td>
<td>both</td>
<td>20-69</td>
</tr>
<tr>
<td>Cerebellum</td>
<td>10</td>
<td>both</td>
<td>22-68</td>
</tr>
<tr>
<td>Substantia Nigra</td>
<td>19</td>
<td>both</td>
<td>25-64</td>
</tr>
<tr>
<td>Cerebral cortex</td>
<td>10</td>
<td>both</td>
<td>20-68</td>
</tr>
<tr>
<td>Medulla oblongata</td>
<td>29</td>
<td>both</td>
<td>18-64</td>
</tr>
<tr>
<td>Hippocampus</td>
<td>20</td>
<td>both</td>
<td>18-54</td>
</tr>
<tr>
<td>Caudate nucleus</td>
<td>23</td>
<td>both</td>
<td>18-55</td>
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<tr>
<td>Nucleus accumbens</td>
<td>6</td>
<td>both</td>
<td>23-56</td>
</tr>
<tr>
<td>Pons</td>
<td>20</td>
<td>both</td>
<td>18-54</td>
</tr>
</tbody>
</table>