Differential serotonin uptake mechanisms at the human maternal-fetal interface

Petra Baković[1,*], Maja Kesić[1,*], Marina Horvatiček[1], Meekha George[2], Maja Perić[1], Lipa Čičin-Šain[1], Gernot Desoye[3], Christian Wadsack[3], Ute Panzenboeck[2], Jasminka Štefulj[1]

[1]Department of Molecular Biology, Ruđer Bošković Institute, Bijenička cesta 54, HR-10000 Zagreb, Croatia; [2]Division of Immunology and Pathophysiology, Otto Loewi Research Center, Medical University of Graz, Heinrichstrasse 31a, A-8010 Graz, Austria; [3]Department of Obstetrics and Gynaecology, Medical University of Graz, Auenbruggerplatz 14, A-8036 Graz, Austria

[*]PB and MK contributed equally to this work

Correspondence

Jasminka Štefulj

Department of Molecular Biology, Ruđer Bošković Institute

Bijenička cesta 54, HR-10000 Zagreb, Croatia

E-mail: jasminka.stefulj@irb.hr

ORCID ID: 0000-0001-8593-9698

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ABSTRACT

Cellular serotonin (5-HT) uptake is central to regulating local levels of 5-HT nearby its molecular targets. Here we studied 5-HT uptake mechanisms in primary placental cells and cord blood platelets, all isolated directly from the human tissues. All cell types took up 5-HT in a time- and temperature-dependent manner. In initial-rate experiments in primary term trophoblasts and cord blood platelets, saturation curves of active 5-HT uptake across multiple 5-HT concentrations were characteristic of the high-affinity transporter-mediated uptake mechanism. In contrast, primary term feto-placental endothelial cells displayed saturation kinetics only over the low-affinity range of 5-HT concentrations. Citalopram, a potent blocker of the serotonin transporter (SERT), inhibited 5-HT uptake in TMT, but not in PEC. In line with this, *SERT* mRNA was abundant in term trophoblasts, but sparse in feto-placental endothelial cells, while the opposite was found for transcripts of the low-affinity plasma membrane monoamine transporter (PMAT). 5-HT uptake into first trimester trophoblasts could not be saturated over the high-affinity range of 5-HT concentrations; as compared to term trophoblasts, first trimester trophoblasts expressed lower and higher levels of *SERT* and *PMAT* mRNAs, respectively. We conclude that 1) placental cells facing maternal and fetal blood at term of human pregnancy use different, low- and high-affinity, respectively, 5-HT uptake systems, 2) fetal platelets possess highly functional high-affinity 5-HT uptake activity, 3) 5-HT uptake mechanisms in trophoblasts change over the course of pregnancy. The multiple molecular mechanisms present for 5-HT uptake highlight the importance of maintaining 5-HT homeostasis at the maternal-fetal interface.

Keywords

cord blood platelets; feto-placental endothelial cells; human placenta; primary trophoblasts; serotonin; serotonin uptake

INTRODUCTION

Serotonin (5-hydroxytryptamine, 5-HT) is a multifunctional biogenic indolamine, best known as the brain modulatory neurotransmitter involved in mood, sleep and appetite regulation as well as in many mental health conditions. Besides, 5-HT acts as an endocrine, paracrine or autocrine agent regulating different peripheral functions including vascular tone, hemostasis, intestinal motility, immune response, bone remodeling and energy metabolism [1, 2]. During pregnancy, 5-HT mediates fine-tuning of embryonic/fetal development [3], and plays important roles in placental physiology [4]. It modulates different processes in placental cells, including proliferation, cell viability, cell cycle progression, apoptosis and estrogen production [5–9]. As a potent vasoactive autacoid, 5-HT also influences utero-placental and feto-placental blood flow [10, 11]. Additionally, in early pregnancy the placenta supplies the developing fetus with maternal and/or placenta-derived 5-HT required for a proper (neuro)development [12–14]. This wide range of functions may account for different studies associating altered placental 5-HT homeostasis with pregnancy disorders such as preeclampsia [15], fetal growth restriction [16], and gestational diabetes mellitus [17], as well as with mental health implications in the offspring [18–22].

5-HT exerts its physiological effects by interacting with plasma membrane-bound 5-HT receptors, which include one ligand-gated ion channel and a variety of metabotropic receptors coupled to diverse intracellular signaling pathways [23]. In addition, 5-HT regulates some physiological functions in a receptor-independent mode, by covalently binding to several extracellular and cytosolic proteins [24]. New findings demonstrate that 5-HT can covalently attach also to histone proteins and regulate gene transcription [25]. All these effects depend on the availability of 5-HT in the vicinity of its molecular targets. Systems for transport of 5-HT across biological membranes efficiently control local 5-HT concentrations and thus play a central role in governing 5-HT actions.

There are two distinct plasma membrane transport systems for 5-HT, uptake-1 and uptake-2, characterized by fundamentally different kinetic properties. Uptake-1 system for 5-HT is represented by the serotonin transporter (SERT), a highly specific, high-affinity / low-capacity 5-HT carrier [26]. SERT is best known for mediating reuptake of 5-HT back into presynaptic neurons. Due to its central role in the clearance of 5-HT from the synaptic cleft, it is a target of various psychotropic drugs, including widely prescribed tricyclic antidepressants (TCAs) and selective serotonin reuptake inhibitors (SSRIs). In adults, SERT also mediates 5-HT uptake into platelets, representing thus a major mechanism that regulates active levels of 5-HT in blood plasma [27]. However, an analogous mechanism for uptake of 5-HT in the fetal circulation has not yet been described. Unlike SERT, carriers of the uptake-2 system, such as the plasma membrane monoamine transporter (PMAT) and organic cation transporter 3 (OCT3), exhibit low-affinity / high-capacity transport kinetics and readily transport 5-HT as well as other organic monoamines [28–31].

The placenta is a complex, heterocellular organ interposed between maternal and fetal blood. Two principal cell types constitute the maternal-fetal blood interface: 1) trophoblasts covering the entire surface of villous trees are directly in contact with maternal blood, and 2) feto-placental endothelial cells forming feto-placental vasculature are in direct contact with the fetal blood. There is evidence of placental uptake of 5-HT from both maternal and fetal circulation at term of human pregnancy. The presence of high-affinity uptake of 5-HT from maternal blood has been first suggested by 5-HT transport studies in plasma membrane vesicles isolated from human term placentas [32]. An analogous 5-HT uptake system, sensitive to TCAs and SSRIs, is present in human trophoblast-like choriocarcinoma cell line JAR [33]. The presence of placental uptake of 5-HT from fetal blood has been recently suggested by *in situ* dual perfusion system in rat term placentas and 5-HT transport studies in plasma membrane vesicles isolated from human term placentas [34]. Yet cell-type specific contributions to 5-HT uptake at the human maternal-fetal interface are not well understood.

Despite the growing interest in the feto-placental and fetal 5-HT system, kinetic and pharmacological properties of 5-HT uptake in primary trophoblasts have not been studied. There is also a clear lack of studies on potential mechanisms of 5-HT uptake into cells that are in direct contact with the fetal plasma, such as feto-placental endothelial cells and fetal platelets. We hypothesized that these cells have functional systems for active uptake of 5-HT. This is important, because such systems will contribute to the regulation of fetal circulating 5-HT levels. Therefore, to expand understanding of the mechanisms maintaining 5-HT homeostasis during human development, we examined and characterized 5-HT uptake activity in primary trophoblasts, feto-placental endothelial cells and cord blood platelets, all isolated directly from the human tissues.

MATERIALS AND METHODS

Materials

Tritium-labelled serotonin creatinine sulphate (^{3}H-serotonin; 28.3 Ci mmol^{-1}, 41.3 Ci mmol^{-1}) and radiocarbon-labelled serotonin binoxalate (^{14}C-serotonin; 54.0 mCi mmol^{-1}) were purchased from Perkin Elmer (Waltham, Massachusetts, USA). Serotonin creatinine sulphate, imipramine hydrochloride, citalopram hydrobromide, 3,4-methylenedioxymethamphetamine hydrochloride (MDMA, 'Ecstasy'), decynium 22 (D22), L-ascorbic acid, and Hanks' Balanced Salts were obtained from Sigma-Aldrich (St. Louis, Missouri, USA). Pargyline hydrochloride was from Cayman Chemical (Ann Arbor, Michigan, USA). Other drugs were generous gifts from the manufacturers: fluoxetine hydrochloride from Eli Lilly and Company (USA), paroxetine hydrochloride from GlaxoSmithKline (England), fluvoxamine maleate from Solvay (Netherlands) and Clomipramine from BASF Schweiz AG (Switzerland).

Isolation and culture of human primary placental cells

Term pregnancy placentas were obtained from women undergoing normal delivery between the 37th and 42nd gestational week (Supplementary Table S1). First trimester placentas were obtained from woman undergoing surgical termination of pregnancy between the 7th and 9th gestational week. Written informed consent was

obtained from all women according to the study protocol approved by the Ethics Committee of the Medical University of Graz, Graz, Austria. Primary cultures of term trophoblasts (TMT) and fetal-placental endothelial cells (PEC) were prepared from villous tissue and feto-placental vessels, respectively, dissected from term placentas, and first trimester trophoblasts (FTT) were isolated from first trimester placentas, all according to previously established protocols [35–37]. Representative cell isolations were routinely tested for identity and purity by immunocytochemical staining of specific cell markers, as described [35–37]. Cells were cultured on gelatine (1%)-coated plates, TMT and FTT in Dulbecco's Modified Eagle's Medium (DMEM; Gibco, Paisley, UK), and PEC in Endothelial Cell Basal Medium (EBM; Lonza, Verviers, Belgium) or Endothelial Cell Growth Medium MV (PromoCell, Heidelberg, Germany), all supplemented with 10% fetal bovine serum and 1% antibiotic. Cultures were maintained at 37°C, 5% CO_2 and 21% O_2.

5-HT uptake in human primary placental cells

For 5-HT uptake studies, cells were seeded onto 24-well plates and allowed to grow until reaching confluency (usually for 1 to 2 days). The uptake buffer consisted of Hanks' Balanced Salt solution (HBSS; 1.25 mM $CaCl_2$, 0.8 mM $MgSO_4$, 3.32 mM KCl, 0.5 mM KH_2PO_4, 66.5 mM NaCl, 0.4 mM Na_2HPO_4, 1 mM D-glucose, 0.03 mM Phenol Red•Na), ascorbic acid (100 μM) and pargyline (10 μM) to prevent 5-HT oxidation and enzymatic degradation, respectively, and a mixture of ^{3}H-labeled and unlabeled 5-HT totaling the required final 5-HT concentration. Following removal of media, cells were washed with HBSS to remove traces of serum. Uptake was initiated by the addition of pre-warmed (37°C) or precooled (4°C) uptake buffer (0.2 mL/well) and incubations were carried out at 37°C or on ice, respectively, for specific amount of time. The uptake was terminated by quick removal of the uptake buffer and addition of ice-cold HBSS (1 mL/well). Cells were then washed with ice-cold HBSS and lysed in 0.3 N NaOH (0.4 mL/well). Radioactivity in cell lysates was quantified on Tri-Carb 2100TR Liquid Scintillation Counter, using Ultima Gold liquid scintillation cocktail (both from Perkin Elmer, Waltham, Massachusetts, USA). Total protein concentration in cell lysates was measured using Qubit™ Protein Assay Kit and Qubit fluorimeter (both from Thermo Fisher Scientific, Waltham, Massachusetts, USA). Active (carrier-mediated) uptake was calculated as the difference between total (at 37°C) and non-specific (on ice) uptake. All assays were performed in triplicates. In initial rate studies, 5-HT uptake was measured within experimentally determined linear time range, at six increasing 5-HT concentrations covering a range typical for high-affinity (0.1, 0.2, 0.4, 0.8. 1.6 and 3.2 μM) and low-affinity (94, 188, 375, 1500 and 3000 μM) uptake mechanism. The initial rates of active 5-HT uptake were fitted into Michaelis-Menten kinetics model using GraphPad Prism software. Best-fit values of Michaelis affinity constant (*Km*) and maximal transport velocity (*Vmax*) were estimated by nonlinear least-squares regression analysis.

Pharmacological studies

Stock solutions of MDMA (10^{-1} M) and D22 (10^{-2} M) were prepared in DMSO, of citalopram (10^{-2} M) in PBS and of all other drugs in HBSS. Following removal of media and rinsing with HBSS, cells were pre-incubated for 10 min in the presence of either drugs or vehicle (control) and then incubated for 10 min in the presence of

^{3}H-5-HT and either drugs or vehicle, respectively. Transport on ice was subtracted from that at 37°C and active uptake in the presence of each drug was expressed as percent of the control (vehicle). There were no significant differences in 5-HT uptake between controls prepared with either DMSO, PBS or HBSS at concentrations equivalent to that in samples with drugs. Half maximal inhibitory concentration values (IC_{50}) along with 95% confidence intervals (CI) were determined by GraphPad Prism software, using nonlinear least-squares regression analysis.

Gene expression analyses

For gene expression analyses, cells were grown on 6-well plates and harvested at the state of confluency. Total RNA was extracted using RNeasy Plus Mini Kit (Qiagen, Hilden, Germany), according to the manufacturer's protocol including optional on-column DNA digestion step. Concentration and purity of RNA was determined spectrophotometrically (NanoDrop, Witec AG, Littau, Germany) and RNA integrity was assessed by 1% agarose gel electrophoresis. Relative levels of specific mRNAs were determined by reverse transcription (RT)-quantitative PCR (RT-qPCR) analysis based on SyberGreen detection chemistry. RT was performed on C1000 Thermal Cycler (Bio-Rad), using uniform amounts of RNA and iScript cDNA Synthesis Kit (Bio-Rad) according to the manufacturer's protocol. Reactions lacking reverse transcriptase (no-RT) were prepared to check for genomic DNA contamination. qPCR assays were performed on CFX 96 Real-Time System using iQ SYBR Green Supermix (both from Bio-Rad) or on 7300 Real Time PCR System using Syber Green Master Mix (both from Applied Biosystems), following manufacturers' recommendation. Sequences of primers targeting genes of interest (*SERT*, *PMAT*, *OCT3*, *MAOA*, *MAOB)* and a reference gene (*ACTB*) are listed in Table S2. The specificity of qPCR products was verified by agarose gel electrophoresis and routinely by melting curve analysis. All reactions were run in triplicates. qPCR efficiencies of genes of interest were about equal to that of *ACTB* (the slope of log input amount versus $\Delta C_q < 0.1$ in all cases) and relative expression levels were calculated using the comparative C_q ($\Delta\Delta C_q$) method [38].

Preparation of cord blood platelet rich plasma

Cord blood samples were obtained from singleton neonates born at term (37 to 42 weeks of gestation) by elective Caesarean section (Supplementary Table S1). Written informed consent was obtained from their mothers in accordance with the study protocol approved by the Ethics Committee of the Clinical Hospital Centre Zagreb and Bioethics Committee of the Ruđer Bošković Institute, Zagreb, Croatia. Cord blood samples were collected via umbilical venipuncture, after baby birth and before delivery of the placenta, and were immediately mixed with acid citrate dextrose (ACD) anticoagulant in a ratio 1 : 5 (ACD : blood). Cord blood platelet rich plasma (CB-PRP) was isolated by centrifugation of anti-coagulated blood for 2 min at 1100 g. Platelet count and volume was determined using DxH 500 hematology analyzer (Beckman Coulter). Platelet counts (mean ± SD, n=9) in whole cord blood and CB-PRP samples amounted to 242 ± 112 x 10^9 L^{-1} and 388 ± 92 x 10^9 L^{-1}, respectively. Mean platelet volume (mean ± SD, n=9) in whole blood and CB-PRP samples amounted to 7.8 ± 0.7 fL and 7.4 ± 0.62 fL, respectively, and were highly correlated (Spearmen's correlation coefficient = 0.94, *P*=0.001, n=9), demonstrating that population of platelets isolated in CB-PRP represented

well platelets in the whole cord blood samples. Platelet protein levels were determined by Bradford's assay, following centrifugation of CB-PRP aliquots and sonication of platelet pellets.

5-HT uptake in cord blood platelets

Uptake of 5-HT in cord blood platelets (CBP) was measured within one hour after CB-PRP preparation, according to a slightly modified protocol used in our previous studies on adults [39, 40]. In brief, aliquots of CB-PRP (60 µL) were added into 840 µL of $CaCl_2$-free Krebs-Ringer phosphate buffer (KRB; 123.2 mM NaCl, 10 mM NaH_2PO_4 x $2H_20$, 1.23 mM $MgSO_4$ x $7H_20$, 4.9 mM, KCl, pH 7.4) and pre-incubated for 10 min in a shaking bath at 37°C. Uptake was initiated by the addition of 100 µL KRB containing ^{14}C-5-HT. Final concentrations of ^{14}C-5-HT in the reaction mixture amounted to 0.15, 0.25, 0.40, 0.70, 1.20 and 2.00 µM. Reactions were carried out in a shaking bath at 37°C for 1 min and were terminated by rapid cooling (addition of 5 mL ice-cold saline) and immediate vacuum (500 mmHg) filtration over a glass microfiber filters (Whatman GF/C, GE Healthcare, Illinois, USA). The filters were rinsed, and the radioactivity retained on them was quantified on Tri-Carb 2810TR Liquid Scintillation Analyzer, using Ultima Gold MV liquid scintillation cocktail (both from Perkin Elmer, Waltham, Massachusetts, USA). Nonspecific uptake was measured by the same procedure, but at around 0°C (ice bath). Assays were performed in duplicates. Active (carrier-mediated) uptake, calculated as a difference between total (at 37°C) and nonspecific (in ice bath) uptake, was expressed per platelet number (for comparison with published data for adult platelets) and per platelet protein (for comparison with TMT and PEC). Values of *Km* and *Vmax* were determined as described for primary placental cells.

Statistical analysis

Statistical analyses were conducted using GraphPad Prism version 8 (GraphPad Software, LLC, San Diego, CA, USA). Normality of data was tested by D'Agostino-Pearson omnibus normality test. Normally distributed data were analyzed using unpaired t-test, one-way analysis of variance (ANOVA) or two-way ANOVA, as appropriate. Data that were not normally distributed were analyzed using Mann-Whitney test. The level of significance was set at 0.05.

RESULTS

Kinetics of 5-HT uptake in term trophoblasts

Human primary term trophoblasts (TMT) showed time-dependent and temperature-sensitive uptake of 5-HT (Figure 1A). Experiments measuring the initial rates of active 5-HT uptake over a high-affinity range of 5-HT concentrations (0.1 to 3.2 µM) demonstrated saturation kinetics compliant with the Michaelis-Menten kinetics model (Figure 1B). The *Km* values calculated in all subjects tested (Figure 1C and Table 1) were typical of high-affinity, SERT-mediated, 5-HT uptake [26].

Kinetics of 5-HT uptake in term feto-placental endothelial cells

Like TMT, human primary fetal-placental endothelial cells (PEC) took up 5-HT in a time- and temperature-dependent manner (Figure 1D), demonstrating the presence of a carrier-mediated process. However, active uptake of 5-HT, measured at submicromolar (10^{-7} M) substrate concentration and expressed per total cellular protein, was lower by up to 70% (P<0.0001) in PEC than TMT (compare also Figures 1A and 1D). Further, the initial rates of active 5-HT uptake in PEC failed to reach saturation over a high-affinity range of 5-HT concentrations (0.1 to 5 μM; data not shown). In contrast, a saturation curve typical for Michaelis-Menten kinetics was obtained at higher 5-HT concentrations (94 to 3000 μM; Figure 1E). Data analysis found *Km* values in PEC (Figure 1F and Table 1) characteristic of a low-affinity (uptake-2) transporters [28–31].

Pharmacologic inhibition of 5-HT uptake in TMT and PEC

To further characterize 5-HT uptake in human primary TMT and PEC, we studied its sensitivity to known inhibitors of uptake-1 (SERT) and uptake-2 (PMAT/OCT3) transporters. Various SERT-targeting antidepressants (citalopram, paroxetine, fluoxetine, fluvoxamine, clomipramine, imipramine; 10^{-7} M) significantly inhibited active transport of 5-HT into TMT (Figure 2A). The SERT-interacting psychostimulant 3,4-methylenedioxy-methamphetamine (MDMA, "Ecstasy") also reduced transport of 5-HT into TMT, although with a lower potency. Specifically, IC_{50} value of MDMA was 1678 (95 % CI 1045 to 2745) nM, while those of citalopram and fluoxetine were only 2 (95 % CI 1 to 7) and 11 (95 % CI 4 to 33) nM, respectively (Figure 2B). Citalopram, a potent and most selective SERT blocker [41], did not affect 5-HT transport into PEC (Figure 3A). However, active uptake of 5-HT into PEC was sensitive to increasing concentrations of decynium 22 (Figure 3B), a common inhibitor of PMAT and OCT3 transporters [28, 31]. These results clearly demonstrate the presence of SERT-mediated 5-HT uptake into TMT. In addition, data also support the involvement of uptake-2 carrier/s and make SERT involvement unlikely in 5-HT uptake into PEC.

Expression of 5-HT regulating genes in TMT and PEC

To support our functional findings and identify molecular players accounting for the different 5-HT uptake systems in TMT and PEC, we analyzed the expression (mRNA levels) of genes encoding SERT, PMAT and OCT3. Consistent with 5-HT uptake findings, TMT were rich in *SERT* mRNA. We found about 10-fold higher *SERT* mRNA levels in TMT than peripheral blood lymphocytes (data not shown), cells known to functionally express SERT [42]. In contrast, RT-qPCR analysis of *SERT* mRNA in PEC from 12 different placentas yielded generally high (mean 31.2) and largely variable (range 26.8 to 36.7) quantification cycle (Cq) values (Figure 4A). The respective Cq values corresponded to relative *SERT* mRNA levels about 1000-fold lower in PEC than TMT and up to 400-fold different between PEC donors (Figure 4B). In line, earlier immunohistochemical studies on the human term placentas reported only a weak staining for SERT protein occasionally observed on the feto-placental endothelium [14, 43]. Combined with our functional (5-HT uptake) results, these findings of very low and variable SERT levels indicate that this transporter is not essential in PEC. Contrary to *SERT*, *PMAT* mRNA levels were about 300-fold higher in PEC than in TMT (P<0.0001; Figure 4C). *OCT3* mRNAs were undetectable (Cq>37) in PEC, suggesting that PMAT, and not OCT3, is responsible for the observed

low-affinity, decynium 22-sensitive uptake of 5-HT into PEC. We also examined mRNAs for monoamine oxidase A (MAOA) and B (MAOB), mitochondrial isoenzymes that catalyze oxidative deamination of various monoamines, showing high and low, respectively, affinity for 5-HT [44]. Both cell types contained high levels of *MAOA* mRNA, with TMT having about 5-fold higher levels than PEC ($P<0.01$; Figure 4D). *MAOB* transcripts were detected only in TMT, albeit at a relatively low levels (not shown).

Kinetics of 5-HT uptake in cord blood platelets

To examine potential further mechanisms contributing to uptake of 5-HT from the feto-placental circulation at term of pregnancy, we investigated 5-HT transport into cord blood platelets (CBP). CBP showed time- and temperature-dependent 5-HT uptake, with initial rates of active 5-HT transport saturable over the high-affinity range of 5-HT concentrations (0.15 to 2.0 μM; Figure 5A). *Km* values in CBP (Figure 5B) were typical for high-affinity uptake [26], approximating those we found here in TMT (Table 1) and previously in adult platelets [39, 40]. *Vmax* values expressed per platelet number were also comparable between CBP (109 ± 35 pmol / 10^8 platelets / min) and adult platelets (142 ± 25 pmol / 10^8 platelets / min^-; [40]). As compared to TMT, *Vmax* values expressed per platelet protein were about 20-fold higher in CBP ($P<0.0001$; Table 1). This is in line with platelets being one of the few cell types with the highest amount of SERT protein in the adult human body [45].

Uptake of 5-HT by human first trimester trophoblasts

To understand if 5-HT uptake characteristics into the trophoblast, i.e. uptake from the maternal circulation, change with gestational age, we measured 5-HT uptake into human first trimester trophoblasts (FTT). 5-HT transport into FTT was temperature-sensitive, but initial rates of active 5-HT uptake in FTT were remarkably lower than that in TMT (on average, by 11- and 2-fold at 0.1 and 3.2 μM 5-HT, respectively) and not saturable over the high-affinity range of 5-HT concentrations (Figure 6A). This suggests that FTT lack the functional high-affinity, SERT-mediated uptake present in TMT. Yet, FTT expressed *SERT* mRNA, albeit at about 10-fold lower levels than TMT ($P<0.01$), while the inverse gestational age difference was found for *PMAT* mRNA levels (Figure 6B).

DISCUSSION

Here we studied 5-HT uptake in two types of human placental cells as well as in cord blood platelets, all isolated directly after birth. To determine uptake kinetics, we carried out comprehensive and rigorous initial rate studies using radiotracer assay. The initial rate period was experimentally determined for each cell type by time-dependent experiments. Initial rates of active (carrier-mediated) 5-HT uptake were measured at multiple substrate concentrations, covering a range typical for both high-affinity (uptake-1) and low-affinity (uptake-2) transport systems. This enabled estimation of kinetic parameters *Km* (approximating affinity of a carrier for 5-HT) and *Vmax* (describing maximal uptake velocity attained when a carrier is fully saturated).

Additionally, we studied pharmacological aspects of 5-HT uptake and examined mRNA expression levels of 5-HT carriers and catabolizing enzymes in primary placental cells.

Our results show that human primary term trophoblasts (TMT) express high levels of *SERT* mRNA and take up 5-HT with a high-affinity transport kinetics characteristic of SERT-mediated process. *Vmax* values of 5-HT uptake in TMT (18 -146 pmol mg^{-1} min^{-1}, n=9) were similar to those published for plasma membrane vesicles isolated from native human placentas (25.6 - 270 pmol mg^{-1} min^{-1}) [32, 46–49]. These findings indicate TMT as a credible physiological model for studying various regulatory features of placental 5-HT uptake from the maternal circulation in an intact cellular system. In contrast, *Vmax* values of 5-HT uptake in JAR cells (0.88 - 1.58 pmol mg^{-1} min^{-1}) [33, 49–51] are much lower than those found in placental plasma membrane vesicles and TMT, indicating a loss of functional SERT protein in JAR cells. It has to be noted that TMT obtained from different donors showed a considerable inter-individual variability in *Vmax* and *Km* values (up to 8.1- and 3.9-fold, respectively; Figure 1C) as well as in levels of *SERT* mRNA (up to 5.3-fold; Figure 5B). This may be result of the influence of various fetal and/or maternal factors that were not controlled for in the present study. Indeed, fetal sex [34] and genotype [52] were reported to influence 5-HT uptake in rat and human placentas, respectively; others and we have found that maternal obesity and glucose tolerance in pregnancy modulate 5-HT system in the human placenta [17, 43, 53, 54], and various other gestational factors, such as maternal genotype, diet, stress, and immune activation, have been shown to influence fetal and placental 5-HT homeostasis [22]. However, it was outside the scope of the present study to understand regulatory influences and factors of the various 5-HT uptake systems in placenta and platelets.

Epidemiological data suggest that SERT-targeting antidepressant medications are increasingly used in pregnancy [55, 56]. Therefore, we have evaluated the ability of different antidepressants to affect 5-HT uptake in TMT. They are the primary cell type on which antidepressants administered to the mother would act in the human feto-placental unit. We have found that 5-HT uptake into TMT was inhibited by nanomolar concentrations of common tricyclic (imipramine, clomipramine) and SSRI (citalopram, paroxetine, fluoxetine, fluvoxamine) antidepressants, with citalopram and paroxetine demonstrating the highest inhibitory effects. In addition, 5-HT uptake in TMT was antagonized by the SERT-interacting recreational psychostimulant MDMA, the use of which also appears to increase among pregnant women [57]. It has been shown that both antidepressants and MDMA may actively traverse the placenta and affect developing fetal organs [58–60]. Our results in primary human trophoblasts together with earlier findings in human placental explants [14] and placental plasma membrane vesicles [32, 46, 47] highlight that placenta itself, specifically, its mother-facing side, may be a target organ of these drugs. Potential downstream consequences of disturbed placental 5-HT homeostasis induced by inhibition of 5-HT uptake in mother-facing trophoblast cells warrant further investigation.

We tested kinetics of 5-HT transport also in the first trimester trophoblasts (FTT). Obtained results suggest that FTT, despite having moderate levels of *SERT* mRNA, lack SERT-mediated uptake activity. A possible

explanation for such findings might be related to cellular internalization of the SERT protein, one of the important mechanisms regulating SERT activity [61]. Indeed, earlier immunohistochemical analysis of the human first trimester placentas showed the presence of SERT protein that was mostly confined to the cytoplasm rather than to the surface of cytotrophoblasts [14]. It should be also mentioned that oxygen level was shown to modulate SERT expression and activity in certain human cells (specifically, pulmonary artery smooth muscle cells) [62]. Given that oxygen level and gestational age both regulate the growth of trophoblasts [63], it remains to be investigated whether oxygen conditions may have influenced the results. Nevertheless, findings of 20-fold higher *PMAT* mRNA levels, as contrasted to 10-fold lower *SERT* mRNA levels, in FTT compared to TMT, support a more important role of uptake-2 than uptake-1 system in early placental development.

Mechanisms involved in uptake of 5-HT from the fetal circulation have only recently gained attention [34]. Here we have demonstrated functional 5-HT uptake system at term of human pregnancy in two cell types that are in direct contact with fetal blood plasma. First, we uncovered a low-affinity (most likely PMAT-mediated) 5-HT uptake activity in feto-placental endothelial cells (PEC). Second, we demonstrated that cord blood platelets (CBP) take up 5-HT via a high-affinity transport mechanism, whose kinetic properties are very similar to those of SERT-mediated 5-HT uptake into adult platelets [39, 40]. Additionally, we identified mRNA for MAOA, a 5-HT catabolizing isoenzyme with a high affinity for 5-HT, in PEC. This suggests that PEC can catabolize 5-HT to an inactive metabolite. In platelets, 5-HT that has been taken up may be either sequestered in dense granules or catabolized by MAOB [64]. Taken together, these findings suggest that both PEC and CBP have systems in place to actively participate in the uptake and deactivation of 5-HT from the fetal circulation.

The *Km* value of 5-HT uptake in CBP (0.654 ± 018 μM) was about three orders of magnitude lower than that in PEC (782 ± 218 μM), suggesting that the two uptake systems operate at different substrate concentrations. We speculate that CBP and PEC cooperatively work to meet requirements for maintaining optimal *in vivo* levels of extracellular 5-HT in different situations. Under basal conditions, where plasma 5-HT is kept at low nanomolar levels [65], uptake of 5-HT is mediated principally by the high-affinity system of CBP. When extracellular 5-HT concentrations increase to levels at which the high-affinity system of CBP is fully saturated, the low-affinity system of PEC, which saturates at much higher concentrations, takes part in helping to bring extracellular 5-HT to low basal levels. A further pathway of 5-HT sequestration from feto-placental circulation, operating especially at high plasma 5-HT levels, may imply 5-HT diffusion through paracellular routes between PEC and placental stroma to reach the syncytiotrophoblast basal membrane. There it might serve as substrate of the low-affinity, OCT3-mediated uptake system [34]. Extracellular 5-HT concentrations may fluctuate locally / transiently towards high levels as a consequence of 5-HT release from fetal platelets or other cells, for endocrine, paracrine or autocrine signaling purposes. Other situations in which the low-affinity 5-HT uptake systems present in PEC and syncytiotrophoblast basal membrane may be important are when the SERT-mediated uptake in CBP is inhibited pharmacologically or compromised due to genetic or pathology-related

reasons. Indeed, *in utero* exposure to SSRI antidepressants has been associated with decreased 5-HT levels in CBP [66], suggesting pharmacological inhibition of 5-HT uptake in CBP. There is evidence that 5-HT clearance system in the brain also employs both high-affinity and low-affinity transporters [67]. Interplay between different 5-HT uptake systems in the feto-placental unit warrants further investigation.

In summary, we comprehensively characterized kinetic and pharmacological aspects of 5-HT uptake into human primary trophoblasts facing maternal blood at the term. Besides establishing these cells as a valuable tool for studying different regulatory features of placental 5-HT uptake from maternal circulation, the results emphasize sensitivity of placental 5-HT transport to inhibitory effects of various psychotropic drugs. Our findings also suggest that 5-HT uptake mechanisms in trophoblasts change over the course of human pregnancy. Further, we demonstrated the presence of a functional 5-HT uptake system, with low substrate affinity, in human feto-placental endothelial cells. These cells on the fetal side of the feto-placental unit are in direct contact with fetal blood and, hence, take up fetal 5-HT. Finally, we show that human fetal platelets express functional high-affinity 5-HT uptake system resembling that in adults. The present identification of multiple membrane transport systems for uptake of extracellular 5-HT at the human maternal-fetal interface (Figure 7) advances our understanding of the relevance of 5-HT for proper fetal development. The results may open opportunities to develop new strategies for the modulation of disorders associated with altered placental 5-HT handling.

AUTHOR CONTRIBUTIONS

JŠ and UP conceived the study. JŠ, UP, CW, LČŠ and GD designed experiments, interpreted results and provided reagents. PB, MK, MH, MG, MP and JŠ conducted experiments and analyzed data. JŠ wrote and GD, LČS and CW critically revised the article. All authors read and approved the final version of the article.

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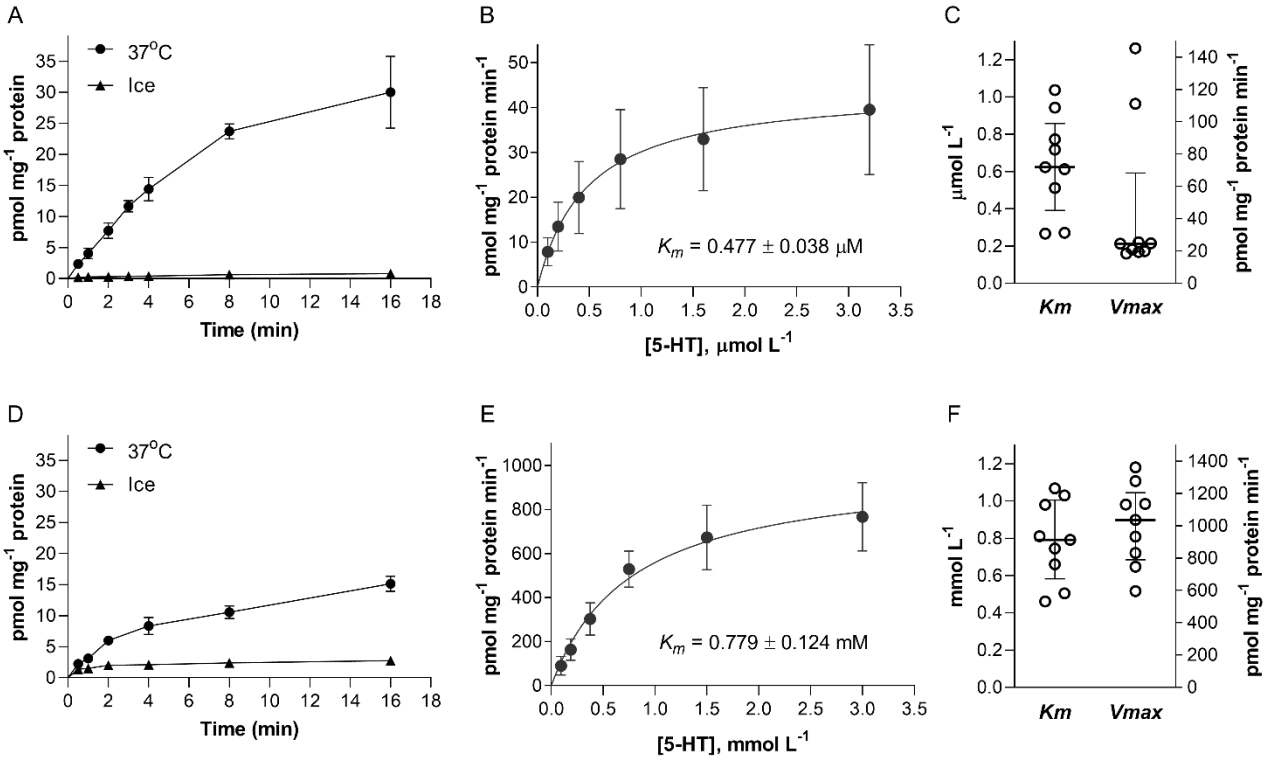
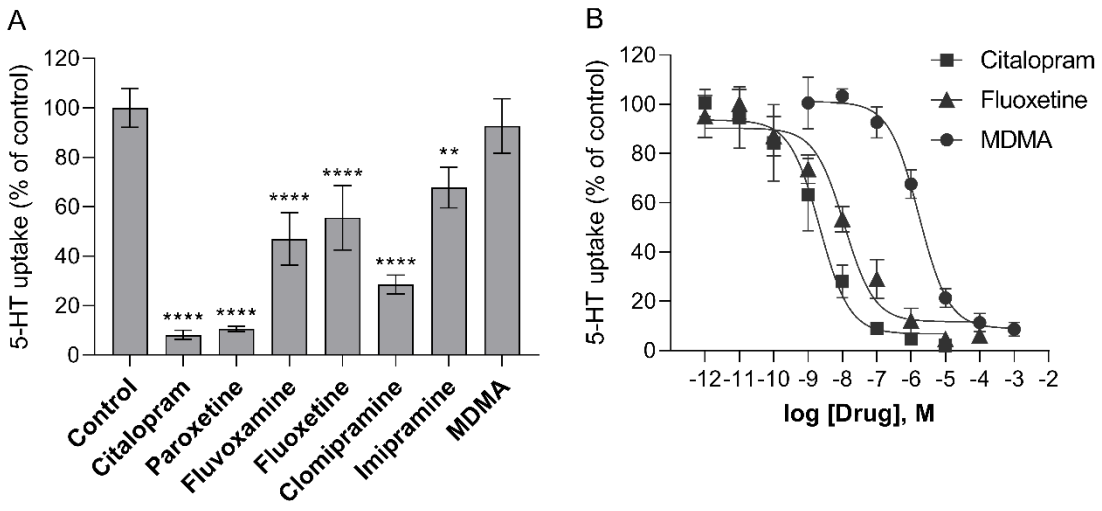
FIGURES

Figure 1



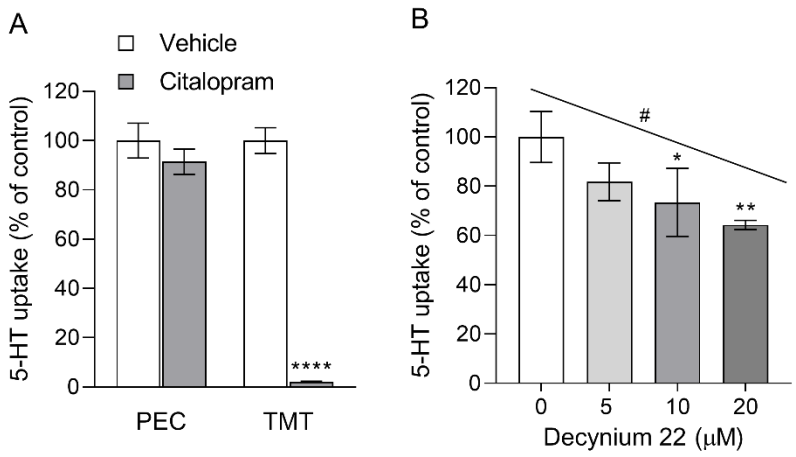
Uptake of serotonin in human term trophoblast (TMT; A-C) and feto-placental endothelial cells (PEC; D-F). (A, D) Time course of cellular 5-HT uptake into (A) TMT and (D) PEC. Cells were incubated at 37oC or on ice in the presence of 3H-5-HT (10-7 M). Data are means ± SD of triplicates; shown is representative of two experiments. (B, E) The initial rates of active 5-HT uptake into (B) TMT and (E) PEC, plotted against substrate concentration and fitted into Michaelis-Menten kinetics model. Cells were incubated in the presence of six increasing 3H-5-HT concentrations ranging from (B) 0.1 to 3.2 µM or (E) 94 to 3000 µM, for 2 or 8 min, respectively. Active transport was calculated as the difference between transport at 37°C and on ice. Data shown are means ± SEM of 9 independent cell preparations, each analysed in triplicate. (C, F) Best-fit values of Michaelis affinity constant (Km) and maximal transport velocity (Vmax) in (C) TMT and (F) PEC preparations from different placentas (n=9 each). Lines represent median and interquartile range.

Figure 2



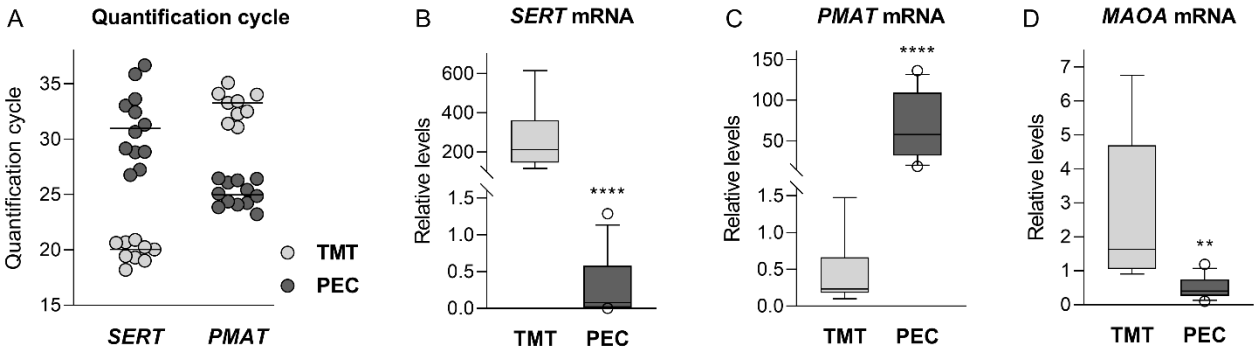
Effects of SERT-targeting drugs on 5-HT uptake in human term trophoblasts. Active uptake of 3H-5-HT (10-7 M) in the presence of (A) 10-7 M indicated drugs or (B) increasing concentrations of citalopram, fluoxetine and 3,4-methylenedioxy-methamphetamine (MDMA). Values are given as a percentage of the control (vehicle without drug). Data are means ± SEM of three separate experiments, each performed in triplicate. **P<0.01, ****P<0.0001 compared to control (Dunnett's test following one-way ANOVA).

Figure 3



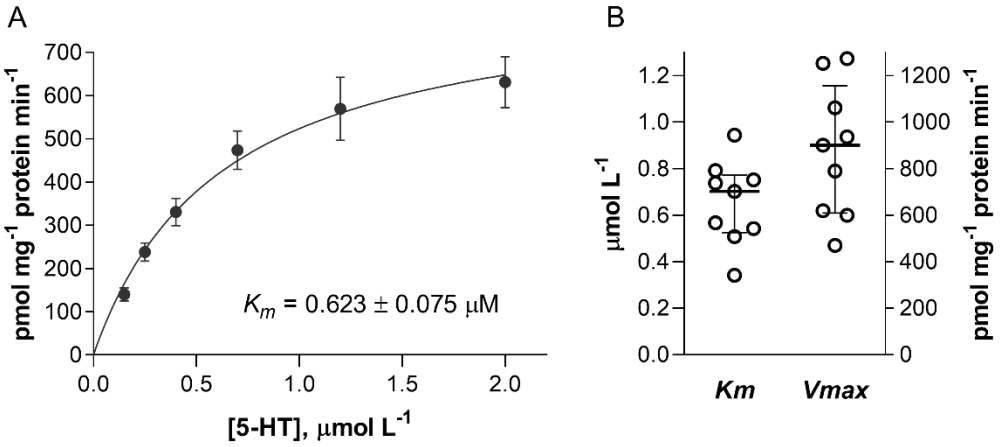
Effects of citalopram and decynium 22 on 5-HT uptake in human term feto-placental endothelial cells. (A) Active uptake of 3H-5-HT (10-7 M) in the presence of 10-4 M citalopram. ****P<0.0001 compared to vehicle (Fisher's LSD test following two-way ANOVA). (B) Active uptake of 3H-5-HT (10-4 M) in the presence of indicated concentrations of decynium 22. *P<0.05, **P<0.01 compared to control (Fisher's LSD test following one-way ANOVA), #P<0.01 for linear trend between column means (test for linear trend following one-way ANOVA). Data are means ± SD of triplicates and shown is representative of two to three experiments.

Figure 4



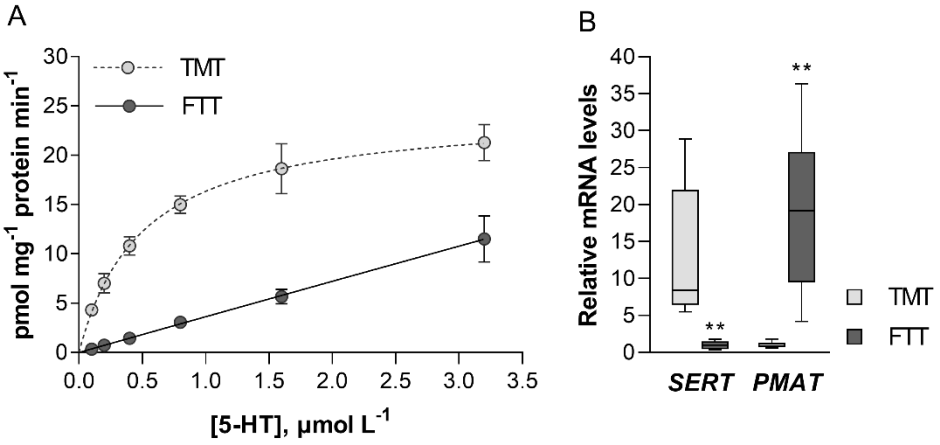
Expression of 5-HT regulating genes in human term trophoblasts and feto-placental endothelial cells. (A) Quantification cycle values obtained by RT-qPCR of serotonin transporter (SERT) and plasma membrane monoamine transporter (PMAT) mRNAs in term trophoblasts (TMT; n=9) and feto-placental endothelial cells (PEC; n=12). Each dot represents a mean of qPCR triplicates. Relative levels of SERT (B), PMAT (C) and monoamine oxidase A (MAOA) (D) mRNAs, normalized to beta-actin (ACTB) mRNA. Data are presented as boxplots with whiskers denoting the 10th and 90th percentiles. **P<0.01, ****P<0.0001, as compared to TMT (Mann-Whitney or t-test, as appropriate).

Figure 5



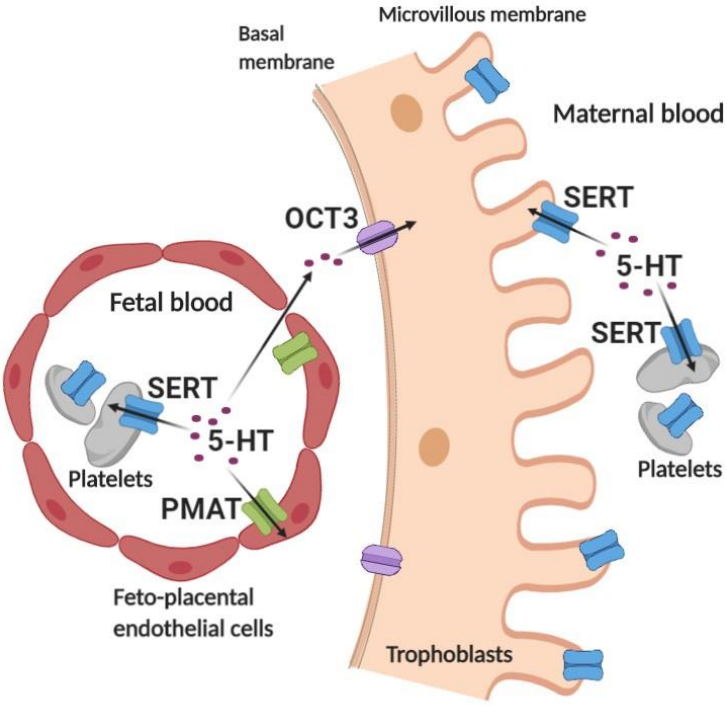
Uptake of serotonin in human cord blood platelets. (A) The initial rates of active 5-HT uptake plotted against substrate concentration and fitted into Michaelis-Menten kinetics model. Cord blood platelets were incubated for 1 min in the presence of six increasing 14C-5-HT concentrations (0.15 to 2.00 □M). Active transport was determined as the difference between transport at 37°C and on ice. Data shown are means ± SEM of 9 subjects. (B) Best-fit values of Michaelis affinity constant (Km) and maximal transport velocity (Vmax) in CBP obtained from different subjects (n=9). Lines represent medians and interquartile range.

Figure 6



Uptake of serotonin in human first trimester trophoblasts. (A) Concentration dependent uptake of 5-HT into first trimester trophoblasts (FTT) and term trophoblasts (TMT). Cells were incubated for 2 min in the presence of increasing 3H-5-HT concentrations (0.1 to 3.2 µM). Active transport was computed as the difference between transport at 37°C and on ice. Shown are means ± SEM of 3 FTT preparations, each analyzed in triplicate. To facilitate comparison, representative experiment on term trophoblast (means ± SD, n=3). (B) Relative levels of serotonin transporter (SERT) and plasma membrane monoamine transporter (PMAT) mRNAs in FTT and TMT preparations. SERT and PMAT mRNA levels, normalized to levels of beta-actin (ACTB) mRNA, are expressed relative to levels in TMT and FTT, respectively, arbitrary set at 1.00. Data are presented as boxplots with whiskers denoting the 10th and 90th percentiles (n=6 per cell type). **P<0.01 as compared to TMT (Mann-Whitney test).

Figure 7



Multiple systems for cellular uptake of serotonin from placental extracellular space at the end of human gestation. Serotonin (5-HT) in fetal plasma is taken up via serotonin transporter (SERT) into fetal platelets and via plasma membrane monoamine transporter (PMAT) into feto-placental endothelial cells. In addition, fetal 5-HT may diffuse through paracellular routes between feto-placental endothelial cells and placental stroma to the basal side of trophoblasts where it serves as a substrate of organic cation transporter 3 (OCT3) [34]. 5-HT in maternal plasma is sequestrated via SERT located on both maternal platelets and apical side of trophoblasts.

TABLES

Table 1. Kinetic parameters of 5-HT uptake in human term trophoblasts (TMT), feto-placental endothelial cells (PEC) and cord blood platelets (CBP). Given values are means ± SD or, where appropriate, median (interquartile range, IQR) of nine independent cell preparations. Data are derived from Figs. 1C, 1F and 5B.

Cells	N	***Km*** (μmol L^{-1})	***Vmax*** (pmol mg^{-1} protein min^{-1})
TMT	9	0.640 ± 0.266	24.5 (48.7)
PEC	9	782 ± 218	1005 ± 251
CBP	9	0.654 ± 0.181	878 ± 286

Km - Michaelis affinity constant, *Vmax* - maximal transport velocity

79–3286.

SUPPLEMENTARY INFORMATION

Supplementary Table S1. Characteristics of term pregnancies involved in the study (mean ± sd, if not indicated otherwise).

Characteristic	TMT	PEC	CBP
Maternal age (years)	29.4 ± 6.5	31.5 ± 5.2	32.8 ± 5.1
Gestational age (weeks)	39.5 ± 1.2	38.8 ± 1.1	39.1 ± 0.7
Labor (vaginal : Cesarean)	5 : 11	10 : 11	0 : 9
Newborn's sex (male : female)	10 : 6	10 : 11	4 : 5
Birth weight (kg)	3462 ± 447	3228 ± 430	3653 ± 616
Birth length (cm)	50.5 ± 2.3	49.6 ± 1.7	50.7 ± 2.3

TMT - term trophoblasts, PEC – placental endothelial cells, CBP – cord blood platelets

Supplementary Table S2. Sequences of gene-specific primers used in RT-qPCR analyses.

Gene symbol	Gene name	Primer sequence (5' - 3')	Source
SERT / SLC6A4	serotonin transporter	f: TGGTTCTATGGCATCACTCAGTTC r: GTTGTGGCGGGCTCATCAG	[1]
PMAT / SLC29A4	plasma membrane monoamine transporter	f: CTGTCCTCCTGAACAACGTCC r: ACACGTCGCAGATGCTGATAA	[2][a]
OCT3 / SLC22A3	organic cation / carnitine transporter 3	f: GCCCTGTTCCAGCAATAAGA r: GAGAGCCAAAAATGTCCCAA	[3][b]
MAOA	monoamine oxidase A	f: GAGCGGCTACATGGAAGGG r: TCACCTTCCCGAGACCATTTA	[4]
MAOB	monoamine oxidase B	f: CTTTTTGGAGAGACATTTGCCC r: TCACAAGTAGCCCCCTTTTGT	[5]
ACTB	actin beta	f: TCCCTGGAGAAGAGCTACG r: GTAGTTTCGTGGATGCCACA	[6]

[a] PrimerBank ID: 100913033c2

[b] RTPrimer database ID: 307

Supplementary References

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