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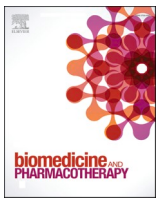

Metformin inhibits OCT3-mediated serotonin transport in the placenta

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ABSTRACT

Proper fetal development requires tight regulation of serotonin concentrations within the fetoplacental unit. This homeostasis is partly maintained by the placental transporter OCT3/SLC22A3, which takes up serotonin from the fetal circulation. Metformin, an antidiabetic drug commonly used to treat gestational diabetes mellitus, was shown to inhibit OCT3. We, therefore, hypothesized that its use during pregnancy could disrupt placental serotonin homeostasis. This hypothesis was tested using three experimental model systems: primary trophoblast cells isolated from the human term placenta, fresh villous human term placenta fragments, and rat term placenta perfusions. Inhibition of serotonin transport by metformin at three concentrations (1 μM, 10 μM, and 100 μM) was assessed in all three models. The OCT3 inhibitor decynium-22 (100 μM) and paroxetine (100 μM), a dual inhibitor of SERT and OCT3, were used as controls. In primary trophoblasts, paroxetine exhibited the strongest inhibition of serotonin uptake, followed by decynium-22. Metformin showed a concentration-dependent effect, reducing serotonin uptake by up to 57 % at the highest concentration. Its inhibitory effect was less pronounced in fresh villous fragments but remained statistically significant at all concentrations. In the perfused rat placenta, metformin demonstrated a concentration-dependent effect, reducing placental serotonin uptake by 44 % at the highest concentration tested. Our findings across all experimental models show inhibition of placental OCT3 by metformin, resulting in reduced serotonin uptake by the trophoblast. This sheds light on mechanisms that may underpin metformin-mediated effects on fetal development.

1. Introduction

Gestational diabetes mellitus (GDM) is one of the most common complications of pregnancy, affecting approximately 13.2–14.0 % of women during the course of gravidity [1,2]. When unsatisfactorily treated, it is associated with poor pregnancy outcomes including higher birth weight [3], increased risk of premature birth or preeclampsia [3,4], and a wide range of metabolic, cardiological, hematological, and neurological disorders [5,6].

Although metformin's safety in pregnancy is still under discussion, it is a frequently used alternative to insulin in GDM treatment [7]. Several studies have indicated that metformin poses no risk of adverse long-term outcomes such as obesity or hypoglycemia when compared to insulin [8]. Similarly, no links between metformin use and major fetal malformations or neonatal death have been reported [9–11]. On the other hand, several recent studies have indicated that prenatal metformin treatment may reduce the birth weight of the offspring, accelerate postnatal growth, and lead to cardio-metabolic adverse effects [12,13]. In addition, long-term treatment of pregnant mice with metformin altered the offspring's social behavior due to changes in placental transporter activity and serotonin (5-hydroxytryptamine, 5-HT) metabolism in the fetal brain [14].

Since metformin crosses the placenta [15–19], its adverse effects on the fetus have mainly been ascribed to its direct effects on the developing fetal organs. However, here we hypothesize that metformin can compromise fetal development and programming even before reaching the fetal organs because of its effects on placental functions – specifically, 5-HT homeostasis in the fetoplacental unit. The placenta, among many other functions, tightly regulates 5-HT levels in the fetoplacental unit during gestation, which is crucial for proper placental and fetal

Abbreviations: 5-HT, serotonin (5-hydroxytryptamine); BM, basal membrane; CTB, cytotrophoblast; D22, decynium 22; GDM, gestational diabetes mellitus; MVM, microvillous membrane; OCT3, organic cation transporter 3; PHT, primary human trophoblast; PRX, paroxetine; SERT, serotonin transporter; STB, syncytiotrophoblast.

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development [20]. Despite not being a neurological organ, the placenta expresses a suite of membrane transporters and cytosolic enzymes similar to those found in the brain [21]. More specifically, a high-affinity/low-capacity serotonin transporter (SERT/SLC6A4) is present in the mother-facing microvillous membrane (MVM) of the trophoblast [22,23], while the low-affinity/high-capacity organic cation transporter 3 (OCT3/SLC22A3) is functionally expressed in the fetus-facing basal membrane (BM) [24,25]. The placenta also expresses 5-HT-producing and 5-HT-breaking enzymes, namely tryptophan hydroxylase (TPH) and monoamine oxidase A (MAO-A), respectively [20, 26,27]. We have recently characterized the interplay between these transporters and enzymes, showing that they are key components of the placental clearance systems regulating 5-HT levels in the fetoplacental unit in both human and rat [24,28].

Metformin is a recognized substrate and inhibitor of OCT3 [29,30] that has been reported to interact with this transporter in both the placenta [19] and brain [31,32]. Consequently, we hypothesize that its effects on 5-HT homeostasis in the fetoplacental unit may be linked to OCT3 inhibition. This dual mechanism could exacerbate disruptions in 5-HT levels, negatively impacting fetal development. We have therefore systematically investigated metformin's effects on 5-HT homeostasis in the fetoplacental unit using three model systems: highly purified primary trophoblast cells isolated from the human term placenta, isolated fresh villous human term placenta fragments, and rat term placenta perfusions.

2. METHODS

2.1. Chemicals and reagents

^{3}H-serotonin (80 Ci/mmol) was obtained from M.G.P. (Zlín, Czech Republic). Metformin hydrochloride was purchased from MedChemExpress (New Jersey, USA). (+)-Sodium L-ascorbate (L-ascorbic acid), phenelzine sulfate salt, paroxetine (positive control, SERT/OCT3 inhibitor [33]), and decynium-22 (D22, positive control, OCT3 inhibitor [34]) were obtained from Sigma-Aldrich (St. Louis, USA). Pierce™ BCA Protein Assay Kit was purchased from Thermo Fisher Scientific (Waltham, United States). All other chemicals were of analytical grade and obtained from Penta Chemicals (Prague, CZ).

2.2. Human term placenta sample collection

Human term placentas were obtained from healthy pregnancies at term (i.e. 38–40 weeks of gestation) promptly after delivery at the University Hospital in Hradec Kralove, with the written informed consent of the donors and approval of the University Hospital Research Ethics Committee (approval no. 201006 S15P). Experiments were conducted in accordance with the Declaration of Helsinki.

2.3. Isolation of PHT cells; evaluation of purity and culture

PHT cells isolations from the human term placentas were performed according to the previously described protocol [27,35,36]. After the tissue was cut and cleaned to remove membranes, vessels, and calcifications, it was subjected to enzymatic digestion three times with 0.25 % trypsin (Gibco; Thermo Fisher Scientific, USA) and 300 IU/ml deoxyribonuclease I (Sigma Aldrich, USA) at 37°C for 30 minutes. CTBs were isolated using Percoll (Sigma Aldrich, USA) density gradient separation. Finally, the cell purity was determined by labeling with specific cell marker antibodies for 1 hour at room temperature, followed by flow cytometric analysis as described previously [27]. The antibodies used in this work were supplied by Novus Biologicals (USA) and included anti-cytokeratin 7 (AF 488®), anti-vimentin (AF 647®), and anti-von Willebrand Factor (AF 647®). For each sample, a minimum of 10.000 cells were scanned using a SA3800 Spectral Analyzer (Sony Biotechnology, USA) and the resulting data were analyzed using the FCS Express package from De Novo Software. Only cells with >90 % purity were used in the study (Supplementary table 1).

The isolated CTBs were cultured in high glucose Dulbecco's Modified Eagle Medium (DMEM) supplemented with GlutaMAX™ (Gibco; Thermo Fisher Scientific, USA), enriched with 10 % FBS and 1 % Penicillin (10.000 U) + Streptomycin (10 mg/ml) (Sigma Aldrich, USA). For subsequent experiments, the cells were kept under incubation conditions at 37°C, with 5 % CO_2 + 95 % air for 12 hours (PHT-CTB) or 72 hours (PHT-STB, medium changed daily).

2.4. In vitro uptake of 5-HT by isolated PHT cells

Experiments were conducted using a 96-well plate setup; 1×10^{5} PHT cells were seeded in Nunc™ Delta-treated surface plates (Nunc; Thermo Fisher Scientific, USA). First, cells were pre-incubated for 10 minutes with Opti-MEM™ containing 0.1 or 10 % DMSO as a negative control or one of three inhibitor solutions: metformin (1, 10, or 100 μM), paroxetine (100 μM in 0.1 % DMSO), or D22 (100 μM in 10 % DMSO). Uptake was then initiated by incubating the cells in the presence of 1 μCi/ml ^{3}H-5-HT. The incubation solution was supplemented with ascorbic acid (1 mM) and phenelzine (100 μM) to prevent oxidation and degradation by MAO-A, respectively. After a 15-minute incubation period, the uptake was terminated by washing with ice-cold Dulbecco's Phosphate Buffered Saline (Sigma Aldrich, USA) at 4°C. Finally, the cells were lysed in 0.5 M KOH and their accumulated levels of ^{3}H-5-HT were measured by scintillation counting with a Tri-Carb 2910 TR instrument (Perkin Elmer, USA). The raw counts were normalized against the protein content, measured using the Pierce™ BCA Protein Assay Kit. The final uptake values are expressed in units of pmol/mg protein or as percentages of the uptake observed in controls; Fig. 1A.

2.5. Ex vivo 5-HT accumulation studies in isolated fresh villous human term placenta fragments

Uptake of 5-HT by the placenta was also evaluated by monitoring the accumulation of ^{3}H-5-HT in fresh villous fragments prepared from human term placenta tissue. To this end, small fragments of villous tissue were dissected immediately after delivery of the placenta and washed in a 1:1 mixture of DMEM and Tyrode's buffer (135 mM NaCl, 5 mM, KCl, 1.8 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM HEPES, 5.6 mM glucose, pH 7.4). The fragments were then tied onto hooks such that the tissue was fully immersed in the buffer and left to equilibrate for 30 minutes at 37°C before the experiment. In the experiments, the fragments were incubated in Tyrode's buffer containing control (blank), metformin (1, 10, or 100 μM), paroxetine (100 μM) or D22 (100 μM) (pre-wash) for 15 minutes at 37°C, then transferred to identical solutions containing 0.5 μCi/ml ^{3}H-5-HT, ascorbic acid (1 mM), and phenelzine (100 μM). After incubation for 15 minutes at 37°C, uptake was terminated and extracellularly bound drug molecules and radioactive species were removed by vigorously washing the fragments with excess buffer at room temperature. The fragments were then immersed in distilled water overnight to release the accumulated ^{3}H-5-HT from the tissue, which was measured by scintillation counting. To measure the protein content in each sample, fragments were lysed in 0.3 M NaOH solution for 6–8 hours at 37°C while shaking, followed by BCA assay measurement. The accumulated radioactivity was normalized against the protein content; Fig. 1B.

2.6. In situ perfusion of the rat term placenta

The experimental animals were pregnant female albino rats (Wistar) provided by the Velaz breeding station (Prague, Czech Republic). All experiments were approved by the Ethical Committee of the Faculty of Pharmacy in Hradec Kralove (approval no. MSMT-4312/2015–8). Experiments were conducted on the 21st day of gestation, as previously described [24,37]. The rats were anesthetized by injecting a

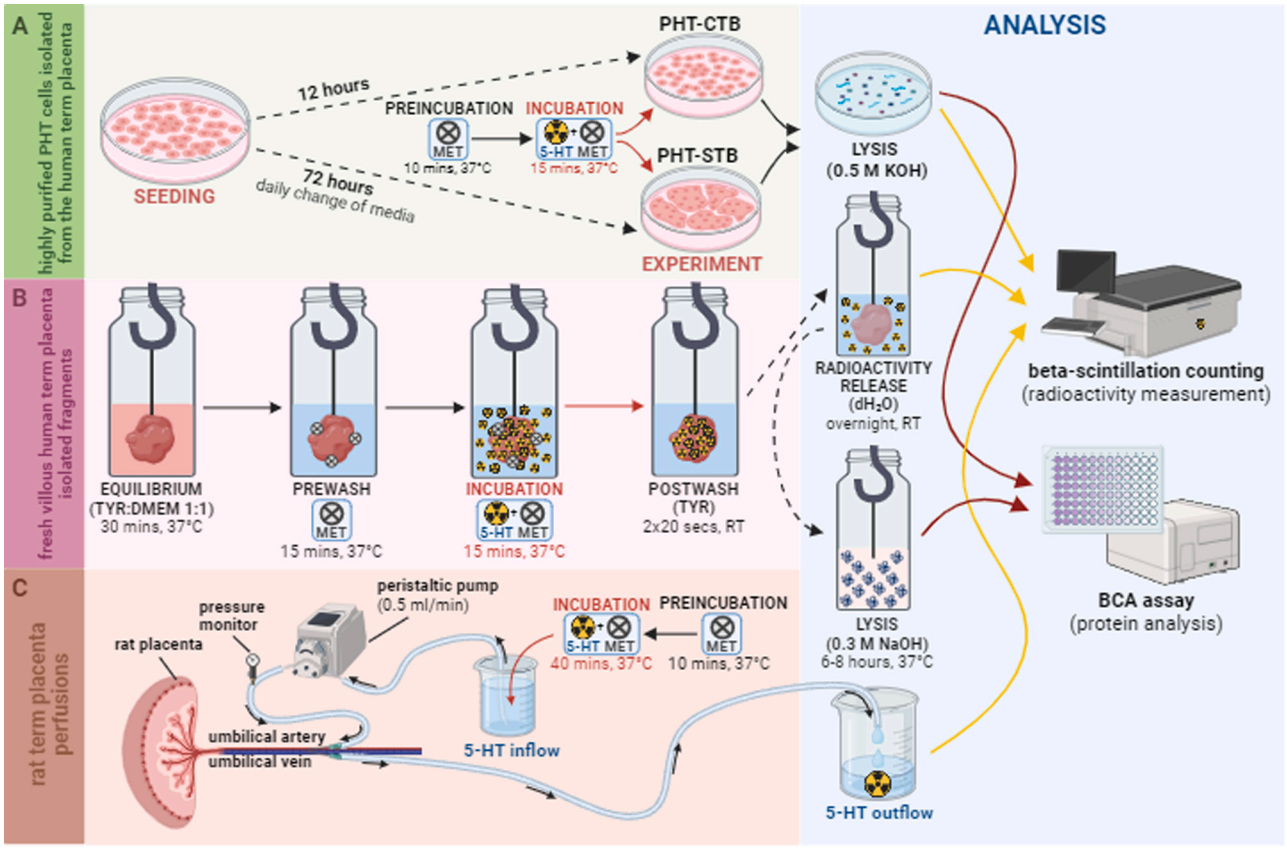

Fig. 1. Schematic representation of experimental methods used. (A) Highly purified PHT cells isolated from the human term placenta were seeded into a 96-well system (a single well is shown). Cells were used in experiments 12 or 72 hours after isolation (representing the PHT-CTB and PHT-STB phases, respectively); in the 72-hour case, the existing media was replaced with fresh media daily. Cells were preincubated with metformin for 10 minutes at RT, followed by a 15-minute incubation with ^{3}H-5-HT at 37°C. After lysis in 0.5 M KOH, their radioactivity and protein contents were measured by scintillation counting and the BCA assay, respectively. (B) Isolated fresh villous human term placenta fragments were tied on hooks and immersed in an equilibration solution (Tyrode's buffer:DMEM, 1:1) for 30 minutes at 37°C. Next, they were preincubated with metformin for 15 minutes at 37°C in a prewash solution and then incubated with ^{3}H-5-HT for 15 minutes at 37°C in the incubation solution. Uptake was terminated by washing twice with a postwash solution (Tyrode's buffer) for 20 seconds each at RT. The fragments were then lysed overnight in distilled water in RT to release the accumulated radioactivity, which was measured by scintillation counting. The protein content was determined by lysing the tissue in 0.3 M NaOH for 6–8 hours at 37°C and then performing a BCA assay. (C) Rat term placentas were perfused for 10 minutes with a metformin solution (preincubation) and then with ^{3}H-5-HT (incubation), both at 37°C. Placental effluent was collected every 5 minutes over a 40-minute period.

pentobarbital solution (Nembutal, 40 mg/kg) into the tail vein. In each uterine horn, one placenta was used for the experiment, and fetal gender was determined by measuring the anogenital distance, with longer distances being indicative of male gender. The umbilical artery and vein were cannulated and connected to a solution reservoir with a flow of 0.5 ml per minute.

Using this setup, the placenta was first prewashed with pure Kreb's solution (1.18 M NaCl, 46.9 mM KCl, 250 mM $NaHCO_3$, 3.9 mM $Na_2HPO_4.12\ H_2O$, 55.5 mM glucose, 1.38 mM $CaCl_2.2\ H_2O$, 0.59 mM $MgCl_2.6\ H_2O$, 0.1 ml heparin/100 ml, 1 g dextran or albumin/100 ml, pH 7.4) as a control or with solutions of metformin (1, 10, and 100 μM), paroxetine (100 μM), or D22 (10 μM). After 10 minutes of prewashing, the reservoir's content was replaced with a control solution of 1 nM ^{3}H-5-HT or with one of 1 nM ^{3}H-5-HT with metformin (1, 10, and 100 μM), paroxetine (100 μM), or D22 (10 μM). All solutions contained ascorbic acid (1 mM) and were maintained at 37 °C in darkness throughout the experiment. The outflowing solution was sampled every 5 minutes over a 40-minute experimental period. After the experiment was terminated, the placenta was weighed and stored at −80°C. The amount of radioactivity in the samples was measured by scintillation counting, and the averaged values were subjected to statistical analysis. The placental extraction ratio (ER) of 5-HT was calculated as $ER = (C_{fa} - C_{fv})/C_{fa}$, where C_{fa} is the 5-HT concentrations in the fetal reservoir entering the placenta via the umbilical artery and C_{fv} is the 5-HT concentration in the umbilical vein effluent; Fig. 1C.

2.7. Statistical analysis

Uptake of ^{3}H-5-HT was evaluated using the paired t-test in the PHT cell studies and the isolated fresh villous human term placenta fragment experiments. In the rat term placenta perfusion studies, ^{3}H-5-HT uptake was evaluated using one-way ANOVA, and sex-dependent uptake was analyzed via two-way ANOVA. Statistical significance is denoted using asterisks: * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$. GraphPad Prism version 9.2.0 was used for all data analysis and graphical presentation of statistical data.

Abbreviations: 5-HT – serotonin, CTB – cytotrophoblast, MET – metformin, PHT – primary human trophoblast, RT – room temperature, STB – syncytiotrophoblast, TYR – Tyrode's buffer

3. RESULTS

3.1. Metformin inhibits 5-HT accumulation in PHT cells

In experiments with PHT cells, paroxetine (100 μM), a dual inhibitor of SERT and OCT3, showed the strongest inhibitory effect, reducing 5-HT uptake by 93 % (PHT-CTB) and 88 % (PHT-STB) (Fig. 2A and Fig. 2B, respectively). The selective OCT3 inhibitor D22 (100 μM) had a

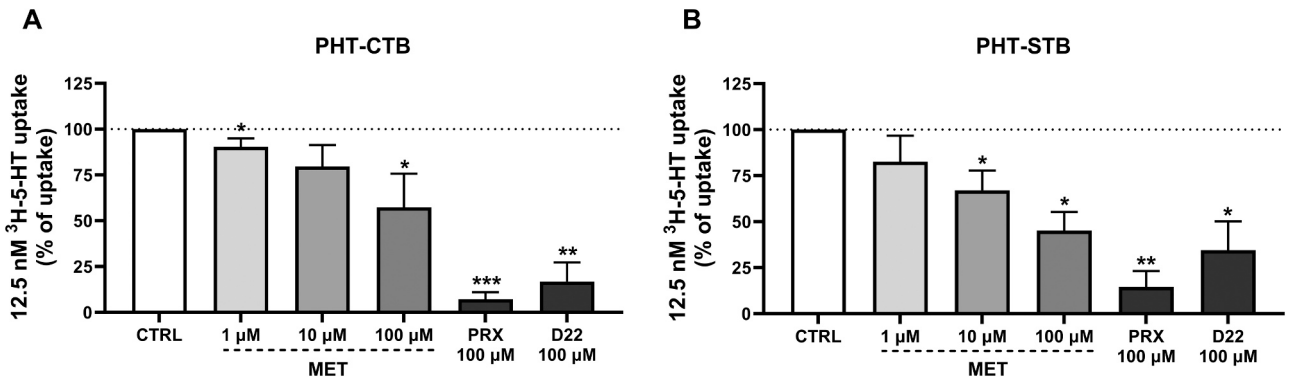

Fig. 2. Inhibition of 5-HT uptake in PHT cells by metformin. Uptake of 5-HT in PHT cells in the undifferentiated CTB (A) and differentiated/fused STB (B) states. Inhibition experiments were performed in the presence of metformin (1, 10, or 100 μM), paroxetine (100 μM), or D22 (100 μM). Values are percentages of the uptake observed in inhibitor-free controls (CTRL). Data are presented as means ± SD (n ≥ 4), with indications of statistical significance based on paired t-tests: *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001. *Abbreviations: 5-HT – serotonin, CTB – cytotrophoblast, CTRL – control, D22 – decynium 22, MET – metformin, PHT – primary human trophoblast, PRX – paroxetine, STB - syncytiotrophoblast.*

slightly weaker effect, reducing 5-HT uptake by 83 % (PHT-CTB) and 65 % (PHT-STB) (Fig. 2A and Fig. 2B, respectively). Metformin had a concentration-dependent effect on 5-HT uptake, reducing it by a maximum of 43 % (PHT-CTB) and 57 % (PHT-STB) at the highest tested concentration of 100 μM (Fig. 2A and Fig. 2B, respectively).

3.2. Metformin reduces 5-HT accumulation in fresh villous fragments isolated from the human term placenta

The results observed in placental fragments were similar to those seen in PHT cells: paroxetine (100 μM) had the strongest inhibitory effect, reducing 5-HT uptake by 62 % (Fig. 3). A less pronounced but still statistically significant effect was observed for D22 (100 μM), which reduced 5-HT uptake by 40 % (Fig. 3). The largest metformin-induced reductions in 5-HT uptake (21 % and 18 %) were observed in the 10 μM and 100 μM treatments, respectively (Fig. 3).

3.3. Metformin reduces placental 5-HT uptake from the fetal circulation in perfused rat term placenta

Paroxetine (100 μM) was the strongest inhibitor in the umbilically perfused rat term placenta, reducing 5-HT uptake by 76 % (Fig. 4A). A statistically significant effect was also observed for D22 (10 μM), which reduced 5-HT uptake by 51 % (Fig. 4A). Metformin had a concentration-dependent effect; at the highest tested concentration (100 μM) its effect

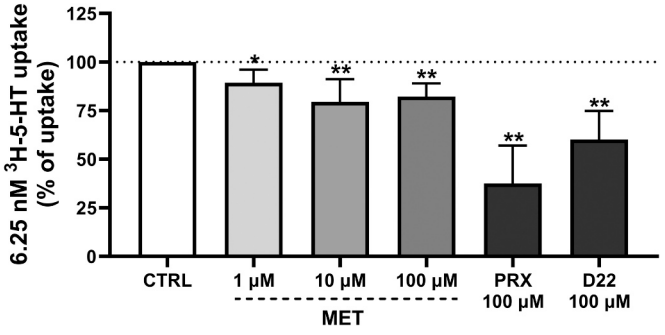

Fig. 3. Inhibition of 5-HT uptake by metformin in isolated fresh villous human term placenta fragments. Uptake of 5-HT in isolated fresh villous human term placenta fragments was evaluated in the presence of metformin (1, 10, or 100 μM), paroxetine (100 μM), and D22 (100 μM). Values are reported as percentages of the uptake observed in inhibitor-free controls (CTRL). Data are presented as means ± SD (n ≥ 9), with indications of statistical significance based on paired t-tests: *p ≤ 0.05, **p ≤ 0.01. *Abbreviations: 5-HT – serotonin, CTRL – control, D22 – decynium 22, MET – metformin, PRX – paroxetine.*

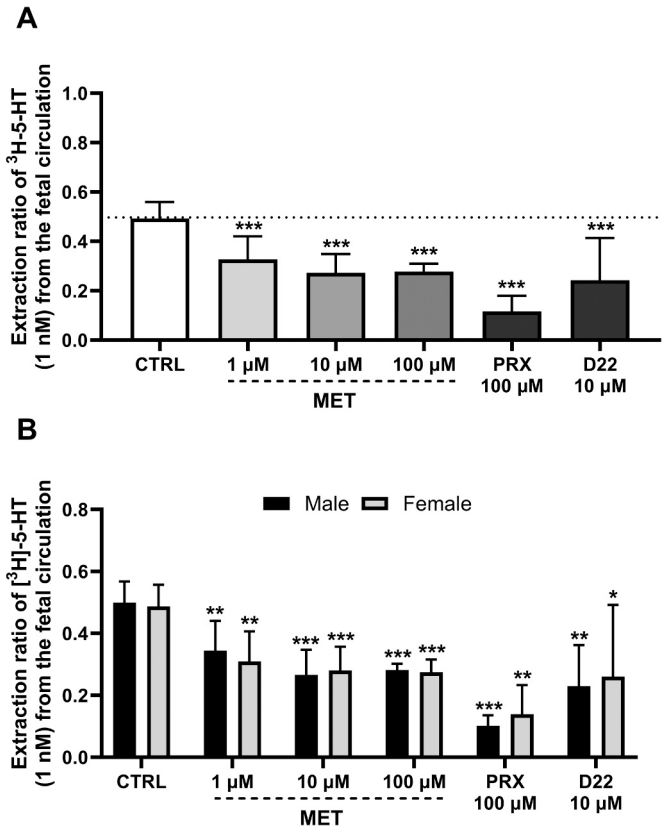

Fig. 4. Inhibition of 5-HT uptake in rat term placenta perfusions by metformin. (A) Uptake of 5-HT in rat term placenta and (B) the effect of fetal sex on 5-HT uptake inhibition was evaluated in the presence of metformin (1, 10, or 100 μM), paroxetine (100 μM) and D22 (10 μM). Data are presented as means ± SD (A) (n ≥ 12) with indications of statistical significance based on One-way ANOVA, and (B) (n ≥ 6) with indications of statistical significance obtained from Two-way ANOVA. (A, B) Values are given as extraction ratios. * p ≤ 0.05, ** p ≤ 0.01, ***p ≤ 0.001. *Abbreviations: 5-HT – serotonin, CTRL – control, D22 – decynium 22, MET – metformin, PRX – paroxetine.*

was comparable to that of D22, reducing placental 5-HT uptake by 44 % (Fig. 4A). When separating male and female placentas, we observed a statistically significant effect of sex for paroxetine and D22 but not for metformin (Fig. 4B). Sex-dependent inhibition of 5-HT uptake from the fetal circulation in rats by paroxetine is consistent with our previous

findings [33].

4. DISCUSSION

5-HT is an essential neurotransmitter in the placenta-brain axis [21] and a key factor in fetal organ development, especially that of the early fetal brain [38]. Levels of this monoamine must be tightly regulated to ensure proper placental function and fetal development. 5-HT is known to contribute to the regulation of organ developmental processes by controlling cell proliferation, differentiation, and migration [39]. During fetal brain development, 5-HT acts as a neurotrophic factor and neuronal maturation regulator [40,41], and also plays a significant role in neuronal brain wiring [42]. Therefore, any disturbance of fetoplacental 5-HT homeostasis during pregnancy may pose a risk to the developing fetus and adversely affect the health of the offspring [43]. We have recently characterized the role of OCT3 transporter in regulating 5-HT levels in the fetoplacental unit in both human and rat [24, 28]. Metformin is a second-line drug for the treatment of GDM [44] and its use is on the rise [45]. Since it is a recognized OCT3 inhibitor [29,30], in this study we investigated its effect on OCT3-mediated uptake of 5-HT by the placenta.

Pharmacological and toxicological studies during pregnancy are difficult to perform due to ethical and technical constraints, so alternative experimental models must be used. Here we adopted a comprehensive approach using three model systems: a) *in vitro*, highly purified PHT cells isolated from the human term placenta (cellular level); b) *ex vivo*, isolated fresh villous human term placenta fragments (tissue level); and c) *in situ*, rat term placenta perfusions (whole-organ level). In each model system, metformin was tested at concentrations of 1 μM, 10 μM, and 100 μM. As positive controls, we used the selective OCT3 inhibitor D22 (10 μM and 100 μM) [24] and paroxetine (100 μM), a dual inhibitor of OCT3 and SERT [33].

The selection of a suitable and representative cell-based model is critical for *in vitro* research on monoamine transport in trophoblasts. Our previous study showed that the frequently used BeWo cell line is an inappropriate model for studying placental monoamine transport because, unlike human trophoblasts, it expresses the dopamine transporter but not OCT3 [36]. Therefore, in this study, we used primary trophoblast cells isolated from healthy human term placentas (PHT). These cells spontaneously differentiate and hence can be used to study the effect of differentiation on the expression and functional characteristics of monoamine transport [36]. We observed a significant and concentration-dependent inhibitory effect of metformin on the uptake of 5-HT by PHT cells. Moreover, a higher degree of inhibition was observed in differentiated PHT-STB cells when compared to the nondifferentiated PHT-CTB stage. We attribute this to the different expression profiles of monoamine transport proteins in the trophoblast before and after cell fusion, as described previously [36]. As expected, the most pronounced inhibition of 5-HT transport was observed with paroxetine because it inhibits both SERT and OCT3. Metformin's inhibitory effect was most pronounced at the highest tested concentration (100 μM) in the PHT-STB stage, which was close to the effect of D22, a specific inhibitor of OCT3. These findings indicate that metformin interferes predominantly with the function of the OCT3 transporter in PHT cells.

To confirm our results in a 3D tissue model, we used fresh villous fragments isolated from the human term placenta. This is an established method for investigating the placental transport of endogenous compounds [46] and has found applications in various functional studies [46–48]. However, to our knowledge, this is the first time it has been used to monitor placental monoamine uptake. Experiments using this model system revealed a concentration-dependent inhibitory effect of metformin on 5-HT uptake by placental tissue, albeit a less pronounced one than was seen in the isolated trophoblast. At the two highest concentrations tested (10 and 100 μM) the inhibition of 5-HT uptake by metformin was comparable to that observed for D22, suggesting that metformin primarily acts on OCT3. The apparent differences in the extent of OCT3 inhibition by metformin in isolated placental cells and placental fragments may arise from differences in cellular heterogeneity, microenvironmental cues, and metabolic activity. Unlike the more homogeneous populations of isolated placental cells, placental fragments retain the complex cellular composition, microenvironmental factors, and intercellular communication networks of the placenta *in vivo*, and may thus exhibit altered sensitivity to metformin-induced OCT3 inhibition [46].

Finally, to verify our *in vivo* and *ex vivo* findings at the whole organ level, experiments were conducted using perfused rat term placentas *in situ* as described in various previously reported physiological, pharmacological and toxicological studies [24,33,49–51]. We have also recently demonstrated the use of perfused rat term placentas as a preclinical model for studying placental homeostasis of monoamines, including 5-HT [24,37]. Since the *in vitro* and *ex vivo* model studies indicated that metformin primarily acts on OCT3, which is localized in the fetus-facing basal membrane in the human and rat placenta, we performed umbilical perfusions to focus on the activity of this transporter [24,25]. Notably, the rat placenta perfusion technique can be used to probe for sexually dimorphic responses [24,33], which was important in this case because several studies have indicated that metformin has sex-dependent effects in various models and organs [52] including the placenta [53] and neural cells [54]. However, the mechanisms underlying these sex-dependent differences have not yet been elucidated. When pooling male and female results, we found that metformin induced a concentration-dependent reduction in 5-HT uptake. At the two highest tested concentrations (10 and 100 μM), metformin reduced placental uptake of 5-HT by 45 % and 44 %, respectively, making its effect comparable to that of D22 (51 %). Statistically significant differences in 5-HT uptake between male and female placentas were observed after treatment with D22 and paroxetine, in accordance with our previous findings concerning the effects of antidepressants on placental 5-HT homeostasis [33]. However, the response to metformin exhibited no such sex-dependence.

There is some controversy in the literature regarding metformin's interaction with SERT. Han et al. [55] suggested that metformin's intestinal absorption is partly mediated by the human SERT. Conversely, Liang et al. [56] observed no significant metformin uptake in a SERT-overexpressing cell line or in HEK cells transiently transfected with the SLC6A4 (SERT) and thus concluded that metformin is a poor substrate of SERT. In our study, metformin's effects were similar to those of the OCT3-selective inhibitor D22, suggesting that its inhibitory effect on 5-HT placenta uptake is mainly mediated by this transporter. However, its interactions with SERT remain to be fully characterized.

In the brain and other tissues, 5-HT is transported also by different carriers, such as OCT1 [57,58], OCT2 [58,59], and plasma membrane monoamine transporter (PMAT) [60,61]. However, the expression of these transporters in the placenta is only negligible [62,63], and their function in the feto-placental unit has not been demonstrated. Although PMAT mRNA has been detected in the human term feto-placental endothelial cells, it was absent in the human first trimester [64] and in term trophoblasts [65,66]. Moreover, no studies have confirmed the presence of PMAT in the placenta at the protein level. On the other hand, OCT3 is abundantly expressed in the placental tissue [24,25]; hence, we attribute the primary effects observed in our study to this transporter.

A strength of this study is that we utilized three different placental models to confirm the effects of metformin on 5-HT transport. This multi-model approach was crucial for validating our findings and ensuring that our results are not dependent on a single model, thereby strengthening the conclusions. However, we are aware that the experimental models reflect only the acute effect of metformin, while metformin is typically administered chronically in clinical practice. Nonetheless, Garbarino et al. subjected pregnant mice to long-term metformin exposure and compared its effects to those of knocking out OCT3 [14]. Behavioral changes in male offspring were observed in both cases, prompting the authors to speculate that both treatments affected

5-HT homeostasis in the brain or placenta [14]. In conjunction with our findings, these results suggest a need for further research to elucidate the long-term effects of metformin exposure on offspring health outcomes and placental function. In particular, longitudinal epidemiological studies assessing the impact of chronic metformin use during pregnancy on neurodevelopmental outcomes, metabolic health, and cardiovascular function in offspring are needed. Investigations into metformin's effects on placental function across different gestational stages, including the first trimester, could also reveal critical windows of susceptibility to medication-induced perturbations. Lastly, while our experimental setup strongly suggests an upstream and downstream relationship between metformin and placental uptake via OCT3, further experiments, such as genetic knockdown or knockout studies, would be required to confirm this conclusively.

In conclusion, we systematically investigated the effect of metformin on placental 5-HT homeostasis at the cellular, tissue, and organ levels using three different experimental models. In all three models, metformin treatment significantly reduced 5-HT uptake by trophoblasts. These findings illustrate the complex mechanisms governing placental functions and highlight how disruptions in 5-HT balance could affect fetal development. They also demonstrate a need for further research on maternal-fetal pharmacology to clarify the impact of metformin treatment during pregnancy on the long-term health of offspring. Moreover, comprehensive risk-benefit assessments are needed to guide clinicians considering the use of metformin in GDM management to ensure optimal maternal health benefits while minimizing risks to the developing fetus.

Author contributions

Study concept and design – R.K., F.S.; Data acquisition – V.V., R.K., F. K.; Formal analysis – V.V., R.K.; Data interpretation – V.V., R.K., F.S.; Paper writing – V.V., R.K., F.S.; Funding and project administration – F. S. The authors read and approved the final manuscript.

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CRediT authorship contribution statement

Kasin Yadunandam Anandam: Writing – review & editing, Supervision, Data curation. **Frantisek Staud:** Writing – review & editing, Writing – original draft, Validation, Supervision, Project administration, Funding acquisition, Conceptualization. **Rona Karahoda:** Writing – review & editing, Writing – original draft, Supervision, Methodology, Formal analysis, Conceptualization. **Cilia Abad:** Writing – review & editing, Writing – original draft, Validation, Supervision. **Veronika Vachalova:** Writing – review & editing, Writing – original draft, Methodology, Investigation, Formal analysis. **Tetiana Synova:** Writing – review & editing, Methodology, Investigation, Formal analysis. **Fiona Kumnova:** Writing – review & editing, Methodology, Investigation, Formal analysis.

Declaration of Competing Interest

none

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Appendix A. Supporting information

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