Charles University

Faculty of Pharmacy in Hradec Králové

Department of Pharmacology and Toxicology

The influence of antidepressants on serotonin homeostasis in placenta

Diploma thesis

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Hradec Králové 2020

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STATEMENT OF AUTORSHIP

I hereby declare that I am the sole author of this diploma thesis and that I have not used any sources other than those listed in the bibliography and identified as references. I further declare that I have not submitted this thesis at any other institution in order to obtain a degree.

In Hradec Králové Veronika Váchalová

Date: 10.5.2020

ACKNOWLEDGEMENT

First of all, I would like to thank to my supervisor Prof. PharmDr. František Štaud, Ph.D. for his professional guidance, willingness, time and support during my whole diploma thesis work. Furthermore, I am extremely grateful to all the members of Placenta in Health and Disease – Charles University Team; specifically Mgr. Rona Karahoda, Mgr. Hana Horáčková and Cilia Abad, Ph.D., whose help was of great significance and high value in completing this research. Last but not least, I would like to thank the Grant Agency of Charles University (GAUK no. 1464119) and Czech Science Foundation (GACR 20-13017S) for their financial support.

ABSTRACT

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Title of diploma thesis: The influence of antidepressants on serotonin homeostasis in placenta

Depression is a serious mental disorder affecting 10-20% of women during pregnancy. Up to 10% of these pregnant women are prescribed antidepressants (ADs), most frequently from the class of selective serotonin (5-HT) reuptake inhibitors (SSRIs). While the safety of this treatment is questionable due to reported impaired pregnancy/fetal outcomes, understanding of potential mechanistic causes is still lacking. During pregnancy, 5-HT is important for normal placental function and proper fetal development and programming. 5-HT homeostasis in the placenta is maintained via the 5-HT transporter (SERT/*SLC6A4*) on the apical side and the recently characterized organic cation transporter 3 (OCT3/*SLC22A3*) on the basal side of trophoblast. These transporters take up 5-HT from the maternal and fetal circulations, respectively into the syncytiotrophoblast (STB) where it is degraded by monoamine oxidase-A (MAO-A). As all ADs interfere with the 5-HT system it is important to study their potential interactions in the feto-placental unit.

Experiments were carried out *in situ* (dually perfused rat term placenta) and *ex vivo* (membrane vesicles isolated from human term placenta). The inhibitory potential of paroxetine, citalopram, fluoxetine, fluvoxamine, sertraline and venlafaxine on 5-HT uptake by placenta was tested. In both models, we observed significant inhibitory potential of selected ADs on SERT and OCT3 mediated transport of 5-HT. In addition, in the rat placenta we observed a pronounced effect of fetal gender on AD-mediated inhibition of OCT3.

Our study suggests that use of ADs in pregnancy may affect both SERT and OCT3 responsible for 5-HT uptake from maternal and fetal circulations, respectively. This could result in disrupted 5-HT homeostasis in the feto-placental unit and thus local toxicity due to the vasoconstrictive properties of 5-HT.

ABSTRAKT

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Název diplomové práce: Vliv antidepresiv na homeostázu serotoninu v placentě

Deprese je vážná duševní porucha postihující 10-20% žen během těhotenství. Až 10% těchto žen užívají antidepresiva (ADs), nejčastěji ze skupiny selektivních inhibitorů zpětného vychytávání serotoninu (SSRIs). Navzdory skutečnosti, že se bezpečnost této léčby v těhotenství jeví jako sporná vzhledem k možným negativním následkům na matku i plod, mechanismy popisující tyto interakce nebyly stále dostatečně popsány. Během těhotenství je serotonin (5-HT) důležitým článkem pro správnou funkci placenty, vývoj a fetální programování plodu. Homeostáza 5-HT v placentě je udržována skrze 5-HT transportér (SERT/SLC6A4) na apikální straně a transportér pro organické kationty 3 (OCT3/SLC22A3) na bazální straně trofoblastu. Tyto transportéry vychytávají 5-HT z maternálního a fetálního krevního oběhu do syncytiotrofoblastu (STB), kde je následně degradován enzymem monoaminoxidázou-A (MAO-A). Vzhledem k tomu, že všechna ADs interferují se 5-HT systémem, je důležité studovat jejich potenciální interkace ve feto-placentární jednotce.

V této práci jsme pomocí *in situ* duálně perfundované potkaní placenty a *ex vivo* membránových vesiklů izolovaných z lidské placenty zkoumali inhibiční potenciál paroxetinu, citalopramu, fluoxetinu, fluvoxaminu, sertralinu a venlafaxinu na vychytávání 5-HT placentou.

V obou experimentálních modelech jsme pozorovali výrazný inhibiční potenciál vybraných ADs na SERT a OCT3 zprostředkovaný transport 5-HT. Navíc, v potkaní placentě jsme pozorovali výrazný vliv fetálního pohlaví na AD-zprostředkovanou inhibici OCT3.

Naše studie naznačuje, že užívání ADs během těhotenství může inhibovat placentární transportéry SERT i OCT3, zodpovědné za vychytávání 5-HT z maternální, respektive fetální krevní cirkulace. To by mohlo mít za následek narušení homeostázy 5-HT ve feto-placentární jednotce, a tím i zvýšení lokální toxicity, kvůli vazokonstrikčním vlastnostem 5-HT.

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1 LIST OF ABBREVIATIONS

5-HT	serotonin, 5-hydroxytryptamine
AD	antidepressant
BCA-assay	bicinchoninic acid assay
BM	basal membrane
DHA	dihydroalprenolol
MAO-A	monoamine oxidase-A
MVM	microvillous membrane
OCT3	organic cation transporter 3
SERT	serotonin transporter
SNRI	selective serotonin and noradrenaline reuptake inhibitor
SSRI	selective serotonin reuptake inhibitor
STB	syncytiotrophoblast
TRP	tryptophan

2 INTRODUCTION

Placenta is a multifunctional organ important for fetal growth, development and programming. As such, it participates in numerous functions including digestion, waste excretion, respiration, endocrine functions and immune protection. Importantly, certain internal and external factors, including inflammation, malnutrition, (epi)genetics, environment and pharmacotherapy in pregnancy, can alter the normal functioning of the placenta. A large number of epidemiological studies have shown that placental adaptation to these insults allows the fetus to survive, yet at the cost of impaired fetal development [1, 2]. This "misprogramming" predisposes the fetus to poor health and higher risk of mental, metabolic and cardiovascular disorders later in life, a concept now known as Developmental Origins of Health and Disease (DOHaD) [3]. To date, the molecular mechanisms involved are largely unexplored [4].

With the rising number of pregnant women experiencing depression during pregnancy, the percentage of those using AD therapy has increased as well [5]. Nevertheless, safety of this therapy for the fetus remains controversial. To optimize pharmacotherapy in pregnancy it is essential to know their transplacental pharmacokinetics and ability to affect placental homeostasis [6]. In this regard, particularly susceptible in the feto-placental unit are the two 5-HT transporting systems, SERT on the apical membrane and OCT3 on the basal membrane. While situated on different sides of the placenta barrier, these transporters are responsible to maintain the precise equilibrium of 5-HT transfer between both mother and fetus [7]. However, their function may be affected by several compounds, including ADs. Impaired 5-HT homeostasis could have deleterious impact on placenta and the fetus due to its vasoconstrictive properties which have been shown to be toxic in elevated levels in the feto-placental unit [8]. Considering the crucial role of 5-HT as a trophic factor, this may represent a molecular mechanism affecting fetal programming, in other words, expose the fetus to different living conditions which might increase the risk of faulty neuronal structure forming and several mental disorders occurrence. Thus, this thesis aimed to evaluate interaction of most frequently prescribed ADs in pregnancy with SERT and OCT3. By employing *ex vivo* and *in situ* methods, we provide a comprehensive overview of the interaction between selected ADs and the two 5-HT transporting systems.

3 BACKGROUND

3.1 Depression

Depression is a serious mental disorder affecting up to 4.8% of the Czech population [9] and approximately 3.4% of the population worldwide [10], with potential irretrievable consequences. The latest data show that at least 10-20% of pregnant women experience depression during the course of pregnancy [11]. Despite that, more than half of depressive patients remain under-treated or treated inefficiently.

Many causations, from genetic factors up to environmental effects participate on its origination. Mechanistically, the monoamine theory suggests deficiency in brain monoamine content, specifically of 5-HT and noradrenaline. Inadequate levels of these monoamines in the synaptic cleft leads in lower levels in the postsynaptic neuron, contributing to depressive symptoms [12]. Additionally, structural modifications such as neuronal atrophy characterized by synaptic connection deprivation in pivotal brain areas (cortex and limbic system) seem to play a role [13, 14].

3.2 AD drugs and their use in pregnancy

ADs are considered the most effective and widely prescribed drugs to a great number of patients not only with depression and anxiety but also many other psychiatric indications. It is estimated that 13% of pregnant women are exposed to at least one AD during the course of pregnancy and the number keeps on rising, despite warnings and risks given by the Food and Drug Administration (FDA) [5]. All currently used ADs usually work through blockade of either SERT or 5-HT metabolizing enzyme MAO-A, thus restoring the physiological synaptic concentrations of 5-HT via inhibition of reuptake or degradation of 5-HT, respectively [12].

When diagnosed with depressive disorder and upon recommendation of AD therapy, pregnant woman are usually prescribed ADs from the group of SSRIs, most frequently: sertraline, citalopram, paroxetine, fluvoxamine or fluoxetine; or the group of selective 5-HT and noradrenaline inhibitors (SNRIs), such as venlafaxine and duloxetine [12]. While administration of ADs usually does not pose particular risk to an adult, when used in pregnancy, they may lead to adverse effects for the developing fetus. Specifically, there have been reports associating prenatal AD use with postnatal adaptation syndrome manifesting shortly after birth

through a range of symptoms like infant irritability, hypertonia, jitteriness and trouble feeding [15]. Other reported impaired pregnancy outcomes include risks of congenital and cardiac malformations [16], fetal pulmonary hypertension [17], gestational hypertension and preeclampsia [18]. Importantly, there is a link between prenatal use of ADs and predisposition to a wide range of neurological and psychiatric disorders e.g. ADHD, autism, depression etc. [19-22].

It is suggested that perturbations of the 5-HT pathway might play a significant role in higher risk of developing neuropsychiatric disorders of the offspring although a detailed molecular pathway remains fully unexplored [23]. Due to their high lipid solubility, ADs cross the biological membranes including placenta barrier with ease thus distributing widely into the placenta itself and the fetus/embryo [24]. This may alter placental/fetal 5-HT homeostasis important for a wide range of functions in many organs, tissues or cell types (brain, heart, lungs, platelets, intestine). Thus, alterations in 5-HT system can contribute to a significant range of abnormalities during pregnancy, such as preterm delivery, pulmonary hypertension, cardiac and gastrointestinal morphology and physiology modification, intrauterine growth restriction and neurobehavioral disturbances in infants (involving autism spectrum disorders, schizophrenia or depression) [23, 25].

3.3 Fetal programming and placental 5-HT theory

Precise fetal development is dependent on *in utero* conditions which, when disturbed, may have serious consequences. This so-called „fetal programming“, pushes the fetus into defense and as it adapts to survive, its physiology, metabolism and morphology start to irrecoverably change. Although the molecular mechanisms of this phenomenon have not yet been sufficiently described several mechanisms have been postulated, centering the placenta as the key component (Fig.1) [26].

The flow of information between the mother and the fetus is facilitated via many regulatory, metabolic and endocrine pathways. In certain conditions such as maternal over/malnutrition, placenta reacts through changes in nutrient and oxygen supply, but also hormonal secretion and nutrient-sensing signaling pathways (e.g. cortisol, insulin and/or insulin growth factors) [26]. In addition, placental mTOR (mammalian target of rapamycin), highly expressed in the placenta, controls the placental nutrient transporters via up- or down-regulation

depending on the nutrient levels in the maternal circulation (Fig. 1) [27, 28]; mTOR may be further influenced by hormones or growth factors (e.g. maternal insulin, leptin, cortisol) [29].

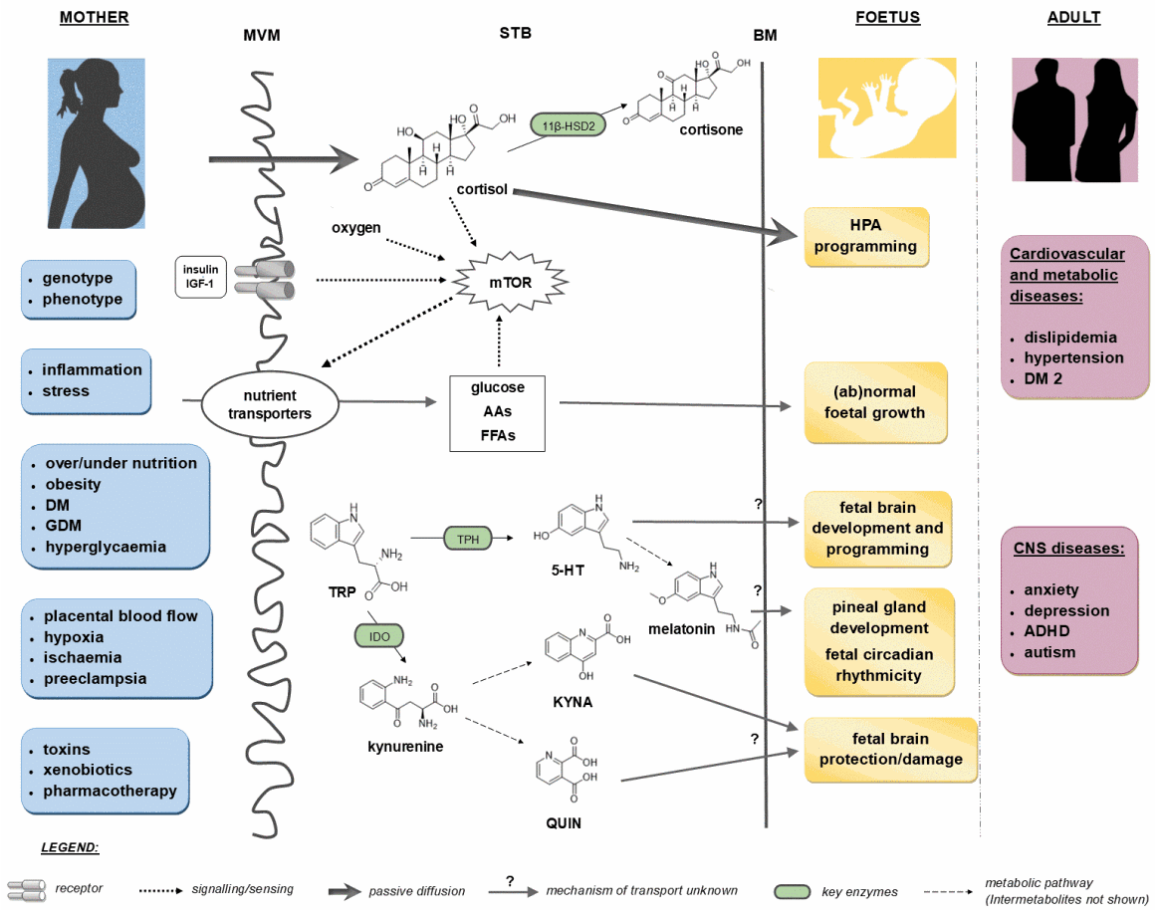

Fig. 1 Proposed mechanisms of fetal programming in the STB.

Altered conditions during pregnancy (in blue) may cause alterations in enzyme/transporter expression/function and mTOR signaling. Modifications of the nutrient supply together with glucocorticoid and TRP systems alter *in utero* conditions which become sub-optimal causing defective fetal programming (in yellow) which may contribute in arise of diseases in adult life (in purple). Adopted from Staud and Karahoda, 2018 [26].

Abbreviations: 11β-HSD2 – 11beta-hydroxysteroid dehydrogenase; 5-HT – serotonin; AAs – amino acids; ADHD – attention-deficit/hyperactivity disorder; BM - basal membrane; DM – diabetes mellitus; DM2 – DM type 2; FFAs- free fatty acids; GDM – gestational DM; HPA – hypothalamic-pituitary-adrenal axis; IDO – indoleamine 2,3-dioxygenase; IGF – insulin-like growth factor; KYNA – kynurenic acid; mTOR – mammalian target of rapamycin; MVM – microvillous membrane; QUIN – quinolinic acid; STB – syncytiotrophoblast; TDO - tryptophan 2,3-dioxygenase; TPH – tryptophan hydroxylase; TRP – tryptophan.

Interestingly, in the recent years, metabolism of tryptophan (TRP) within the placenta has also been suggested as a mechanism of fetal programming through its crucial role in fetal brain development (Fig. 1). Two main pathways have been identified, namely: the 5-HT [30] and kynurenine pathway [31], generating metabolites of neuroactive and immunoprotective properties. Altered maternal conditions such as inflammation, stress, depression, polymorphisms and xenobiotics can contribute to changed expression and activity of the TRP metabolic enzymes in placenta thus modifying the TRP catabolism and resulting in changed production of neuroactive and immunoprotective metabolites [26]. This may predispose the fetus to metabolic, cardiovascular and also CNS pathologies later in life, as a feedback mechanism to an altered fetal programming [26].

3.4 Placenta – structure and function

Placenta is a highly specialized organ of a discoid shape interlaced with blood vessels and connected to the fetus via the umbilical cord. It is considerably important for the proper fetal development, including providing oxygen and nutrients to the fetus, excreting its waste products as well as protecting against the maternal immune system. It also plays a very important endocrine and transport function [26].

In the human placenta both fetal and maternal blood circulations are kept isolated by two layers of cells - the fetal capillary endothelium and placental trophoblast. Two major segments form the tissue of placenta, maternal decidua basalis and fetal chorionic plate (or chorionic villi). Decidual septa further segregates the decidua basalis into 20-40 compartments, known as cotyledons, working as individual and fully functional vascular units of the placenta. Every cotyledon is composed of villous trees whose surface is covered with fused mononucleated cytotrophoblasts creating a polarized multinucleated STB layer, representing the semipermeable placental barrier. Maternal blood washes the chorionic villi where the fetal blood circulates and the STB barrier allows the nutrients, hormones and other material needed to pass through the umbilical cord into the fetus (vice versa for the waste products) (Fig. 2) [26].

The STB further consists of two distinct membranes: basal (fetus-facing; BM) and apical/microvillous (mother-facing; MVM) membrane (Fig. 2). Together they form a rate-limiting barrier for the transfer of both endo- and exogenous molecules. The difference between BM and MVM is both structural and functional. MVM has much larger surface area

than BM and both membranes consist dissimilar variety of enzymes, receptors and transporters [32].

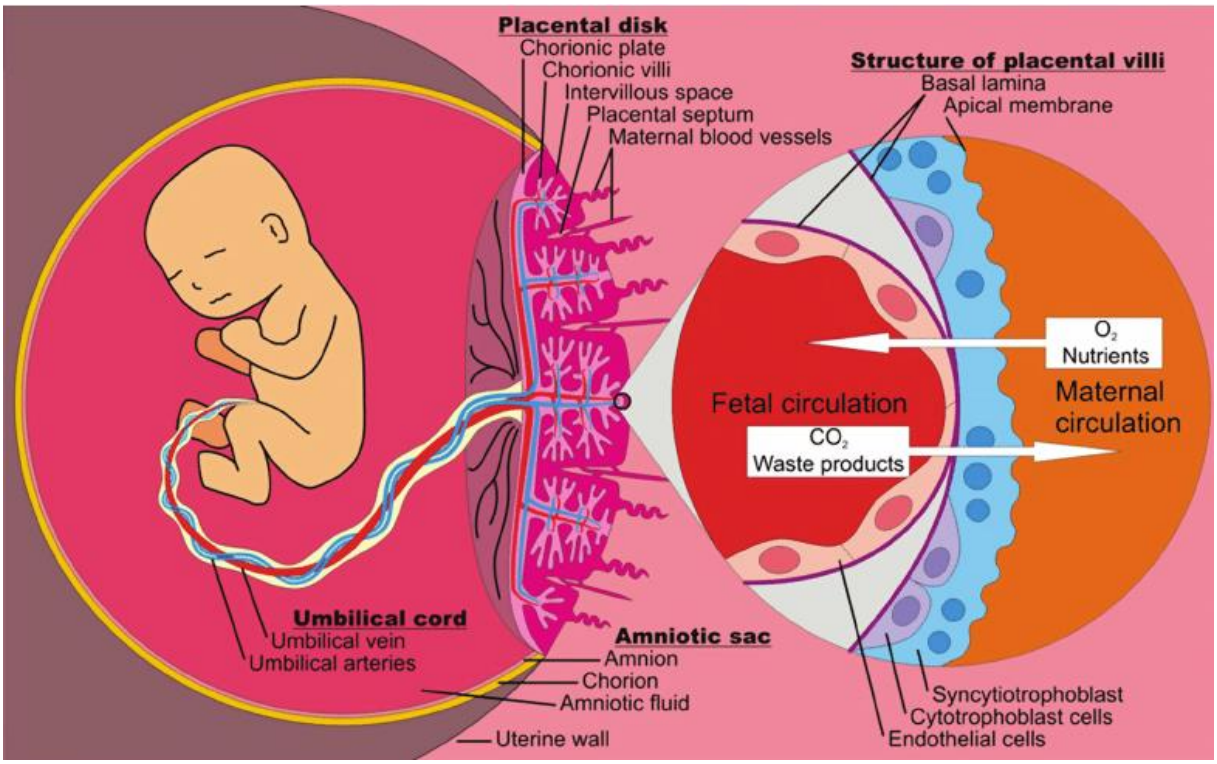

Fig. 2 Schematic representation of the placental structure and barrier.

Placental septa segregate the decidua into cotyledons consisting of villous trees covered with a fused mononucleated cytotrophoblasts and creating a layer of a polarized multinucleated STB standing as a semipermeable placental barrier between maternal blood space and fetal endothelium. The placental villi consist of basal and apical membranes allowing the waste products and nutrients to flow between the maternal and fetal unit. Adopted from Elad et al., 2014 [33]

3.4.1 Endocrine function

One of the main functions the placenta plays during the nine months is its endocrine function. Placenta produces numerous endogenous compounds such as steroid hormones (estrogens, progesterone), proteins (human chorionic gonadotropin, somatotropin, growth hormone, adipokines) and neurotransmitters (5-HT), all crucial for successful gestation [26]. Disturbances in hormone production may lead to various pathologies such as pre-eclampsia, chromosomal anomalies, intrauterine growth restriction or gestational diabetes mellitus [34].

3.4.2 Transport function

Thanks to the presence of a wide range of specialized transporters in the STB, placenta is capable to transport many endogenous and exogenous compounds [6]. Through the transport of nutrients (e.g. glucose, amino acids), steroids (cortisol), monoamines (5-HT) the placenta controls *in utero* homeostasis [26]. Many transporters have been characterized on the mother-facing MVM and fetus-facing BM, responsible for fetal protection and detoxification. These proteins can recognize and provide transplacental transfer of various substrates, in the mother-to-fetus or fetus-to-mother direction. Two major families of transporters are described – 1) the ATP binding cassette transporter family (ABCs), responsible mostly for efflux of various drugs and 2) solute carrier transporter family (SLCs) facilitating transport of nutrients [6].

3.5 5-HT in the feto-placental unit

5-HT is a monoamine neurotransmitter participating in neural activity and many neuropsychological processes. Nevertheless, 5-HT is not only found within the brain; majority of 5-HT occurs abundantly outside of the CNS, taking care of a vast number of biological processes, such as function of the cardiovascular system, bowel motility, bladder control etc. [35].

In the developing brain 5-HT is important in neuronal cell proliferation, migration and brain wiring [30, 36]. Current studies also demonstrate that 5-HT plays a key role in the thalamocortical wiring of the fetal forebrain and experiments performed on animal models demonstrated that 5-HT may play a significant role also in cardiac morphogenesis of the fetus [37]. Interestingly, while the receptors, transporters and degrading enzymes for 5-HT are already present in the fetal brain early in development, the 5-HT innervation and synthesis are absent [30]. With no 5-HT synthetic capacity early in gestation, the embryo/fetus is dependent on exogenous delivery of 5-HT, for which maternal and placental sources have been described [30, 38, 39].

3.5.1 Synthesis from TRP and degradation

5-HT is synthesized from TRP via the enzyme TRP-hydroxylase (TPH). Synthesized 5-HT can further be metabolized to melatonin or degraded to 5-hydroxyindoleacetic acid via the activity of MAO-A. In 2011, a breakthrough study showed that the placenta can also produce its own 5-HT, which is the main source of fetal 5-HT early in gestation [30]. However, as the pregnancy

advances into later stages, the fetus gains 5-HT synthetic ability from maternally-delivered TRP [40, 41].

3.5.2 Transport mechanisms

Two main 5-HT transport mechanisms have been described in the placenta: 1) SERT on the MVM [42, 43] and 2) the recently reported OCT3 on the BM [7]. SERT is a high-affinity and low-capacity membrane transporter protein present in the MVM responsible for facilitating 5-HT transport in the mother-to-fetus direction [42, 44]. Activity of SERT is dependent on Na^+ [42] and is the prime target of most ADs. OCT3 is poly-specific transporter highly expressed on the BM of the placental barrier as the only isoform of the OCT family [45-47]. Contrary to SERT, it transports 5-HT in a low-affinity but high-capacity manner [48]. Recent reports suggest that ADs may affect OCT3 function in the brain, a potential new mechanism of AD action [49].

While extent literature described SERT-mediated 5-HT uptake from the maternal circulation into STB, a recent study from our research team has shown that at term, human and rat placenta massively extract 5-HT from the fetal circulation through OCT3-mediated transport [7]. Within the STB, 5-HT is subsequently degraded by MAO-A into inactive metabolite and thus providing fetal protection against excessive amounts of 5-HT [7].

Considering that placental handling of 5-HT is dependent on various transport and metabolic proteins, it is important that their normal function is maintained throughout pregnancy. However, these proteins are all prone to alterations not only by genetic factors (such as polymorphisms and up- or down-regulation of gene expression) but also through pharmacological or environmental interferences [7]. As perturbations in the 5-HT pathway and alterations of 5-HT concentrations in the fetal tissues can cause serious developmental and functional defects [8], it is important to study the mechanisms which can contribute to a wide range of neuropsychiatric disorders, for instance schizophrenia, affective disorders, anxiety and disorders of the autism spectrum later in life.

4 AIM OF STUDY

The goal of this study was to determine whether and to what extent might commonly used ADs interfere with placental 5-HT homeostasis; in particular, we focused on inhibition of SERT and OCT3-mediated transport of 5-HT in rat and human term placenta (Fig. 3). The following drugs have been tested: paroxetine (PRX), sertraline (SRL), fluoxetine (FOX), fluvoxamine (FVX), citalopram (CIT), venlafaxine (VLF).

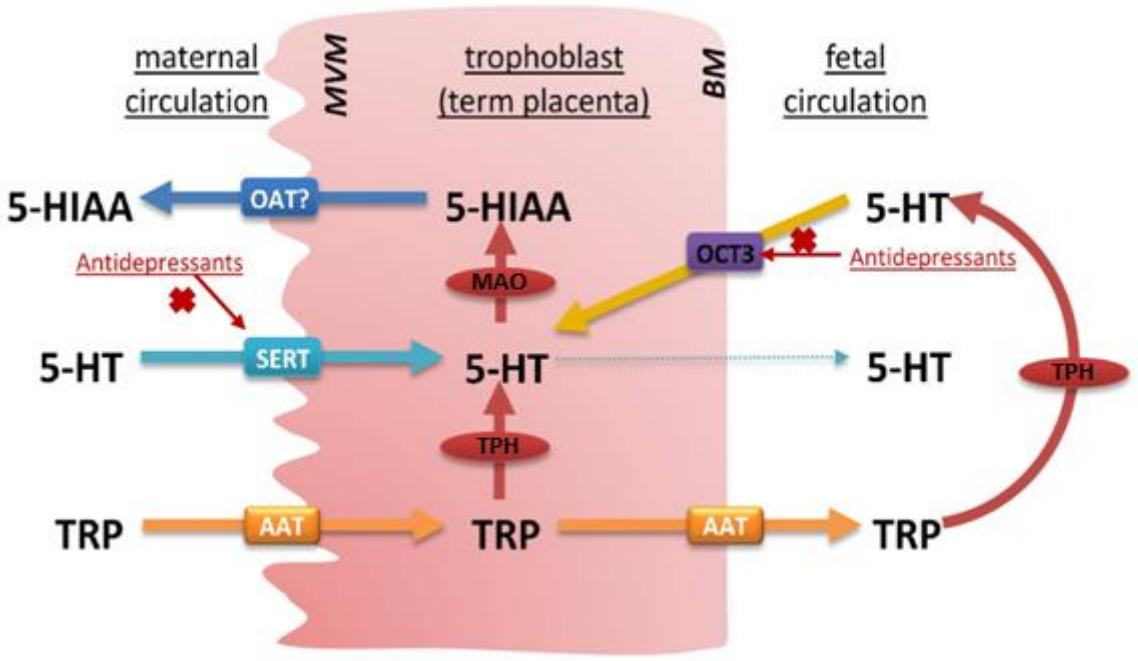

Fig. 3 Placental 5-HT homeostasis and the transporters/enzymes as possible targets of AD drugs.

At term, 5-HT is mainly taken up by the placenta from the maternal (SERT-mediated) and fetal (OCT3-mediated) circulation for enzymatic deactivation into 5-hydroxyindoleacetic acid (5-HIAA) by MAO-A. Due to their physical and chemical properties, ADs cross the placenta barrier with ease and may interact with the transporters (SERT/OCT3) or biodegradation enzymes (MAO-A) within the trophoblast thus alternating the 5-HT homeostasis in the placenta.

Abbreviations: 5-HIAA – 5-hydroxyindoleacetic acid; 5-HT – serotonin; AAT – amino acid transporter; AD – antidepressant; BM - basal membrane; MAO-A – monoamine oxidase-A; MVM - microvillous membrane; OAT – organic anion transporter; OCT3 – organic cation 3 transporter; SERT – serotonin transporter; TRP - tryptophan; TPH - tryptophan hydroxylase

5 EXPERIMENTAL METHODS

As investigation of placental function *in vivo* is limited due to technical and ethical constraints, many alternative approaches have been applied to imitate the materno-fetal barrier, including *in vitro* cell cultures, *ex vivo* human placenta-derived models and/or *in situ* animal (e.g. rat) placenta perfusion [6].

Unfortunately, due to the perplexity of the placental architecture, use of a single method mentioned above does not bring information satisfactory enough about all the human placental functions. Therefore, a combination of various distinctive research methods for examination of transplacental pharmacokinetics and functions of transporters expressed in placental trophoblast is recommended [6]. A summary figure of all experimental approaches used in this study can be found in Fig. 4. For a detailed description the reader is referred to the respective sections.

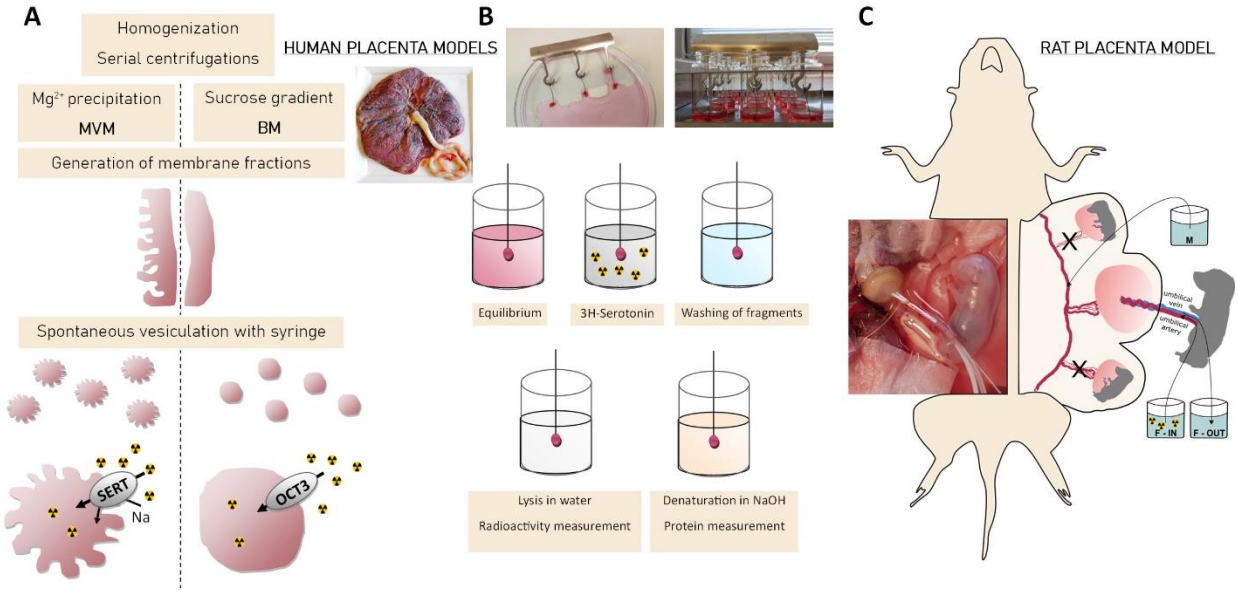

Fig. 4 Summary of the experimental methods used.

During the study three experimental methods were conducted: A) *ex vivo* uptake studies by MVM and BM vesicles; B) *ex vivo* uptake studies in fresh villous fragments isolated from a human term placenta; and C) *in situ* perfusion of rat term placenta.

5.1. Materials and chemicals

[^{3}H]5-hydroxytryptamine ([^{3}H]-5-HT), 80 Ci/mmol and [^{3}H]dihydroalprenolol ([^{3}H] DHA), 80 Ci/mmol were purchased from American Radiolabeled Chemicals, Inc. (St. Louis, MO, USA). Unlabeled 5-HT hydrochloride, paroxetine hydrochloride, citalopram hydrobromide, fluoxetine hydrochloride, fluvoxamine maleate, sertraline hydrochloride, phenylmethylsulfonyl fluoride, propranolol, p-nitrophenylphosphate, diethanolamine, DMSO were purchased from Sigma-Aldrich (St. Louis, MO, USA). Pentobarbital was purchased from Abbott Laboratories (IL, USA). Bicinchoninic acid assay (BCA assay) reagents were purchased from Thermo Fisher Scientific (Rockford, MA, USA). All other chemicals were of analytical grade and were purchased from Penta Chemicals (Prague, CZ).

5.2 Human placenta sample collection

Human term placentas were obtained from uncomplicated pregnancies at term (38-40 weeks of gestation) immediately after delivery at the University Hospital in Hradec Kralove, after obtaining the women's written informed consent and approval of the University Hospital Research Ethics Committee (approval no. 201006 S15P).

5.3 Isolation of placental MVM and BM vesicles from human term placenta

5.3.1 Composition of solutions used

Table 1. The composition of Placenta solutions 1/2

Solutions	Compounds	Final concentration
Placenta Solution 1	**Tris-Hepes 1M pH 7.2***	50 mM
	EGTA 100 mM	5 mM
	EDTA 100 mM	5 mM
	PMSF	1 mM
	Sucrose	250 mM
Placenta Solution 2	**Tris-Hepes 1M pH 7.2***	10 mM
	Sucrose	250 mM

**pH adjusted with Tris-Base 1M pH 10*

Table 2. Sucrose gradient preparation

Compounds	Final concentration/density	Compounds	Final concentration/density
Sucrose	1.165 gr/cc	**Sucrose**	1.19 gr/cc
Tris Hepes 1M pH 7.2*	10 mM	**Tris Hepes 1M pH 7.2***	10 mM

**pH adjusted with Tris-Base 1M pH 10*

Table 3. The composition of EVB and IVB

Extravesicular buffer (EVB)		**Intravesicular buffer (IVB)**	
Compounds	**Final concentration**	**Compounds**	**Final concentration**
Tris	5 mM	**Tris**	5 mM
Hepes	5 mM	**Hepes**	5 mM
NaCl	145 mM	**Sucrose**	290 mM

Table 4. The composition of Stop solution

Compounds	Final concentration
NaCl	130 mM
Na_2HPO_4	10 mM
KCl	4.2 mM
$MgSO_4$	1.2 mM
$CaCl_2$	0.75 mM

5.3.2 Experimental procedure

After receiving, the placenta was constantly kept cool during transportation and vesicles preparation as the isolation procedure is temperature sensitive. While working on an ice tray, the placenta was cut into 4-5 pieces (i.e. cotyledons), washed with saline (0.9% NaCl, physiological solution) and collected in a beaker with saline. After that, fetal chorionic membrane and maternal decidua basalis were removed from the cotyledons. Using dissection scissors, the prepared cotyledons were cut into approximately 0.5x0.5 cm pieces during which time we removed the vessels and possible calcificated areas. The minced tissue was subsequently washed on a mesh with saline until no longer bloody. The tissue was then weighed and resuspended in three volumes (ml/g) of Placenta Solution 1 (Table 1). The suspension was

homogenized using a Keenwood blender for roughly two minutes and filtered through a double folded gauze. Two millilitres of this homogenate were stored aside as Placenta Starting Homogenate, for further vesicle characterization. The rest of the homogenate was centrifuged for 15 minutes at 10 000 g at 4°C. The supernatant (S1) was collected and kept in ice while the precipitate was resuspended in Placenta Solution 1. The resuspended precipitate was then homogenized using a Teflon tip homogenizer and centrifuged again for 15 minutes at 10 000 g at 4°C. The supernatant (S2) was collected, joined with S1 and filtered through a double folded gauze. After centrifuging for 60 minutes at 47 500 g at 4°C, the supernatant was discarded and the precipitate was resuspended in total 1200 µl of Placenta Solution 1. Once again, the resuspended precipitate was homogenized using a Teflon tip homogenizer. An aliquot of this suspension was taken and the protein concentration was determined using the BCA-assay kit and samples were diluted to 10-15 mg/ml. Subsequently, freshly-prepared $MgCl_2$ solution was added to a final concentration of 12 mM. The suspension was incubated on ice for 20 minutes, while shaking. Finally, it was centrifuged for 15 minutes at 2500 g at 4°C. As Mg^{2+} precipitates the BM, at this point, the supernatant consisted of MVM and precipitate consisted of BM.

From this moment, the MVM and BM preparation was separated. For the MVM the suspension was centrifuged for 30 minutes at 47 500 g at 4°C, supernatant discarded and precipitate resuspended in Placenta Solution 2 (Table 1). The process of centrifugation and resuspension was repeated two times. Subsequently, the suspension was homogenized using a small hand glass homogenizer. Protein concentration was determined using the BCA-assay kit and finally the sample was diluted to 1.5 mg/ml concentration.

For the BM, the precipitate was resuspended in Placenta Solution 2 and homogenized using a Teflon tip homogenizer. An aliquot of the suspension was taken and the protein concentration was determined; subsequently, sample was diluted to 1.5-2 mg/ml concentration. Then the suspension was added into an ultracentrifuge tube in a discontinuous sucrose gradient (Table 2) and ultracentrifuged with T865 (Sorvall) rotor for 70 minutes at 141 000 g (i.e. 44 200 RPM) at 4°C. Afterwards, the interphase was collected, resuspended in Placenta Solution 2 and centrifuged again for 30 minutes at 47 000 g at 4°C. The precipitate was resuspended in IVB (Table 3) and homogenized using a small hand glass homogenizer. Finally, the protein concentration was determined using the BCA-assay kit and the sample was diluted to 1.5 mg/ml concentration.

Eventually, both membranes were vesiculated by passing 15 times through a 25-gauge needle. The prepared vesicles were stored at 4°C and used in uptake experiments within 3 days of isolation or frozen at −80°C for later use.

All isolation procedure involved the use of the following centrifuges: Avanti J-30 I (Beckman Coulter, USA) and Sorvall WX 80 (Thermo Fisher Scientific, USA).

5.3.3 Membrane fractions purity and enrichment determination

5.3.3.1 Alkaline phosphatase activity assay

As the enzyme alkaline phosphatase may be found solely in the MVM, this assay determines the purity of the separated membranes with ease as the enzymatic activity can be observed at much greater extent in the MVM then BM.

The measurement of alkaline phosphatase in Placental Starting Homogenate and the isolated MVM and BM was performed by adding 5 µl of samples to 250 µl of DEA (diethanolamine) buffer (20 mM diethanolamine, 12 mM $MgCl_2$) in a microplate well and mixed very well. The reaction was initiated by addition of 25 µl commix (dissolved tablet of p-nitrophenylphosphate). The increase in absorbance (A) was measured at times 0, 2, 4 and 6 at or near 410 nm. Afterwards, difference between A at every 2 minutes was calculated. Finally, the phosphatase activity was adjusted to protein content as in Eq. 1.

$$\textbf{phosphatase activity} = \frac{\Delta A_{2\ min}}{\text{protein content}} \text{ (Eq. 1)}$$

With the knowledge of phosphatase activity in the samples (MVM, BM and homogenate) it was possible to calculate the enrichment (Eq. 2).

$$\textbf{enrichment factor} = \frac{\text{phosphatase activity of the MVM/BM}}{\text{phosphatase activity of the homogenate}} \text{ (Eq. 2)}$$

5.3.3.2 DHA binding assay

The principle of this method is that DHA binds to the beta-adrenergic receptor expressed solely by the BM (not MVM). Propranolol inhibits the binding and thus the extent of binding in control conditions can be estimated.

The experiment was initiated by adding 50 µl of MVM, BM or homogenate to U-bottom shaped test tube. The reaction was started by adding 50 µl of [^{3}H]-DHA in EVB (Table 3) (with or without propranolol) and stopped after 30 minutes using Stop Solution (Table 4). Rapid vacuum filtration approach [50] was used to filter the sample through a 0.45 µm mixed cellulose ester filter (MF-Millipore membranes filter HAWP00010). The filter was consequently washed with 20 ml of Stop Solution and the filter radioactivity was measured by liquid scintillation counting.

The obtained CPMA values (counts per minute) were averaged and blanks were subtracted. The samples were subsequently compared to the standards and binding was calculated by subtracting the sample with the propranolol added from the sample with solely the DHA. Finally, the results were adjusted to protein content (Eq. 3).

$$\textbf{DHA binding} = \frac{\text{binding}_{\text{control}} - \text{binding}_{\text{propranolol}}}{\text{protein content}} \quad \text{(Eq. 3)}$$

The enrichment factor was subsequently calculated using the data from the binding per protein content of the homogenate compared to the MVM or BM (Eq. 4).

$$\textbf{enrichment factor} = \frac{\text{DHA binding (MVM or BM)}}{\text{DHA binding (homogenate)}} \quad \text{(Eq. 4)}$$

5.4 *Ex vivo* 5-HT uptake by MVM and BM vesicles

For the examination of [^{3}H]5-HT uptake into the prepared MVM and BM vesicles a rapid vacuum filtration technique was performed at room temperature where 10 µl of either MVM or BM vesicles (10-20 mg/ml) were incubated for indicated times (time dependency studies), various concentrations of unlabelled 5-HT (concentration dependency studies) or presence/absence of inhibitors (ADs) and Na^+.

Six ADs (paroxetine, sertraline, citalopram, fluoxetine, fluvoxamine and venlafaxine – all 100 µM concentration) and one control compound (Decynium-22) were tested during the experiment. To determine the unspecific binding of the tracer to the filter or/and plasma membranes measuring of protein free controls (using the IVB instead of membrane vesicle protein) and time zero uptakes were carried out.

The experiment started by 10 minutes of pre-incubating the vesicles with the ADs/control inhibitor. Afterwards, [^{3}H]5-HT (24 µCi/ml) was added and 1-minute uptake experiment was then initiated. After 1 minute, the uptake was stopped by adding 2 ml of ice-cold stopping buffer. The sample was filtered through a 0.45 µm mixed cellulose ester filter (MF-Millipore membranes filter HAWP00010) and washed with 20 ml of Stop Solution (Table 4); radioactivity of the filter was measured by liquid scintillation counting (results as CPMA).

After the values from the scintillation counting were obtained, the non-protein control (NCP) was subtracted from the data. The average uptake (pmol/mg protein) was calculated using Eq. 5. Subsequently the uptake inhibition was calculated using these values.

$$\textbf{average uptake} = \frac{\frac{CPMA_{sample} \times concentration_{standard}}{CPMA_{standard}}}{\text{protein content}} \quad \text{(Eq.5)}$$

5.5 *In situ* perfusion of rat term placenta

5.5.1 Experimental animals

Healthy female albino rats of Wistar species, as the most used strain of the outbred rats, were obtained from Velaz breeding station (CZ). All experiments were approved by the Ethical Committee of the Faculty of Pharmacy in Hradec Kralove (approval no. MSMT-4312/2015-8; Charles University, Czech Republic). Experiments were conducted on rats 21 days after mating.

5.5.2 Preparation of solutions

On each day of the experiments, a Krebs solution was prepared. Krebs stock solution (10 ml; Table 5) was diluted with 90 ml distilled water. Afterwards, 0.7 ml of $CaCl_2.2H_2O$ stock solution (136 mM) and 0.5 ml of $MgCl_2.6H_2O$ stock solution (98 mM) was added. The solution was mixed and pH was adjusted to 7.2. Finally, 0.1 ml of heparin and 1 g of dextran was added. The solution was mixed, filtered and heated to 38°C.

Table 5. The composition of Krebs's stock solution

Compound	Final concentration
NaCl	1000 mM
KCl	27 mM
$NaHCO_3$	238 mM
$Na_2HPO_4.12H_2O$	10 mM
Glucose	56 mM

5.5.3 Perfusion procedure

For the anaesthesia of the rat, a solution of pentobarbital (Nembutal) was used at the very beginning of the experiment. 40 mg/kg of the anaesthetic was administered into the tail vein. Two experiments were performed on each rat, by using one placenta from each uterine horn. During the whole experiment rat´s vital functions, temperature of the solutions, pressure in the placenta, bubbles occurrence and time was watched, as any perturbations might ruin the experiment.

To distinguish the gender of the fetus the anogenital distance was measured (female for shorter distance). The umbilical artery and vein were cannulated and connected with respective reservoirs. The speed of the flow was rated (0.5 ml per minute) or adjusted for that desired value.

In the first 10 minutes of the experiment, the placenta was prewashed with Krebs's solution (control) or 100 µM AD solution. Then the solutions were exchanged for either a solution of solely 1 nM [^{3}H]5-HT (control) or a solution of 1 nM [^{3}H]5-HT and 100 µM AD. All the solutions used were maintained and controlled at a temperature of about 37°C. The outcoming samples were collected into individual vials in 5 mins intervals. After 40 minutes the experiment was terminated. Finally, the placenta was cut off, cleaned from the excess tissue, weighed and stored for the further experiments (RNA isolation and gene expression). Samples were measured by scintillation counting, averaged and used for statistical analysis.

Using these results it was possible to calculate the extraction ratio for each time interval (Eq. 6), where c_{fa} is the 5-HT concentration in the fetal reservoir entering the placenta via the umbilical artery and c_{fv} is the drug concentration in the umbilical vein effluent.

$$\textbf{extraction ratio} = \frac{c_{fa} - c_{fv}}{c_{fa}} \text{ (Eq. 6)}$$

5.6 *Ex vivo* uptake studies in fresh villous fragments isolated from human term placenta

Pilot experiments on *ex vivo* uptake of [^{3}H]-5HT (0.5 µCi/ml) into human placental villous fragments were carried out to establish this method for 5-HT uptake studies. Specifically, time-, concentration- and Na^+-dependent studies were accomplished.

Table 6. The composition of Tyrode's buffer

Compound	Final concentration
NaCl *(choline chloride^x)*	135 mM
KCl	5 mM
$CaCl_2$	2 mM
$MgCl_2$ ($6H_2O$)	1 mM
HEPES	10 mM
Glucose	6 mM

**pH adjusted to 7.4 with NaOH 10 M*
^{x}Na$^+$ free medium

On the first day of the experiment (meaning the arrival of the placenta) 4-5 pieces of villous material were removed from the middle section of the placenta between the basal and chorionic plate trying to avoid sampling the tissue from the edges. Subsequently, the tissue was placed into the equilibrium solution (a 1:1 mixture of DMEM and Na^+/Na^+-free Tyrode's buffer, Table 6). While kept bathed in the equilibrium solution the chunks were dissected into smaller pieces, tied onto hooks and placed into the vials with equilibrium solution heated at 37°C and kept to equilibrate for 30 minutes. The uptake measurement was initiated by moving the fragments to the uptake solution with the radioactive isotope. The reaction was stopped after 15 minutes and fragments were moved quickly into the post-wash solutions (Na^+/Na^+-free Tyrode´s buffer). The tissue was agitated vigorously in the post-wash solution two times (for 15-20 seconds each) in order to terminate accumulation and remove drugs bounded extracellularly. In the last step, the tissue was put into the vials containing pure distilled water and left overnight at room temperature so that the radioactive isotope would release from the fragments and could be counted subsequently.

On day two of the experiment the fragments were removed from the water and put into the vials filled with NaOH 0.3 M. The vials were then placed into a shaking incubator set on 37°C for 6-8 hours. Lastly, the BCA-assay and scintillation counting were performed. Average uptake was calculated according to Eq. 5.

5.7 Radioisotope analysis

Concentrations of [^{3}H]5-HT (80 Ci mmol/1) and [^{3}H]DHA (80 Ci mmol/1) in experimental samples were measured by liquid scintillation counting using a Tri-Carb 2910 TR instrument (Perkin Elmer, Waltham, USA).

5.8 Statistical analysis

Effects of tested inhibitors *in situ* and *ex vivo* were assessed using Mann-Whitney test, implemented in GraphPad Prism 8.1 software (GraphPad Software, Inc., San Diego, USA). Asterisks in the figures indicate significance levels: * ($p \leq 0.05$), ** ($p \leq 0.01$), and *** ($p \leq 0.001$).

6 RESULTS

6.1 *Ex vivo* uptake studies on MVM and BM

6.1.1 Purity of isolated membrane vesicles

As exclusive isolation of membranes is rather impossible, various markers are used to assess the enrichment of a membrane preparation. MVM, in contrast to BM, expresses the enzyme alkaline phosphatase, thus by measuring its activity in MVM and BM we can estimate the success of the isolation procedure. The MVM membranes used in this study showed five times higher activity of the enzyme compared to BM (Table 7), with enrichment levels being in line with previous publications [51, 52] and thus confirming minimal contamination with BM.

On the other hand, receptors for DHA, can be found solely in the BM. Thus, binding of DHA to this receptor should be observed in much greater extent in the BM, in contrast to MVM. Results showed almost 11 times more extensive binding of the DHA to the BM (Table 7), which is also in line with reported literature [51, 52].

Table 7. Term placenta vesicles purity and enrichment assessment

Measured parameter	**MVM**		**BM**	
	mean	**SD**	**mean**	**SD**
Alkaline phosphatase activity	16.63	5.50	3.27	0.67
Dihydroalprenolol (DHA) binding	1.68	0.95	17.33	2.99

6.1.2 Time-dependent uptake of 5-HT in MVM and BM

Uptake of [^{3}H]5-HT by the MVM was shown to increase up to 15 minutes, and then decrease with time (Fig. 5A). This is known as “overshoot-phenomenon”, typical for transporters such as SERT, dependent on Na^+, whose gradient is lost with time [53, 54]. On the other hand, uptake in the BM increases with time reaching a plateau phase after 5 minutes (Fig. 5B). This indicates that the transport mechanism on the BM is independent of Na^+. Subsequently, a 1-minute uptake was chosen for concentration-dependent studies and testing of inhibitors.

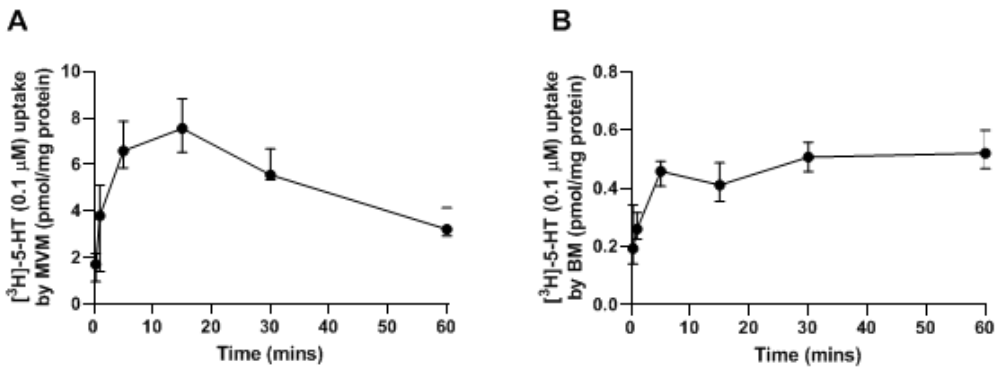

Fig. 5 Time dependency studies of the [^{3}H]5-HT uptake into the A) MVM vesicles and B) BM vesicles.

Due to the Na^+-dependent transport into the MVM vesicles the uptake shows a continual decrease over time as Na^+ gets depleted (A). Contrariwise, the uptake into the BM vesicles remains stable after reaching the plateau phase, indicating that the transport is not Na^+-dependent (B). Data are presented as median ± interquartile range; n = 3

6.1.3 Concentration-dependent uptake of 5-HT in MVM and BM

We observe that the vesicular uptake of 5-HT significantly decreases with increasing concentration of the substrate, indicating a transporter-mediated mechanism. Interestingly, the uptake in the MVM was affected at a much higher rate upon increasing concentrations of 5-HT, compared to the BM (Fig. 6). Subsequently, a 0.1 µM concentration was chosen for testing of inhibitors.

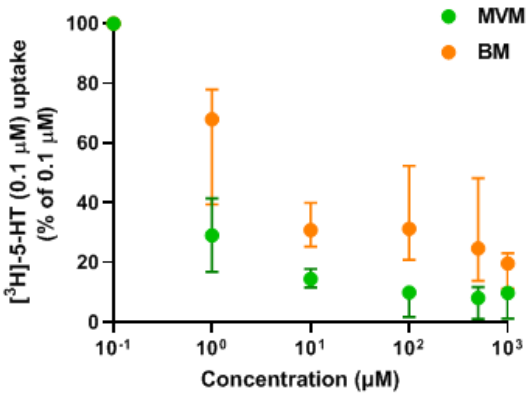

Fig. 6 Concentration dependency studies of the [^{3}H]5-HT uptake into the MVM vesicles and BM vesicles.

Increasing concentrations of unlabelled 5-HT affect differently the transport mechanisms in MVM and BM. Specifically, the transporter in MVM shows saturation at lower concentrations of 5-HT, whereas the uptake in the BM remains high even at 1 mM 5-HT. Data are presented as median ± interquartile range; n=5.

6.1.4 Binding assays

In order to determine also to what extent does the non-specific binding of 5-HT to the membrane take place during the experiments, uptake of 5-HT at time 0 was investigated. We observed similar binding properties in the MVM and BM accounting to 10.6% for MVM and 12.5% for BM (Fig. 7).

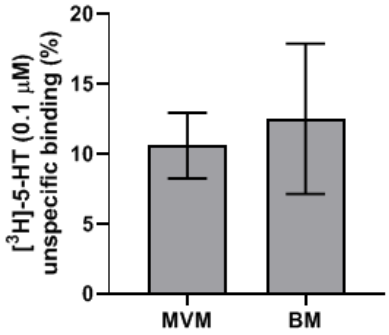

Fig. 7 The binding assay of the [^{3}H]5-HT in the MVM and BM vesicles.

Similar binding properties were observed for both MVM and BM. Data are presented as median ± interquartile range; n > 1.

6.1.5 Na^+-dependency of 5-HT uptake

Uptake studies in presence or absence of Na^+ were performed to differentiate transporters responsible for 5-HT uptake in the MVM and BM. An expected decrease in uptake upon Na^+ loss was observed in the MVM vesicles (Fig. 8A). On the other hand, uptake by the BM was not changed absence of Na^+ (Fig. 8B), indicating that the uptake of [^{3}H]5-HT into the BM vesicles is not by a Na^+-dependent mechanism.

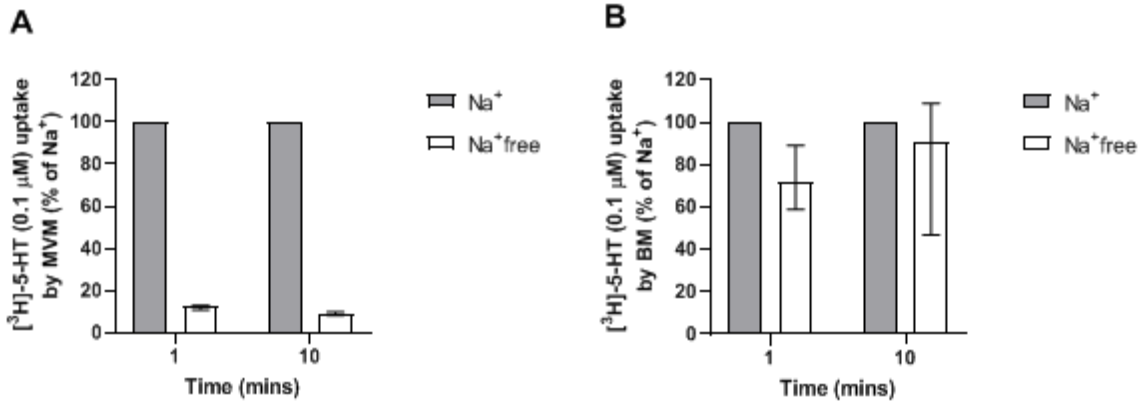

Fig. 8 The Na^+-dependency assay of the A) MVM vesicles and B) BM vesicles.

A distinctive decrease in [^{3}H]5-HT Na^+-free uptake in the MVM indicates that the uptake mechanism is Na^+-dependent (A), unlike BM (B). Data are presented as median ± interquartile range; n=3.

6.1.6 Effect of ADs on 5-HT uptake by MVM and BM

Six inhibitors were examined: five SSRIs (paroxetine, sertraline, fluoxetine, fluvoxamine, citalopram) and one SNRI (venlafaxine) for their potential to inhibit 5-HT uptake *in vitro*. The results indicate rather aligned inhibition of 5-HT uptake for all ADs examined compared to control (Fig. 9). Comparing MVM and BM, a higher extent of inhibition is observed in MVM vesicles (Fig. 9A) against BM vesicles (Fig. 9B).

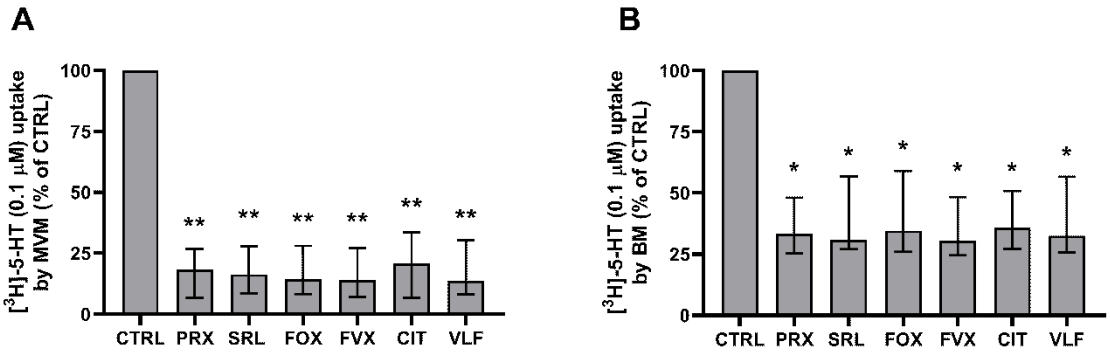

Fig. 9 Effect of ADs on [^{3}H]5-HT uptake into the A) MVM vesicles and B) BM vesicles.

All ADs examined (100 µM concentration) showed an inhibitory effect on the [^{3}H]5-HT uptake, no significant differences between ADs were observed. A higher extent of inhibition was expressed in MVM vesicles compared to BM vesicles. The extent of inhibition is demonstrated via percentage of inhibition compared to control. Data are presented as median ± interquartile range; n>4. Statistical significance was evaluated using non-parametric Mann-Whitney test. * $p<0.05$; ** $p<0.01$.

Abbreviations: CIT – citalopram; CTRL – control; FOX – fluoxetine; FVX – fluvoxamine; PAR – paroxetine; SRL – sertraline; VFX – venlafaxine

6.2 Effect of ADs on 5-HT extraction in dually perfused rat term placenta

Seven ADs were examined: five SSRIs (paroxetine, sertraline, fluoxetine, fluvoxamine, citalopram), one SNRI (venlafaxine) and decynium-22 as a control inhibitor for their potential to inhibit placental 5-HT uptake from the fetal circulation. Results illustrate two ADs to have a considerable inhibitory effect on [^{3}H]5-HT uptake from the fetal circulation: paroxetine and fluvoxamine (Fig. 10)

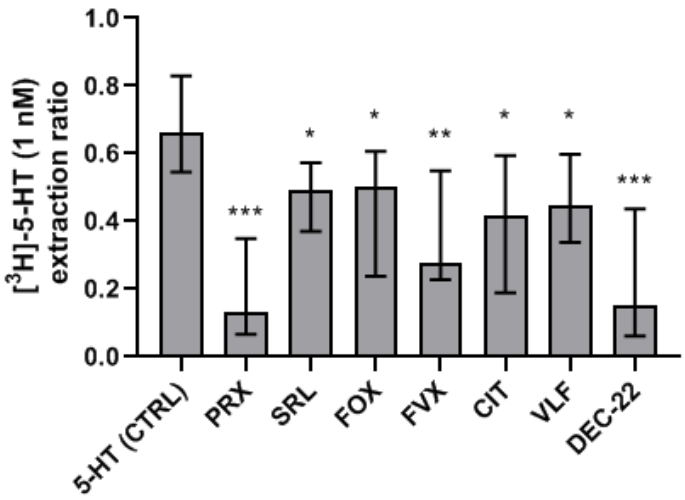

Fig. 10 Effect of ADs on 5-HT uptake from fetal circulation in rat term placenta.

All ADs (100 μM concentration) show statistically significant inhibitory effect on the [^{3}H]5-HT uptake from fetal circulation. While the majority of ADs affect the uptake partially, paroxetine and fluvoxamine revealed the most potent inhibitory potential. Data are presented as median ± interquartile range; n>6. Statistical significance was evaluated using non-parametric Mann-Whitney test. * $p<0.05$; ** $p<0.01$; *** $p<0.001$.

Abbreviations: CIT – citalopram; CTRL – control; DEC-22 – decynium-22; FOX – fluoxetine; FVX – fluvoxamine; PRX – paroxetine; SRL – sertraline; VLF – venlafaxine

Nevertheless, high inter-individual variability was noted, visible by the large deviations of the samples from the median value. Thus, we investigated the possibility that fetal sex could be responsible for the variability observed. Results indicate that inhibition of [^{3}H]5-HT uptake from the fetal circulation by ADs is largely dependent on the fetal sex. Specifically, when separating the sexes, all ADs showed significant inhibitory effect only in the placentas of male fetuses (Fig. 11).

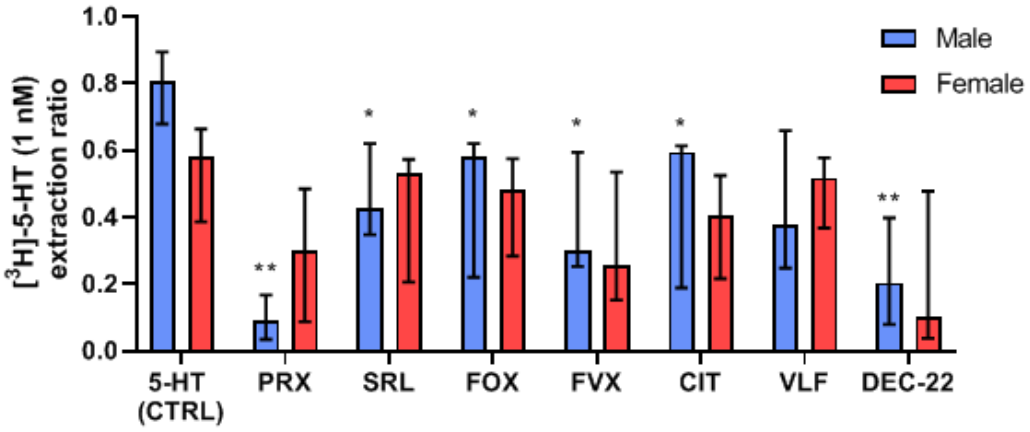

Fig. 11 Sex-dependent inhibition of 5-HT uptake *in situ*.

Results indicate the [^{3}H]5-HT uptake inhibition to be sex dependent, specifically male fetuses are more susceptible to inhibition of 5-HT uptake from fetal circulation in presence of ADs (100 µM concentration). Data are presented as median ± interquartile range; n>3. Statistical significance was evaluated using non-parametric Mann-Whitney test. * $p < 0.05$; ** $p < 0.01$. *Abbreviations: CIT – citalopram; CTRL – control; DEC-22 – decynium-22; FOX – fluoxetine; FVX – fluvoxamine; PRX – paroxetine; SRL – sertraline; VLF – venlafaxine*

6.3 *Ex vivo* uptake studies on placental villous fragments

6.3.1 Time-dependent uptake of 5-HT

Due to the complexity of placental villous fragments preparation, only MVM is accessible for uptake examination, whereas BM is not. Based on the previous results of a Na^+-dependent transporter in MVM, time-dependent uptake of [^{3}H]5-HT was investigated in presence and absence of Na^+. Uptake of [^{3}H]5-HT by the fragments in the presence of Na^+ increases up to 60 minutes and then reaches a plateau phase. After the conditions of the surrounding medium change for Na^+-free environment, uptake markedly decreases indicating the transport to be also Na^+-dependent (Fig. 12). Subsequently, a 10-minute uptake was chosen for concentration-dependent studies.

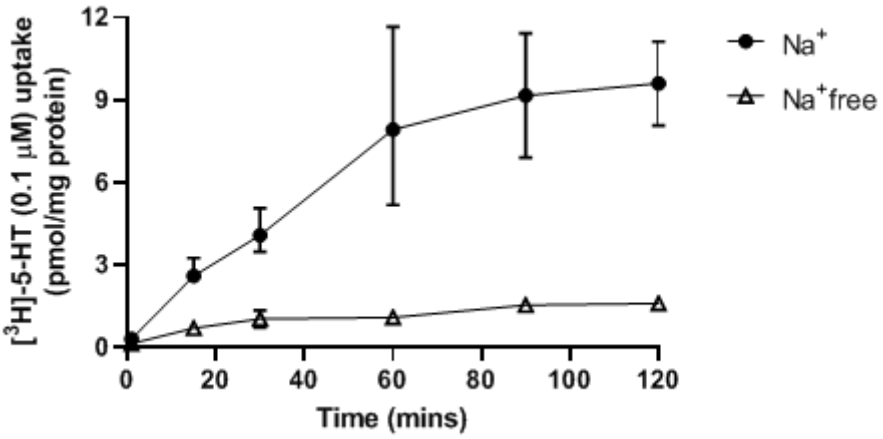

Fig. 12 Time dependent [^{3}H]5-HT uptake into the villous placenta fragments.

A significant decrease in [^{3}H]5-HT uptake into placental villous fragments was observed in Na^+-free medium confirming the Na^+-dependent transport mechanism in MVM. Data are presented as median ± interquartile range; n > 2.

6.3.2 Concentration-dependent uptake of 5-HT

5-HT uptake by placental fragments rapidly decreases at 5-HT concentrations above 100 µM, indicating transporter-mediated mechanism (Fig. 13). Subsequently, a 0.1 µM concentration was determined as optimal for future testing of inhibitors.

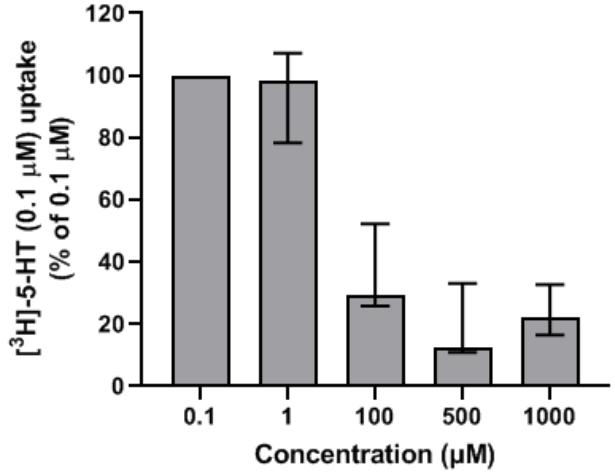

Fig. 13 Concentration dependent [^{3}H]5-HT uptake into the villous placenta fragments.

Uptake of [^{3}H]5-HT in the range of 1-500 µM concentrations of unlabelled 5-HT indicates a saturable, transporter-mediated uptake of 5-HT in placental villous fragments. Data are presented as median ± interquartile range; n = 4.

7 DISCUSSION

Depression in pregnancy, when left untreated, has been shown to be harmful for both, the mother and the fetus. Particularly, women with untreated depression are in greater danger of building up an alcohol or tobacco addiction and have a worse nutrition [55]. Neonates who are born to these mothers are more likely to be delivered preterm, have a lower birth weight, show difficulty in forming a social interactions later in life and show differences in the developmental and emotional aspects [55]. Thus, use of ADs during pregnancy has significantly increased in the recent years with the most commonly prescribed ones being from the group of SSRIs: sertraline, citalopram, paroxetine, fluvoxamine or fluoxetine [56] and SNRIs such as venlafaxine and duloxetine [57, 58].

Current state of knowledge suggests that ADs may affect placental 5-HT transport (by inhibition of SERT) and/or metabolism (by inhibition of MAO), resulting in suboptimal 5-HT concentrations *in utero*; however, direct evidence of the molecular mechanisms underlying these perturbations remain lacking. Fetal development is linked closely with precise regulation of 5-HT levels in the feto-placental unit due to its role in various functions including neuronal tissue organization or cardiovascular system development [30, 35]. Inadequate elevation of this monoamine in the feto-placental unit may result in either altered fetal development [36] or inadequate perfusion of the placenta due to vasoconstrictive effects of the 5-HT [59].

In this work, the inhibitory potential of SSRIs and venlafaxine on 5-HT uptake by placental membranes was evaluated *ex vivo* (though isolated human term placental membranes) and *in situ* (perfusion of rat term placenta). The advantage of the isolated human placental membranes is the possibility of simultaneous isolation of both MVM and BM from the same placenta and thus allowing direct comparison between the two membranes. To our knowledge, only one study using MVM vesicles from human term placenta evaluated the effect of fluoxetine on 5-HT uptake by MVM [60]; the extent of inhibition observed in the study is comparable to our findings. Nevertheless, we show that not only fluoxetine, but all examined ADs showed an inhibitory effect in both MVM and BM vesicles. Through a series of method-establishing studies and consisted with the work published by our team [7], we are confident that the inhibitory effect observed in the BM is due to inhibition of OCT3.

On the other hand, in our *in situ* studies in rat term placenta perfusions, solely fetal side of the placenta was examined. This is due to the fact that at term there is negligible transfer

of 5-HT in the mother-to-fetus direction as a result of high metabolism in the placenta [7]. The effect of tested ADs on 5-HT uptake from fetal circulation in rats was less pronounced compared to studies *ex vivo* in the BM vesicles; nevertheless, all tested compounds significantly affected OCT3 function in rat term placenta. Interestingly, we observed sex-dependent differences in the effect of ADs on OCT3 function. Sex-dependent differences in OCT3 mediated uptake of 5-HT from fetal circulation was also reported before and was not attributable to differences in the gene expression of the transporter [7]. Yet, these findings are interesting and may explain the sex-dependent effects observed in behavioral studies upon prenatal treatment with ADs [61]. In addition, there is link between ADs treatment and higher risk of ADHD, autism, depression and other cognitive difficulties, behavioral problems and delayed motor development, more significantly observed in males [19, 21, 62]. Further studies in other species or experimental models are required to fully understand this phenomenon.

The most potent AD visible from perfusion studies was paroxetine. This drug was categorized by the US FDA as a class D drug for use in pregnancy, due to an increased risk of septal heart defects of the offspring [55] and increased incidence of cardiovascular malformations [37]. Additionally, use of paroxetine during pregnancy was associated with an increased risk of congenital and cardiac malformations but also neonatal withdrawal including symptoms of respiratory depression, poor feeding, lethargy, and jitteriness [63]. Similar reports are also found regarding prenatal use of fluoxetine [37] and a recent study suggests that citalopram and sertraline ought to be the number one chosen pharmacotherapy from the SSRIs for depression and anxiety of the pregnant women [64].

Based on current literature, the role of placenta in 5-HT homeostasis changes with the course of pregnancy. During the first trimester, placental synthesis and transport of 5-HT represents the main source for fetus [30], whereas in later stages external 5-HT supply is no longer necessary as the fetus becomes competent in its own 5-HT synthesis from maternally-derived TRP [40]. A recent study from our research group shows that indeed at term, placenta no longer provides 5-HT to the fetus; instead it takes it up from both maternal and fetal circulation via SERT- and OCT3-mediated mechanism, respectively [7]. The simultaneous increase in MAO-A activity observed at term (Karahoda, Abad et al., manuscript submitted) efficiently metabolizes 5-HT within the STB presumably to detoxify the placenta from high 5-HT levels. Thus, along with SERT, found expressed in MVM, we show that ADs can affect OCT3-mediated 5-HT uptake in BM. To date, only one study using human OCT3 transfected cells

have been conducted with SSRIs and SNRIs which confirmed an inhibitory effect of these substances on OCT3 activity [49]. Collectively, outcomes of this work suggest that ADs may disturb the 5-HT homeostasis in placenta through their inhibitory action on both SERT and OCT3. This may contribute to the disproportional amount of 5-HT in the feto-placental unit during different stages of pregnancy.

It should be noted that the concentrations of ADs used in this study (100 μM) were based on the rationale of observing the potential of inhibition by several drugs. We realize, that achievable plasma concentrations for ADs during pregnancy are significantly lower [65-68] and ongoing experiments are being carried out with concentrations closer to the pharmacological levels.

Part of this work also focused on establishing a model of placental villous fragments to study 5-HT uptake by the term human placenta [69]. We managed to establish time dependent 5-HT uptake into the villous placenta fragments; experiments using ADs as inhibitors are being validated for follow-up studies.

8 CONCLUSION

Collectively, here we propose a mechanism that could account for the poor neonatal outcomes reported with prenatal use of ADs. Our *in situ* and *ex vivo* data indicate that ADs block both 5-HT transporters in the placenta, i.e. SERT on the mother-facing apical membrane and OCT3 on the fetus-facing basal membrane. Considering the role of 5-HT in fetal development/programming, ADs may disrupt placental homeostasis of 5-HT and lead to faulty feto-placental development. Additionally, in the rat placenta, we observed sex-dependent effects on the inhibitory potential of ADs towards OCT3. Further studies conducted in different species and experimental models are required in order to fully understand this phenomenon. Furthermore, dose-dependent studies are necessary to show the clinical relevance of our findings.

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